The Cloning Of Novel Gonadotropin-Releasing Hormone Receptors By Polymerase Chain Reaction

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DECLARATION

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ABSTRACT

The cloning of novel Gonadotropin-releasing hormone receptors by PCR

Gonadotropin-releasing hormone (GnRH), a central regulator of reproductive function in all vertebrates, exerts its effects via binding to the GnRH receptor (GnRHR) in the pituitary gonadotrophs. The GnRHR is a member of the G-protein coupled receptor (GPCR) superfamily. A second form of the GnRHR (type II), other than the pituitary gonadotrope GnRHR (type I) has been proposed to exist and to play a role other than the classical endocrine role of the pituitary GnRHR. Elucidation of amino acid residues of the GnRHR that are crucial for ligand binding, activation of the receptor, and coupling to the G-protein, is important in understanding structure-function relationships towards the design of drugs for therapeutic intervention. Such information can often be deduced by a comparison between conserved and non-conserved amino acid residues of GnRHRs from different species.

At the start of this project no non-mammalian or invertebrate, and only some of the eutherian mammalian type I GnRHRs had been cloned. The aim of this project was to clone novel GnRHRs, i.e. type I and type II GnRHRs from redbait and mole and type II mouse and human GnRHRs using polymerase chain reaction (PCR) strategies. PCR was performed with degenerate primers designed to human type I GnRHR to areas that are not conserved between GPCRs in general, but are conserved between mammalian GnRHRs.

No positive results were obtained with any of the primer pairs tried on redbait. The reason this strategy did not work on redbait, might be that the redbait GnRHR differs much from that of the mammalian GnRH receptors. Another possibility is that there is no GnRHR in redbait. However, this is very unlikely, since GnRH has been detected in tunicates.

A fragment of a novel type I mole GnRHR was obtained, corresponding to exon 1 of the human type I GnRHR. Exon 1 of type I mole GnRHR 89.5% is homologous to human type I GnRHR on the DNA level, and 93% on the amino acid level, and differed from the human type I GnRHR in only 4 amino acids out of a total of 96. These were all conservative changes that occurred in the transmembrane domains. This result was rather surprising given the

evolutionary distance between the mole, a member of the non-placental eutherians, and humans, but showed that a strict requirement exists for conserved amino acids in the type I GnRHR for widely divergent mammals. Attempts to clone the putative type II GnRHR from mole or mouse were unsuccessful.

Human genome data base searches revealed the presence of putative human type II GnRHR DNA sequences, indicating that a gene for a type II GnRHR exists in the human genome. Using gene specific primers designed to this sequence, it was shown that several human tissues and cell lines expressed a type II GnRHR RNA. In most tissues this mRNA contained an intron. An intron-less novel type II human GnRHR cDNA was cloned from human testes RNA, and corresponded to exons 2 and 3, minus intron 1, of the type I human GnRHR. The deduced amino acid sequence is 68% homologous to type I human GnRHR. An interesting feature of the type II GnRHR, is the presence of a C-terminal tail which is absent from all the type I mammalian GnRHRs cloned to date. This result shows that a processed RNA transcript (i.e. not containing the equivalent intron 2) coding for part of a type II GnRHR i.e. putative exons 2 and 3, is indeed expressed in at least one human tissue. This processed transcript is clearly of much lower abundance than the intron-containing transcript. Using a primer designed to span the exon 2/exon 3 boundary to selectively amplify processed RNA rather than intron-containing RNA, it was shown that several other human tissues may also express the processed type II GnRHR RNA. Human genomic DNA clones containing putative exons 2 and 3 of the type II GnRHR were purchased commercially. Analysis of these clones confirmed the existence of putative type II GnRHR exons 2 and 3 sequences in the human genome. The presence of type II GnRHR sequences corresponding to exon I of the type I GnRHR could not be detected in either human cDNAs from several tissues or in the human genomic clones. The inability to detect the presence of "full length" type II GnRHR transcripts may mean that they do not occur, or that we were unable to detect them due to their low abundance, or that they are not expressed in the tissues examined. From the results it was not possible to determine whether the processed transcript is the sense or the antisense strand. It is possible that the transcripts that were detected are antisense transcripts which could have a regulatory function in supressing transcription of the sense transcript. It remains to be shown whether the type II human GnRHR RNA expressed in testes and possibly in other tissues, and corresponding to exons 2 and 3 of the type I GnRHR, is

expressed as a functional protein.

ABBREVIATIONS

Amp	Ampicillin
bp	DNA base-pair
BSA	Bovine serum albumin
cGnRHII	Chicken gonadotropin-releasing hormone II
DAG	Diacylglycerol
DEPC	Diethylpyrocarbonate
DMSO	Dimethylsulfoxide
ds	Double stranded
DTT	Dithiothreitol
EC	Extracellular loop
EST	Expressed sequence tag
EtBr	Ethidium bromide
EC	Extracellular loop
EDTA	Ethylenediaminetetra-acetic acid tetrasodium salt
FSH	Follicle stimulating hormone
xg	Gravitational force
gDNA	Genomic DNA
GnRH	Gonadotropin-releasing hormone
GnRHR	Gonadotropin-releasing hormone receptor
GPCR	G-protein coupled receptor
GSP	Gene specific primer
IC	Intracellular loop
IP_3	Inositol triphosphate
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	Kilo base
LB	Luria-Bertani medium
LD PCR	Long distance polymerase chain reaction
LH	Luteinizing hormone
min	Minute
MMLV	Moloney Murine Leukemia Virus

MOPS	Morpholinopropanesulfonic acid
MW	Molecular weight
oligo(s)	Oligodeoxyribonucleotide(s)
ORF	Open reading frame
PCI	Phenol:Chloroform:Isoamyl alcohol
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PIPES	Piperazine-N,N'-bis-[2-ethanesulfonic acid]
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl fluoride
PVP	Polyvinylpyrrolidone
RACE	Rapid amplification of cDNA ends
RNase A	Ribonuclease A
RT	Reverse transcriptase
sec	Second
SDS	Sodium dodecyl sulphate
TBE	Tris-borate-EDTA
TdT	Terminal deoxytransferase
TE	Tris-EDTA
TEMED	N,N,N',N'-Tetramethylethylene-diamine
TM	Transmembrane helix/domain
Tris	Tris(hydroxymethyl)aminomethane
T4PNK	T4 polynucleotide kinase
U	Unit
UTR	Untranslated region
UV	Ultraviolet
vol	Volume
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside

CONTENTS

CHAPTER 1

INTRODUCTION

Gonadotropin releasing hormone (GnRH) is a hypothalamic decapeptide that is the key neural regulator of the reproductive process. It is synthesized by hypothalamic neurons, secreted in a pulsatile manner, and carried to the anterior lobe of the pituitary gland where it binds to receptors in the pituitary gonadotrophes. There, GnRH stimulates the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH), which, in turn, regulate the gametogenic and hormonal functions of the gonads.

The GnRH receptor (GnRHR) is a member of the G protein-coupled receptor (GPCR) family (Spiegel, 1989). They are a variety of cell-surface receptors that mediate their intracellular actions by a pathway that involves activation of one or more guanine nucleotide-binding regulatory proteins (G proteins) (Strader *et al.*, 1994). These receptors form a large and functionally diverse superfamily. Receptors that belong to this class respond to a variety of hormone and neurotransmitter agonists. The signalling pathway of this family normally involves the binding of the agonist to its receptor, causing conformational changes in the receptor (Strader *et al.*, 1994). These changes allow the receptor to interact with the G protein to form an agonist-receptor-G protein complex. A wide variety of second-messenger molecules are influenced by the stimulation of G protein-coupled receptors with specific hormone agonists, including adenylyl cyclase, guanylyl cyclase, phospholipases C (PLC) and A2, and Ca^{2+} and K^{+} channels. Both the COOH- and NH_2-termini of GnRH interact with the receptor, resulting in high affinity binding (King and Millar, 1995). The NH_2-terminal sequence is essential for activating the receptors to allow initiation of the transducing signals (King and Millar, 1995). The GnRHR activates PLC through a G protein, which gives rise to the generation of inositol triphosphate (IP_3), which in turn mobilizes intracellular Ca^{2+}. PLC activation by GnRH also results in the generation of diacylglycerol (DAG) and consequent activation of protein kinase C (PKC).

Species	GnRH Structure					
Agnatha						
Lamprey (*Petromyzon marinus*)			LI		LIII	
Hagfish						
Heptatretus hexatrema						U
Myxine glutinosa						U
Chondrichthyes						
Ratfish (*Hydrolagus colliei*)	CII					
Ray (*Torpedo marmorata*)	CII			Df		
Dogfish						
Squalus acanthias	CII			Df		U
Scyliorhinus canicula	CII			Df		U
Poroderma africanum	CII					U
Osteichthyes						
Reedfish (*Calamoichthys calabaricus*)	CII	M				
Sturgeon (*Acipenser transmontanus*)	CII	M				
Alligator gar (*Lepisosteus spatula*)	CII	M				
Bowfin (*Amia calva*)		M				
Eel (*Anguilla anguilla*)	CII	M				
Catfish						
Clarias gariepinus	CII				Cf	
Clarias macrocephalus	CII				Cf	
Herring (*lupea harengus pallasi*)	CII		S			
Milkfish (*Chanos chanos*)	CII		S			
Goldfish (*Carassius auratus*)	CII		S			
Salmon						
Oncorhynchus keta	CII		S			
Oncorhynchus masou	CII		S			
Carp (*Cyprinus carpio*)	CII		S			
Trout (*Salmo gairdneri*)	CII		S			
Hake (*Merluccius capensis*)	CII		S			U
Codfish			S			
Molly			S			
Snook			S			U
Seabass	CII		S			
Mullet	CII		S			
Tilapia	CII		S			U
Platyfish	CII		S			
Wrasse	CII		S			
Cichlid	CII		S			U
Lungfish						
Neoceratodus forsteri	CII	M				
Protopterus annectens	CII	M				

Figure 1 Taxonomic distribution of GnRHs in vertebrates (King and Millar, 1995). CII, chicken GnRH II; M, mammalian GnRH; S, salmon GnRH; CI, chicken GnRH I; Cf, catfish GnRH; Df, dogfish GnRH; LI, lamprey GnRH I; LIII, lamprey GnRH III; U, an unidentified form of GnRH. Where identification of known GnRHs was not conclusive, the GnRH has been designated as an unidentified form of GnRH.

Species	GnRH structure			
Amphibia				
Newt				
Taricha granulosa	CII	M		
Ambystoma gracile	CII	M		
Ambystoma mexicanum	CII	M		
Clawed toad (*Xenopus laevis*)	CII	M		
Frogs				
Hyla regilla	CII	M		
Rana pipiens	CII	M		
Rana catesbeiana		M		
Rana esculenta	CII	M		
Rana ridibunda	CII	M		
Reptilia				
Turtle				
Pseudemys scripta	CII		CI	
Trachemys scripta	CII		CI	
Lizards				
Podarcis secula sicula				U
Cordylis nigra	CII			U
Skink (*Calcides ocellatus*)	CII			
Snake (*Thamnophis sirtalis parietalis*)			CI	
Alligator (*Alligarot missisippiensis*)	CII		CI	
Crocodile (*Crocodylus niloticus*)	CII		CI	
Aves				
Chicken (*Gallus domesticus*)	CII		CI	
Turkey (*Gallus meleagris*)	CII		CI	
Ostrich (*Struthio camelus*)	CII		CI	
Quail (*Coturnix coturnix*)	CII		CI	
Starling (*Sturnus vulgaris*)	CII		CI	
Song sparrow (*Melospiza melodia*)			CI	

Figure 1 (Continued). Taxonomic distribution of GnRHs in vertebrates. (King and Millar, 1995). CII, chicken GnRH II; M, mammalian GnRH; S, salmon GnRH; CI, chicken GnRH I; Cf, catfish GnRH; Df, dogfish GnRH; LI, lamprey GnRH I; LIII, lamprey GnRH III; U, an unidentified form of GnRH. Where identification of known GnRHs was not conclusive, the GnRH has been designated as an unidentified form of GnRH.

Species	GnRH structure		
Mammalia			
Echidna (*Tachyglossus aculeatus*)			U
Opussum (*Monodelphis domestica*)	CII	M	
Possum (*Trichosurus vulpecula*)	CII	M	
Quoll (*Dasyurus veverrinus*)	CII	M	
Bandicoot (*Isoodon macrourus*)	CII	M	
Wallaby (*Macropus eugenii*)		M	
Musk shrew (*Suncus murinus*)	CII	M	
Mole (*Chrysochloris asiatica*)		M	
Bat (*Miniopterus schreibersii*)		M	
Pig		M	
Sheep		M	
Rat		M	
Mouse		M	
Dormouse		M	
Guinea pig		M	
Marmoset		M	
Human		M	

Figure 1 (Continued). Taxonomic distribution of GnRHs in vertebrates. (King and Millar, 1995). CII, chicken GnRH II; M, mammalian GnRH; S, salmon GnRH; CI, chicken GnRH I; Cf, catfish GnRH; Df, dogfish GnRH; LI, lamprey GnRH I; LIII, lamprey GnRH III; U, an unidentified form of GnRH. Where identification of known GnRHs was not conclusive, the GnRH has been designated as an unidentified form of GnRH.

Structural variants of GnRH

Two or more molecular forms of GnRH, of which chicken GnRHII (cGnRHII) (first isolated from chicken by Miyamoto *et al.*, 1984) is the most universally present form (Figure 1), are present in the brains of species from all the major non-mammalian vertebrate groups. This implies that there was a gene duplication in the earliest vertebrates and that cGnRHII has been highly conserved during vertebrate evolution (Millar and King, 1994; King and Millar, 1992, 1995). Until recently only the mammalian form of GnRH (mGnRH) had been isolated in placental mammals. An interesting observation is that two forms are present in metatherian species (such as possum, *Trichosurus vulpecula*) and early-evolved eutherian species (musk shrew, *Suncus murimus*, and mole, *Chrysochloris asiatica*). Recently cGnRHII has been

reported to exist in primate brains (Lescheid *et al.*,1996).

The data suggest that the primary role of the cGnRHII is not as a gonadotropin-releasing factor. Instead, the peptide probably acts as a neurotransmitter in the medial habenula and in other regions (Rissman *et al.*, 1995).

The different GnRH variants are highly conserved in length and in the NH_2- and COOH-terminal sequences (Figure 2) (Millar and King, 1994). Position 8 is the most variable, followed by positions 5, 7, and 6. The existence of at least two different types of GnRH in most vertebrates (Millar and King, 1994) suggests the possibility of different GnRH receptor subtypes.

	1	2	3	4	5	6	7	8	9	10	
MAMMAL	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly	NH_2
CHICKEN I	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	Gly	NH_2
SEA BREAM	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	Gly	NH_2
CATFISH	pGlu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	Gly	NH_2
SALMON	pGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	Gly	NH_2
DOGFISH	pGlu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	Gly	NH_2
CHICKEN II	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly	NH_2
LAMPREY II	pGlu	His	Trp	Ser	His	Asp	Trp	Lys	Pro	Gly	NH_2
LAMPREY I	pGlu	His	Tyr	Ser	Leu	Glu	Trp	Lys	Pro	Gly	NH_2
TUNICATE I	pGlu	His	Trp	Ser	Asp	Tyr	Phe	Lys	Pro	Gly	NH_2
TUNICATE II	pGlu	His	Trp	Ser	Leu	Cys	His	Ala	Pro	Gly	NH_2

Figure 2 Primary structure of the eleven GnRHs isolated from vertebrate brain. Boxes show highly conserved residues (King and Millar, 1995; Sherwood, 1995).

Function of GnRH

The role of GnRH in stimulating gonadotropin release has been established in various vertebrate species. However, it has become evident that GnRH has additional functions. GnRH has been shown to affect the secretion of other pituitary hormones such as growth hormone in some fish species, as well as the activity of cells of a number of non-pituitary tissues (Millar and King, 1994). The existence of eleven structural variants of GnRH, together with the occurrence of more than one form of GnRH in most species, i.e. cGnRHII and a second form which varies in the different species (mGnRH in mammals), could be indicative of diversity in functional regulation by GnRHs (Millar and King, 1994).

In the musk shrew brain the distribution of cGnRHII is very different from the distribution of mGnRH fibres and cells (Rissman *et al.*, 1995). Also, there is no overlap in the distribution of neurons that contain mGnRH and cGnRHII (Dellovade *et al.*, 1993)(Figure 3). In addition, there is also little overlap in the projection sites of these neurons. The majority of the forebrain neurons that produce mGnRH project along a midline ventral path from the olfactory regions toward the median eminence (Silverman, 1988; Dellovade *et al.*, 1993). In contrast, the major projection site of the cGnRHII cells is the medial habenula. Taken together, the data strongly suggest that the primary role of the cGnRHII form of GnRH is not as a gonadotropin-releasing factor, but instead, the peptide probably acts as a neurotransmitter in the medial habenula and in other regions.

The GnRH subtype dominant in the hypothalamus (mGnRH for mammals) seems to serve a role in regulating pituitary gonadotropins. This GnRH subtype varies in different species. The conserved cGnRH II subtype, more prevalent in extrahypothalamic brain areas and probably peripheral nervous system, may serve as a neurotransmitter or neuromodulator within the nervous system (Millar and King, 1994; King and Millar, 1995). This opinion is based on several findings: (i) GnRH is distributed throughout the brain (Mikami *et al.*,1988; Katz *et al.*, 1990; Sharp *et al.*, 1990); (ii) GnRH receptors are present in several brain areas; (iii) GnRH has direct effects on reproductive behaviour; and (iv) GnRH affects the function of amphibian sympathetic ganglion neurons.

Outside the central nervous system, cGnRHII has paracrine effects in the gonads, where it affects steroid hormone production, and in the placenta, where it simulates secretion of chorionic gonadotropin (Hsueh and Schaeffer, 1985). In breast tumor cells GnRH seems to have an autocrine regulatory role. The GnRH gene is expressed here (Harris *et al.*, 1991), GnRH receptors are present (Barron *et al.*, 1985), and GnRH antagonists inhibit cell growth (Eidne *et al.*, 1987).

In different tissues, specificity of action of GnRH is thought to be achieved through anatomical arrangements that ensure exclusive delivery of effective concentrations to the target cells. Hypothalamic GnRH is transported to the anterior pituitary via portal vessels. In mammals, the concentrations of this GnRH is too low in general circulation to allow binding to GnRHRs in other tissues (Millar and King, 1994). Gonadal and placental GnRHs are thought to be secreted by specific cells to affect adjacent cells in a paracrine fashion, and the same exclusive communication is retained when the hormones function as neurotransmitters or as autocrine regulators (Millar and King, 1994).

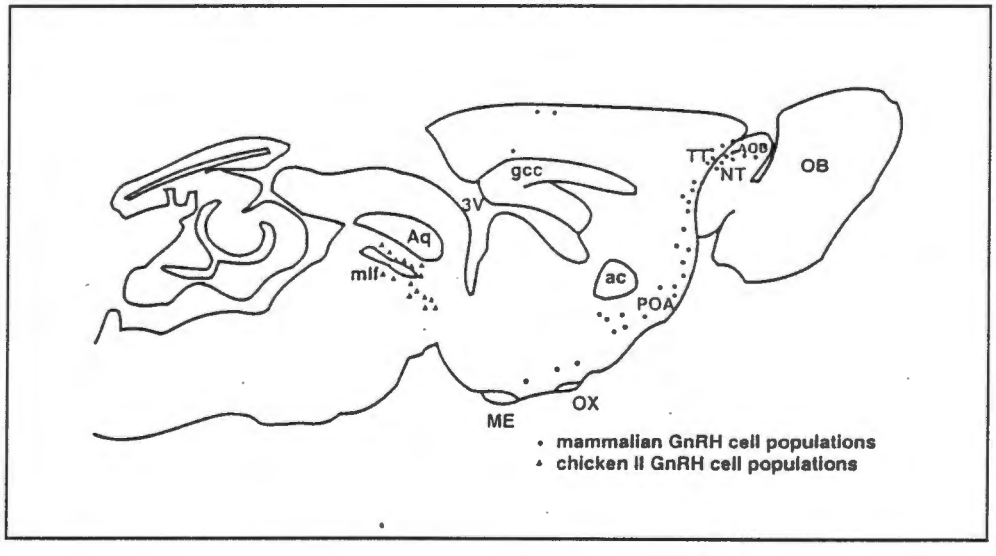

Figure 3 Schematic drawing of a parasagittal musk shrew brain. Each dot represents at least five GnRH-immunoreactive neurons that contain mGnRH, and each triangle represents at least five cGnRH-II-ir neurons. AOB, accessory olfactory bulb; ME, median eminence; NT, nervus terminalis; OB, olfactory bulb; POA, preoptic area; TT, tenia tecta; ac, anterior commissure; Aq, aqueduct; gcc, genu corpus callosum; mlf, medial longitudinal fasciculuc; ox, optic chiasm; 3V, third ventricle (Rissman *et al.*, 1995).

Receptor structure

The GnRHR is a cell-surface receptor, sharing the typical structural formation of the G-protein coupled receptors. The receptor is characterized by seven hydrophobic stretches of 20-25 amino acids, predicted to form transmembrane α-helices, connected by alternating extracellular and intracellular loops (Figure 4). Most of the primary sequence homology among this family of receptors is contained within the hydrophobic transmembrane domains, with the hydrophilic loop regions being more divergent (Strader *et al.,* 1994; Millar *et al.*, 1993).

The % homology between the transmembrane domains of 2 different G protein-coupled receptors at amino acid level, can be a good indication of the relationship between the 2 receptors (Strader *et al.*, 1994). A 85-95% identity indicates species homologues, whereas 60-80% is indicative of related subtypes of the same receptor. Other members of the same family are 35-45% identical, whereas unrelated G protein-coupled receptors are 20-25% identical.

G protein-coupled receptors are glycoproteins. All GPCRs cloned to date, have one or more consensus sequences for N-linked glycosylation (Asn-X-Ser/Thr) in the extracellular domain. These sites are usually located near the N terminus of the protein, although putative glycosylation sites have also been found in the second extracellular loop (Strader *et al.*, 1994).

A number of cysteine residues have been conserved in G protein-coupled receptors, some of which appear to play a role in the receptor structure (Strader *et al.*, 1994). The high degree of structural similarity of the family of G protein-coupled receptors is indicative of the common mechanism of action of these different receptors (Strader *et al.*, 1994).

The most notable difference of the GnRH receptor compared to other G protein-coupled receptors, is the absence of an intracellular C-terminal tail (Millar *et al.,*1993; Stojilkovic *et al.*, 1994*).* Other unique features include the lack of a conserved aspartate/glutamate in the second transmembrane (TM2) domain (GnRH receptor has an asparagine), the length and highly basic nature of the first cytoplasmic loop, and the short overall length of the receptor

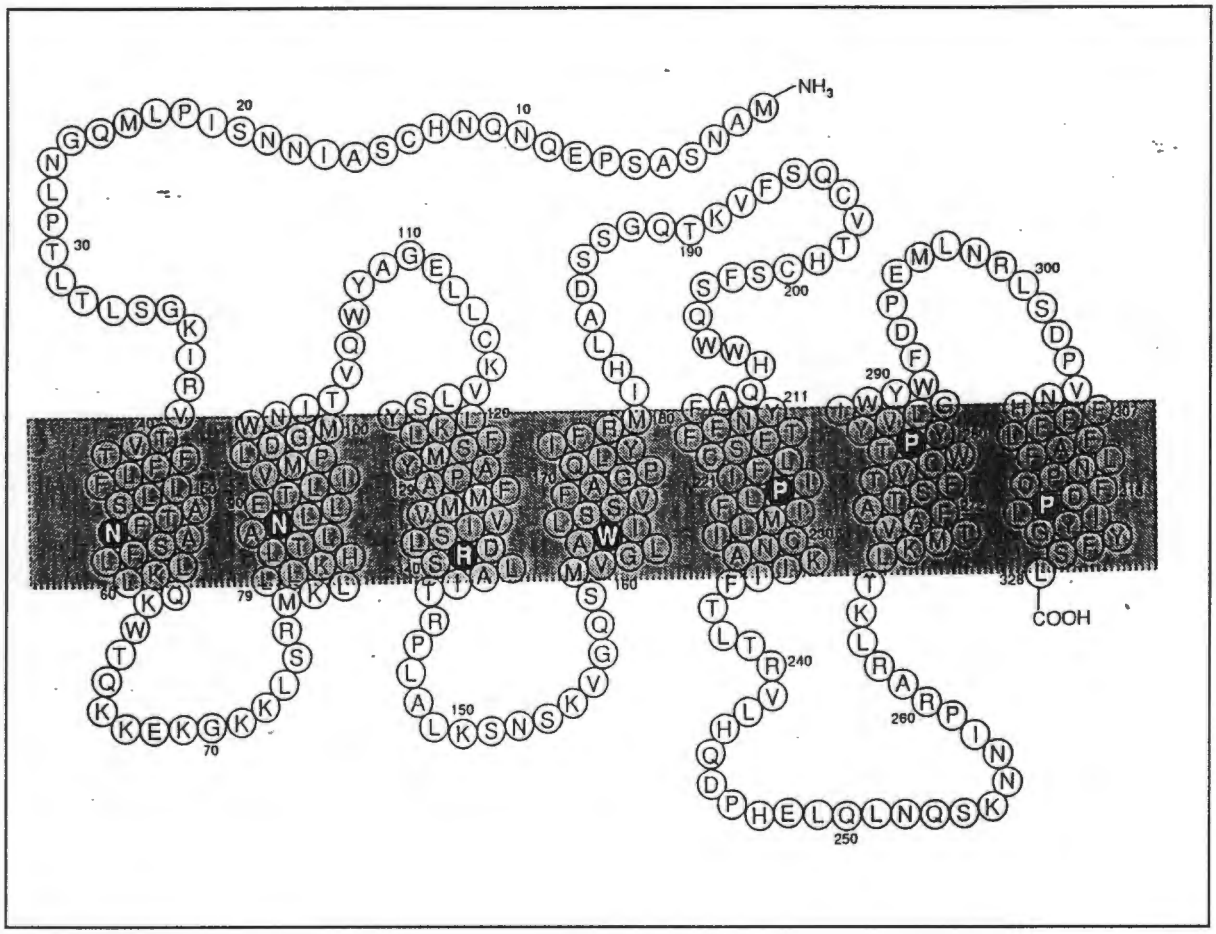

Figure 4 Proposed structure of the human pituitary (Type I) GnRH receptor. The shaded areas indicate amino acids that are conserved within the G protein-coupled receptor superfamily.

protein. (Millar *et al.*, 1993). Another difference between the GnRH receptor and other G protein-coupled receptors, is the change of the highly conserved DRY sequence to DRS at the TM2 / intracellular loop 2 junction (Millar *et al.*, 1993).

Most of the GPCRs are buried in the membrane, exposing only a small surface to the cytoplasm. This surface is composed predominantly of the ends of the transmembrane helices and the connecting loops. Much of this intracellular surface is thought to be involved in the interaction with G proteins. Studies have implicated residues at the cytoplasmic ends of all helices and the portions of the loops closest to the membrane as critical for overall interaction and specificity determination. Other lines of evidence point to the importance of the intracellular loops in activating the G proteins (Strader *et al.*, 1994).

Distribution of GnRH receptors

GnRH receptors have been characterized as specific binding sites with high affinity for GnRH and its potent agonist or antagonist analogs in the pituitaries of several species, including rat (Naor *et al.*, 1980), human (Wormald *et al.*, 1985), mouse (Pal *et al.*, 1992), rabbit (Limonta *et al.*, 1986), goldfish (Peter *et al.*, 1992), and the African catfish, *Clarias gariepinus* (Schulz *et al.*, 1993). The presence of GnRH receptors have also been confirmed using radiolabelled GnRH analogues by autoradiography in the brain, specifically the hippocampus, lateral septal nucleus, anterior angulate cortex, subiculum, and entophinal cortex. GnRH receptor expression has also been detected in the testis (Clayton *et al.*, 1980; Bourne *et al.*, 1980), ovary (Clayton *et al.*, 1979; Hazum and Nimrod, 1982; Pati and Habibi, 1992), the human placenta, human breast tumor (Eidne *et al.*, 1985; Eidne *et al.*, 1987), human epithelial ovarian carcinomas (Pahwa *et al.*, 1989; Emons *et al.*, 1992), as well as in prostate tumors (Qayum *et al.*, 1990). The existence of GnRH and GnRHR mRNA's in normal non-reproductive tissues suggests that in addition to regulating the secretion of gonadotropins from the anterior pituitary, GnRH and GnRHR may play an important role in the regulation of cellular functions in an autocrine or paracrine manner.

Cloning of GnRH receptor cDNAs

The GnRHR was first cloned in mouse by Tsutsumi *et al.* in 1992. The authors isolated a GnRHR cDNA from the gonadotrope cell line , αT3-1, using a PCR-based strategy. αT3-1 RNA was used as a template for the synthesis of cDNA with degenerate oligo's designed to conserved regions of the GPCRs. PCR products were subcloned and sequenced, whereupon antisense oligo's were synthesized for a hybrid-arrest assay. Two oligo's were found to completely abolish the expression of GnRHR in oocytes when coinjected with αT3-1 and rat brain RNA. These two oligo's represented different segments of a specific clone (WZ7). Clone WZ7 was then used as a probe to screen an αT3-1 cDNA library. Seven positive plaques were detected, of which the largest insert was 1.3 kb (clone WZ25). To check if WZ25 encoded a functional GnRHR, it was subcloned, and injected into oocytes. These oocytes, when exposed to GnRH, demonstrated a large depolarizing response characteristic of GnRHR expression. The pharmacology of the response obtained was in agreement with

expression of the mammalian GnRHR.

Following the cloning of the first GnRHR, the GnRHR has been cloned in a variety of placental mammalian species, including human (Chi *et al.*, 1993; Kakar *et al.*, 1992), mouse (Reinhart *et al.*, 1992), rat (Eidne *et al.*, 1992; Kaiser *et al.*, 1992), cattle (Kakar *et al.*, 1993), pig (Weesner and Matteri, 1994) and sheep (Brooks *et al.*, 1993; Illing *et al.*, 1993). The analysis of the different GnRH receptors revealed that the GnRHR is highly conserved between different mammalian species (Figure 5). cDNA library screening was used for the cloning of these different GnRH receptors.

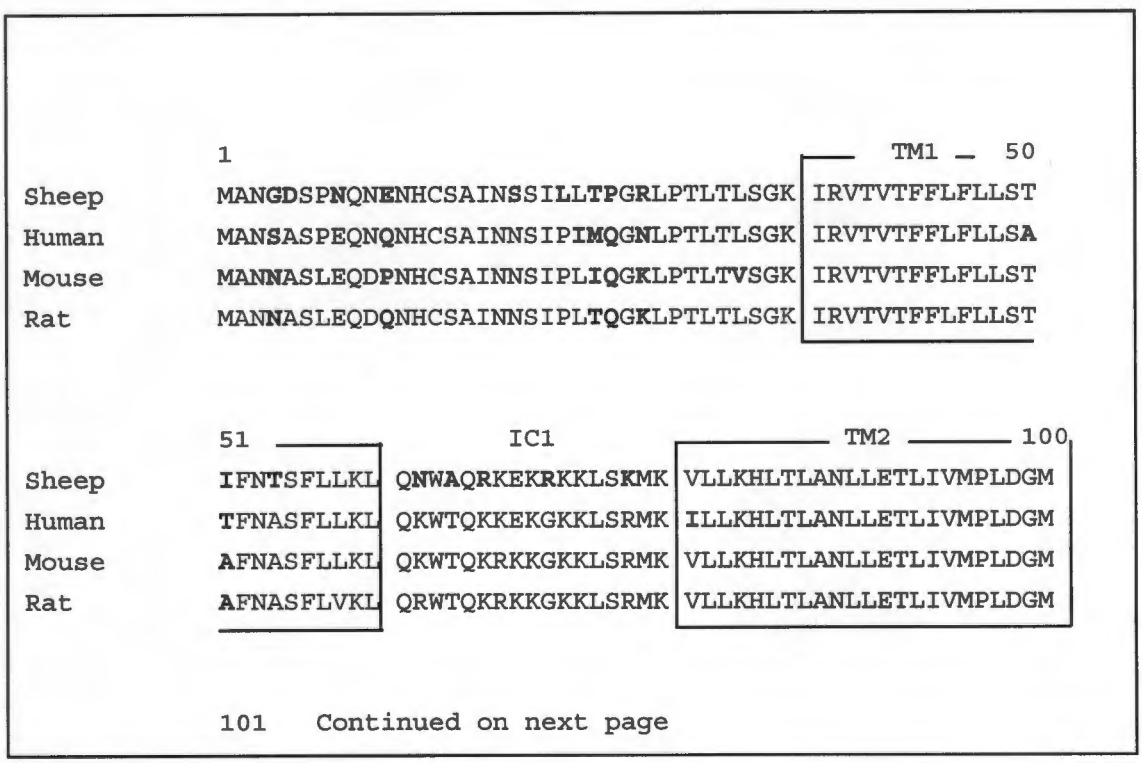

Figure 5 Comparison of the amino acid sequences of the sheep, human, mouse, and rat GnRHR. Amino acids are shown in one-letter code. Amino acids that are not conserved between different species are printed in bold. Transmembrane (TM) helices are indicated with boxes.

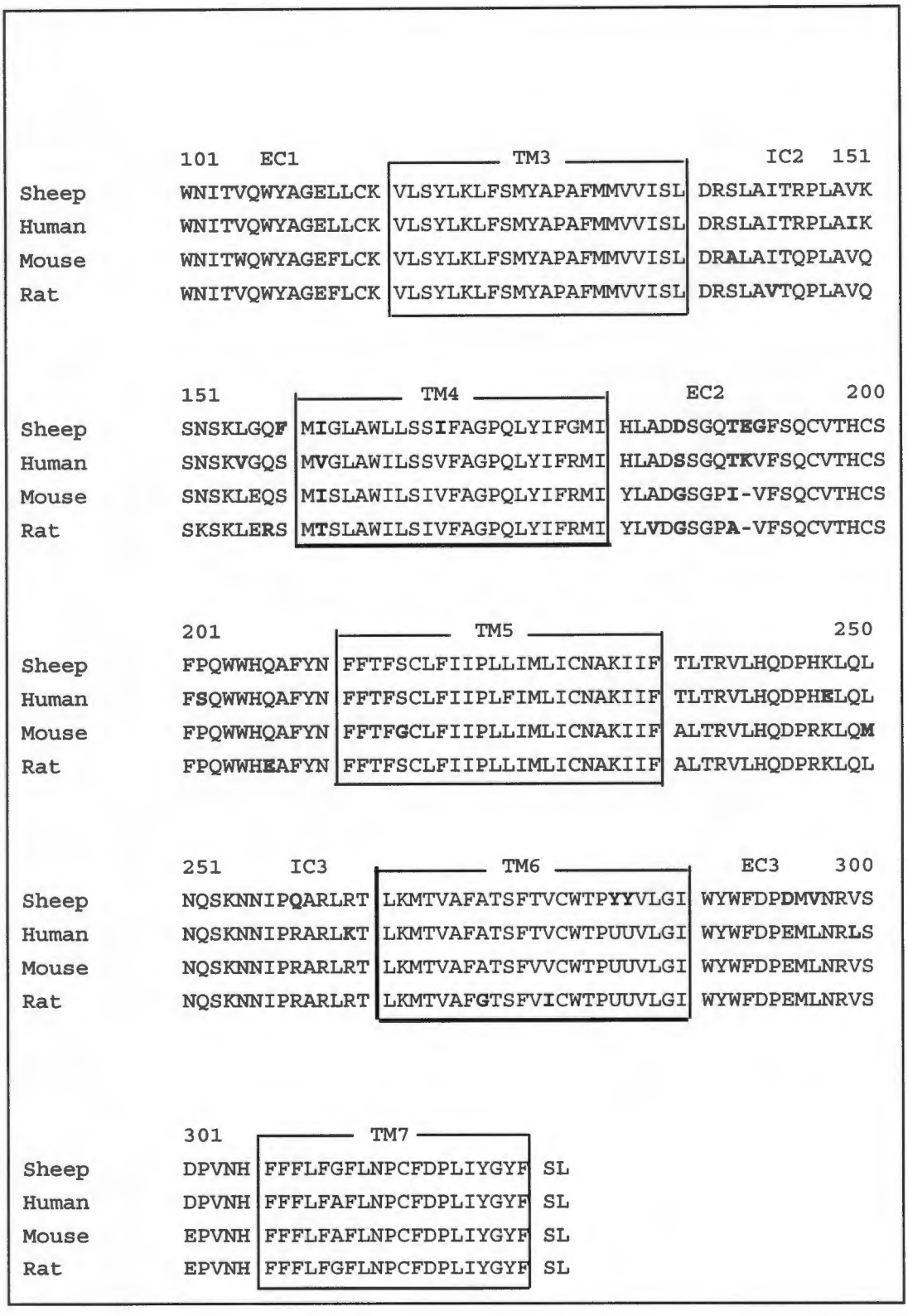

Figure 5 (continued) Comparison of the amino acid sequences of the sheep, human, mouse, and rat GnRHR. Amino acids are shown in one-letter code. Amino acids that are not conserved between different species are printed in bold. Transmembrane (TM) helices indicated with boxes.

Gene structure of the GnRHR

The type I human GnRHR gene consists of at least three exons and 2 introns and spans over 18.9 kb (Fan *et al.*, 1994)(Figure 4). Exon 1 consists of the 5'-untranslated region (5'UTR) and part of the open reading frame (ORF) encompassing transmembrane (TM) domains 1 to 3, and a portion of TM4. Exon 2 is only 219 bp in size, and codes for the remainder of TM4, as well as for TM5. Exon 3 encodes the COOH-terminal part of the ORF (TM6 + TM7) and the 3'UTR. The presence of 2 introns within the protein coding region is quite interesting, since many GPCR genes that have been examined are intronless (Probst *et al.*, 1992).

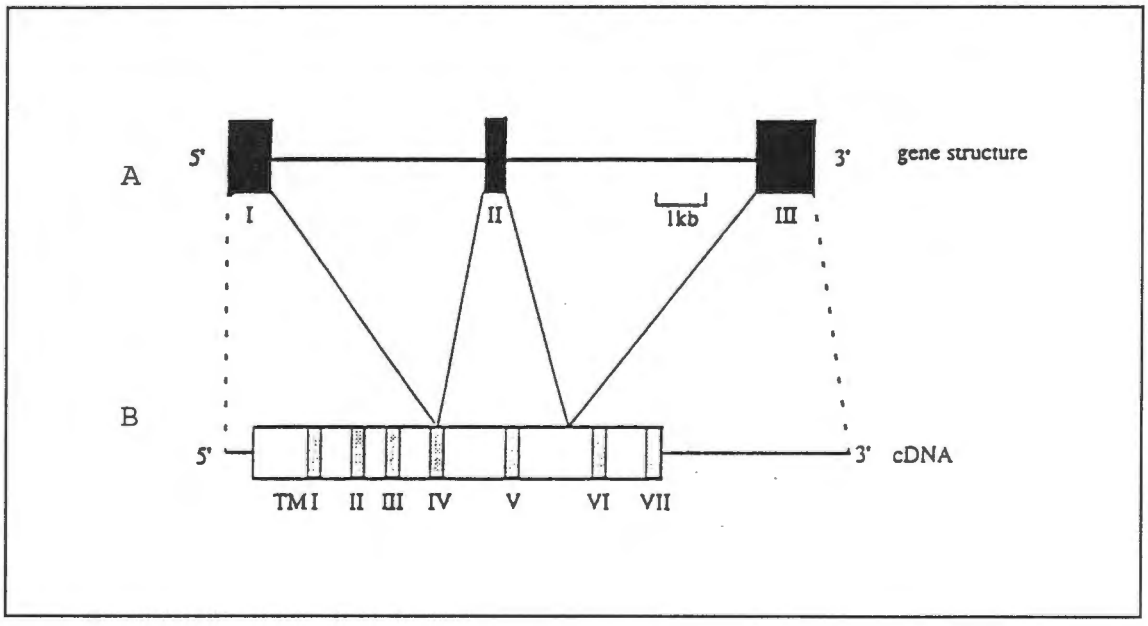

Figure 6 Schematic representation of the human GnRHR gene (Fan *et al.*, 1994). (A) Exon-intron localization (B) The structure of human GnRHR cDNA. Open box indicates the protein coding regions and hatched boxes are the putative transmembrane domains.

Aim of study

The aim of this study was to attempt to clone novel GnRH receptors from redbait and mole using a PCR strategy. Two or more molecular forms of GnRH had been identified in the brains of most of the non-mammalian vertebrate groups, but until recently, only the mammalian form of GnRH (mGnRH) had been isolated in placental mammals. The existence of more than one molecular form of GnRH in a single species led us to believe that more than one form of the GnRHR was probably also present. The early-evolved eutherian species are the most highly evolved mammals for which good evidence exists for the presence of cGnRHII (Figure 1). The mole, a species readily available in our region, is a member of the early-evolved eutherians and as such is a good candidate to use to search for the presence of a novel mammalian GnRHR for cGnRHII that might differ substantially from the type I mammalian GnRHRs characterized to date. Another aim of this study was to find a type II GnRHR in higher mammals.

Recent evidence shows that the primitive tunicate, *Chelyosoma productum* (sea squirt), contains GnRH (Powell *et al.*, 1996). The cloning of a GnRHR from such primitive tunicates would be very interesting since the species is evolutionary far separated from mammals, and one would expect that regions critical for receptor function would be selectively conserved whereas other regions may differ substantially from the mammalian GnRHRs. Redbait is a tunicate that is readily available in our region and is thus a good candidate for the search of novel GnRHRs from primitive tunicates.

A series of degenerate oligonucleotide primers was designed (J.P. Hapgood) to the areas of the type I mammalian GnRHR that are not conserved between GPCRs in general, but are conserved within mammalian GnRHRs. PCR would thus hopefully amplify novel GnRH receptors and not any other GPCR. As far as possible, codons were chosen with the least degeneracy. This would make the PCR more specific, and thus lessen the chance of non-specific amplifications. Also, primer pairs were chosen within an exon, given that successful

amplification of exons across large introns was unlikely. This strategy is based on the assumption that the gene structure of the GnRHR is conserved within the different mammalian species.

Knowledge of the primary structure of the GnRHR from other mammals will allow us to identify important conserved and variant amino acids, and to correlate this with the difference in pharmacology of the receptors. The cloning of GnRHRs will allow rapid progress of our understanding of the molecular mechanisms of receptor regulation and the role these play in reproductive physiology. There have already been indications that GnRH plays an important role in reproductive behaviour in the musk shrew (Rissman *et al.*, 1995).

The reason for cloning novel GnRH receptors includes importance in development of therapeutic drugs. Residues that are conserved are likely to be important in receptor configuration, ligand induced molecular switching, and coupling to G-proteins. A variety of GnRH analogues have already successfully been used in the treatment of a variety of tumours and endocrine disorders. The more we know about the structure of GnRH receptors, the more impact this could have in the medical field. Knowledge of the GnRHR structure, its interaction with GnRH and GnRH analogues, and its physiological regulation is vital to our understanding of the functioning of the hypothalamic-pituitary-gonadal axis.

CHAPTER 2

MATERIALS AND METHODS

1. AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis was performed according to the specifications described by Sambrook *et al.*, 1989.

Agarose was weighed and placed into an Erlenmeyer flask, to which the appropriate volume of 1x gel running buffer was added. Generally, agarose gels were run in 1xTBE, except low melting point agarose gels which were run in 1xTAE. The Erlenmeyer flask was sealed with clingwrap which was pierced to prevent the buildup of steam. The agarose mix was then heated in a microwave oven in the minimum time required to allow all of the grains of agarose to dissolve. The solution was cooled to 50°C, and ethidium bromide (EtBr, 10 mg/ml) was added to a final concentration of 0.5 μg/ml. The solution was swirled to allow the EtBr to mix, and then poured into the gel mold. Gels were generally between 5 mm and 8 mm thick and were allowed to set at room temperature. Minigels were 66x100 mm, and midigels were 110x150 mm.

Gels were run in just enough 1x electrophoresis buffer to cover the gels to a depth of about 1 mm. Gels were run at a voltage of 1 - 5 V/cm (measured as distance between electrodes). DNA on the gels was visualised by ultraviolet (UV) light.

The necessary precautions outlined in Sambrook *et al*, 1989, was taken when working with EtBr or UV light.

2. ISOLATION OF GENOMIC DNA

Redbait (*Pyura stolonifera*) was collected at Kalkbaai with the assistance of W. Liebrich and J. Hapgood. The samples were put in a bucket with saltwater (seawater) immediately after

removal from the rocks. The redbait was dissected at the laboratory by E. Hutchinson, J. Hapgood, and W. Liebrich. Tissues were placed on dry ice immediately after dissection, and then stored at -70°C.

Moles (*Chrysochloris asiatica*) were donated by Kirstenbosch Gardens. Mouse and mole dissections were performed by John Morta. Tissues were placed in liquid nitrogen immediately after dissection, and then stored at -70°C.

Human genomic DNA was kindly donated by Geneè Harmse.

Preparation of genomic DNA (gDNA) was performed under RNase-free conditions. RNase-free solutions and plugged tips were used throughout the procedure.

As soon as possible after excision the tissue was frozen in liquid nitrogen. This is essential in order to minimize the activity of endogenous nucleases. Digestion buffer (100 mM NaCl; 10 mM Tris.Cl (pH 8); 25 mM EDTA (pH 8); 0.5% sodium dodecyl sulfate (SDS); 0.1 mg/ml proteinase K) was added (1.2 ml per 100 mg tissue). EDTA is included in the digestion buffer to inhibit DNases. The tissue was then ground in a homogeniser until no clumps remained. It is important that the tissue be well dispersed and not left in large clumps to permit rapid and efficient access to proteinase K and SDS. The samples were then incubated with shaking at 50°C for 12 to 18 hours in tightly capped tubes. After an overnight incubation the samples were thoroughly extracted with an equal volume of phenol/chloroform/isoamyl alcohol (PCI) (25:24:1 vol/vol) and centrifuged at 1700xg for 10 minutes in a swinging bucket rotor. This procedure was repeated three times. The aqueous layer was then transferred to a new tube and ½ vol 7.5 M ammonium acetate and 2 vol 100% ethanol added. The DNA should immediately form a stringy precipitate. DNA was recovered by centrifugation at 1700xg for 2 minutes. The pellet was rinsed with 70% ethanol and air dried. DNA was resuspended in TE (10 mM Tris.HCl, 1 mM EDTA, pH 8.0) until dissolved. To remove residual RNA 0.1% SDS and 1 μg/ml DNase-free RNase A was added. The DNA was then incubated at 37°C for 1 hour. This was followed by organic extraction and ethanol precipitation, as described above. The integrity of the genomic DNA was checked by agarose gel electrophoresis. No degradation of gDNA could be detected, as the gDNA all appeared

to be bigger than 20 kb when checked on an agarose gel.

3. SOUTHERN HYBRIDIZATION

The objective of a Southern transfer is to set up a flow of liquid from the reservoir through the gel and the nylon membrane, so that DNA fragments are eluted from the gel and are deposited onto the nylon membrane (Figure 7).

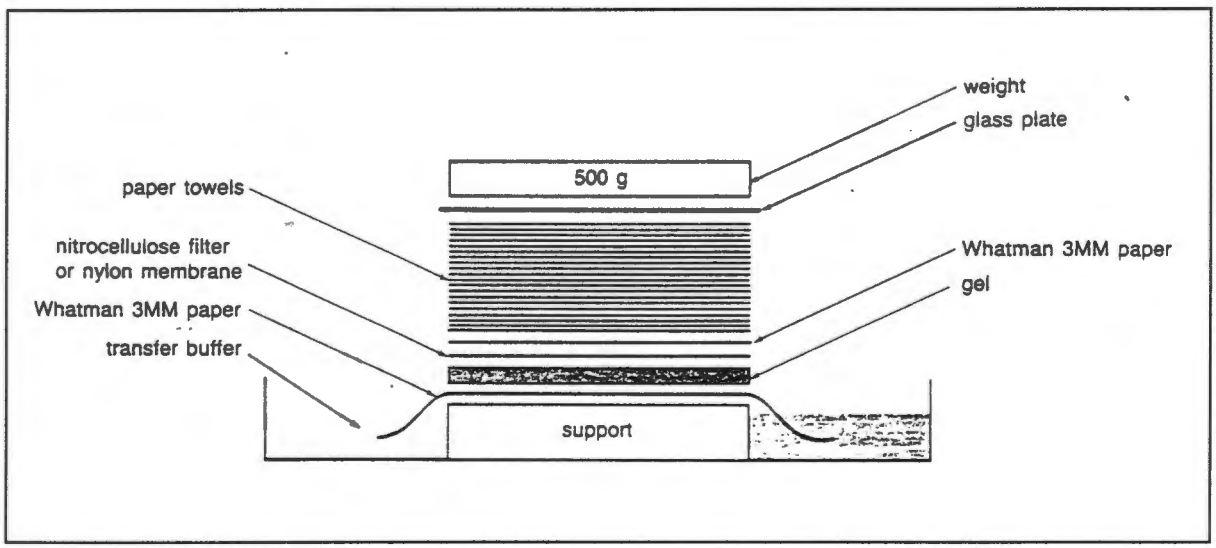

Figure 7 Capillary transfer of DNA from agarose gels. Buffer is drawn from a reservoir and passed through the gel into a stack of paper towels. The DNA is eluted from the gel by the moving stream of buffer and is deposited on a nylon membrane. A weight applied to the top of the paper towels helps to ensure a tight connection between the layers of material used in the transfer system (Sambrook *et al.*, 1989).

3.1 Preparation of the gel

After electrophoresis was completed, gels were photographed. A ruler was placed alongside the gel so that the distance that any given band of DNA migrated could be read directly from the photographic image. The gel was then transferred to a glass dish and any unused areas of the gel were trimmed away with a razor blade.

3.2 Gel denaturation

The DNA was denatured by soaking the gel in 1.5 M NaCl and 0.5 M NaOH for 30 min at room temperature with constant shaking. This was followed by a short wash in distilled water. The gel was neutralized by soaking in several volumes of 1.5 M NaCl, 0.5 M Tris.Cl (pH 8.0) and 0.001 M EDTA (pH 8.0) for 15 min at room temperature with shaking. This was followed by a distilled water wash. The neutralization step was repeated for another 15 min, followed by another water wash.

3.3 Capillary Transfer

A glass dish was filled with 20 x SSC (3 M NaCl, 0.3 M Na_3citrate). SSC acts as the transfer buffer. A glass plate was used as a platform. The support should be longer and wider than the gel. This was covered with 2 sheets of Whatman 3MM paper, saturated with transfer buffer (20xSSC). Air bubbles were smoothed out with a pipette. It is also important to ensure that the wick does not touch the sides of the glass dish.

The gel was inverted so that its original underside was uppermost and placed on the damp 3MM paper. Air bubbles between the gel and the 3MM paper were removed with a pipette. A piece of nylon membrane (Hybond N^+, Amersham) cut to 1-2 mm bigger than the gel in both length and width and soaked in 2x SSC until wet, was placed on top of the gel. Bubbles between the gel and the membrane were removed. Three sheets of 3MM paper cut to the same size as the membrane and wetted in buffer was placed on top of the membrane. Air bubbles were removed again. This was all covered with clingwrap. A hole was cut in the clingwrap so that the gel was uncovered. The clingwrap prevents evaporation of the transfer buffer. A stack of absorbent paper towels (± 5-10 cm high), of similar size to that of the 3MM paper, was then placed on top of the 3MM paper. A glass plate was placed on the paper towels. This was weighed down by a 500 - 750 g weight. Transfer was allowed to proceed overnight (16-24 hours). The rate of transfer of DNA depends on the size of the DNA fragment and the porosity of the gel.

After the overnight transfer, the paper towels and the 3MM filters above the gel were

removed. The positions of wells and the edges of the gel were marked on the membrane with a soft pencil. The membrane was then rinsed briefly (< 1 min) and carefully in 2 x SSC to remove any adhering agarose. DNA was fixed on the membrane by alkaline fixation.

3.4 Alkaline fixation of DNA on membrane

The DNA was fixed on the membrane according to the specifications of the manufacturers.

The membrane was placed DNA side up on a pad of absorbent filter paper (Whatman 3MM), 3 pieces thick, for 20 min at room temperature. The absorbent filter paper had been soaked in 0.4 M NaOH. The membrane was then rinsed in 5x SSC with gentle agitation for no more than 1 min. Excess fluid was allowed to drain from the membrane. The membrane was then dried at room temperature on 3MM paper.

If the membrane was used immediately, it was placed in prewarmed prehybridization buffer immediately. Membranes not used immediately were vacuum sealed and stored at 4°C.

3.5 Preparation of the human type I GnRHR probe

The human type I GnRHR cDNA was cloned into Bluescript. Restriction enzyme digestions were performed with *Eco*RI and *Xho*I to cut out the insert of 1.2 kb (Section 4, Materials and Methods). The digestion mix was run on a low melting point agarose gel to separate the 1.2 kb insert from the 3.0 kb vector. The insert was cut out with a sterile razor blade. The gel slice was stored in a 1.5 ml eppendorf tube at -20°C. Labelling of the human type I GnRHR probe was performed in gel.

3.6 ^{32}P Labelling of Probe

When working with ^{32}P, all necessary precautions were taken, as outlined by Ausubel *et al.*, 1987. Gloves and lab overcoats were worn at all times. Reactions were performed behind a

plexiglass screen. The equipment was checked with a Geiger counter regularly to monitor contamination.

Three different techniques were used to label probes.

3.6.1 [γ-^{32}P] dATP end-labelling of a single-stranded oligo with T4 polynucleotide kinase

The following were combined in a 1.5 ml eppendorf tube: 100 pmoles of oligo; 4 μl of 10x PNK buffer; 5 μl of [γ-^{32}P] dATP (50 μCi) and 5 μl of T4 PNK(400). The volume was adjusted to 40 μl with distilled water. The mixture was then incubated at 37°C for 45 min, followed by a 10 min incubation at 68°C. The mixture was then placed on ice. The volume was adjusted to 100 μl with STE (TE (pH 8.0) containing 0.1 M NaCl). Unincorporated [γ-^{32}P] dATP and excess salt was removed by centrifugation through Sephadex G-50 packed in disposable syringes (Section 2.6.4, Materials and Methods).

3.6.2 [α-^{32}P] dCTP labelling of a double-stranded probe in agarose with Amersham's Megaprime Kit

Probes were labelled with [α-^{32}P] dCTP (10 μCi/μl). The protocol recommended by the manufacturers was followed.

The required components from the Megaprime kit (not the enzyme) were thawed at room temperature. To melt the gel slice containing the DNA, it was placed in boiling water in a 1.5 ml tube for 30 sec. The required volume (25 ng DNA in a maximum of 21 μl) was added to a clean tube and 5 μl of primer was added. This mixture was incubated at 95°C for 5 min and then spun down. The following mixture was added at room temperature: 4 μl dATP, 4 μl dGTP, 4 μl dTTP, 5 μl reaction buffer, 2 μl Klenow enzyme, and "x" μl of water to a final volume of 45 μl. This solution was mixed and spun down. Radioactive dNTP, 5 μl of [α-^{32}P] dCTP (50 μCi), was added carefully. The tube was incubated at 37°C for 10 min. The labelling reaction was stopped by the addition of 5 μl of 0.2 M EDTA. The labelled probe was purified on a G-50 Sephadex spin column (Section 2.6.4, Materials and Methods).

3.6.3 [α-^{32}P] dCTP labelling of a probe by PCR

The following mixture was added to a 0.5 ml tube: 2.5 μl 50 mM $MgCl_2$, 5 μl 10x PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 4 μl dNTP's (dATP, dGTP, dTTP), 5 μl [α-^{32}P] dCTP (50 μCi), 1 μl primer 1, 1 μl primer 2, "x" μl DNA, 0.5 μl Taq (5U/μl Taq DNA polymerase from *Thermus aquaticus* YT1, Gibco BRL), and "x" μl of water to a final volume of 50 μl. The mixture was covered with 50 μl of mineral oil. The PCR cycle used was as follows: 93°C for 10 min, followed by 35 cycles of 1 min at 93°C, 2 min at 55°C and 3 min at 72°C. This was followed by a 10 min extension period at 72°C. The PCR mixture was separated from the oil on parafilm. Unincorporated [α-^{32}P] dCTP and excess salt was removed by centrifugation through Sephadex G-50 packed in disposable syringes (Section 2.6.4, Materials and Methods).

3.6.4 Preparation of a G-50 Sephadex Spin Column

Sephadex G-50 equilibrated in STE was prepared to a bed volume of 0.9 - 1.0 ml in a 1-ml disposable syringe plugged with a small amount of sterile glass wool. The syringe was placed into a disposable centrifuge tube and centrifuged at 400xg for 1 min, causing the Sephadex to pack down. The column was washed with 0.1 ml STE and recentrifuged at exactly the

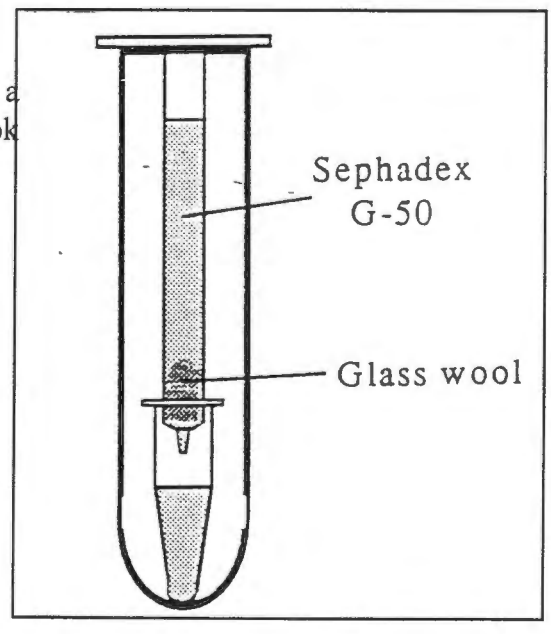

Figure 8 Schematic diagram of a Sephadex G-50 spin column (Sambrook *et al.*, 1989).

same speed and for exactly the same time as before. This was repeated three times. The DNA sample was then applied to the column in a volume of 0.1 ml. This was recentrifuged at exactly the same speed and for exactly the same time as before. The 100 μl effluent from the syringe was collected in a decapped eppendorf tube. The unincorporated [^{32}P] dNTP's remain in the syringe, which should be carefully discarded.

3.7 **Hybridization of Southern filters**

Three different hybridization conditions were used. The choice of hybridization solution depended on the probe used, as well as the stringency required. The stringency is dependant on the GC content of the probe, as well as the percent homology of the probe to the target. The hybridizations were performed in a closed flat-bottomed plastic container. ± 0.25 ml prehybridization/hybridization solution was used per cm^2 membrane. The membranes were washed until no background "noise" remained.

3.7.1 Hybridization conditions for [γ-^{32}P] dATP end-labelled oligo

Prehybridization solution (6x SSC (3 M NaCl, 0.3 M sodium citrate); 0.4% SDS; 5x Denhardt's solution (0.1% (w/v) bovine serum albumin (BSA); 0.1% (w/v) Ficoll™, 0.1% (w/v) polyvinylpyrrolidone (PVP)); 20 mM NaH_2PO_4 (pH 7.5); 0.5 mg/ml denatured herring sperm DNA) was prewarmed to 60°C in a waterbath in a flat-bottomed plastic container with lid. The filter was immersed in this solution and incubated at 60°C for 1 hour with shaking.

The prehybridization solution was discarded, and prewarmed (60°C) hybridization solution (6x SSC; 20 mM NaH_2PO_4 (pH 7.5); 0.4% SDS; 0.5 mg/ml denatured herring sperm DNA; [γ-^{32}P]-labelled probe DNA) was added. The filter was incubated at 60°C for 3 hours for hybridization.

After the 3 hour hybridization period, the filter was removed and immediately submerged in a container containing a solution of 6x SSC and 0.1% SDS prewarmed to 42°C. The filter was incubated at 42°C for 10 min with shaking. It is very important that the filter must not

be allowed to dry out at any stage during the washing procedure. After 10 minutes the filter was placed in a fresh solution of 6x SSC and 0.1% SDS (prewarmed to 42°C) and incubated at 42°C for 10 min with shaking. This was repeated once more.

This was followed by three 10 min washes at 60°C in a solution containing 1x SSC and 0.1% SDS. These washes were followed by three 10 min washes at 65°C in a solution containing 0.1x SSC and 0.1% SDS.

After the washes, the filter was briefly dried at room temperature on a sheet of 3MM Whatman. The filter was then sealed in clingwrap and exposed to pre-flashed X-ray film (Hyperfilm MP, Amersham) in a light-excluding cassette with intensifying screen to obtain an autoradiographic image. The cassette was placed at -70°C.

3.7.2 Low stringency hybridization conditions

Prehybridization solution (6x SSC; 5x Denharts; 0.5% SDS; and 100 μg/ml denatured herring sperm DNA) was prewarmed to 50°C in a waterbath in a flat-bottomed container. The filter was immersed in this solution and incubated at 50°C with shaking for at least 1 hour.

After an hour the prehybridization solution was discarded and prewarmed (50°C) hybridization solution (6x SSC; 0.5% SDS; 100 μg/ml denatured herring sperm DNA; denatured probe) was added. The filter was incubated overnight at 50°C with shaking.

All the washes were performed in a flat-bottomed plastic container with shaking. When hybridization was complete, the membrane was removed and immediately placed in a container with 2x SSC, 0.1% SDS. After 20 min at room temperature, the solution was replaced with 1xSSC, 0.1% SDS, prewarmed to 50°C. After a further 20 min, this solution was replaced with 0.5x SSC, 0.1% SDS, also prewarmed to 50°C. The membrane was washed in this solution for 20 min.

After the washes, the filter was dried briefly at room temperature on a sheet of 3MM

Whatman. The filter was then sealed in clingwrap and exposed to pre-flashed X-ray film (Hyperfilm MP, Amersham) in a light-excluding cassette with intensifying screen to obtain an autoradiographic image. The cassette was placed at -70°C.

3.7.3 High stringency hybridization conditions

Prehybridization solution (2x PIPES (0.8 M NaCl, 0.02 M PIPES (Piperazine-N, N'-bis-[2-ethanesulfonic acid]), pH 6.5); 50% deionized formamide; 0.5% SDS; 100 μg/ml denatured herring sperm DNA) was prewarmed to 42°C in a waterbath in a flat-bottomed container. The filter was immersed in this solution and incubated at 42°C with shaking for at least 1 hour.

After an hour, the denatured probe was added to the prehybridization solution. Hybridization was performed overnight at 42°C with shaking.

All the washes were performed in a flat-bottomed plastic container with shaking. When hybridization was complete, the membrane was removed and immediately placed in a container with 2x SSC, 0.1% SDS. After 20 min at room temperature, the solution was replaced with 1x SSC, 0.1% SDS, prewarmed to 50°C. After a further 20 min, this solution was replaced with 0.5x SSC, 0.1% SDS, also prewarmed to 50°C. The membrane was washed in this solution for 20 min.

After the washes, the filter was dried briefly at room temperature on a sheet of 3MM Whatman. The filter was then sealed in clingwrap and exposed to pre-flashed X-ray film (Hyperfilm MP, Amersham) in a light-excluding cassette to obtain an autoradiographic image. The cassette was placed at -70°C.

3.8 **X-ray film development**

After exposure to the membrane, the X-ray film was removed from the cassette and placed in developing solution (Metalex, Champion). After approximately 5 min the film was

transferred to water for 2 min and then to fixing solution (Fixaplus, Champion) for a further 2 min. The X-ray film was then rinsed with water and hung up to air dry or alternatively, dried in an oven at 50°C for about 5 min.

3.9 **Membrane stripping protocol**

For successful removal of probes, membranes must never be allowed to dry during or after hybridization and washing.

Probe was removed from the membrane by pouring a boiling solution of 0.5% (w/v) SDS onto the membrane. This was allowed to cool to room temperature while shaking. To check that the probe has been removed, the filter can be autoradiographed for the normal exposure time. The filter can then be prehybridized and hybridized with a new probe.

3.10 **Colony/plaque blotting**

Magna-lift membranes (Micron Separations inc.) were used for colony blots. The colonies were grown on gridded LB/Amp plates.

The membrane was carefully placed on the agar surface. To ensure correct orientation of colonies, the membranes and agar were marked with a sterile needle. The membrane was removed after 1 min and placed, colony side up, on a pad (2 pieces thick) of absorbent filter paper soaked in denaturing solution (0.5 M NaOH, 1.5 M NaCl). After 7 min the membrane was transferred to a pad of absorbent filter paper soaked in neutralizing (1.5 M NaCl, 0.5 M Tris (pH 7.2), 0.001 M EDTA (pH 8)) solution. The membrane was left for 3 min, and then transferred to a fresh pad soaked in the same solution. After a further 3 min, the membrane was rinsed briefly (< 60 sec) in 2x SSC, and then fixed by alkaline fixation, as described above (section 2.4, Materials and Methods). A second membrane was placed on the agar and marked through the hole used to align the first membrane. This membrane was left for 2 min before transfer to denaturing solution and the solutions following.

4. ISOLATION OF PLASMID DNA

Plasmid DNA was isolated with an alkaline-lysis method. Depending on the amount and quality of DNA that was needed, different protocols of isolation were used.

4.1 Medium-scale (midi-prep) method

4.1.1 Midi-preps using the Qiagen Plasmid Midi Kit

The protocol recommended by the manufacturers was followed.
Overnight cultures (LB or 2xYT) of 100 ml containing the antibiotic of interest (0.1 mg/ml ampicillin) was grown.

To pellet the cells, the overnight culture was centrifuged for 10 min at 10000 rpm. The bacterial pellet was then resuspended in 4 ml of buffer P1, containing RNase A (10 mg/ml). To this 4 ml of buffer P2 was added, and the mixture was incubated at room temperature for 5 min. After 5 min, 4 ml of chilled buffer P3 was added. The sample was then incubated on ice for 15 min, and centrifuged at 4°C for 30 min at $\geq$ 30000xg. The supernatant was removed promptly and applied onto a QIAGEN-tip 100 equilibrated with 4 ml of buffer QBT. This was followed with 2x 10 ml washes of buffer QC. The DNA was eluted with 5 ml of buffer QF. To precipitate the DNA, 0.7 volumes of isopropanol was added. The sample was then centrifuged at $\geq$ 15000xg at 4°C for 30 min. The DNA was washed with 5 ml of cold 70% ethanol, air dried for 5 min, and redissolved in a suitable volume of TE or water.

4.1.2 Midi-preps using the Nucleobond Plasmid Purification System

The protocol recommended by the manufacturers was followed. Buffers were supplied by the manufacturers. A 100 ml overnight culture containing the antibiotic of interest (0.1 mg/ml ampicillin) was grown for the midi-prep.

To pellet the bacterial cells, the culture was centrifuged at 5000 rpm for 15 min. The bacterial pellet was resuspended in 4 ml buffer S1 (50 mM Tris.HCl, 10 mM EDTA, 100

μg/ml RNase A, pH 8.0). 4 ml Buffer S2 (200 mM NaOH, 1% SDS) was added, mixed gently, and incubated at room temperature for 5 min. Buffer S3 (2.8 M Potassium acetate, pH 5.1), 4 ml, was added, mixed, and incubated on ice for 5 min. The sample was then centrifuged at high speed (>10000xg, 4°C). The supernatant was removed from the white precipitate, and loaded onto a Nucleobond cartridge equilibrated with 2 ml of buffer N2 (100 mM Tris, 15% ethanol, 900 mM KCl adjusted with H_3PO_4 to pH 6.3). The cartridge was washed with 2x 4ml of buffer N3 (100 mM Tris, 15% ethanol, 1150 mM KCl adjusted with H_3PO_4 to pH 6.3). The plasmid DNA was eluted from the column with 2 ml of buffer N5 (100 mM Tris, 15% ethanol, 1000 mM KCl adjusted with H_3PO_4 to pH 8.5). To precipitate the DNA, 0.7 vol isopropanol was added, and the sample was centrifuged at >10000xg at 4°C for 30 min. The DNA pellet was washed with 70% ethanol for 10 min. The air-dried pellet was then dissolved in a suitable volume of water or TE.

4.2 **Small-scale (mini-prep) method**

4.2.1 Mini-preps using the Wizard Minipreps DNA Purification System

The protocol suggested by the manufacturers (Promega) was followed.

A 3 ml overnight culture containing antibiotics (0.1 mg/ml ampicillin) was grown. To pellet the cells it was centrifuged for 1 - 2 min at 12000xg at room temperature in eppindorf tubes. The pellet was resuspended in 200 μl of cell resuspension solution. To lyse the cells, 200 μl of cell lysis solution was added and mixed by inversion. The solution was neutralized with 200 μl of neutralization solution and mixed by inversion. The sample was then centrifuged at 12000xg for 5 min and the supernatant transferred to a new tube. 1 ml of Wizard minipreps purification resin was added to the supernatant. For each prep a different column was prepared. A 5 ml syringe barrel was attached to the minicolumn and the resin/DNA mix was added to the syringe. The syringe plunger was inserted, and the slurry gently pushed into the minicolumn. The syringe was detached, and the barrel re-attached to the column. The column was washed by pushing 2 ml of column wash solution through. The syringe was then removed. The column was transferred to an eppendorf tube and centrifuged for 2 min to dry the resin. The column was transferred to a new tube. 50 μl of water/TE was applied to the

column. After 1 min the column was centrifuged for 20 sec to elute the DNA. The column was removed and discarded.

4.2.2 Mini-prep method described by Sambrook *et al.*, 1989

A 1.5 - 5 ml overnight culture was grown, containing the plasmid of interest in LB with antibiotics (0.1 mg/ml ampicillin). Bacterial cells were pelleted by centrifugation at 12000xg at room temperature. The medium was removed by aspiration, leaving the bacterial pellet as dry as possible. The pellet was resuspended in 100 μl cold solution I (50 mM Glucose; 25 mM Tris (pH 8); 10 mM EDTA (pH 8)) by vigorous vortexing. To this 200 μl of freshly prepared solution II (0.2 N NaOH; 1% SDS) was added. The sample was mixed by inverting the tube, and then placed on ice. 150 μl of cold solution III (60 ml 5 M potassium acetate; 11.5 ml glacial acetic acid; 28.5 ml H_2O) was added. After gently mixing the sample, it was stored on ice for 3 - 5 min. The sample was centrifuged at 12000xg for 5 min at 4°C. The supernatant containing the plasmid DNA was transferred to a fresh tube, where an equal volume of phenol/chloroform was added. The sample was mixed well by vortexing, and centrifuged at 12000xg for 2 min at 4°C to separate the phases. To precipitate the plasmid DNA, 2 vol of room temperature ethanol was added. The sample was then incubated at room temperature for 2 min. DNA was precipitated by centrifugation at 12000xg for 5 min at 4°C. After removing the supernatant, 70% ethanol was added to wash the pellet. It was then centrifuged as described above. The pellet was air dried and dissolved in 50 μl TE (pH 8.0) containing DNase-free RNase (20 μg/ml).

4.2.3 Mini-prep method described by Stephan *et. al.*, 1990

Overnight bacterial cultures (3 ml) was grown in selective medium (LB containing 0.1 mg/ml ampicillin). The cells were pelleted by centrifugation. The supernatant was removed and cells were resuspended in 200 μl of a solution of 25 mM Tris (pH 8), 50 mM glucose and 10 mM EDTA. This was incubated at room temperature for 5 min. A fresh solution of 0.2 N NaOH and 1% SDS (400 μl) was added to this, mixed by inverting the tube, and incubated on ice for 5 min. To this, 300 μl of 8 M ammonium acetate was added. After mixing gently, the sample was incubated on ice. After 10 min the sample was centrifuged and the

supernatant transferred to a fresh tube. To this 0.6 vol of isopropanol was added. After a 10 min incubation at room temperature, the DNA was precipitated by centrifugation for 10 min. The pellet was washed with 75% ethanol, dried, and dissolved in an appropriate amount of TE (pH 8.0).

4.2.4 A PEG precipitation method described by Kraft *et al.*, 1988

A 3 - 5 ml overnight culture was grown in selective medium (LB containing 0.1 mg/ml ampicillin). To pellet the cells, the bacterial culture was centrifuged for 2 min. As much as possible of the supernatant was removed, and 100 μl of ice-cold solution I (50 mM Glucose; 10 mM EDTA; 25 mM Tris-HCl (pH 8)) was added. The pellet was resuspended by vortexing. After a 5 min incubation at room temperature, 200 μl of solution II (0.2 N NaOH; 1% SDS) was added and the contents of the tube mixed by inversion. 150 μl ice-cold potassium acetate (pH 4.8) was added after a 5 min incubation on ice. The sample was incubated on ice for another 5 min and then centrifuged for 5 min at 4°C. The supernatant was transferred to a new tube and re-centrifuged. Once again the supernatant was transferred to a new tube. RNase A was added to a final concentration of 50 μg/ml. The sample was then incubated at 37°C for 30 min to 90 min. After the RNase digestion an equal volume of phenol/chloroform was added. The sample was vortexed vigorously and centrifuged for 2 min. The aqueous phase was transferred to a fresh tube where 2.5 vol of ice-cold 100% ethanol was added. The sample was vortexed well and incubated at -70°C for 10 - 30 min. To precipitate the DNA, the sample was centrifuged at 4°C for 5 min. The supernatant was removed and the pellet was washed with ice-cold 70% ethanol. The pellet was dried under vacuum and dissolved in 16.8 μl dH_2O. To this, 3.2 μl 5 M NaCl and 20 μl 13% PEG was added and mixed very well by vigorous vortexing. The DNA was incubated on ice in a cold room (4°C) for a minimum of 30 min, and precipitated by centrifugation at 13000xg for 10 min at 4°C. The pellet was washed with cold 70% ethanol, dried under vacuum, and dissolved in dH_2O.

4.2.5 A PEG precipitation method described in the PRISM Sequenase^{R} Terminator Double-Stranded DNA Sequencing Kit Protocol

This method yields high-quality, super-coiled plasmid DNA that is relatively free of contaminating chromosomal DNA and RNA. Throughout the procedure, the use of a vortex must be avoided, so as to minimize shearing of the contaminating chromosomal DNA. Samples are mixed by pipeting up and down. The protocol has been modified slightly. All manipulations were carried out in 1.5 ml eppindorf tubes. Centrifugation was performed at 12000xg at room temperature, unless stated otherwise.

Overnight cultures were incubated overnight at 37°C in Terrific Broth, with an appropriate amount of antibiotic (0.1 mg/ml ampicillin). To pellet the culture, it was centrifuged for 1 min in a microcentrifuge. After removal of the supernatant, the bacterial pellet was resuspended in 200 μl of GTE buffer (50 mM Glucose; 25 mM Tris-HCl (pH 8.0); 10 mM EDTA (pH 8.0)). 300 μl of freshly prepared 0.2 N NaOH/1% SDS was added, and the contents of the tube was mixed by inversion until the solution cleared. The sample was then incubated on ice for 5 min. The solution was neutralized by adding 300 μl of 3.0 M potassium acetate, pH 4.8, mixed by inversion, and incubated on ice for 5 min. Cellular debris was removed by centrifuging the sample at room temperature for 10 min, and then transferring the supernatant to a clean tube. RNase A (DNase-free) was added to a final concentration of 20 μg/ml and the sample was incubated at 37°C for 20 min. After the RNase A treatment, the supernatant was extracted with 3 x 400 μl of chloroform. The layers were mixed by hand for 30 s after each extraction. The phases were separated by centrifuging the tube for 2 min. The aqueous layer was transferred to a new tube and total DNA was precipitated by adding an equal vol of 100% isopropanol and immediately centrifuging the tube at room temperature for 10 min. The DNA pellet was washed with 500 μl of 70% ethanol and then dried under vacuum for 3 min. The pellet was dissolved in 32 μl of dH_2O. The plasmid DNA was precipitated by first adding 8 μl of 4 M NaCl, and then adding 40 μl of autoclaved 13% PEG_{8000}. After thorough mixing, the sample was incubated on ice for at least 30 min. The plasmid DNA was then pelleted by centrifugation at 4°C for 30 min. The supernatant was removed carefully, and the pellet was rinsed with 500 μl of 70% ethanol. The pellet was dried under vacuum for 3 min and resuspended in an appropriate amount of

dH_2O.

5. RESTRICTION ENDONUCLEASE DIGESTIONS

Restriction digests were carried out using restriction buffers and enzymes as recommended by the manufacturers. The enzyme volumes never exceeded 1/10 of the total reaction volume, as the enzyme contains glycerol which may inhibit the reaction. Two restriction enzymes could be combined in a single digest provided their salt requirements were compatible. If this was not the case, the DNA was first digested with one enzyme, ethanol precipitated and dissolved in dH_2O, and then digested with the second enzyme. Plasmid DNA was usually digested at 37°C for at least 60 min. However, when the restriction enzyme *BclI* was used, incubations were performed at 55°C.

Reactions were terminated by the addition of 0.2 M EDTA (pH 8.0) or 6x loading buffer and analysed by agarose gel electrophoresis.

6. ISOLATION OF DNA FRAGMENTS FROM AGAROSE

6.1 Phenol/Chloroform purification method

Gel slices were melted at 67°C for 10 min. Then 4 - 5 vol of prewarmed (67°C) TE buffer were added. The sample was mixed well and kept at 67°C until ready for phenol extraction. One vol of phenol was added, whereafter the sample was mixed well by vigorous vortexing. The phases were separated by centrifugation at 1600xg for 3 min. The aqueous phase was transferred to a fresh tube and 1 vol of phenol/chloroform/isoamyl alcohol (PCI) (25:24:2 vol/vol) was added. Centrifugation was repeated as described above. The PCI extraction was repeated, followed by a chloroform extraction. At this stage the interface was clear. To precipitate the DNA, 2½ vol 100% ethanol and 1/10 vol of 3 M sodium acetate was added. The sample was mixed well and incubated on dry ice. After 10 - 30 min, the sample was centrifuged at 15000 rpm for 30 min at 4°C. The pellet was rinsed with 70% ethanol and air dried. The pellet was then dissolved in an appropriate vol of dH_2O.

6.2 Wizard PCR preps DNA purification system

The protocol recommended by the manufacturers (Promega) was followed.

The agarose slice was incubated at 67°C in a 1.5 ml tube until the agarose was melted completely. 1 ml of Magic PCR Preps Resin was added to the melted agarose slice. This was mixed by vortexing for 20 sec. For each PCR product, one Wizard minicolumn was prepared. The syringe barrel of a 5 ml syringe was attached to the luer-lock extension of each column. The Resin/DNA mix was pipetted into the syringe barrel, and the slurry was gently pushed into the column with the syringe plunger. After removing the syringe from the column and then removing the plunger, the syringe was reattached to the column. 2 ml of 80% isopropanol was pipetted into the syringe and pushed through the column. The syringe was then removed, and the column was transferred to a 1.5 ml tube. The minicolumn was centrifuged at 12000xg for 20 sec to dry the resin. The column was transferred to a new tube, to which 50 μl of water or TE was applied. After 1 min the column was centrifuged at 12000xg for 20 sec to elute the bound DNA fragment. The column was removed and discarded and the purified DNA was stored at -20°C.

7. CLONING OF A PCR PRODUCT

Amersham's pMOS*Blue* T-vector kit provides an efficient system for direct cloning of a PCR product. The pMOS*Blue* vector allows for blue-white screening, with recombinant colonies appearing white when plated on X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside) and IPTG (Isopropyl-β-D-thiogalactopyranoside) indicator plates.

All the cloning was performed using this kit and the solutions provided. The protocol recommended by the manufacturers was followed.

7.1 Ligation

For optimal cloning efficiency a vector to insert ratio of 1:5 to 1:10 was used.
For each PCR product to be cloned, the following ligation reaction was set up : 1 μl 10x

ligase buffer, 0.5 μl 100 mM DTT, 0.5 μl 10 mM ATP, 1.0 μl 50 ng/μl vector, "x" μl amplified product, and 0.5 μl T4 DNA ligase (2-3 Weiss Units). The volume was made up to 10 μl with nuclease-free water. The ligation reaction was stirred gently with a pipette tip and incubated overnight at 16°C.

7.2 Transformation

Competent cells (MOS*Blue* cells supplied in the kit) were thawed on ice and mixed evenly to suspend the cells. 20 μl of competent cells was pipetted into a pre-chilled tube and 1 μl of ligation mix was directly added to the cells. After mixing gently, the tube was left on ice for 30 min. The cells were then heat-shocked for exactly 40 sec in a 42°C water bath and immediately placed on ice. After 2 min on ice, 80 μl of room temperature SOC medium was added. The tube was then shaken at 200-250 rpm at 37°C for 1 hour. During this hour the X-gal/IPTG plates were prepared as follows: 35 μl of 50 mg/ml X-gal and 20 μl 100 mM IPTG was spread per L agar antibiotic plate. The plates were then left to soak for at least 30 min prior to plating. After the 1 hour incubation at 37°C, the 50 μl of the transformation was spread onto the L agar plates. The plates were incubated overnight at 37°C in and inverted position.

8. PCR SCREENING OF COLONIES

White colonies were picked off plates with sterile toothpicks and inoculated in 20 μl sterile dH_2O. This was mixed (vortexed) very well. Sterile dH_2O containing no bacteria was used as controls. To 5 μl of bacterial suspension, the following mix was added: 9.4 μl dH_2O, 0.5 μl 10 mM dNTP, 2 μl 10x PCR Buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 1 μl primer 1 (25 pmoles), 1 μl primer 2 (25 pmoles), 0.6 μl 50 mM $MgCl_2$, and 0.5 μl Taq DNA Polymerase (5U/μl). This was mixed well. The solution was overlayed with 50 μl of mineral oil. The PCR cycle used, was as follows: 30 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C. This was followed by a 72°C extension for 10 min. The PCR products were analysed by agarose gel electrophoresis.

9. RNA ISOLATIONS

Latex gloves were worn for all manipulations involving RNA. Plugged tips were used at all times. Pipettes, centrifuge tubes and homogenizers were treated with 3% H_2O_2 for an hour, rinsed with methanol, washed with DEPC H_2O and air dried. They were then autoclaved. All solutions used were RNase-free. Water was treated with diethylpyrocarbonate (DEPC). The RNA isolations were performed in another laboratory to avoid any contamination.

9.1 Small scale isolation of Total RNA

Small scale isolations were performed with the QIAGEN RNeasy Kit. The protocol recommended by the manufacturers was followed.

Lysis buffer RLT was added to the tissue (350 μl buffer to 30 mg tissue) and the tissue was then homogenized. A maximum of 600 μl is advised. The sample was then centrifuged for 3 min at 12000xg. The supernatant was transferred to a clean tube, where 1 vol of 70% ethanol was added. The sample was mixed by pipeting. The sample was applied onto a RNeasy spin column and centrifuged at 8000xg for 15 min. The column was washed with 700 μl wash buffer RW1 and centrifuged as described above. This was followed by 2x 500 μl washes with buffer RPE, and centrifugation for 2 min. To elute the RNA, 30 - 50 μl H_2O was applied to the column, followed by 60 sec of centrifugation.

9.2 Large scale isolation of total RNA

Large scale RNA isolations were performed with the guanidine isothiocyanate/acid-phenol method. The REX™ Total RNA Extraction Kit (United States Biochemical) was used. The protocol recommended by the manufacturers was followed.

Tissue was homogenized in 10 ml denaturing solution in a hand held homogeniser. After homogenization, the following was added: 1 ml 2 M sodium acetate (pH 4.0), 10 ml water-saturated phenol, and 2 ml chloroform/isoamyl alcohol (24:1). The sample was shaken well and allowed to cool on ice. After 15 min the sample was centrifuged at 10000xg for 15 min at 4°C. The supernatant was transferred to a new tube and re-extracted if necessary. An equal volume of 100% isopropanol was added to the sample and it was incubated at -20°C

for 1 hour to precipitate the RNA. The sample was then centrifuged at 10000xg for 15 min at 4°C. The RNA pellet was resuspended in 2 ml 4M LiCl. The tubes were then centrifuged at 3000xg for 10 min at 4°C to pellet the RNA. The pellet was dissolved in 2 ml REX™ Buffer. An equal volume of chloroform/isoamyl alcohol was added, vortexed well, and the sample was centrifuged at 3000xg for 10 min. The aqueous phase was transferred to a new tube, to which an equal vol of isopropanol was added. After a 15 min incubation at -20°C, the sample was centrifuged at 10000xg for 15 min. The pellet was rinsed with 5 ml 75% cold ethanol, dried under vacuum (at room temperature) and dissolved in TE.

9.3 **Small scale mRNA isolation**

Small scale isolations of mRNA were performed with Promega's Poly A Tract mRNA Isolation System. This isolation system uses a biotinylated-oligo(dT) probe that is captured with a magnet. The protocol recommended by the manufacturers was followed.

9.3.1 Annealing of probe

1 mg of Total RNA in a volume of 500 μl was used for mRNA isolation.

The RNA was denatured by incubation at 65°C for 10 min. 3 μl of biotinylated-oligo(dT) probe and 13 μl 20x SSC was added. The sample was mixed gently, and allowed to cool to room temperature for annealing of the probe to the RNA.

9.3.2 Washing of Streptavidin-paramagnetic particles (SA-PMPs)

The SA-PMP's were resuspended by flicking the tube and captured on the magnetic stand. The supernatant was removed carefully. The SA-PMP's were then washed with 3x 0.3 ml of 0.5x SSC. After the final wash, the SA-PMP's were resuspended in 0.1 ml 0.5x SSC.

9.3.3 Capture and washing of annealed oligo(dT)-mRNA Hybrids

The entire contents of the annealing reaction was added to the tube containing the washed SA-PMP's. The tube was incubated at room temperature for 10 min. The SA-PMP's were captured on the magnetic stand, and the supernatant was removed. The particles were washed with 4x 0.3 ml 0.1x SSC. After the final wash, as much as possible of the aqueous phase was removed.

9.3.4 Elution of mRNA

The final SA-PMP pellet was resuspended in 0.1 ml RNase-free H_2O by gentle flicking of the tube. The SA-PMP's were captured magnetically. The eluted mRNA aqueous phase was transferred to a sterile tube. The elution step was repeated with 0.15 ml H_2O. The two aqueous phases were pooled.

9.4 **RNA concentration and integrity**

RNA concentrations were checked in cuvettes treated with 50 mM NaOH and DEPC H_2O. The integrity of the RNA was checked by denaturing agarose/formaldehyde electrophoresis, and visualised by EtBr staining.

10. cDNA SYNTHESIS

10.1 **Synthesis of double-stranded cDNA**

cDNA was synthesised from human testes RNA with the Clontech Marathon cDNA Amplification Kit. Human testes RNA was obtained from post mortem material (adult male, ~ 25 years old) by J. Hapgood, with permission from the UCT Ethics Committee. Two different total RNA batches and a poly A RNA batch was used. A control reaction was also included. All reactions were performed in 0.5 ml tubes in a thermocycler. The protocol recommended in the kit user manual was followed.

10.1.1 First-strand cDNA synthesis

In a 0.5 ml tube, 1 μg RNA, 1 μl 10 μM cDNA synthesis primer, and dH_2O to 5 μl were added. This was mixed well, spun down, and incubated at 70°C. After 2 min the tubes were placed on ice for an additional 2 min. The following mix was then added to the tubes: 2 μl 5x first strand buffer, 1 μl 10 mM dNTP, 1 μl [α-^{32}P] dCTP (1 μCi/μl), and 1 μl MMLV (Moloney Murine Leukemia Virus) reverse transcriptase (100 U/μl). The sample was mixed gently by pipetting, and incubated at 42°C for 1 hour. The tubes were then placed on ice to terminate the first-strand synthesis.

[α-^{32}P] dCTP is optional. Addition of the isotope facilitates tracking of the cDNA through subsequent steps and troubleshooting cDNA synthesis.

10.1.2 Second-strand cDNA synthesis

All components and reaction vessels should be pre-chilled on ice.

The following mixture was added to the 10 μl of the first-strand reaction: 48.4 μl sterile H_2O, 16 μl 5x second- strand buffer, 1.6 μl 10 mM dNTP mix, and 4 μl 20x second-strand enzyme cocktail. The contents was mixed thoroughly by gentle pipetting. The tubes were spun briefly to collect the contents at the bottom. The tubes were then incubated at 16°C. After 90 min, 2 μl (10 U) T4 DNA polymerase was added. The contents was mixed by pipeting. The tubes were then incubated at 16°C for a further 45 min. To terminate second-strand synthesis, 4 μl EDTA/glycogen mixture was added. 100 μl PCI was added to this and vortexed thoroughly. The tubes were then centrifuged at 14000 rpm for 10 min. The supernatant was transferred to a new tube, where 100 μl of chloroform/isoamyl alcohol (24:1) was added. The sample was mixed well and then centrifuged at 14000 rpm for 10 min. The supernatant was transferred to a clean tube. To precipitate the DNA, ½ vol 4 M ammonium acetate and 2½ vol 95% room temperature ethanol was added. The sample was vortexed thoroughly and centrifuged at 14000 rpm for 20 min at room temperature. The supernatant was removed carefully. If the DNA precipitated efficiently, the pellet should contain 1-10% of the total radioactivity in the sample. The pellet was rinsed with 300 μl of 80% ethanol and centrifuged at 14000 rpm for 10 min. The pellet was air dried, and dissolved in 10 μl of H_2O.

The yield and size of the ds cDNA was estimated by comparing it to the positive control. This was done by running 2 μl of the cDNA on a 1% agarose gel.

The agarose gel was dried using a vacuum gel drying system and exposed to X-ray film.

10.1.3 Adaptor ligation

This procedure uses 5 μl of the 10 μl ds cDNA obtained after second-strand synthesis. The 5x ligation buffer must be completely thawed at room temperature for at least 30 min before use.

The following mixture was combined in a 0.5 ml tube: 5 μl ds cDNA, 2 μl Marathon cDNA Adaptor (10 μM), 2 μl 5x DNA ligation buffer, and 1 μl of T4 DNA ligase (1 U/μl). The contents was mixed by vortexing, and incubated overnight at 16°C, after which the ligase was inactivated by heating the tube to 70°C for 5 min. The adaptor-ligated ds cDNA was diluted with Tricine-EDTA buffer and stored at -20°C.

10.2 **Synthesis of single-stranded cDNA**

cDNA was synthesised from testes poly A RNA template with the Gibco BRL 5'RACE System for Rapid Amplification of cDNA Ends (See figure 22).

10.2.1.1 First-strand cDNA synthesis - SuperScriptII RT

This procedure is designed to convert specific RNA sequences from a background of up to 1 μg of total RNA into first strand cDNA.

cDNA was synthesised from 1 μg of poly A RNA isolated from human testes. RNA ($\leq$ 1 μg), 1 μl (2.5 pmoles) gene specific primer 1 (GSP1), and DEPC-treated H_2O sufficient for a final volume of 14 μl were added to a 0.5 ml tube. The mixture was incubated at 70°C for 5 to 10 min to denature RNA. After chilling the tube on ice for 1 min, it was centrifuged briefly to collect the contents of the tube. The following mixture was then added: 2.5 μl 10x

reaction buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 3 μl 25 mM $MgCl_2$, 1 μl 10 mM dNTP mix, 2.5 μl 0.1 M DTT, and 1 μl RNase inhibitor. The mixture was mixed gently and incubated at 42°C for 2 min, after which 1 μl of SuperScript II RT was added. After gentle mixing, the tube was incubated at 42°C for 30 min, and then placed at 65°C - 70°C. After 15 min, the tube was centrifuged briefly to collect the contents, and then placed at 55°C. To degrade the mRNA template, 1 μl of RNase H was added. After a further 10 min incubation at 55°C, the reaction was collected by brief centrifugation, and the tube was placed on ice.

10.2.1.2 First-strand cDNA synthesis-Expand™ RT (Boehringer Mannheim)

cDNA was synthesised from 1 μg of poly A RNA. RNA (≤ 1 μg), 1 μl (2.5 pmoles) GSP1, and DEPC-treated H_2O sufficient for a final volume of 12.5 μl were added to a 0.5 ml tube. The mixture was incubated at 70°C for 10 min to denature RNA. After chilling the tube on ice for 1 min, it was centrifuged briefly to collect the contents of the tube. The following mixture was then added: 5 μl 5x Expand buffer (2.5 mM $MgCl_2$, 20 mM KCl, 25mM Tris), 2.5 μl 100 mM DTT, 2.5 μl 10 mM dNTP mix, 2 μl Expand RT (100 units), and 0.2 μl RNase inhibitor (RNaseIn) (101 U/μl). The solution was mixed gently and collected by centrifugation. The tube was then incubated at 42°C for 60 min, followed by a 15 min incubation at 68°C. After a quick spin, the tube was placed at 55°C, 1μl of RNase H was added, and the tube was incubated for 10 min. The contents of the tube was then collected by brief centrifugation, and the tube was placed on ice.

10.2.2 Purification of cDNA

cDNA was purified with Promega's Wizard PCR preps DNA purification system. The protocol recommended by the manufacturers was followed.

1 ml of Magic PCR Preps Resin was added to the cDNA. This was mixed by vortexing for 20 sec. For each cDNA sample, one Wizard minicolumn was prepared. The syringe barrel of a 5ml syringe was attached to the luer-lock extension of each column. The Resin/cDNA mix was pipetted into the syringe barrel, and the slurry was gently pushed into the column with the syringe plunger. After removing the syringe from the column and removing the

plunger, the syringe was reattached to the column. 2 ml of 80% isopropanol was pipetted into the syringe and pushed through the column. The syringe was then removed, and the column was transferred to a 1.5 ml tube. The minicolumn was centrifuged at 12000xg for 20 sec to dry the resin. The column was transferred to a new tube, and 50 μl of water or TE (pH 8.0) was added. After 1 min the column was centrifuged at 12000xg for 20 sec to elute the bound cDNA. The column was removed and discarded. After elution, the sample was placed in a vacuum drier until the volume was reduced to 10 μl.

10.2.3 TdT Tailing of cDNA

The following mixture was added to the cDNA sample: 7.5 μl DEPC-treated H_2O, 2.5 μl 10x reaction buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 1.5 μl 25 mM $MgCl_2$, and 2.5 μl 2mM dCTP. The sample was then incubated at 94°C. After 3 min the tube was transferred to ice for a further 1 min. To collect the contents of the tube, it was centrifuged briefly. 1 μl of TdT (10 U/μl) was added to the tube and the contents mixed gently. The mixture was incubated at 37°C for 10 min. The TdT was heat inactivated by incubation at 65-70°C for 10 min. The contents of the tube was collected by brief centrifugation.

11. SEQUENCING OF DOUBLE-STRANDED TEMPLATES

All the kits use the chain-terminating DNA sequencing method.
Different sequencing kits were used for sequencing plasmid DNA.

11.1 IsoTherm™ DNA Sequencing Kit (Epicentre Technologies)

11.1.1 Alkaline denaturation of template

2 μg of plasmid DNA in a total volume of 10 μl was used as template. 10 μl of 0.4 N NaOH, 0.4 mM EDTA was added to this and mixed gently. The sample was then incubated at 65° for 15 min, after which the following were added: 2 μl 2 M ammonium acetate (pH 4.5), and 50 μl room temperature 95% ethanol. The sample was incubated at room

temperature for 5 min, and then centrifuged for 5 min. The supernatant was removed carefully, and the pellet was washed with 70% ethanol, dried, and resuspended in 8 μl H_2O.

11.1.2 Sequencing protocol

0.5 pmol primer and 1.0 μl 10x reaction buffer were added to the denatured dsDNA. The tube containing this mixture was labelled as the "premix" tube. It was incubated at 70°C for 5 min. The mixture was then allowed to cool to room temperature on the benchtop. Then 1.0 μl IsoTherm DNA Polymerase and 1.5 μl [α-^{35}S]dATP (5 μCi) was added to the premix and mixed gently. 2 μl of each termination mixture was transferred to a clearly labelled tube, and 3 μl of premix was added to each termination tube. The tubes were then incubated at 65°C. After 2 min 2 μl of Chase Mix was added. The tubes were incubated at 65°C for a further 2 min. The reaction was stopped by adding 4 μl stop/loading buffer to each reaction. Before loading the samples on a sequencing gel, they were incubated at 70°C for 5 min.

11.2 **T7 Sequenase Quick Denature Plasmid Sequencing Kit**

This kit is made especially for plasmid DNA, and includes solutions for denaturing of the template. The protocol recommended by the manufacturers was followed.

11.2.1 Alkaline Denaturation

0.5 - 3 μg of DNA in a maximum volume of 8 μl was used as a template. 2 μl 1.0 M NaOH, 1 μl primer (0.5-2 pmol), and H_2O to 11 μl were added to the template. After thorough mixing, the sample was incubated at 37°C for 10 min, and then placed on ice. 2 μl 1.0 M HCl and 2 μl plasmid reaction buffer were added to the sample.

11.2.2 Annealing

To allow the primer to anneal, the template/primer/buffer mixture was incubated at 37°C for 10 min, and then placed on ice.

While annealing, preparations for the labelling were done. The labelling mix was diluted 5-fold with water, and 2.5 μl of each of the termination mixtures were transferred to clearly labelled tubes. The termination tubes were pre-warmed at 37°C.

11.2.3 Labelling reaction

The following mixture was added to the ice-cold annealed DNA mixture: 1 μl 0.1 M DTT, 2 μl diluted labelling mix, 0.5 μl [α-^{35}S] dATP (5 μCi), and 2 μl T7 Sequenase plasmid sequencing formulation. The sample was mixed well and incubated at room temperature for 2 to 5 min.

11.2.4 Termination reactions

4.5 μl of labelling reaction was transferred to each termination tube and mix. The mixture was incubated at 37°C for a further 5 min. To stop the reaction, 4 μl of stop solution was added. The samples were heated to 75°C for 2 min immediately before loading onto a sequencing gel.

11.3 **Sequenase Version 2.0 DNA Sequencing Kit**

Two different protocols were followed when sequencing with this kit. They are both slight modifications of the protocol recommended by the manufactures.

11.3.1 Standard sequencing protocol

11.3.1.1 Denaturing

Two different methods were used for denaturing plasmid DNA

11.3.1.1.1 Denaturation of plasmid DNA by boiling

Plasmid DNA (3-5 μg in a maximum volume of 7 μl) was denatured by boiling the sample

for 3 min and then snap-freezing it on dry ice for 30-60 sec. The sample was then spun briefly to collect the contents of the tube.

11.3.1.1.2 Alkaline denaturation of plasmid DNA

5 μg of plasmid DNA in a total volume of 10 μl was used as template. 10 μl of 0.4 N NaOH, 0.4 mM EDTA was added to this and mixed gently. The sample was then incubated at 65° for 15 min, after which the following was added: 2 μl 2 M ammonium acetate (pH 4.5), and 50 μl room temperature 95% ethanol. The sample was incubated at room temperature for 5 min, and then centrifuged for 5 min. The supernatant was removed carefully, and the pellet was washed with 70% ethanol, dried and resuspended in 7 μl H_2O.

11.3.1.2 Annealing

1 μl primer (10 ng), 2 μl reaction buffer, and H_2O to 10 μl were added to the plasmid DNA. The mixture was incubated at 37°C for 15 min for annealing of the primer to the template, and then placed on ice.

During the 15 min annealing, solutions were prepared for the labelling reaction. 2.5 μl of each termination mix was transferred to a clearly labelled tube. These tubes were incubated at 37°C for at least 1 min. Labelling mix was diluted 5-fold with H_2O. The Sequenase Version 2.0 enzyme was diluted 1:8 in ice-cold enzyme dilution buffer.

11.3.1.3 Labelling reaction

The following mixture was added to the annealed template-primer (on ice): 1.0 μl 0.1 M DTT, 2.0 μl diluted labelling mix, 0.5 μl [α-^{35}S]dATP, and 2.0 μl diluted Sequenase. The sample was mixed thoroughly and incubated at room temperature for 2 to 5 min.

11.3.1.4 Termination reaction

When labelling was complete, 3.5 μl of mix was removed and transferred to the tubes

containing the termination mixes. These were then incubated at 37°C for a further 5 min. To stop the reaction, 4 μl of stop solution was added to each termination reaction and mixed thoroughly. The samples were heated to 80°C for 2 min just before loading on a sequencing gel.

11.3.2 A modified sequencing protocol using DMSO

This protocol is basically the same as the one described above, except for the use of DMSO. DMSO is added to the sequencing reaction for clearer, darker bands on the autoradiograph. This method was obtained from Brigitte Blackman.

11.3.2.1 Alkaline denaturation of template

1 μl of 2 M NaOH was added to 9 μl DNA (3-5 μg) and mixed well. The sample was incubated at 37°C for 15 min, after which 10 pmoles of primer (1 μl of 10 μM stock) was added. The sample was vortexed briefly. To neutralize the NaOH, 3 μl of 3M potassium acetate was added. To precipitate the DNA, 75 μl of ethanol was added. The sample was then centrifuged at 13000 rpm for 10 min. The pellet was washed with 100 μl of 75% ethanol and dried under vacuum.

11.3.2.2 Sequencing protocol

The pellet was resuspended in 8 μl H_2O and 2 μl reaction buffer. The termination mixes were prepared as follows: 2.5 μl termination mix and 0.25 μl DMSO were combined in a tube and prewarmed to 45°C. The following mixture was added to the sample: 1 μl DTT, 2 μl diluted labelling mix, 0.5 μl [α-^{35}S] dATP (5 μCi), 2 μl diluted Sequenase, and 1.7 μl DMSO. The sample was incubated at room temperature for 5 min, after which 4.3 μl of mixture was transferred to each of the termination mixes. The samples were incubated at 45°C for 5 min. To stop the reaction, 5 μl of stop solution was added. The samples were heated to 80°C for 2 min before loading on a sequencing gel.

11.4 **Denaturing acrylamide gel electrophoresis**

Samples were run on a 6% acrylamide, 7 M urea gel in 1xTBE at 90 Watts. Gels were 0.46 mm thick. Gels were run between 1½ to 4 hours.

5.7 g acrylamide, 0.3 g bisacrylamide, 42 g urea, and 20 ml 5xTBE were combined in a beaker. H_2O was added to a volume of 100 ml. The solution was mixed until dissolved, and then filtered. 100 μl TEMED and 1 ml 10% ammonium persulfate were added to the mixture before pouring the gel.

12. POLYMERASE CHAIN REACTION (PCR)

All PCR reactions were set up in a laboratory where no post-PCR products or plasmids were present. Gloves were worn at all times, and frequently changed. Plugged tips were used for all reactions to avoid aerosol contaminants. Typically, 50 μl reactions were set up. The reaction mixture and PCR cycling conditions varied with the template and primers used.

For amplification of gDNA with degenerate primers, the following reaction was used: 1 μl 10 mM dNTP, 5 μl 10x reaction buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 2 - 3 μl 50 mm $MgCl_2$, 0.25 - 1 μg of each primer (depending on the degeneracy of the primer, 0.5 μl Taq (5U/μl Taq DNA from *Thermus aquaticus* YT1, Gibco BRL), 1 μg gDNA, and sterile water to 50 μl. The reaction mixture was covered with 50 μl mineral oil. The following PCR cycle was used: 94°C for 1 min (denaturation), followed by 55°C for 2 min for annealing of the primer, and 3 min at 72°C for the extension of the product. Typically, 35 cycles were performed.

For amplification of cDNA with gene specific primers the following reaction was used: 1 μl 10 mM dNTP, 5 μl 10x reaction buffer (400 mM Tricine -KOH (pH 9.2), 150 mM potassium acetate, 35 mM magnesium acetate, 750 μg/ml bovine serum albumin), 10 - 20 μM of each primer, 1 μl 50x Klentaq polymerase mix (KlenTaq-1 DNA polymerase, TaqStart Antibody), cDNA, and sterile water to 50 μl. Touchdown cycling conditions were normally used, in order to maximize the specificity of the reaction. The typical PCR cycle was as follows: 94 °C for 1 min, followed by 5 cycles of 94°C for 30 sec, 72°C for 4 min. This was followed by another 5 cycles of 94°C for 30 sec, 70°C for 4 min, followed by 25 cycles of 94°C for

30 sec, 68°C for 4 min. This was followed by a 10 min extension at 72°C.

13. ELIMINATING CONTAMINATION DURING PCR

All the solutions used were aliquoted into small aliquots when opened. PCR reactions were set up in another laboratory, where nobody worked with the GnRHR, or any other plasmids. The PCR solutions were also stored in this laboratory. All the reactions were performed with plugged tips, to avoid aerosol contaminants. The set of pipettes used to set up the reactions, were used for PCR only. The tips, as well as the eppendorf tubes, were stored in a UV-lightbox. Also, gloves were changed frequently. When setting up a PCR reaction, a negative (no DNA) control was always included. An icebox was also set apart for the sole purpose of setting up PCR reactions.

To minimize template contamination, all genomic DNA isolations were performed in another laboratory, with sterile solutions and plugged tips. The tubes containing the genomic DNA were never opened in the laboratory used for plasmid work or post-PCR.

CHAPTER 3

RESULTS

1. OPTIMISATION OF DEGENERATE PRIMERS

A series of degenerate primers was designed to the type I GnRHR sequences that were conserved between different mammalian receptors (J. Hapgood) (Figure 9).

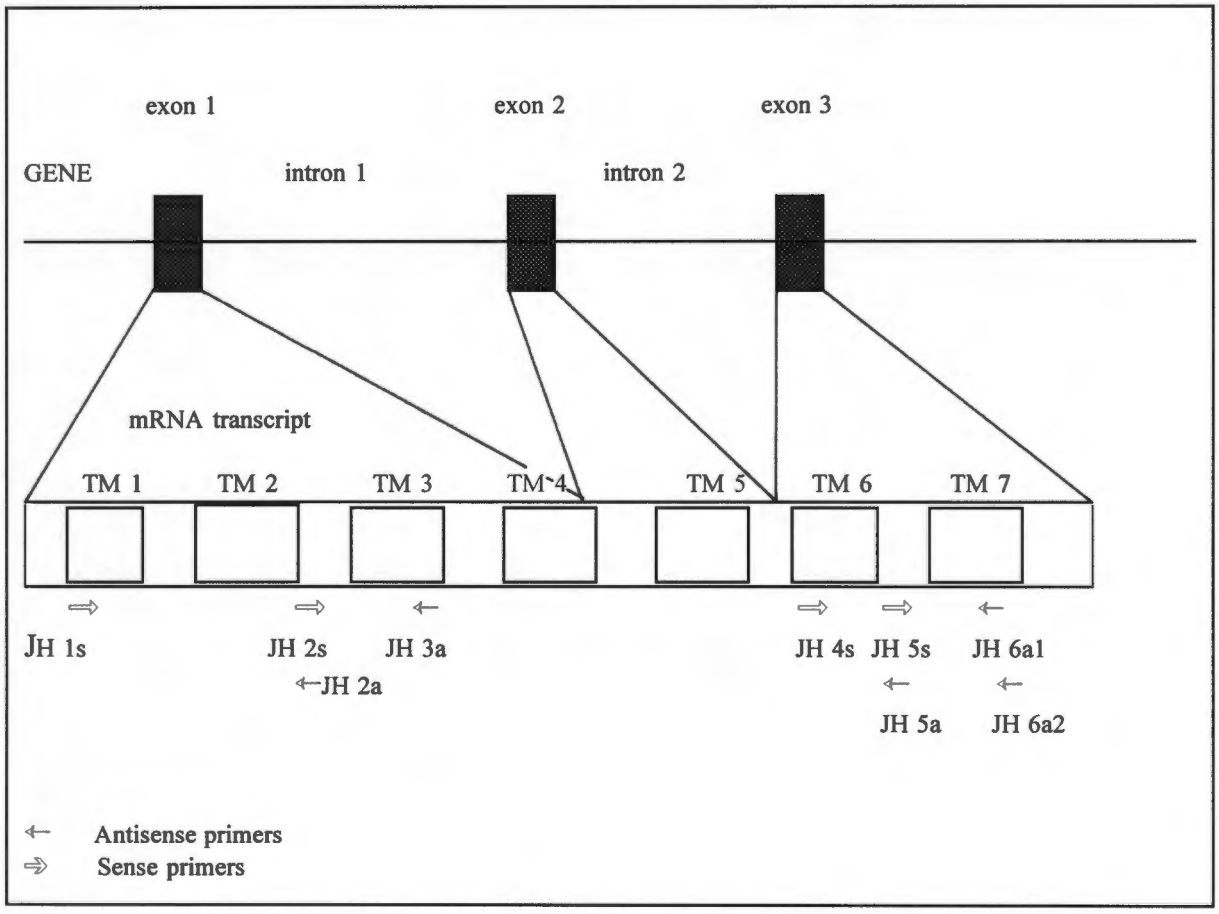

Figure 9 Schematic diagram to indicate the relation of the degenerate primers to the type I human GnRHR gene.

The different primer pairs were tested on human gDNA. Mouse GnRHR cDNA was used as a positive control, since we knew what size PCR product to expect. The primers were originally designed to mammalian type I GnRHR. If the primers did not work on human gDNA and mouse GnRHR cDNA (in other words if this did not produce a PCR product of the expected size), we did not expect them to work on redbait gDNA, or any other species' gDNA. The PCR was performed twice, in order to confirm our results. PCR products of the expected size, i.e. 134 bp, 170 bp, 309 bp, 120 bp, 233 bp, and 95 bp, were obtained with primer pairs 5S/6A2, 4S/6A2, 1S/3A, 2S/3A, 1S/2A, and 4S/5A, respectively (Figure 10).

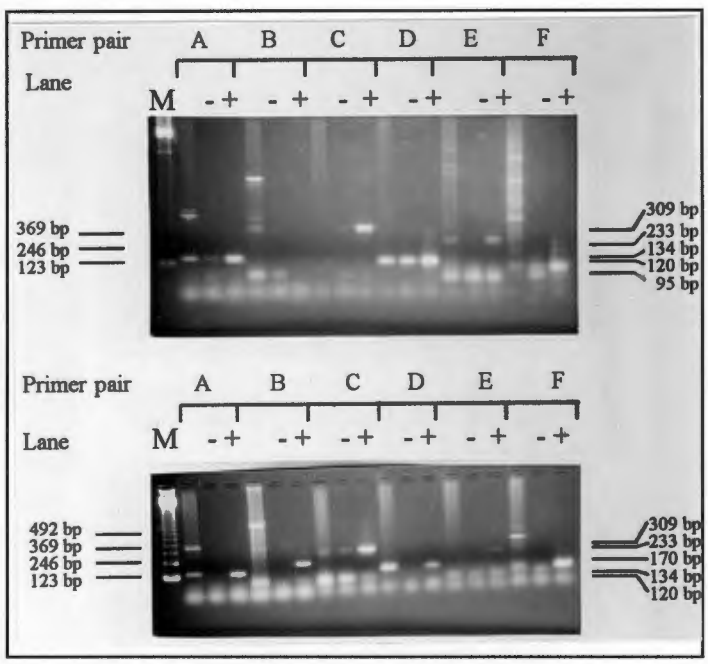

Figure 10 The photographs represent 2 independent experiments. Different combinations of degenerate primer pairs were tested on human gDNA. 20 µl of PCR product was electrophoresed on a 1.5 % agarose gel. A 123 bp ladder is included as a molecular weight marker. The 1st of each group of 3 lanes is the PCR performed on human gDNA, (-) indicates a negative control (no DNA or water blank), and (+) indicates a positive control PCR performed on mouse GnRHR cDNA. Bands obtained that were of the expected size are marked. Primer pair A = 5S/6A2, B = 4S/6A2, C = 1S/3A, D = 2S/3A, E = 1S/2A, and F = 4S/5A.

All the primer pairs tested produced bands of the expected size, although some primer pairs produced better results than others (a clearer band on the EtBr-stained agarose gel). Note that a band of the expected size can be seen in lane 2 of A in the first photograph whereas in the second photograph that lane is clear. The opposite is seen in C where a band is seen in the negative control sample in the second photograph but not in the first. The bands present in the negative controls are due to contamination, which despite taking all proper precautions could sometimes not be avoided (See section 13, Materials and Methods). These results established that the degenerate primers were able to specifically amplify the GnRHR sequences from human gDNA and mouse GnRHR cDNA.

2. ISOLATION OF A PUTATIVE REDBAIT GnRHR GENE WAS UNSUCCESSFUL

Genomic DNA (gDNA) was isolated from the redbait gonads as described in Current Protocols in Molecular Biology. To assess the integrity of the gDNA, 1 μg of DNA was run on an analytical agarose gel with a molecular weight marker (Figure 11).

The DNA was mostly >11.5 kb, therefore DNA did not appear to be degraded. PCR was performed on the redbait gDNA with degenerate primers designed to areas of the GnRH receptor that are highly conserved between different mammals. Six different combinations of primers were tested. They are JH5S + JH6A2, JH1S + JH3A, JH2S + JH3A, JH1S + JH2A, JH4S + JH5A and JH4S + JH6A2. The PCR products were checked by electrophoresis on an analytical agarose gel. Only primer pairs 1S/2A (233 bp), 4S/5A (95 bp) and 4S/6A2 (170 bp) showed products of the expected size (Figure 12). Primer pairs 5S/6A2, 1S/3A, 2S/3A did not appear to work (did not produce a PCR product of the expected size). A Southern blot was performed on this gel (Figure 13), probing the membrane with human type I GnRHR cDNA probe (See preparation in section 2.5, Materials and Methods).

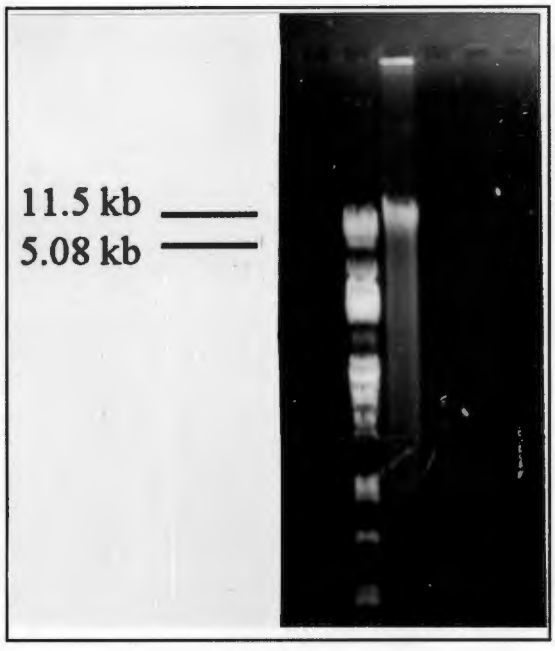

Figure 11 1 μg of redbait genomic DNA (right lane) was electrophoresed on a 0.6% EtBr-stained agarose gel. λ DNA digested with *Pst* I is used as a size marker (left lane).

The 1S/2A and 4S/5A products gave a clear positive signal on the autoradiograph, and the 4S/6A2 a weaker signal. These products were also checked by restriction enzyme digestions, to check if the positive signals were contamination, or if they were real. The logic behind the use of restriction enzyme digestions as an analytical tool, is that different GnRH receptor species are bound to differ in some of the restriction enzyme sites. By doing digestions with a few different enzymes, we would be able to tell if our isolated fragments differed from mouse and human GnRH receptor, by looking at the pattern of the digested DNA. The products of restriction enzyme digestions were different to mouse and human digests, suggesting that the products were not mouse or human contaminants (data not shown).

Subsequent PCR reactions performed on redbait gDNA with primer pair 5S/6A2, did produce a product of the expected size, i.e. 134 bp (data not shown).

These PCR products were isolated by electrophoresis on a low melting agarose gel and excision of the fragments of interest. The 1S/2A and 4S/6A2 PCR products were cloned into pMOS*Blue* T-vector and transformed into pMOS bacterial cells. 4S/5A PCR products were not cloned, since it proved difficult to obtain sufficient product for cloning.

In order to obtain sufficient DNA for further analysis of the cloned products, small-scale isolation of plasmid DNA was performed. These plasmids were then digested with restriction enzymes and checked by Southern blot. The Southern was probed with a human GnRHR cDNA probe, stripped, and reprobed with a mouse PCR-radiolabelled GnRHR cDNA probe. The digestion of the plasmids differed from the human and mouse cDNA digestions, indicating that we did not have any mouse or human contamination. We then sequenced several of the samples, in order to find out exactly what we had isolated. The sequencing data was analysed by comparison to the human GnRHR sequence. The same cloning strategy was followed for 5S/6A2 PCR products.

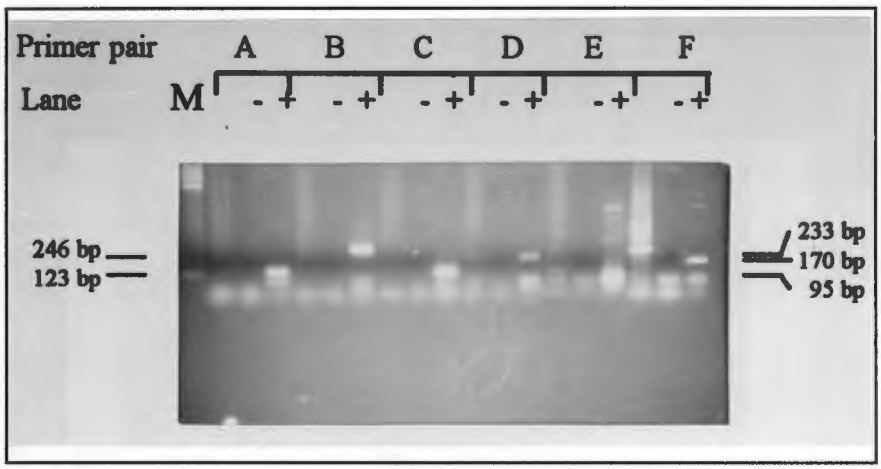

Figure 12 PCR on redbait genomic DNA using different combinations of degenerate primers. The bands obtained that were of the expected size are marked. Lane M contains the 123 bp marker. Primers used were, A = 5S/6A2, B = 1S/3A, C = 2S/3A, D = 1S/2A, E = 4S/5A, and F = 4S/6A2. The first of each group of 3 lanes is the PCR performed on redbait gDNA, (-) indicates a negative control (no DNA or water blank), and (+) indicates a positive control PCR performed on mouse GnRHR cDNA.

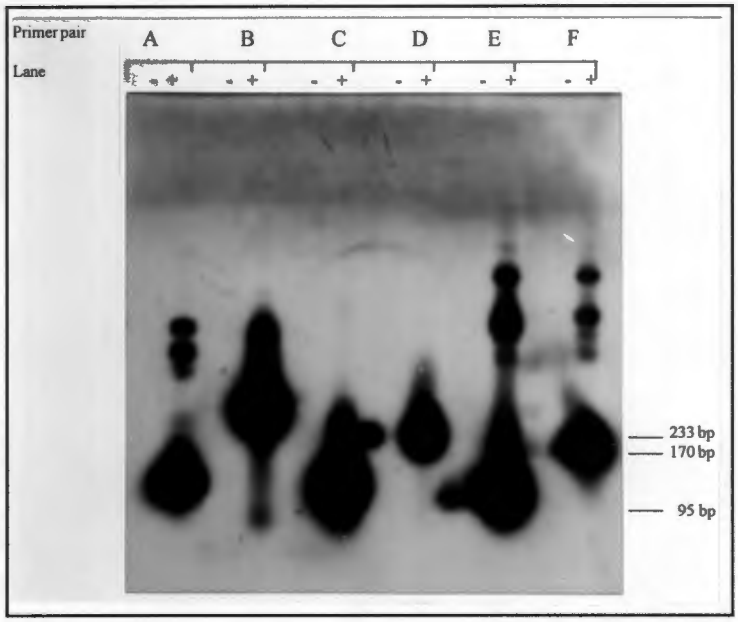

Figure 13 Autoradiograph of a Southern blot performed on the gel in Figure 12. PCR was performed on redbait gDNA using different combinations of degenerate primers (See Figure 7). A Southern blot was performed on this gel, probing with human type I GnRHR cDNA. The first lane of each primer pair is the PCR performed on the redbait gDNA, (-) is the negative (no DNA) control, and (+) is the PCR performed on mouse GnRHR cDNA (positive control). The expected sizes for the different primer pairs are as follows: 5S/6A2 - 134 bp; 1S/3A - 309 bp; 2S/3A - 120 bp; 1S/2A - 233 bp; 4S/5A - 95 bp; 4S/6A2 - 170 bp. These signals are clearly visible as large overexposed "blobs" for the positive control lanes for mouse cDNA. The figure clearly shows the positive signals obtained for redbait gDNA with primer pairs 1S/2A (D)(233 bp), 4S/5A (E)(95 bp), and 4S/6A2 (F)(170 bp). No bands were visible with redbait gDNA for primer pairs 5S/6A2 (A), 1S/3A (B), and 2S/3A (C). No positive signals were visible for the negative controls, indicating that there was no contamination of PCR reactions. Lines indicate bands obtained that were of the expected size.

Another way of analysing the cloned colonies, was by use of direct PCR of the colonies with primers located in the vector. We used primer pair T7 and U19, which are located in the polylinker of the pMOS*Blue* T-vector. This primer pair was a very handy tool, since it allowed us to immediately identify the size of our cloned PCR fragments. These PCR products were transferred to Hybond N^+ by Southern blot, and probed with type I chicken GnRHR[1]. Positive colonies were sequenced.

A total of 43 1S/2A colonies, 34 4S/6A2 colonies, and 36 5S/6A2 colonies were sequenced. The sequencing data was analysed on GCG by comparison to the human GnRH receptor sequence. Unfortunately, most of the 5S/6A2 colonies were contaminating *Xenopus* or Goldfish GnRHR[2]. Several of the colonies were not contaminants, but were found not to be related to GnRHR or any other previously identified protein in the database.

3. ISOLATION OF EXON 1 OF TYPE I GnRHR GENE FROM MOLE GENOMIC DNA

Genomic DNA (gDNA) was isolated from mole gonads by the method described by Ausubel *et al.*, 1987, in Current Protocols in Molecular Biology. From 600 mg of tissue 2.6 mg of gDNA was isolated. The quality of the gDNA was analysed by agarose gel electrophoresis.

After the integrity of the DNA was confirmed, PCR was performed on mole gDNA with primer pair JH1S + JH2A. In order to find the optimum conditions for amplification of the

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[1]The Type I GnRHR was cloned from chicken gDNA by with degenerate primer pair 5S/6A2 by Yuh-man Sun.

[2]In parallel to this study, other researchers in the laboratory were busy cloning *Xenopus* and Goldfish GnRHRs using the same degenerate primers. It is possible that the primers became contaminated with plasmid or PCR products present in the laboratory (Section 11, Materials and Methods).

1S/2A fragment, an increasing concentration range of $MgCl_2$ (from 2 mM to 3.5 mM) was used in the PCR experiment. Primer pair 1S/2A is designed to amplify a 233 bp fragment of exon 1 from TM1 to TM2. Upon analysis of PCR products on an analytical agarose gel, a fragment of the expected size was detected (Figure 14).

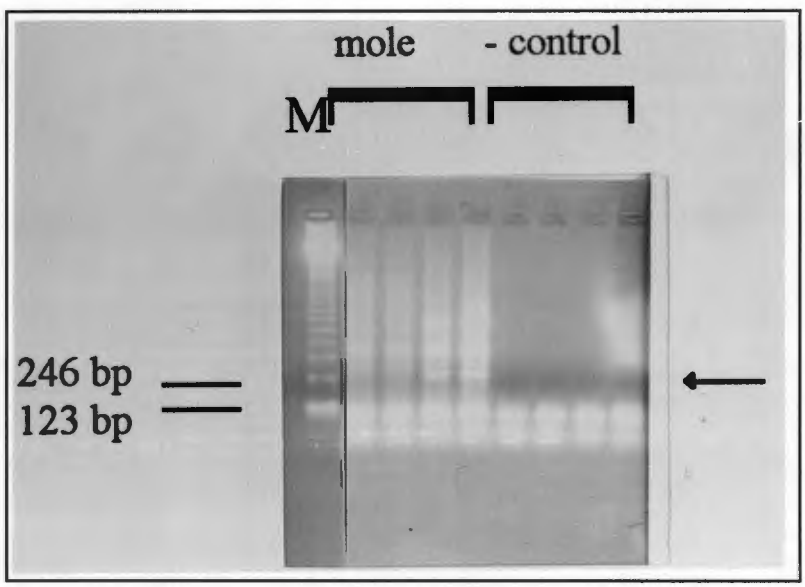


Figure 14 PCR was performed on mole gDNA, and no DNA as negative control (- control) with primer pair JH1S + JH2A. 20 μl of each 50 μl PCR reaction was loaded on an EtBr-stained 1.5 % agarose gel. A 123 bp ladder was used as a size marker (lane M). Four lanes can be seen for each sample. This is four different $MgCl_2$ concentrations, i.e. 2 mM, 2.5 mM, 3 mM, and 3.5 mM. The arrow indicates a band of approximately 240 bp. No bands were visible in the negative (no DNA) control, indicating that there were no contaminants in the PCR.

The remaining PCR mixture was pooled and the fragment of interest (233 bp) was isolated by electrophoresis on a low melting point agarose gel and excision. The DNA was purified from the agarose by the phenol/chloroform extraction method (Section 5.1, Materials and Methods), and dissolved in water. With the use of the pMOS*Blue* cloning kit, the DNA was ligated into the pMOS*Blue* T-vector, and transformed into pMOS bacteria.For further analysis of the PCR generated inserts, twenty colonies were picked at random and subjected to plasmid preparations to obtain sufficient DNA for analysis by DNA sequencing (Figure 15).

The sequencing data obtained was analysed by comparison to the human type I GnRHR. Comparisons were done on both the DNA level, and the amino acid level. The comparisons revealed that six (30%) of the total of twenty colonies sequenced, were exon 1 of the mole type I GnRHR. The remaining fourteen (70%) colonies all contained inserts that were non-specific amplifications. Sequence analysis revealed that exon 1 of the mole type I GnRHR is 89.5% homologous to human GnRHR on the DNA level, and 93% on the amino acid level (Figure 16).

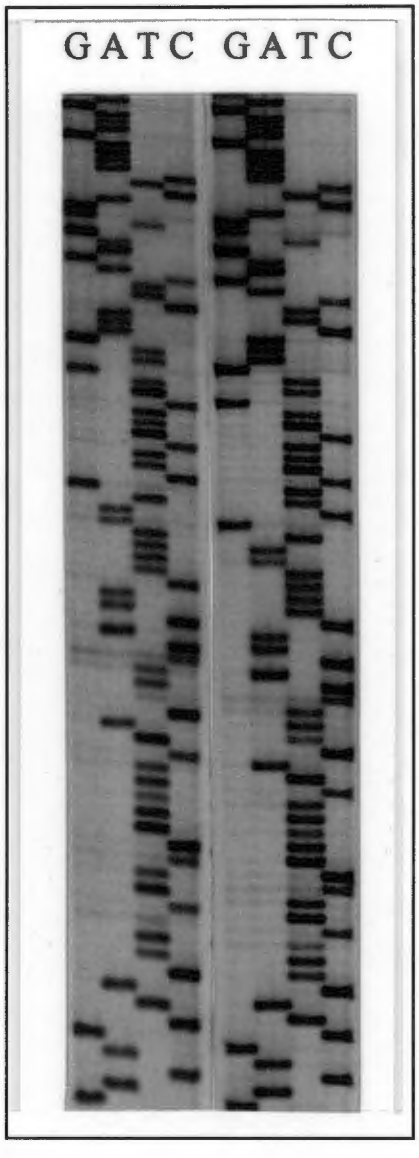


Figure 15 The 233 bp PCR products shown in Figure 14 were cloned into a vector. Several colonies were picked and plasmid DNA was isolated. The DNA was sequenced by the Sanger di-deoxy method with ^{35}S and exposed to film. An autoradiograph of the sequencing gel is shown. The 2 sequences represent 2 identical sequences of 2 representative mole clones. Part of exon 1 of the mole GnRHR DNA sequence (see Figure 16) is visible.

DNA sequence comparison:

```
Human:  GGAAAGATCCGAGTGACGGTTACTTTCTTCCTTTTTCTGCTCTCTGCG
Mole :  GGTAAGATTAGGGTGACAGCTACTTTCTTCCTTTTTCTACTTTCCACA
                          □ →
Human:  ACCTTTAATGCTTCTTTCTTGTTGAAACTTCAGAAGTGGACACAGAAG
Mole :  ACTTTTAATGCTTCTTTCTTGTTGAAACTTCAGAAGTGGACTCAAAAG

Human:  AAAGAGAAAGGGAAAAAGCTCTCAAGAATGAAGCTGCTCTTAAAAC
Mole :  AAAGAGAAAGGGAAAAAGCTCTCAAGAATGAAGGTGCTTTTAAAAC
           ← □
Human:  ATCTGACCTTAGCCAACCTGTTGGAGACTCTGATTGTCATGCCACTGG
Mole :  ATCTGACCTTAGGCAATCTGTTGGAGACTCTGATTGTCATGCCATTGG

Human:  ATGGGATGTGGAACATTACA
Mole :  ATGGAATGTGGAACATCACC
```

Amino Acid Comparison:

```
Human:  GKIRVTVTFFLFLLSATFNASFLLKLQKWTQKKEKGKKLSRMKLLL
Mole :  GKIRVTATFFLFLLSTTFNASFLLKLQKWTQKKEKGKKLSRMKVLL

Human:  KHLTLANLLETLIVMPLDGMWNITVQ
Mole :  KHLTLGNLLETLIVMPLDGMWNITVH
```

Figure 16 Part of exon 1 of mole GnRHR consensus sequence derived by comparison of 6 individual clones. Differences between the mole and human sequences are highlighted. Underlined sequences indicate the primers that were used in the PCR reactions. The DNA sequence corresponding to the sequence on the autoradiograph in Figure 15 is marked (□ → to ← □).

4. IDENTIFICATION AND CLONING OF PART OF A TYPE II GnRH RECEPTOR GENE IN HUMANS.

4.1 Detection of a type II GnRH receptor cDNA in the database by homology searches

Human genome data bases were searched for a type II GnRH receptor homologue (D. Conklin). The searches were performed with the sequence of a putative type II GnRHR from reptile (E. Rumback, personal communication). The type II GnRHR in reptile differs quite strikingly in amino acid composition and length from mammalian type I GnRHR in extracellular loop 3 (EC 3). The searches revealed the presence of antisense human type II GnRHR EST cDNA clones in several human tissues, showing that a gene for a type II GnRHR exists in the human genome. The novel type II sequences obtained in the data base corresponded to exons 2 and 3 of the type I human GnRHR, and contained an intron corresponding to intron 2 of type I human GnRHR (Figure 17). A series of gene specific primers was designed (See Figure 18) to the sequence found in the database (R.P. Millar).

Finding these cDNA sequences in the data base raised the following questions:

(i) Is the "sense" RNA (i.e. minus intron) actually expressed in human tissues?

(ii) In which tissues is the sense RNA expressed?

(iii) Does the full length cDNA contain the equivalent of exon 1 of the type I GnRHR?

(iv) Is there a mouse equivalent type II GnRHR?

I attempted to answer some of these questions as part of this MSc thesis.

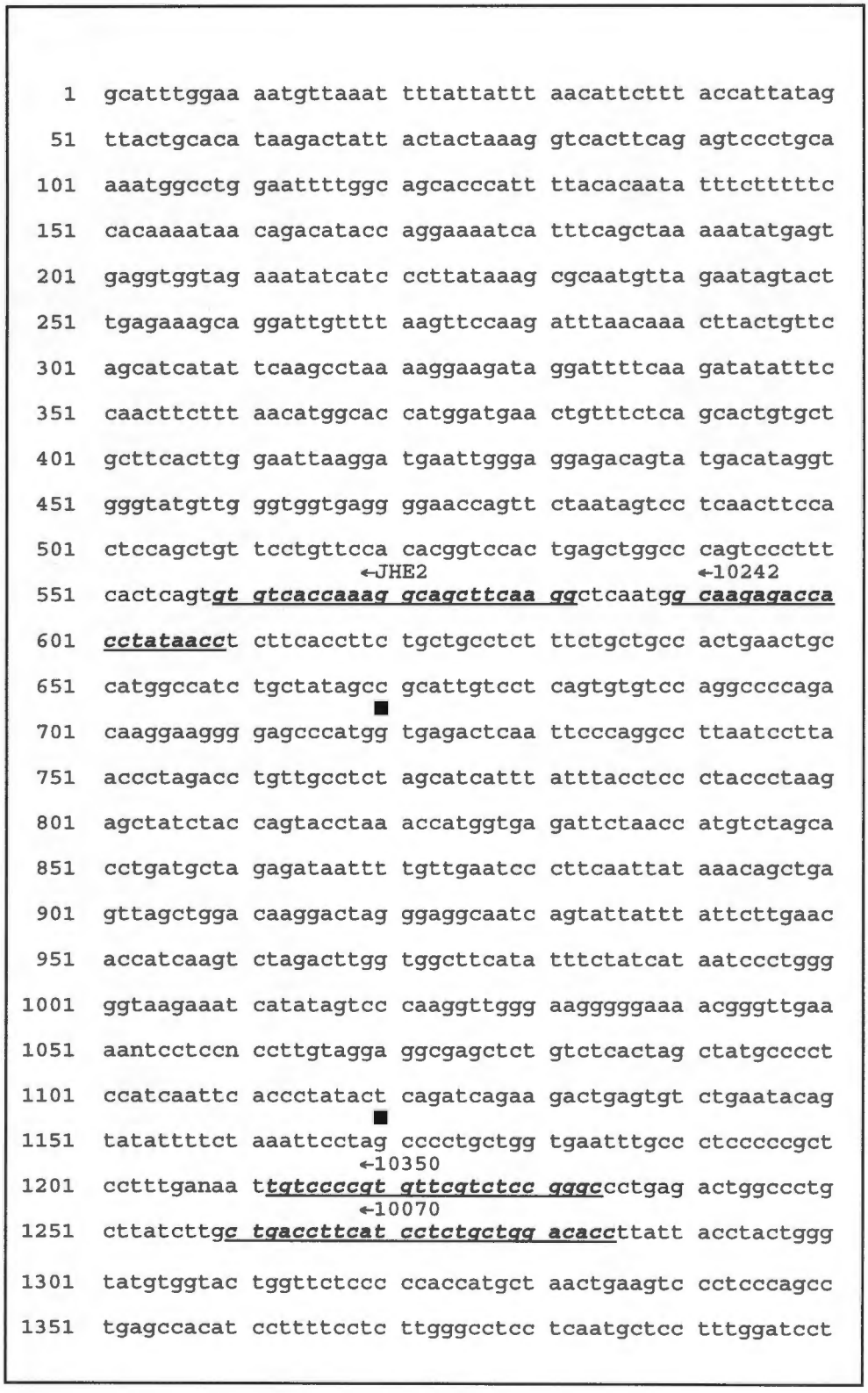


Figure 17 Genomic DNA sequence of the human type II GnRHR found in the data base. The gene specific primers (see Figure 18) designed to this sequence are underlined and printed in bold italics. ■ indicates the intron/ exon boundaries determined experimentally.

```
1401  ctcctctatg gggccttcac ccttggctgc cgaagagggc accaagaact
                                 ←10068
1451  tagtatagac tcttctaaag aagggtctgg gagaatgctc caagaggaga

1501  ttcatgcctt tagacagctg gaagtacaaa aaactgtgac atcaagaagg

1551  gcaggagaaa caaaaggcat ttctataaca tctatctgat cctaacagag

1601  tatgtaggaa cagaatagta agtctttagt gccataagat cttaacatct
            ←10071
1651  cacttctact cctgctctcc
```

Figure 17 (continued). Genomic DNA sequence of the human type II GnRHR found in the data base. The gene specific primers (see figure 18) designed to this sequence are underlined and printed in bold italics.

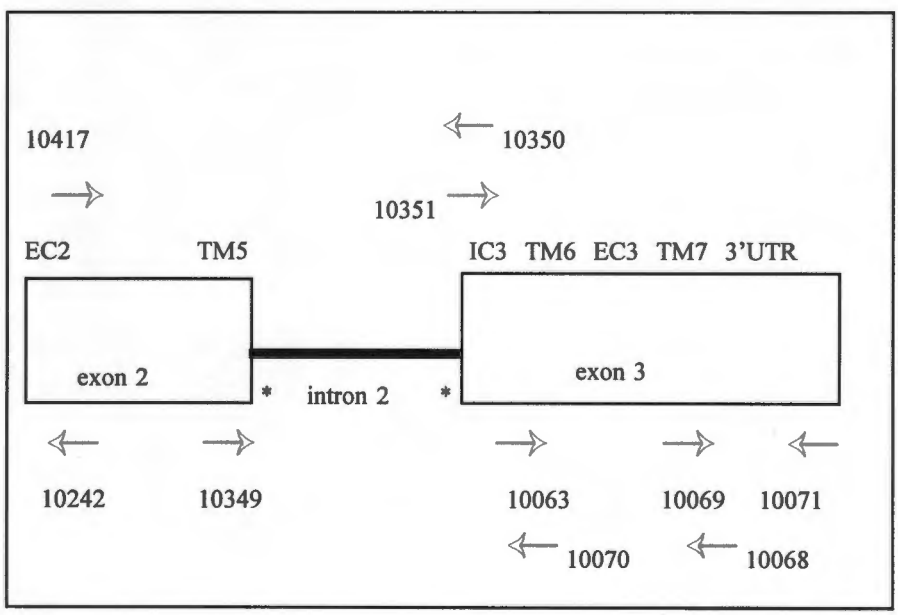


Figure 18 Diagram indicating the location of gene specific primers designed to the human type II GnRH receptor sequence found in the data base. TM - transmembrane domain, EC - extracellular loop, IC - intracellular loop, UTR - untranslated region. Note the precise exon 2/exon 3 boundary (marked with *) could not be predicted at this stage.

4.2 Detection and sequencing of intron-less processed RNA of part of human type II GnRHR

From the type II GnRHR DNA sequence in the database, it was clear that the open reading frame for putative exons 2 and 3 was interrupted by an intron. However, the precise location of the exon/intron boundaries could not be deduced just from the sequence. Several possible intron/exon splice junctions were possible as predicted from the DNA sequence.

To determine where RNA for the type II GnRHR is expressed, PCR was performed on cDNA's with gene specific primers (10417 + 10070) designed across the intron/exon boundary of the human type II GnRHR sequence found in the database. Using primers 10417 + 10070, intron-containing RNA would result in a band of 660 bp, while RNA in which the intron was removed or processed, would result in smaller products (Figure 19). Since we did not know the position of the putative exon 2/exon 3 boundary, the exact size of the processed RNA PCR product could not be predicted at this stage. This strategy is based on the assumption that the gene structure of the type II GnRHR is the same as that of the type I GnRHR.

cDNA from several different human tissues and cell lines was obtained from R.P. Millar at Zymogenetics Inc., Seattle. In addition, cDNA was also prepared from various different human tissues by J.P. Hapgood and E. Hutchinson (Section 9, Materials and Methods). In total, RNA was analysed from the following cell lines and tissues: T47D (breast carcinoma), prostate, bone marrow, uterus, hvvec 2 (umbilical cord), testis, retina, insulinoma, MCF-7 (breast carcinoma), fetal brain, thyroid, lymphnode, spleen, placenta, daudi (B lymphoma), small intestine, kidney, HUH-7, adult brain, CaCO2 (colon tumor), liver, pons, medulla, midbrain, pituitary, hypothalamus, pBMc1, and lung.

PCR products were separated on agarose gels and stained with EtBr (Figure 20). The result showed that unprocessed intron-containing RNA (660 bp) is present in almost all adult human tissues and cell lines examined, as well as fetal tissue. For some of the samples, bands of molecular weight lower than 660 bp were also observed, raising the possibility that some processed RNA had been amplified. In order to positively identify type II GnRHR PCR products, the PCR products were subjected to Southern blotting, probing the membrane with

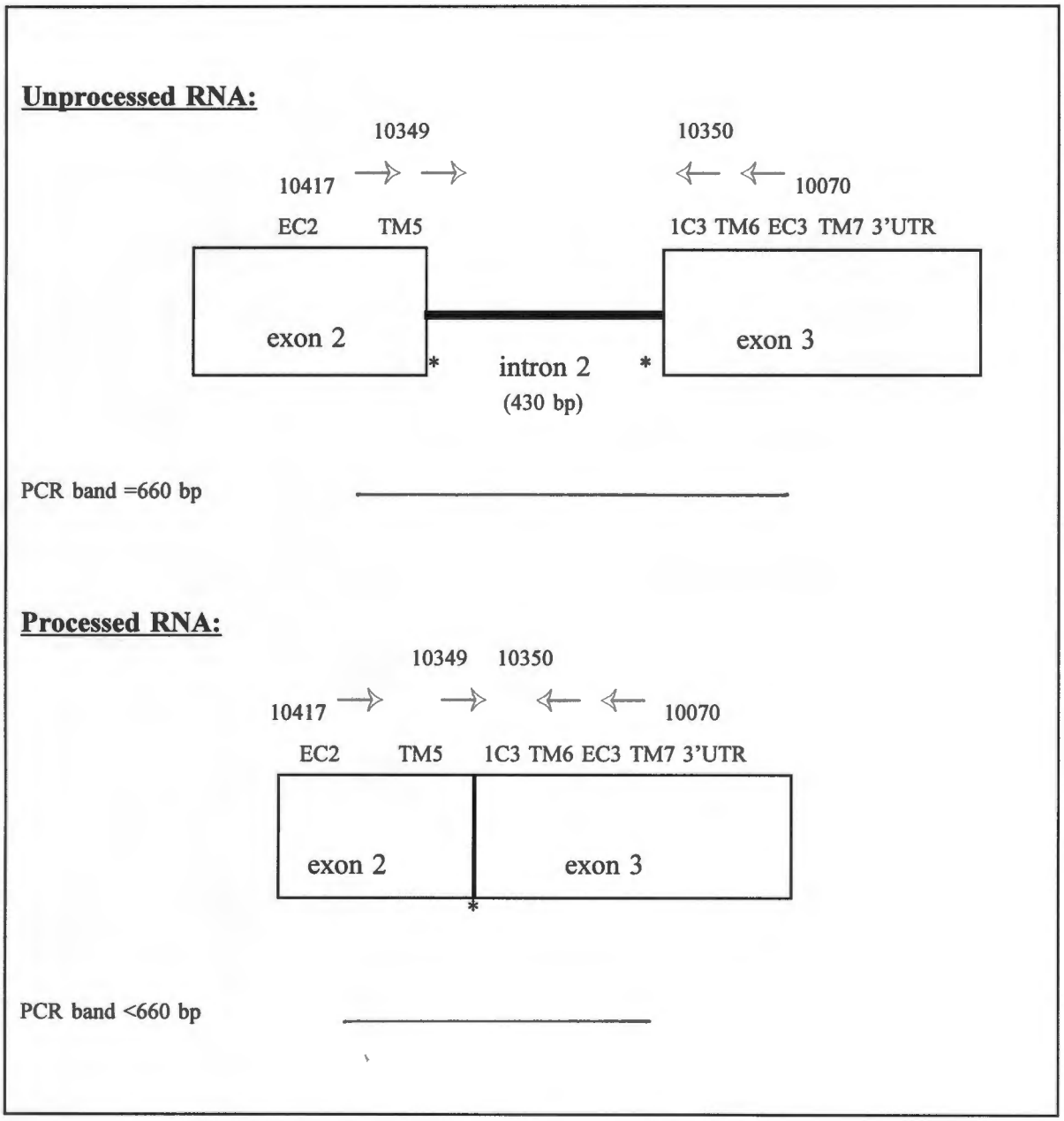


Figure 19 Schematic illustrating the rationale used to distinguish between unprocessed and processed (intron-less) RNA transcript by PCR with primers 10417 + 10070. Note the precise exon 2/exon 3 boundary (marked with *) could not be predicted at this stage.

an internally nested primer, 10350. Positive signals at 660 bp were obtained for almost all the samples. On close examination of the Southern, a minor band of 200 - 300 bp was detected for the PCR reaction on testes cDNA, which would be consistent with the presence

of a processed transcript (Figure 21).

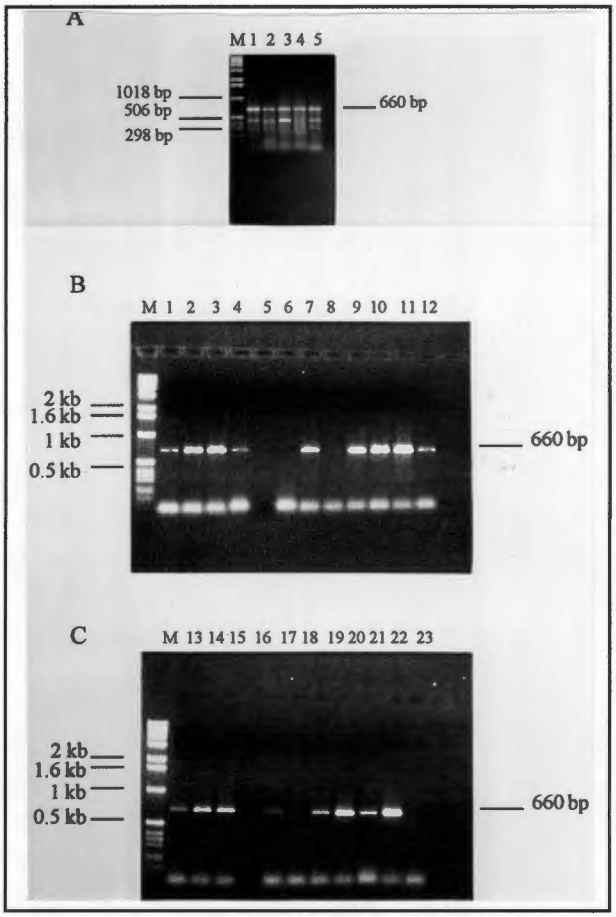


Figure 20 PCR was performed on different human tissues and cell lines with primer pair 10417 + 10070 designed across the intron/exon boundary of the human type II GnRHR sequence found in the database. PCR samples were run on 1%agarose gels. The result showed that unprocessed intron-containing RNA (660 bp) is present in almost all of the human tissues and cell lines examined. For some of the samples, bands of <660 bp were also observed. The 660 bp bands obtained are marked on the figure. 1 kb ladder was used as a size marker (lane M).

A: (1) Cerebellum (2) adrenal (3) testes (4) lung (5) cortex

B: (1) T47D (2) prostate (3) bone marrow (4) uterus (5) hvvec2 (6) no sample (7) retina (8) insulinoma (9) MCF-7 (10) fetal brain (11) thyroid (12) lymphnode

C: (13) spleen (14) placenta (15) daudi (16) small intestine (17) kidney (18) HUH-7 (19) adult brain (20) CaCO2 (21) liver (22) uterus (23) liver

A: Minigel B+C: Midi gels (Section 1, Materials and Methods)

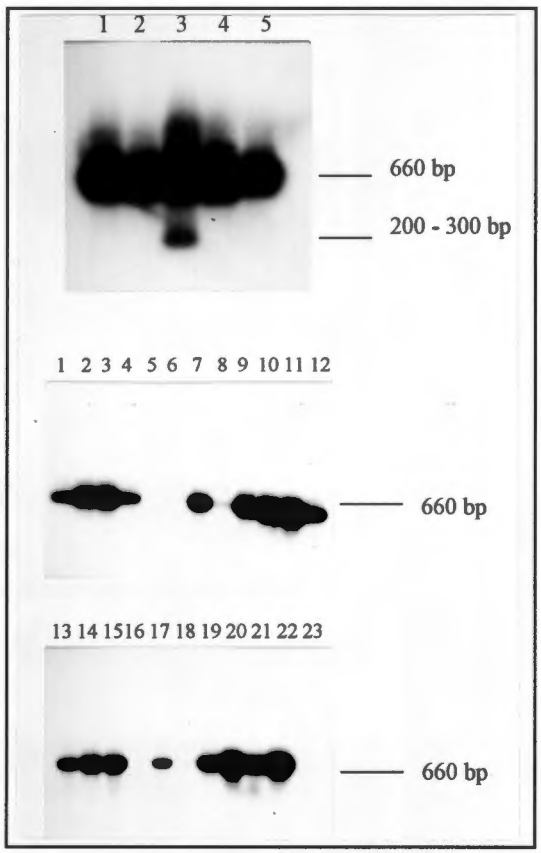


Figure 21 Autoradiographs of Southern blots performed on the gels in figure 20. PCR was performed on various human tissues and cell lines with primer pair 10417 + 10070 (Figure 20). Southern blots were performed on these gels, probing the membranes with an internally nested primer, 10350 (see figure 19). The result shows that unprocessed intron containing RNA (660 bp) is present in almost all adult human tissues and cell lines examined, as well as fetal tissue. A minor band of 200 - 300 bp was detected in human testes. Lanes as for Figure 20.

Attempts were made to isolate and clone the 200 - 300 bp DNA band which showed positive on the Southern (Figure 21). Because the resolution of the Southern blot was only ± 50 bp, we were not sure of the exact size of the positive PCR product. In addition, several DNA bands < 660 bp were visible on the EtBr-stained gel, and some were very faint. To obtain more DNA of the correct product for cloning, the region of the band was isolated from low melting point agarose, and reamplified with primers 10417 + 10070. Following this second PCR reaction, a smear of DNA bands of 200 - 300 bp was again visible by EtBr staining on an agarose gel (Figure 22). Southern blotting of this gel and probing with the internal primer 10350 confirmed that the reamplified DNA still contained a DNA product of 200 - 300 bp containing type II GnRHR sequences (data not shown). The DNA bands from 200 - 300 bp were isolated by electrophoresis on a low melting point agarose gel. The DNA was isolated, cloned into a vector, and transformed into bacteria. Several colonies were picked and analysed by PCR (see Methods, section 7) to check the size of the inserts (Figure 23). Note that the actual size of the inserts in Figure 23 is 140 bp smaller than the bands on the gel due to amplification of part of the pMOS vector using this method. The clones contained several inserts in the range of 200 to 400 bp. Southern blotting of this gel revealed that several of the clones contained a DNA insert of 230 bp which contained type II GnRHR sequences (data not shown). Plasmid preparations were performed on some of these colonies (eg. number 13), and the DNA was sequenced. The sequencing data revealed that the 230 bp fragment contained DNA amplified from an intron-less processed RNA of putative type II GnRHR, and contained exon 3 and part of exon 2 (Figure 24).

The finding that RNA for type II GnRHR, without putative intron 2, was expressed in testes tissue as a minor product in the presence of excess unprocessed intron-containing RNA, and that most of the tissues examined appeared to express the unprocessed intron-containing RNA for the type II GnRHR, raised the possibility that other tissues also express the processed RNA. To test this possibility, PCR primers which span the exon 2 exon 3 boundary were designed (i.e. JHE2E3A and JHE2E3S). These primers should selectively amplify processed RNA and provide a more sensitive method of analysis. The primers should not anneal very efficiently to the intron-containing RNA, since the 3'end of the primer would not base pair to the intron (Figure 24).

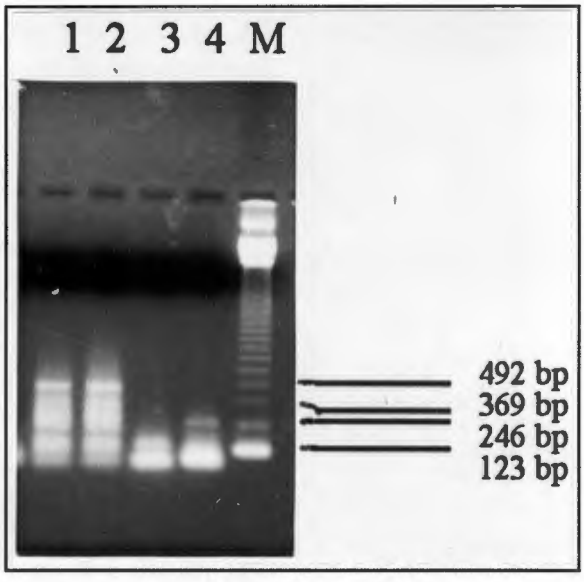


Figure 22 The gel-purified DNA products of 200 - 300 bp visible in Figure 20 were reamplified with primer pair 10417 + 10070, and checked by electrophoresis on a 1% EtBr-stained agarose gel. Lanes 1 & 2 are testes products, and lanes 3 & 4 are pituitary products. 123 bp ladder was used as a molecular weight marker (lane M).

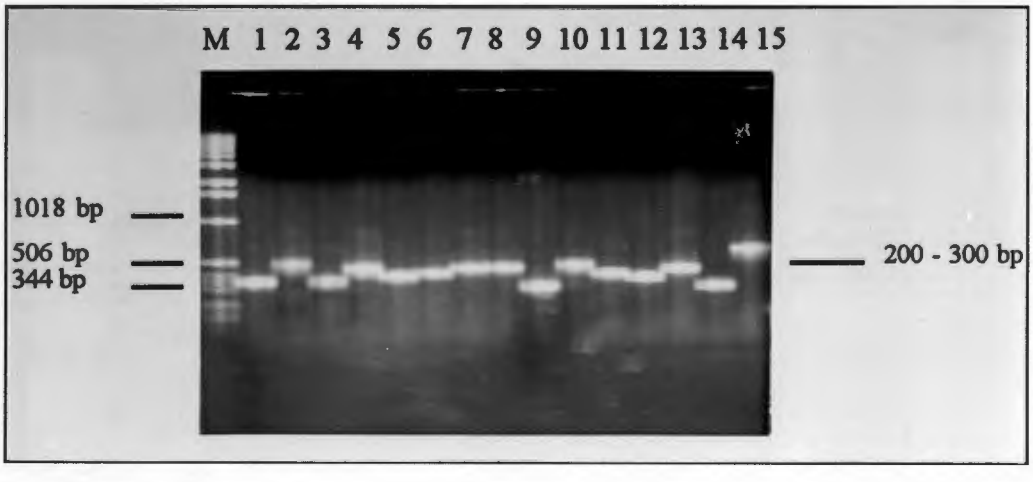


Figure 23 EtBr-stained agarose gel of PCR performed on clones in pMOS with polylinker primers T7 and U19 to check the insert sizes of the colonies. Lanes 1 to 15 correspond to the colony numbers. The line on the right side indicates the actual size of the inserts cloned, and not the size of the PCR product. Note that the actual sizes of the inserts are 140 bp smaller than the bands on the gel due to amplification of part of the pMOS vector using this method. 1 kb ladder was used as a size marker (lane M).

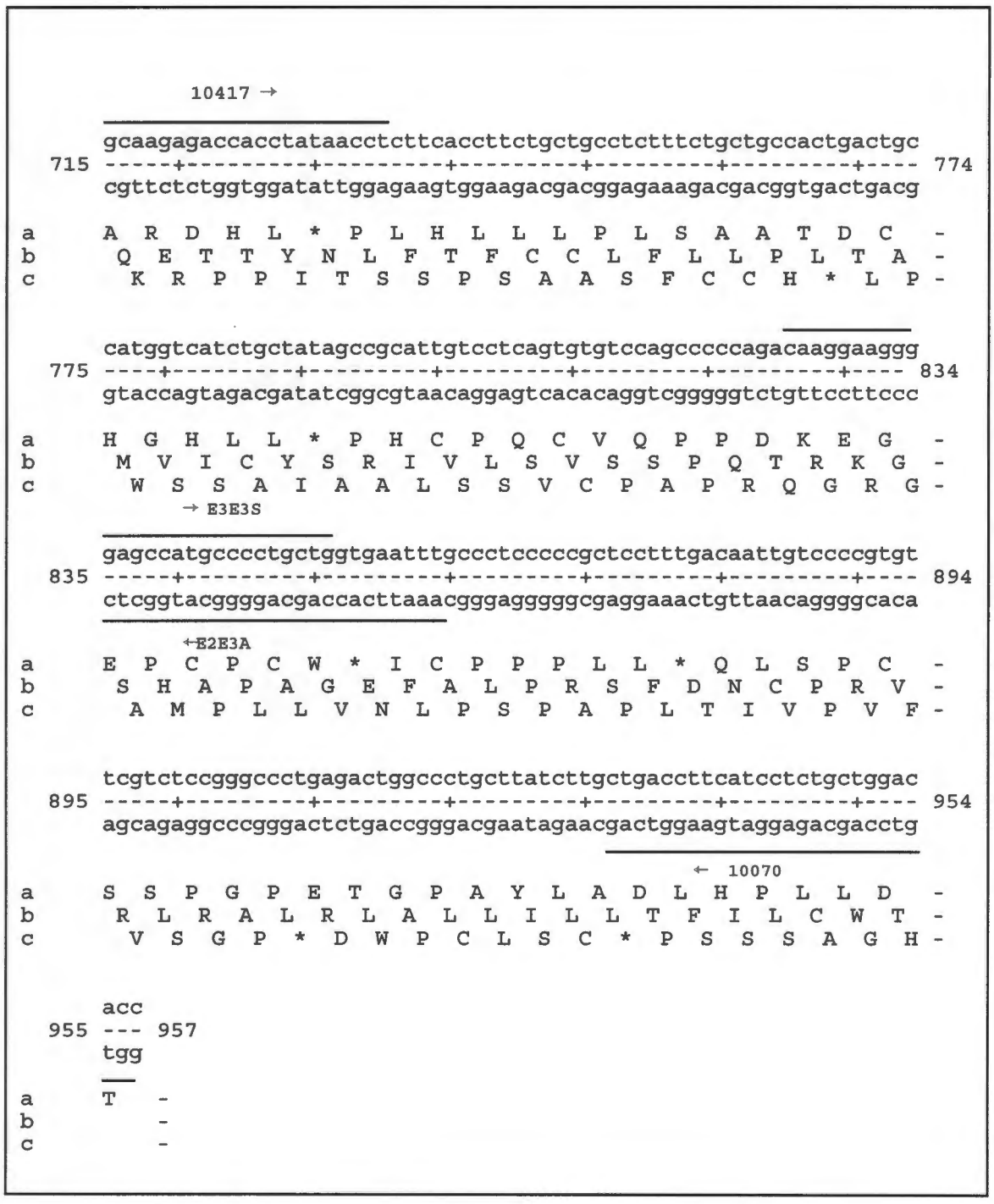


Figure 24 DNA sequence and protein translation of the processed human type II GnRHR from 10417 to 10070. Frame b corresponds to the correct frame. The sequence of the primers is underlined. The sequence was obtained by sequencing 3 independent clones.

To check for the presence of processed transcript in our available cDNA's, PCR was performed with primers JHE2E3A and 10417 (located in EC2 in exon 2). Processed RNA would thus amplify a PCR product of 144 bp, which is 430 bp (size of intron 2) smaller than that obtained for unprocessed RNA (Figure 25). PCR was performed on the available human cDNA's from a total of 28 different human tissues and cell lines. The results showed the presence of a ~130 bp product in almost all the cDNAs, which is indicative of the absence of an intron (Figure 26). The products were also checked by Southern blotting, probing the membrane with an internally nested oligo (10349) (Figure 27). The size of positives on the autoradiograph was consistent with the size of major bands seen on the EtBr-stained agarose gel at 130 bp. In appears from Southern blotting that the expression levels of T47D, fetal brain, placental, pituitary, and testis is high, and that the kidney has a very low signal. However, no accurate quantification of relative levels of expression can be deduced from this PCR reaction.

To confirm our results showing the presence of processed RNA, the PCR was repeated with a different set of primers, i.e. JHE2E3S and 10068 (located in TM7 in exon 3) (Figure 25). PCR was repeated on all 40 different cDNA samples. The products were checked by agarose gel electrophoresis (Figure 28), as well as by Southern blotting, probing the membrane with an internally nested oligo (10350)(Figure 29). The sizes of the positives on the autoradiograph were consistent with the size of major bands seen on the EtBr-stained agarose gel at 350 bp, confirming the presence of processed intron-less RNA for the human type II GnRHR in almost all human tissues tested. Although this strategy did confirm the presence of processed intron-less RNA, we were not able to distinguish between "sense" and "antisense" RNA transcripts.

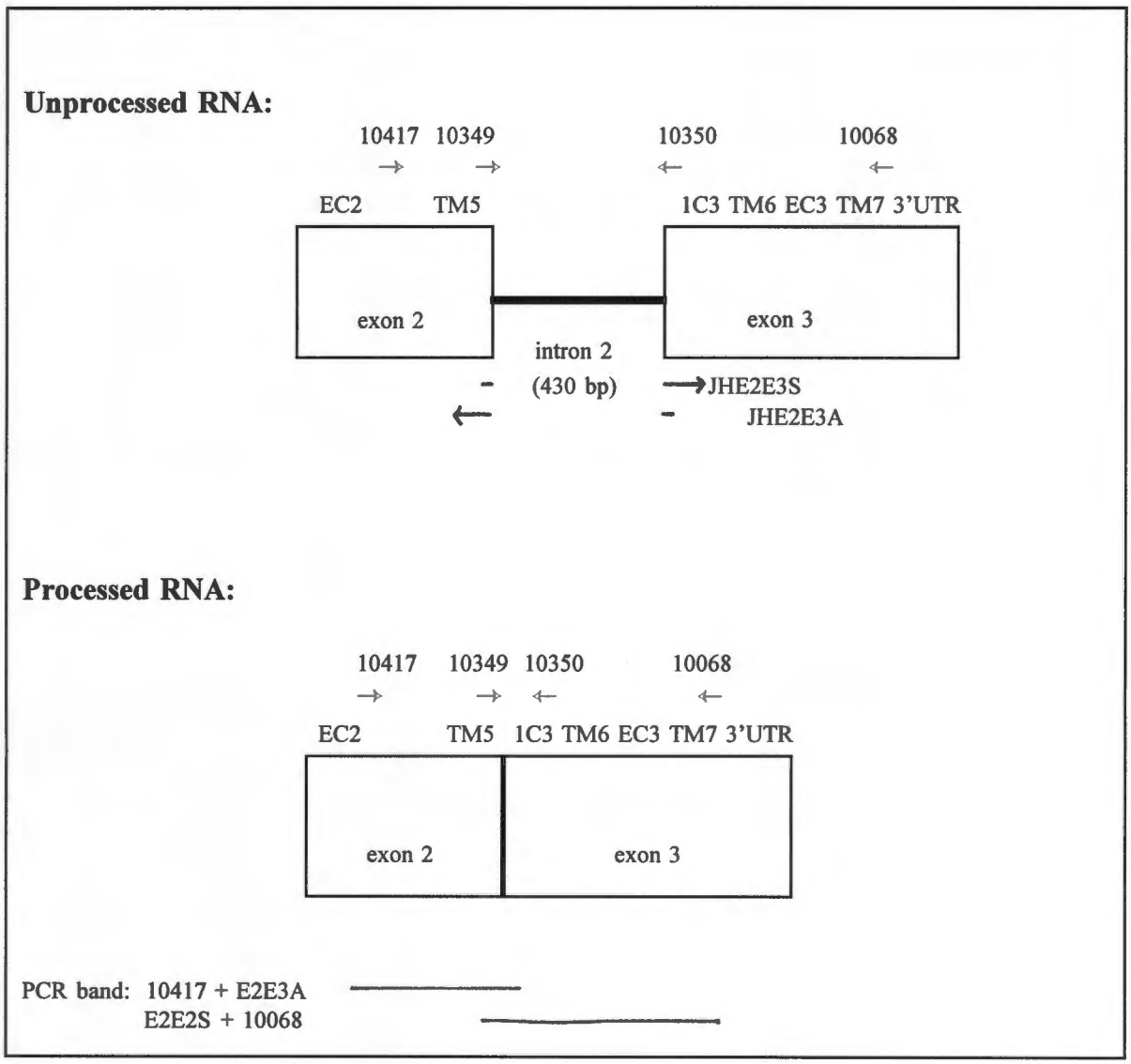


Figure 25 Schematic illustrating the PCR strategy used to selectively amplify intron-less RNA, by use of PCR primers (JHE2E3S & JHE2E3A) designed across the exon2/exon3 boundary determined for the human type II GnRHR. This strategy did not distinguish between "sense" and "antisense" RNA transcripts. PCR band expected with 10417 + JHE2E3A = 144 bp, and JHE2E3S + 10068 = 337 bp for processed (intron-less) type II GnRHR cDNA. No PCR band was expected for unprocessed intron-containing cDNA (3' end of JHE2E3A only hybridised to 5 bp, and 3' end JHE2E3S only to 9 bp).

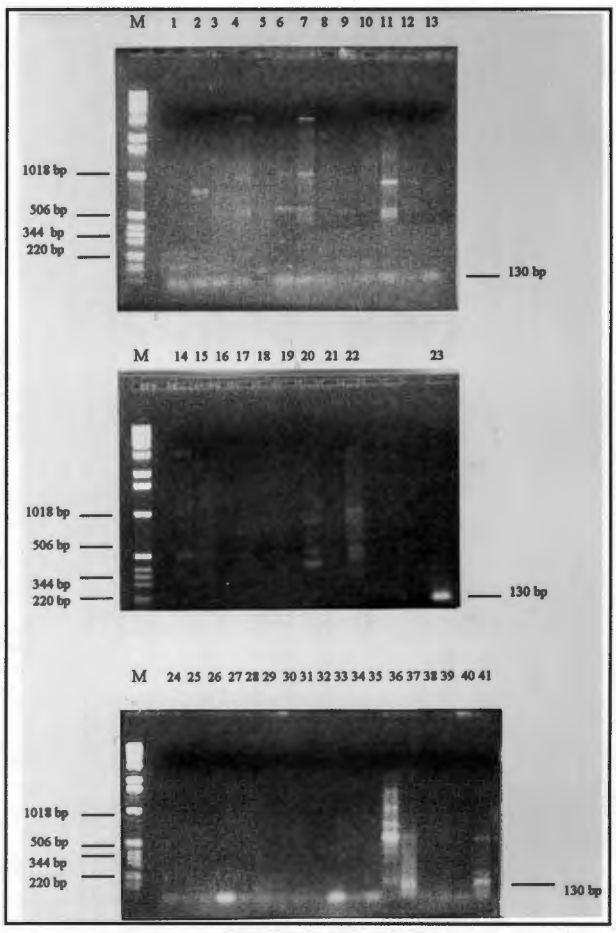


Figure 26 EtBr-stained agarose gel of PCR performed on various human cDNA's with primer pair JHE2E3A and 10417. The 130 bp band of the expected size that was obtained is marked on the figure. 1 kb ladder was used as a size marker (lane M). In some of the samples the 130 bp band is not visible in the photograph, but was faintly visible by eye.

(1) T47D (2) prostate (3) bone marrow (4) uterus (5) hvvec 2 (6) testes (7) retina (8) insulinoma (9) MCF-7 (10) fetal brain (11) thyroid (12) lymphnode (13) spleen (14) placenta (15) daudi (16) small intestine (17) kidney (18) HUH-7 (19) adult brain (20) $CaCO_2$ (21) liver (22) uterus (23) positive control (10417/10070 cloned cDNA) (24) pons (25) pons (26) pons (27) pons (28) medulla (29) medulla (30) medulla (31) midbrain (32) midbrain (33) midbrain (34) pituitary (35) hypothalamus (36) pBMc1 (37) testes (38) hypothalamus (39) liver (40) pituitary (41) lung.

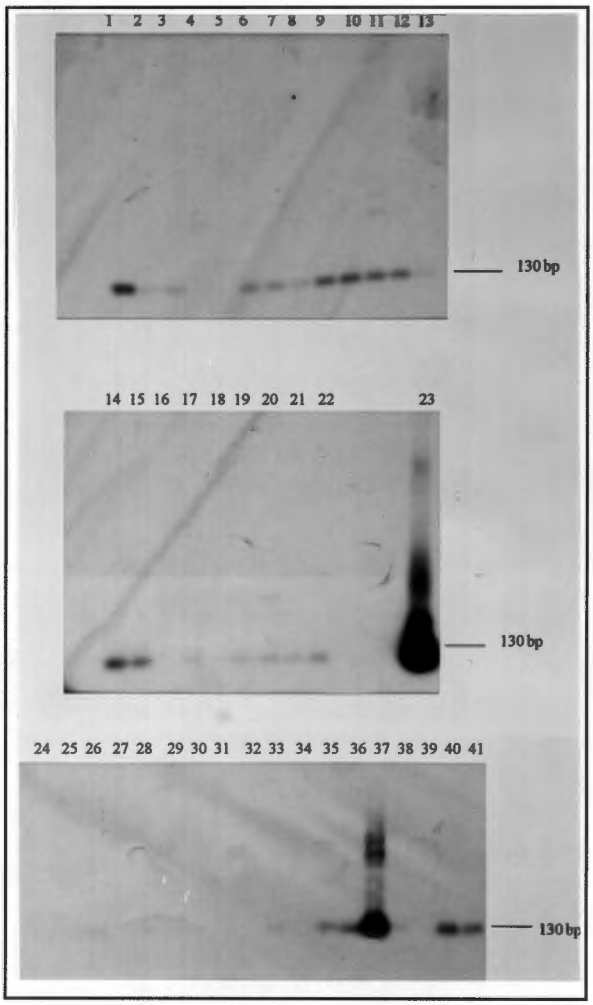


Figure 27 Autoradiographs of Southern blots performed on the gels in Figure 26, probing with 10349. The presence of a band of 130 bp containing part of processed intron-less type II GnRHR cDNA in various human tissues is marked on the figure. Lanes as for Figure 26.

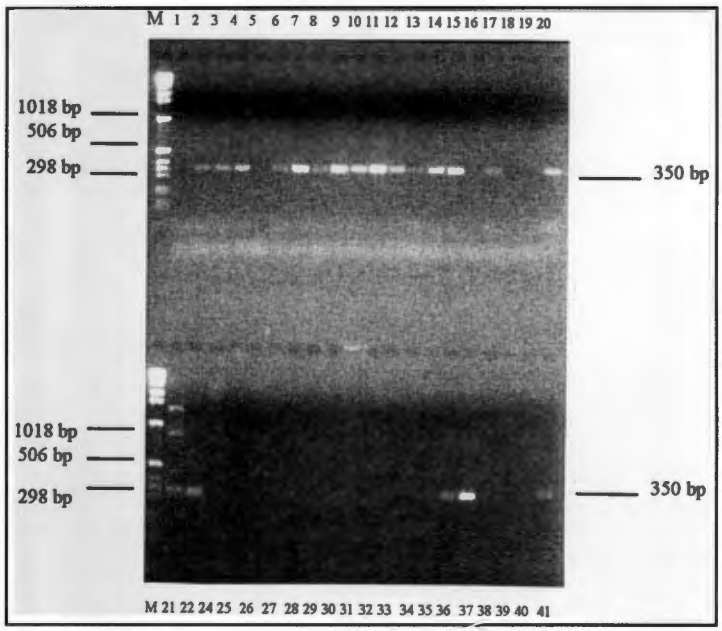


Figure 28 Products of PCR performed on human cDNA's with primer pair JHE2E3S + 10068 were checked on a 1.5 % EtBr-stained agarose gel. A very clear ~350 bp band, indicated by arrows, is visible in almost all of the human tissues and cell lines tested, indicating the presence of "processed" intron-less cDNA. 1 kb ladder was used as a size marker (lane M).

(1) T47D (2) prostate (3) bone marrow (4) uterus (5) hvvec2 (6) testes (7) retina (8) insulinoma (9) MCF-7 (10) fetal brain (11) thyroid (12) lymphnode (13) spleen (14) placenta (15) daudi (16) small intestine (17) kidney (18) HUH-7 (19) adult brain (20) $CaCO_2$ (21) liver (22) uterus (24) pons (25) pons (26) pons (27) pons (28) medulla (29) medulla (30) medulla (31) midbrain (32) midbrain (33) midbrain (34) pituitary (35) hypothalamus (36) pBMc1 (37) testes (38) hypothalamus (39) liver (40) pituitary (41) lung.

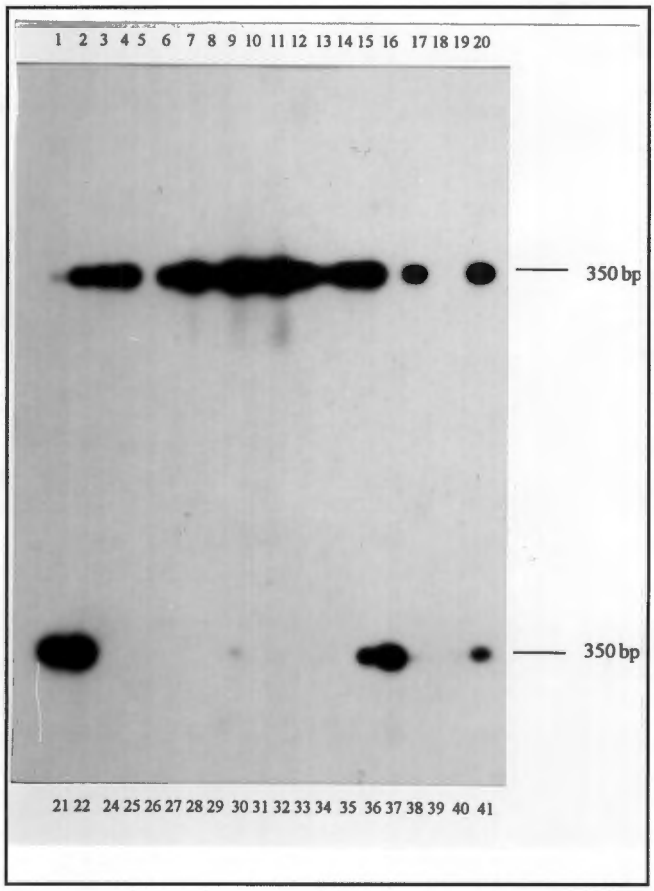


Figure 29 Autoradiograph of a Southern blot performed on the gel in Figure 28. The membrane was probed with 10350. The lines indicate the presence of a positive signal at 350 bp, confirming the presence of processed intron-less cDNA for the human type II GnRHR in almost all human tissues tested. Lanes as for Figure 28.

4.3 Generation of a testes cDNA clone containing type II human GnRHR from 10417 to 10071

PCR was performed on human testes cDNA with primer pair 10351 and 10071 to isolate a fragment containing sequences which overlapped on the 3' end with the fragment obtained from 10417 + 10070. The two fragments were then joined by use of restriction enzymes. This fragment was prepared in order to construct a full length cDNA. We now had a clone that contained part of putative exon 2 and putative exon 3 of a possible type II GnRHR gene, but lacking putative exon 1. Attempts to obtain exon 1 are described below.

4.4 Attempts to obtain exon 1 of human type II GnRHR cDNA

Additional data base searches with exon 2 sequences did not reveal any exon 1-like sequences for the type II GnRHR (J.P. Hapgood, personal communication). It was decided to perform 5'RACE in order to try and clone the full length receptor. The primer JHE2E3A (see section 4.2) was exploited in our strategy.

Different strategies were followed, to ensure that we had as many options as possible for cloning the full-length receptor. cDNA was prepared using several different RNA priming methods, i.e. priming with gene specific primers, random priming, and dT priming. The 5'RACE was performed on cDNA's from several tissues, based on the results of section 4.2. Two different 5'RACE methods were used, namely 5'RACE using the Gibco BRL Kit (Figure 30), and Marathon 5'RACE (Clontech Kit). Marathon RACE reactions are capable of amplifying much larger templates than can be amplified with conventional RACE methods, since the protocol uses enzyme mixes designed for long distance PCR (LD PCR). The marathon RACE reaction uses a combination of proofreading and non-proofreading Taq polymerases designed to obtain high fidelity PCR. Also, Taq Start Antibody provides an automatic form of hot start PCR. The essential features of the two different RACE methods are summarized in Figure 31. Where possible, we often reamplified the original PCR products with internally nested gene specific primers, ensuring that the products we amplified were as specific as possible.

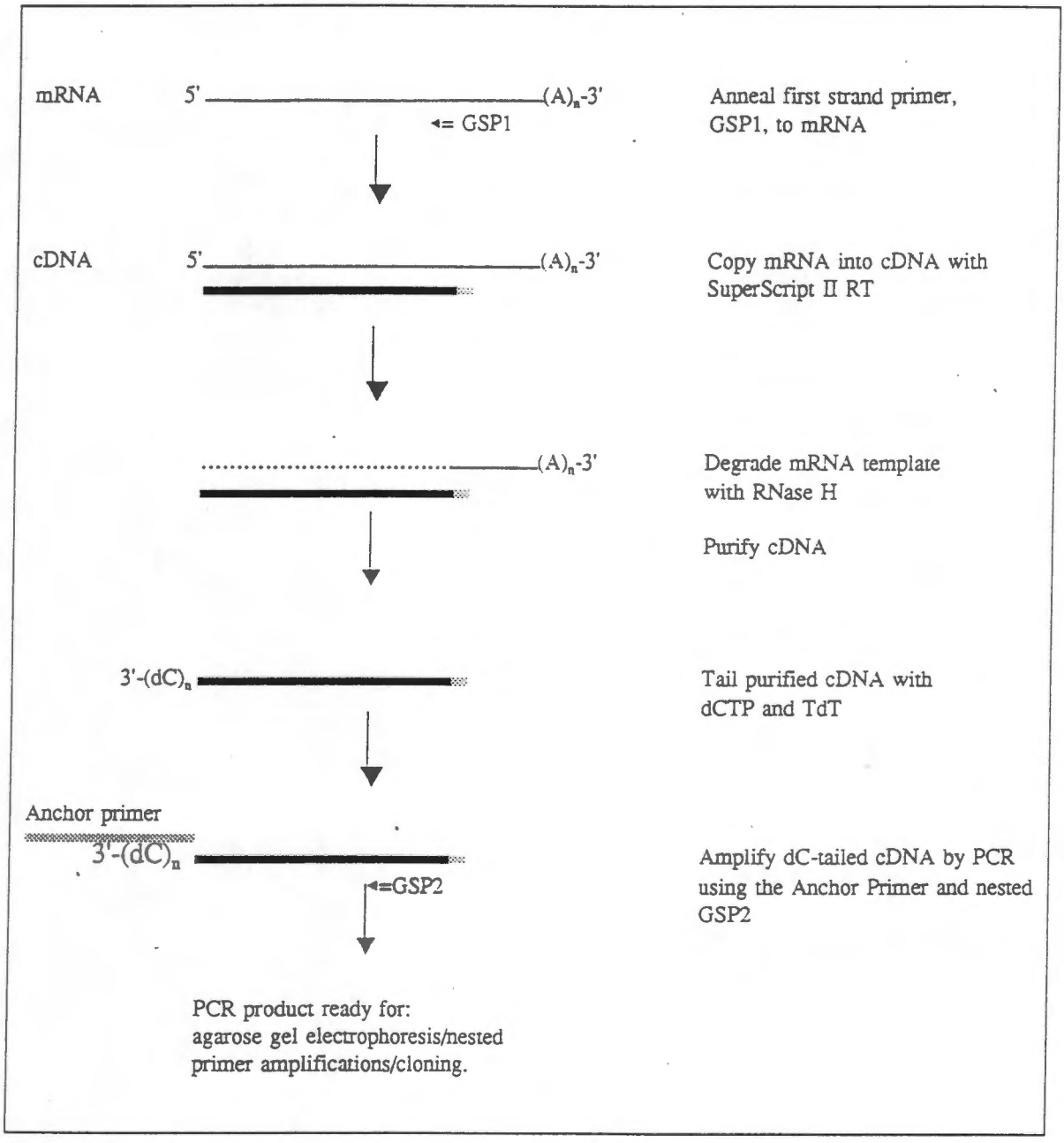


Figure 30 Overview of the Gibco BRL 5'RACE Protocol.
GSP = gene specific primer, RT = reverse transcriptase, TdT = terminal deoxytransferase

5'RACE using Gibco BRL Kit	Marathon 5'RACE
● Uses single-stranded cDNA synthesis (We tried priming with GSPs, random priming, and dT priming)	● Uses double-stranded cDNA (dT primed)
● dC-tailed (3') with TdT	● Adaptor ligation (T4 DNA ligase)
● Amplifies target cDNA with anchor primer (sense) and GSP (antisense)	● Amplifies target cDNA with adaptor primer containing dTs and GSPs

Figure 31 Summary of the essential features of the two 5'RACE methods. GSP = gene specific primer.

We performed marathon 5'RACE with the JHE2E3A primer (designed across the putative exon 2/exon 3 boundary), in order to selectively amplify intron-less cDNA in the presence of an excess of intron-containing cDNA's. Marathon 5'RACE on testes cDNA produced a fragment of ~3.5 kb visible by EtBr-staining on an analytical agarose gel (Figure 32). Southern blotting with an internally nested oligo (10242) produced a signal consistent with the size of the major band seen on the analytical gel at ~3.5 kb (Figure 33). This fragment was isolated by electrophoresis on a low melting point agarose gel excision and elution (See section 5, Materials and Methods). After purification, the DNA fragment was reamplified with different combinations of internally nested oligo's, i.e. AP1 + JHE2E3A, AP1 + JHE2, AP2 + JHE2E3A, and AP2 + JHE2 (AP = Marathon adaptor primer). Upon analysis on an analytical agarose gel, a ~2.5 kb band was detected, and not 3.5 kb as expected (Figure 31).

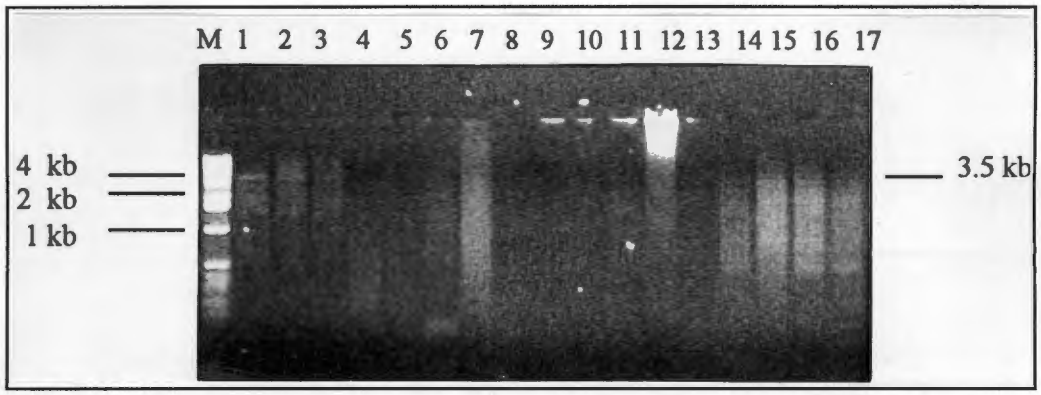


Figure 32 EtBr-stained agarose gel of the products of marathon 5'RACE performed with JHE2E3A. A faint 3.5 kb band (indicated by line) is visible for testes. 1 kb ladder was used as a size marker (lane M).

(1) - (9) Testes cDNA (different preparations), (10) T47D, (11) testes, (12) MCF-7, (13) fetal brain, (14) thyroid, (15) placenta, (16) daudi, (17) lymphnode

Figure 33 Autoradiograph of a Southern blot performed on the gel in Figure 32. The membrane was probed with 10242. A clear signal can be detected at ~3.5 kb for testes, as well as for some of the other samples.

(1) - (9) Testes (different preparations), (10) T47D, (11) testes, (12) MCF-7, (13) fetal brain, (14) thyroid, (15) placenta, (16) daudi, (17) lymphnode.

Figure 34 Reamplification of the 3.5 kb fragment visible in Figure 32 with
(1) AP1 + JHE2E3A
(2) AP1 + JHE2
(3) AP2 + JHE2E3A
(4) AP2 + JHE2.
A clear band of ~2.5 kb (indicated by line) is visible by EtBr-staining of the agarose gel. 1 kb ladder was used as a size marker (lane M).

This led us to suspect that the 3.5 kb band visible on the Southern blot was a minor component of the 3.5 kb PCR product detected by EtBr staining. We therefore attempted to isolate the minor 3.5 kb PCR product by analysing several clones obtained by cloning DNA PCR products in the region of the 3.5 kb band isolated from low melting point agarose.

Different methods were used for analysis of the colonies, namely:

a) Direct PCR screening of colonies with primers located in the vector, followed by Southern blotting, probing with an internally nested oligo.

b) Colony blots, probing with an internally nested oligo. This method allows us to probe a great many colonies at once. In excess of 150 colonies were screened by this method.

No positive colonies were detected in any of the Southern blots performed.

Additional primer pairs were tried in the 5'RACE reaction, i.e. first reaction with 10350 and adaptor primer, followed by nested PCR with JHE2E3A and adaptor primer, using the marathon RACE kit. cDNA from testes was used as template. Electrophoresis on an agarose gel and staining with EtBr revealed a faint 1.6 kb PCR product on the second reaction (data not shown). Southern blotting, probing with an internally nested oligo (10242) produced a strong signal corresponding to the band detected on the gel at 1.6 kb (Figure 35). The fragment was isolated by electrophoresis on a low melting point agarose gel, followed by purification of the fragment from the agarose (See section 5.2, Materials and Methods). After cloning into pMOS and PCR screening of colonies (Section 7, Materials and Methods), a Southern blot was performed, probing with an oligo designed to putative intron 2 sequences

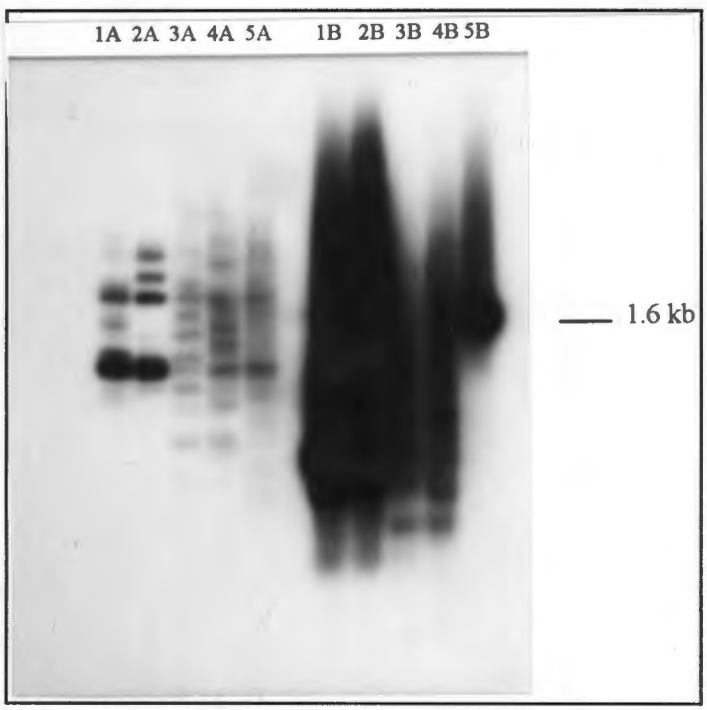


Figure 35 PCR was performed on testes cDNA with AP1 + 10350 (A). A second reaction was performed with internally nested primers AP2 + JHE2E3A (B). The figure shows an autoradiograph of a Southern blot performed on the EtBr-stained agarose gel, probing with internally nested oligo 10242. A strong signal (marked on the figure) corresponding to 1.6 kb, is visible. Lanes 2A to 5A, and 2B to 5B shows the result of reactions of different batches of testes cDNA, and lanes 1A and 1B shows the results of reactions of T47D cells (see section 4.5)

(JHi2S). No positive signals were detected on the autoradiograph, indicating that the 1.6 kb fragment did not contain any intron 2 sequences (data not shown).

In order to obtain sufficient DNA of the 1.6 kb fragment for cloning, the gel-purified fragment was reamplified with internally nested oligo's (AP2 + JHE2E3A, or AP2 + JHE2). This produced very clear bands of ~1.6 kb on an EtBr-stained analytical agarose gel (Figure 36). The 1.6 kb fragments were isolated from low melting point agarose gels, cloned into a vector, and transformed into bacteria. Analysis of the clones by colony PCR screening, followed by Southern blot, probing with an internally nested oligo (JHE2) produced a very strong signal at 1.6 kb for several of the clones, corresponding to the size of the fragments detected on the agarose gel (data not shown). Positive colonies on Southern blots were subjected to plasmid preparations to obtain sufficient DNA for analysis by DNA sequencing. Figure 37 shows the sequence of the full length 1.6 kb 5'RACE product. The sequence was confirmed by sequencing several individual clones, in both directions, with several different primers. The sequencing results showed that the open reading frame 5' to putative exon 2 is not in frame with the open reading frame in putative exon 2, and a stop site occurs between the two open reading frames. The open reading frame 5' to putative exon 2 has no similarity to exon 1 of the human type I GnRHR, or to any other known receptor or protein. The sequencing data was analysed by doing homology searches on data bases on both the amino acid level, and the DNA level.

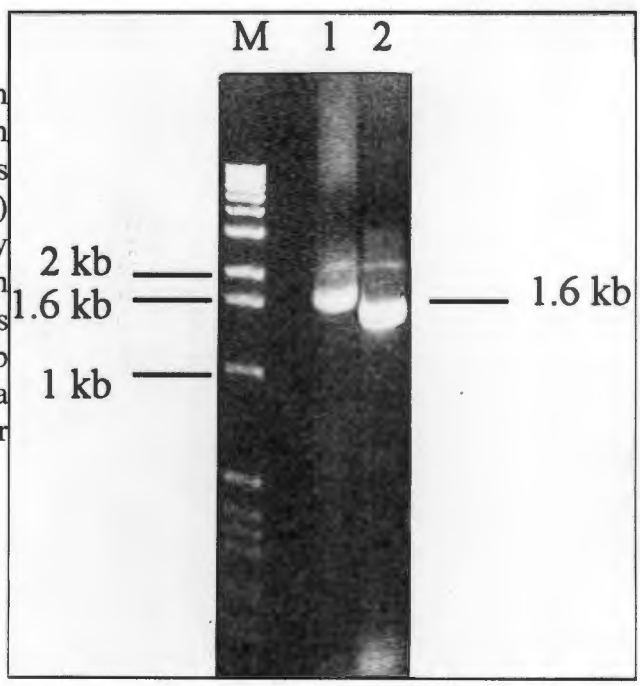


Figure 36 Reamplification of the 1.6 kb band visible in Figure 35 with primer pairs (1) AP2 + JHE2E3A, and (2) AP2 + JHE2 produced very clear bands of ~ 1.6 kb on an EtBr-stained agarose gel, as indicated by the line. 1 kb ladder was used as a molecular weight marker (lane M).

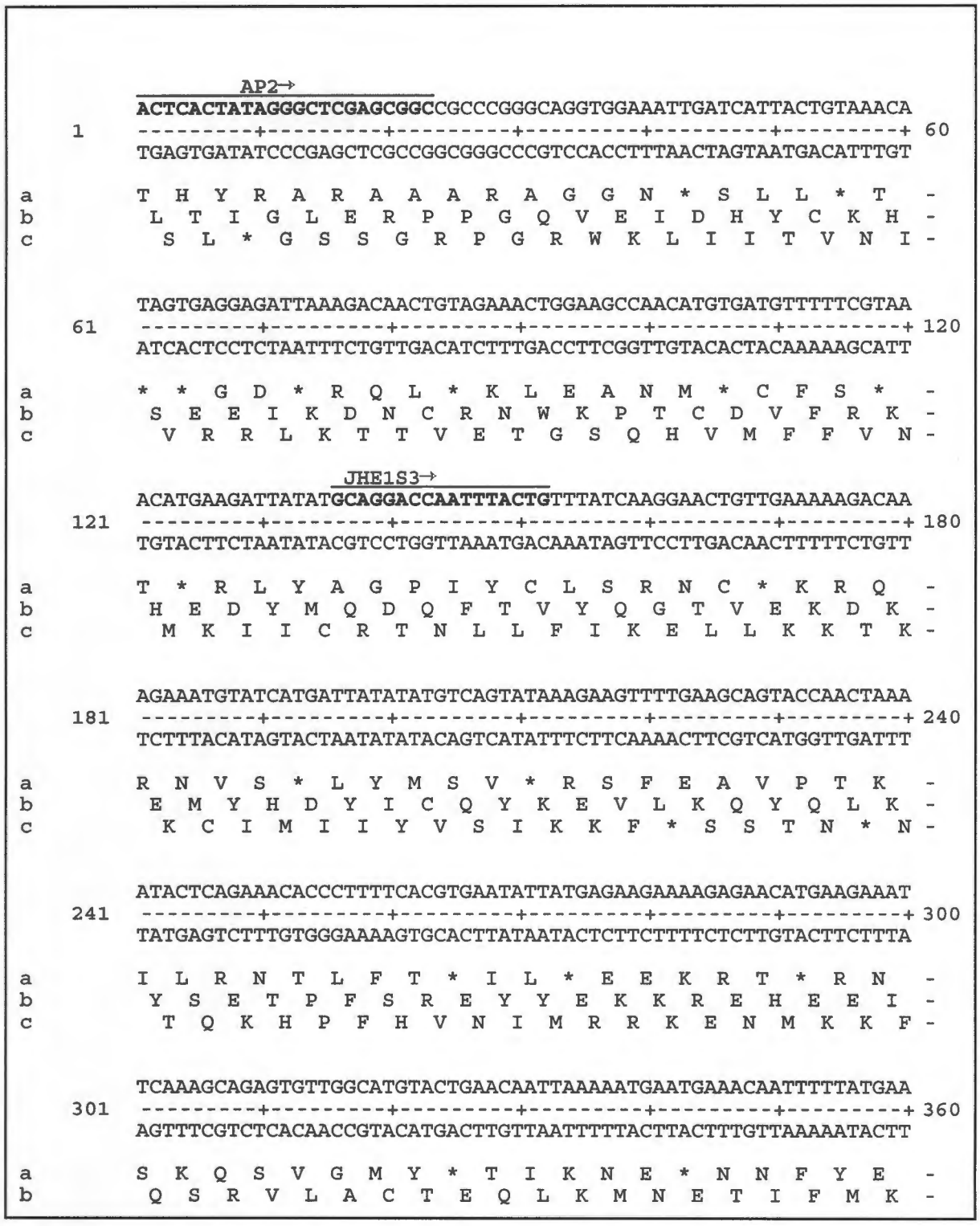
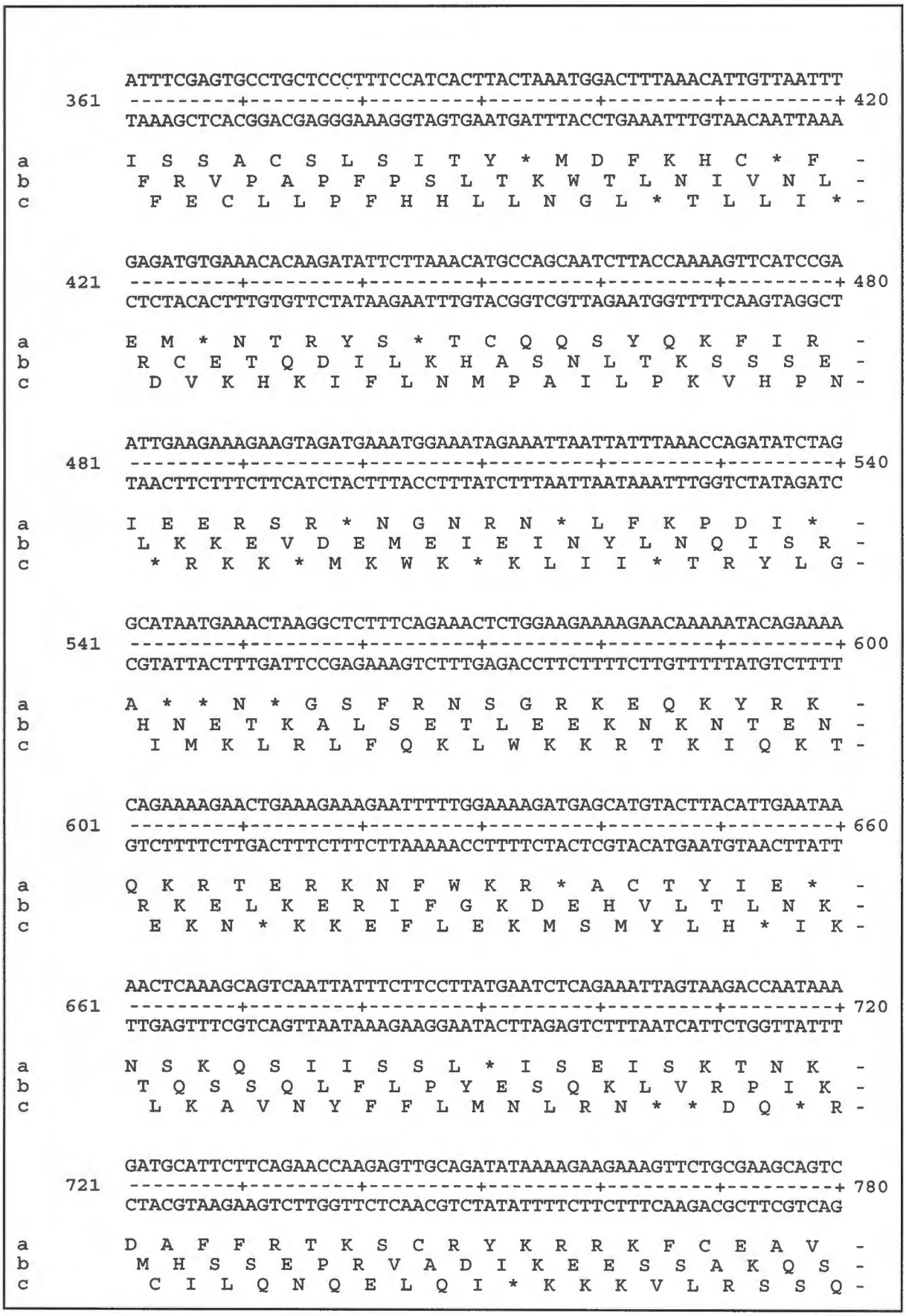


Figure 37 DNA and amino acid sequence of the full length 1.6 kb 5'RACE product from testes (visible in Figure 36). The position of the primers used in the 5'RACE reactions are indicated in bold and by solid lines. The positions of putative extracellular loop 2 (EC2), transmembrane domain 5 (TM5), and intracellular loop 3 (IC3) are marked (see pages 82 + 83). Translation frame c is the correct reading frame for EC2 to IC3, i.e. putative exons 2 and 3.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361
TAAAGCTCACGGACGAGGGAAAGGTAGTGAATGATTTACCTGAAATTTGTAACAATTAAA
420
a I S S A C S L S I T Y * M D F K H C * F -
b F R V P A P F P S L T K W T L N I V N L -
c F E C L L P F H H L L N G L * T L L I * -
GAGATGTGAAACACAAGATATTCTTAAACATGCCAGCAATCTTACCAAAAGTTCATCCGA
421
CTCTACACTTTGTGTTCTATAAGAATTTGTACGGTCGTTAGAATGGTTTTCAAGTAGGCT
480
a E M * N T R Y S * T C Q Q S Y Q K F I R -
b R C E T Q D I L K H A S N L T K S S S E -
c D V K H K I F L N M P A I L P K V H P N -
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481
TAACTTCTTTCTTCATCTACTTTACCTTTATCTTTAATTAATAAATTTGGTCTATAGATC
540
a I E E R S R * N G N R N * L F K P D I * -
b L K K E V D E M E I E I N Y L N Q I S R -
c * R K K * M K W K * K L I I * T R Y L G -
GCATAATGAAACTAAGGCTCTTTCAGAAACTCTGGAAGAAAAGAACAAAAATACAGAAAA
541
CGTATTACTTTGATTCCGAGAAAGTCTTTGAGACCTTCTTTTCTTGTTTTTATGTCTTTT
600
a A * * N * G S F R N S G R K E Q K Y R K -
b H N E T K A L S E T L E E K N K N T E N -
c I M K L R L F Q K L W K K R T K I Q K T -
CAGAAAAGAACTGAAAGAAAGAATTTTTGGAAAAGATGAGCATGTACTTACATTGAATAA
601
GTCTTTTCTTGACTTTCTTTCTTAAAAACCTTTTCTACTCGTACATGAATGTAACTTATT
660
a Q K R T E R K N F W K R * A C T Y I E * -
b R K E L K E R I F G K D E H V L T L N K -
c E K N * K K E F L E K M S M Y L H * I K -
AACTCAAAGCAGTCAATTATTTCTTCCTTATGAATCTCAGAAATTAGTAAGACCAATAAA
661
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720
a N S K Q S I I S S L * I S E I S K T N K -
b T Q S S Q L F L P Y E S Q K L V R P I K -
c L K A V N Y F F L M N L R N * * D Q * R -
GATGCATTCTTCAGAACCAAGAGTTGCAGATATAAAAGAAGAAAGTTCTGCGAAGCAGTC
721
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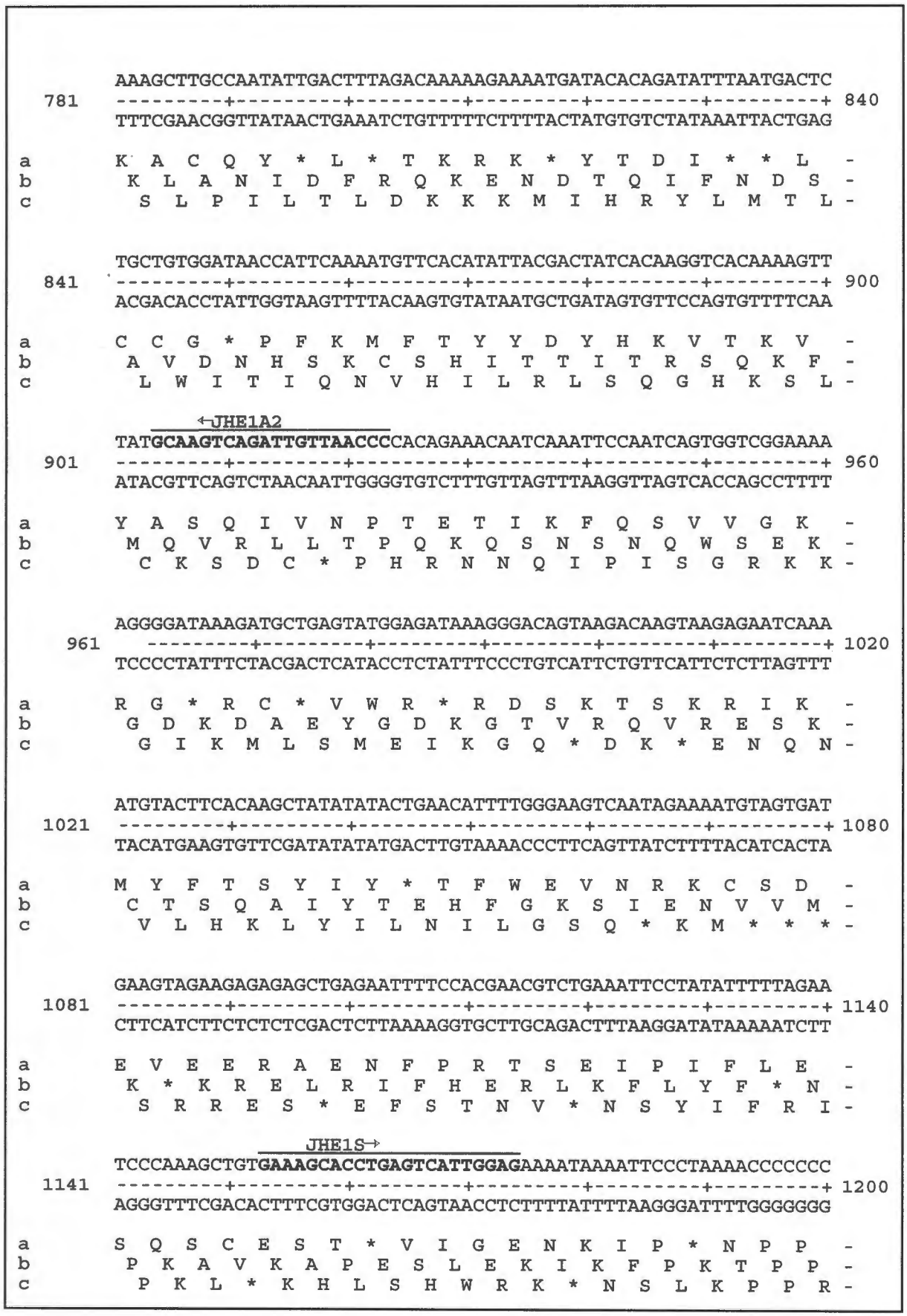
780
a D A F F R T K S C R Y K R R K F C E A V -
b M H S S E P R V A D I K E E S S A K Q S -
c C I L Q N Q E L Q I * K K K V L R S S Q -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781
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840
a K A C Q Y * L * T K R K * Y T D I * * L -
b K L A N I D F R Q K E N D T Q I F N D S -
c S L P I L T L D K K K M I H R Y L M T L -
TGCTGTGGATAACCATTCAAAATGTTCACATATTACGACTATCACAAGGTCACAAAAGTT
841
ACGACACCTATTGGTAAGTTTTACAAGTGTATAATGCTGATAGTGTTCCAGTGTTTTCAA
900
a C C G * P F K M F T Y Y D Y H K V T K V -
b A V D N H S K C S H I T T I T R S Q K F -
c L W I T I Q N V H I L R L S Q G H K S L -
←JHE1A2
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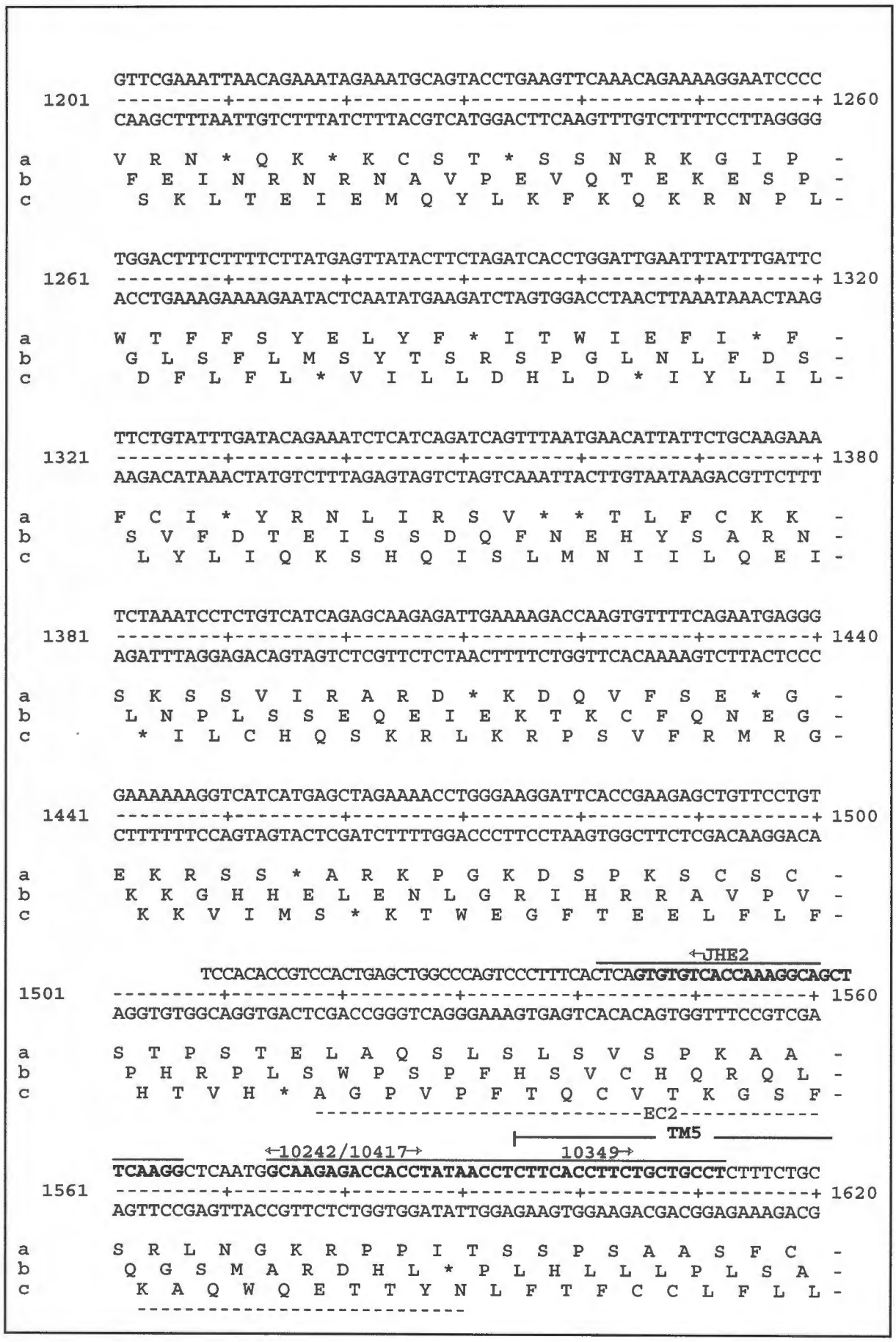
901
ATACGTTCAGTCTAACAATTGGGGTGTCTTTGTTAGTTTAAGGTTAGTCACCAGCCTTTT
960
a Y A S Q I V N P T E T I K F Q S V V G K -
b M Q V R L L T P Q K Q S N S N Q W S E K -
c C K S D C * P H R N N Q I P I S G R K K -
AGGGGATAAAGATGCTGAGTATGGAGATAAAGGGACAGTAAGACAAGTAAGAGAATCAAA
961
TCCCCTATTTCTACGACTCATACCTCTATTTCCCTGTCATTCTGTTCATTCTCTTAGTTT
1020
a R G * R C * V W R * R D S K T S K R I K -
b G D K D A E Y G D K G T V R Q V R E S K -
c G I K M L S M E I K G Q * D K * E N Q N -
ATGTACTTCACAAGCTATATATACTGAACATTTTGGGAAGTCAATAGAAAATGTAGTGAT
1021
TACATGAAGTGTTCGATATATATGACTTGTAAAACCCTTCAGTTATCTTTTACATCACTA
1080
a M Y F T S Y I Y * T F W E V N R K C S D -
b C T S Q A I Y T E H F G K S I E N V V M -
c V L H K L Y I L N I L G S Q * K M * * * -
GAAGTAGAAGAGAGAGCTGAGAATTTTCCACGAACGTCTGAAATTCCTATATTTTTAGAA
1081
CTTCATCTTCTCTCTCGACTCTTAAAAGGTGCTTGCAGACTTTAAGGATATAAAAATCTT
1140
a E V E E R A E N F P R T S E I P I F L E -
b K * K R E L R I F H E R L K F L Y F * N -
c S R R E S * E F S T N V * N S Y I F R I -
JHE1S→
TCCCAAAGCTGTGAAAGCACCTGAGTCATTGGAGAAAATAAAATTCCCTAAAACCCCCCC
1141
AGGGTTTCGACACTTTCGTGGACTCAGTAACCTCTTTTATTTTAAGGGATTTTGGGGGGG
1200
a S Q S C E S T * V I G E N K I P * N P P -
b P K A V K A P E S L E K I K F P K T P P -
c P K L * K H L S H W R K * N S L K P P R -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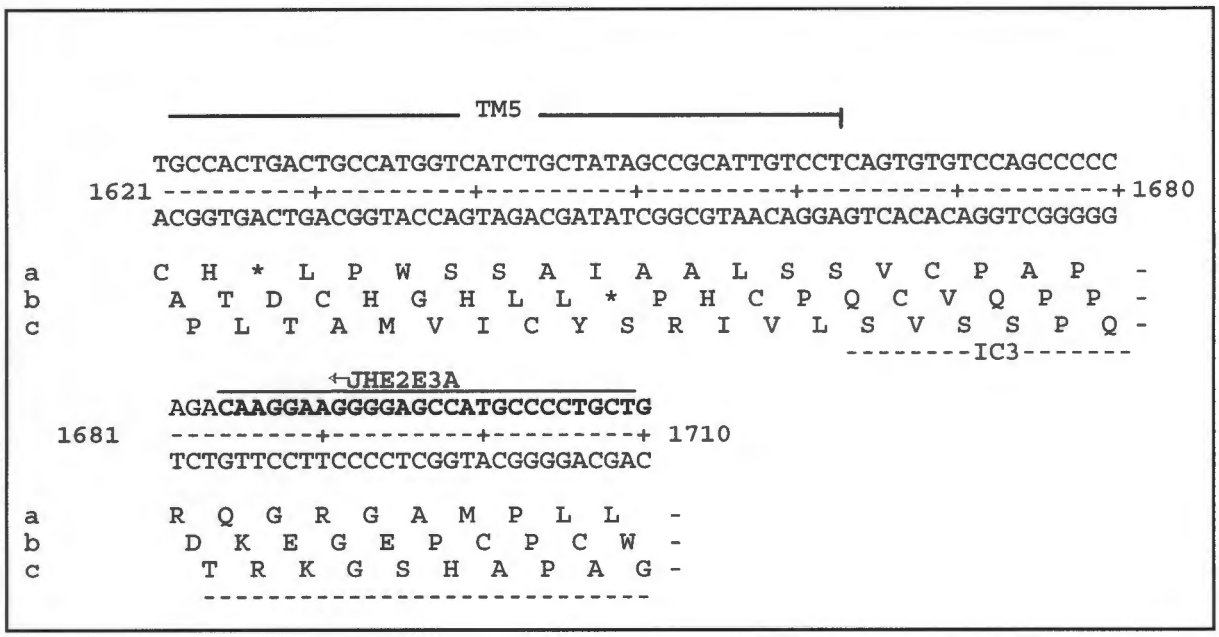
1201
1260
CAAGCTTTAATTGTCTTTATCTTTACGTCATGGACTTCAAGTTTGTCTTTTCCTTAGGGG
a V R N * Q K * K C S T * S S N R K G I P -
b F E I N R N R N A V P E V Q T E K E S P -
c S K L T E I E M Q Y L K F K Q K R N P L -
TGGACTTTCTTTTCTTATGAGTTATACTTCTAGATCACCTGGATTGAATTTATTTGATTC
1261
1320
ACCTGAAAGAAAAGAATACTCAATATGAAGATCTAGTGGACCTAACTTAAATAAACTAAG
a W T F F S Y E L Y F * I T W I E F I * F -
b G L S F L M S Y T S R S P G L N L F D S -
c D F L F L * V I L L D H L D * I Y L I L -
TTCTGTATTTGATACAGAAATCTCATCAGATCAGTTTAATGAACATTATTCTGCAAGAAA
1321
1380
AAGACATAAACTATGTCTTTAGAGTAGTCTAGTCAAATTACTTGTAATAAGACGTTCTTT
a F C I * Y R N L I R S V * * T L F C K K -
b S V F D T E I S S D Q F N E H Y S A R N -
c L Y L I Q K S H Q I S L M N I I L Q E I -
TCTAAATCCTCTGTCATCAGAGCAAGAGATTGAAAAGACCAAGTGTTTTCAGAATGAGGG
1381
1440
AGATTTAGGAGACAGTAGTCTCGTTCTCTAACTTTTCTGGTTCACAAAAGTCTTACTCCC
a S K S S V I R A R D * K D Q V F S E * G -
b L N P L S S E Q E I E K T K C F Q N E G -
c * I L C H Q S K R L K R P S V F R M R G -
GAAAAAAGGTCATCATGAGCTAGAAAACCTGGGAAGGATTCACCGAAGAGCTGTTCCTGT
1441
1500
CTTTTTTCCAGTAGTACTCGATCTTTTGGACCCTTCCTAAGTGGCTTCTCGACAAGGACA
a E K R S S * A R K P G K D S P K S C S C -
b K K G H H E L E N L G R I H R R A V P V -
c K K V I M S * K T W E G F T E E L F L F -
←JHE2
TCCACACCGTCCACTGAGCTGGCCCAGTCCCTTTCACTCAGTGTGTCACCAAAGGCAGCT
1501
1560
AGGTGTGGCAGGTGACTCGACCGGGTCAGGGAAAGTGAGTCACACAGTGGTTTCCGTCGA
a S T P S T E L A Q S L S L S V S P K A A -
b P H R P L S W P S P F H S V C H Q R Q L -
c H T V H * A G P V P F T Q C V T K G S F -
EC2
TM5
←10242/10417→
10349→
TCAAGGCTCAATGGCAAGAGACCACCTATAACCTCTTCACCTTCTGCTGCCTCTTTCTGC
1561
1620
AGTTCCGAGTTACCGTTCTCTGGTGGATATTGGAGAAGTGGAAGACGACGGAGAAAGACG
a S R L N G K R P P I T S S P S A A S F C -
b Q G S M A R D H L * P L H L L L P L S A -
c K A Q W Q E T T Y N L F T F C C L F L L -



4.5 Attempts to clone the full-length receptor from tissues other than testes

5'RACE, using the Marathon kit, was performed with primer pair JHE2E3A + AP1 on cDNA from tissues other than testes, i.e. T47D, MCF-7, fetal brain, thyroid, placenta, daudi, and lymphnode. As found for testes (see section 4.4, Figure 33), a 3.5 kb band was also detected by Southern blotting, probing with 10242 (Figure 38). A strong signal was also detected at 1.6 kb in the T47D sample. Since attempts to clone the 3.5 kb testes fragment were unsuccessful, we decided to attempt to clone the 1.6 kb fragment detected in T47D. The fragment was isolated by electrophoresis on low melting point agarose, followed by purification of the DNA from the agarose. The 1.6 kb fragment was cloned into a vector, and transformed into bacteria. The colonies were analysed by colony PCR screening, followed by agarose gel electrophoresis with molecular weight markers. Bands on the EtBr-stained agarose gel corresponded with the expected size (i.e. 1.6 kb) of the insert (Figure 39). A Southern blot was performed on this gel, probing the membrane with an internally nested oligo (10242). No positive signals were detected on the autoradiograph (data not shown). Reamplification of the product isolated from the agarose gel also did not produce the expected results. To confirm that none of the colonies did contain the full-length receptor cDNA, colonies were screened by colony Southern blotting, allowing us to screen in excess of 150

colonies at once. Once again no positives were detected on the autoradiographs. We then decided to abandon further efforts on the T47D sample.

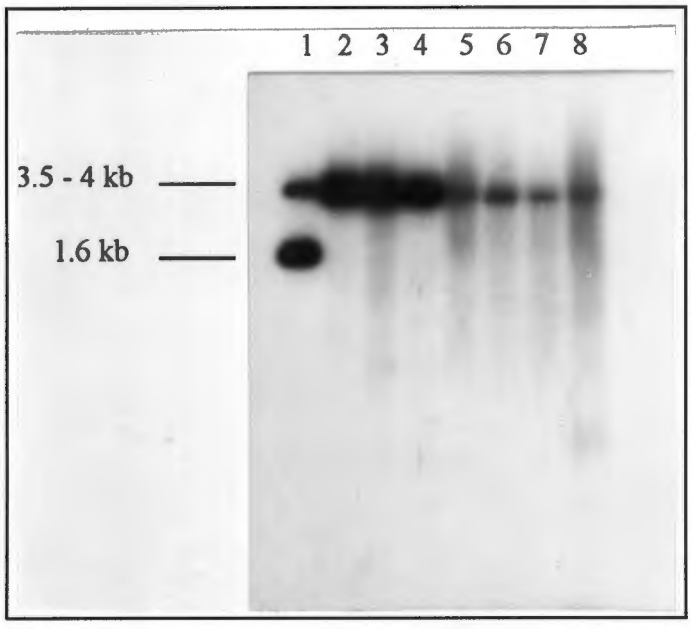


Figure 38 5'RACE was performed on different human tissues and cell lines with AP1 + JHE2E3A, followed by Southern blotting with 10242. A clear 3.5 kb band is visible in all the samples, but only T47D shows a 1.6 kb signal. Note that the particular testes RNA in this experiment did not show the 1.6 kb band, unlike the result shown in Figure 35.
(1) T47D, (2) testes, (3) MCF-7, (4) fetal brain, (5) thyroid, (6) placenta, (7) daudi, (8) lymphnode.

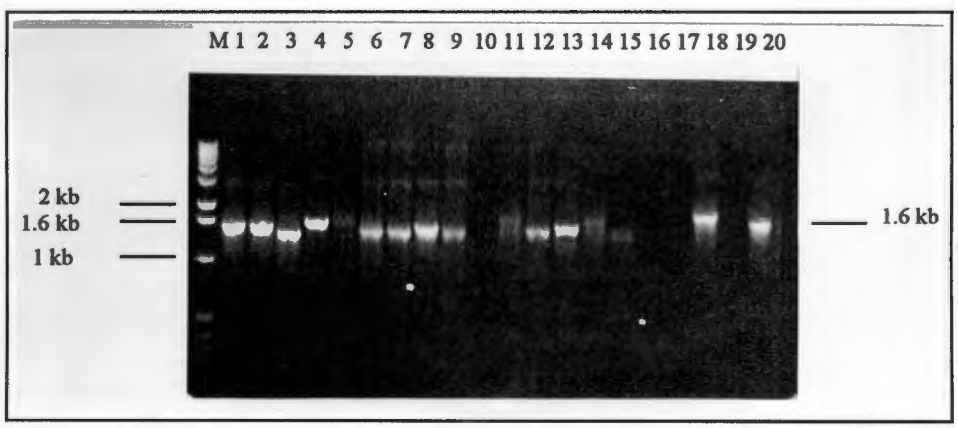


Figure 39 Colony PCR screening of 1.6 kb T47D colonies with pMOS polylinker primers T7 and U19. The arrow indicates the 1.6 kb band. Lanes 1-20 correspond to colony numbers. Lane M is marker.

5. ANALYSIS OF GENOMIC CLONES FOR HUMAN TYPE II GnRH RECEPTOR

P1 clones containing human genomic DNA inserts of ~70 kb were purchased from Genome Systems Inc. These were obtained by screening a human genomic DNA library with a PCR product generated with 10417 + 10070, i.e. putative exon 2/intron 2/exon 3 sequences. Three clones were obtained, i.e. P1A, P1B, and P1C.

The rationale for using these genomic clones was twofold:

a) To confirm that the type II GnRHR cDNA we cloned does exist in the human genome and is thus not an artifact.
b) It provided us with an alternative strategy to obtain exon 1 of the type II human GnRHR.

a) In an initial analysis of the genomic clones, we wanted to confirm the presence of putative exon 2/intron 2/exon 3 sequences found in the data base and in our cDNA sequences (minus intron). PCR was performed on clones P1A, P1B, and P1C with different combinations of gene specific primers (designed to the sequence found in the data base) for confirmation of the presence of exons 2 and 3. These primer pairs are 10063 + 10068 (IC 3 to TM 7), 10349 + 10070 (TM 5 to IC 3), and 10349 + 10068 (TM 5 to TM 7). Agarose gel electrophoresis (Figure 40) and Southern blotting (Figure 41) confirmed that the sequences isolated from cDNA containing exon 2 and exon 3 from TM 5 to TM 7 are present in the genomic DNA.

b) To look for the presence of exon 1, PCR was performed with different combinations of degenerate primers designed to the type I human GnRH receptor. These were 1S + 2A (designed to amplify TM1 - TM2), 1S + 3A (TM1 - TM3), and 1S + 10607 (TM1 -TM2) Agarose gel electrophoresis and Southern blotting confirmed that none of the primer pairs amplified a fragment indicative of exon 1. The positive controls (human genomic DNA) showed bands of the expected size on EtBr-stained agarose gels, indicating that the conditions used for the PCR reactions were specific enough to amplify exon 1 of the human type I GnRHR if it were present.

PCR was performed on the genomic clones with gene specific primers designed to the cloned 1.6 kb 5' RACE testes product 5' to exon 2 (see section 4.4), i.e. putative exon 1 sequences. Our intent was to see if the 5' RACE sequences 5' to exon 2 occurs in the genomic clone 5' to exon 2. If this occurred, we could be reasonably sure that the 5'RACE product was authentic. Five different combinations of primers were used, i.e. JHE1S3 + JHE1A, JHE1S3 + 10242, JHE1S3 + JHE1A2, 10351 + 10071, and JHE1S + 10242. The products were checked by electrophoresis on an agarose gel , as well as by Southern blotting, probing the membrane with an internally nested oligo. No positive signals were obtained on the autoradiographs (data not shown), although some faint bands could be seen on EtBr-stained agarose gels.

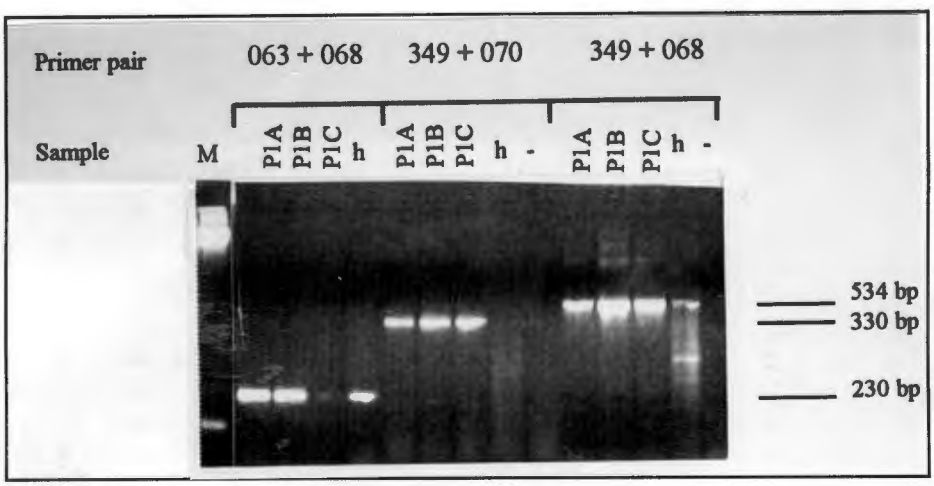


Figure 40 PCR performed on P1 clones with gene specific primers confirmed the presence of putative exon 2 and 3 of the type II GnRHR. Human genomic DNA (lanes marked h) was included in the PCR experiment as a positive control. Lanes that contain the negative controls (no DNA) are marked (-). The lines indicate the correct sized bands on the gel.
Sizes expected are: 10063 + 10068 = 230 bp; 10349 + 10070 = 330 bp, and 10349 + 10068 = 534 bp.

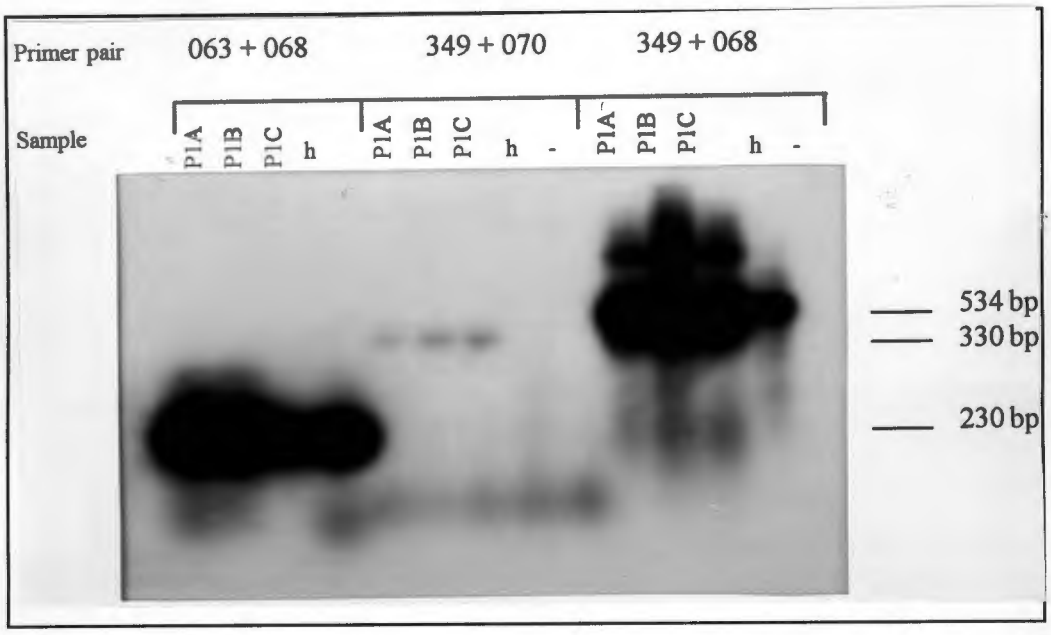


Figure 41 Autoradiograph of Southern blot performed on the gel in Figure 40 with a 10063 + 10068 PCR-generated probe (see section 2.6.3, Materials and Methods). The sizes of the positive signals obtained are consistent with the sizes of the bands indicated with lines in the EtBr-stained agarose gel. P1A, P1B, and P1C refer to the 3 different genomic clones. Lane (-) is the negative (no DNA) control, and lane h the positive (human genomic DNA) control.

This could be as a result of a few possibilities:

a) The genomic clones do not contain the sequences from the 5'RACE clone 5' to exon 2, because these sequences occur further 5' to the 70 kb genomic clone.

b) The genomic clones do contain sequences from the 5'RACE clone 5' to exon 2, but intron 1 might be too big to PCR across.

c) The 1.6 kb 5'RACE product was the result of an artifactual amplification of another gene with the exon 2 to exon 3 region of the GnRHR gene, and the sequences 5' to exon 2 are therefore not part of the GnRHR gene.

In all 3 cases, the appearance of PCR products by EtBr staining would be due to non-specific annealing of the primers to the genomic DNA, as confirmed by the lack of positive signals on the Southern blot.

CHAPTER 4

DISCUSSION

GnRH is a central reproductive hormone in all vertebrates, and exerts its effects via binding to the GnRHR. This investigation was part of a larger effort to clone GnRHRs from different species by a PCR approach. Insights about structure-function relationships are frequently obtained by a comparison of conserved and non-conserved amino acids of receptors from different species. Prior to commencement of this project, only eutherian mammalian GnRHRs had been cloned, and no non-mammalian GnRHRs. These mammalian GnRHRs were all highly conserved in both the TMs and loops (see Figure 5, Introduction), and were the so-called type I GnRHR found in the pituitary gonadotrophs and responsible for the classical endocrine response of LH and FSH release due to binding to mGnRH. During the course of this project, a second form of the GnRHR was identified in reptile and toad (*Xenopus*), the so-called type II GnRHR due to the presence of several non-conserved amino acids relative to the type I GnRHR. The function of this type II GnRHR is not known, but it was thought to be the receptor for the CII GnRH hormone involved in non-pituitary neurotransmitter actions. From these results, and also since mammals were recently found to contain two different forms of GnRH, i.e. mGnRH and CII GnRH, it was proposed that mammals also express a type II form of GnRHR. Therefore, in this project attempts were made to find a type II GnRHR in higher mammals, i.e. mouse and human. In addition, the mole was investigated since, as an example of a more primitive non-placental mammal, its receptor(s) may be particularly interesting. In addition, redbait was investigated as an example of a tunicate, since GnRH had been found in other tunicates, but no receptor had been identified. A GnRHR from an organism so far separated from mammals in evolutionary development would be expected to yield important structure/function information.

The aim of this project was to clone novel GnRHRs, i.e. type I and type II GnRHRs from redbait and mole and type II mouse and human GnRHRs using a PCR strategy. PCR was performed with degenerate primers designed to human type I GnRHR to areas that are not conserved between GPCRs in general, but are conserved between mammalian GnRHRs. No positive results were obtained with any of the primer pairs tried on redbait. A fragment of

a novel type I mole GnRHR was obtained. This cloned fragment corresponds to exon 1 of the human type I GnRHR, from TM 1 to TM 2. Attempts to clone a type II GnRHR from mole or mouse were unsuccessful. We managed to clone a part of a novel type II GnRHR from human tissues. The cloned receptor corresponds to exons 2 and 3 (TM 4 to TM 7) of the type I human GnRHR. A 1.6 kb 5'RACE type II GnRHR product was successfully isolated from human testes cDNA.

A parallel investigation by other members of the laboratory on other species has shown that these different primer pairs have been successful in the isolation of other non-mammalian GnRHR's, including the GnRHR's from *Xenopus*, goldfish, and chicken (unpublished). Primer pair 5S/6A2 has been used successfully to clone a part of type I and type II GnRHR from *Xenopus* as well as goldfish (Brigitte Troskie, personal communication). To date, only a type I GnRHR has been cloned from chicken (Yuh-man Sun, personal communication).

4.1 Redbait GnRHR

PCR was performed on redbait genomic DNA using degenerate primers designed to areas of the GnRH receptor that are highly conserved between different mammals. Although PCR products of the expected size were obtained for some of the primer pairs, as well as some positive signals upon analysis by Southern blotting (Chapter 3, Section 2), all attempts to clone a novel GnRHR from redbait gDNA failed. The positive signals were found to be due to contamination, possibly via plasmids in the laboratory.

The reason this strategy did not work on redbait, might be that the redbait GnRHR differs substantially from that of the mammalian GnRH receptors. If the redbait GnRHR is very different from the other cloned receptors (in other words, if the redbait GnRHR has a very low homology to other GnRH receptors), PCR might not be the best method of isolating this receptor. If we lower the specificity of the PCR reaction too much, we would probably get a lot of non-specific amplification, and still not amplify the redbait GnRHR. A different cloning strategy would thus be necessary, for instance one that is based on expression screening of cDNAs with GnRH ligand.

Another possibility is that there is no GnRHR in redbait. However, this is very unlikely, since we know that GnRH has been detected in tunicates (Powell *et al.*, 1996). The presence of GnRH implicates the presence of GnRHR.

4.2 Type I GnRHR gene from mole

Sequence analysis revealed that this fragment was indeed exon 1 of the mole type I GnRHR. This exon 1 type I mole GnRHR fragment is 89.5% homologous to human type I GnRHR on the DNA level, and 93% on the amino acid level (see Figure 16 and Figure 43). The exon 1 fragment of the type I mole GnRHR differed from the human type I GnRHR in only 4 amino acids out of a total of 96. Furthermore, these were all conservative changes, i.e. V to A, A to T, L to V, and A to G. Also, these changes all occurred in the transmembrane domains, and not in the loop areas, as one would expect. Important amino acids conserved, are the asparigine in TM 1, since this amino acid is conserved in all known GPCRs, as well as the leucine in TM 2, which is conserved in most of the known GPCRs. Other conserved amino acids include the VMPLD sequence in TM 2, which is conserved in all the mammalian GnRHRs. Other amino acids that are conserved in mole, as in all the other mammalian GnRHRs, include R, V, T, L, N, and L in TM 1, and three L's and T in TM 2.

We decided not to continue our efforts on isolating the rest of the type I GnRHR from mole genomic DNA, since the fragment we had isolated was very similar to the human type I GnRHR, and the results were unlikely to be very interesting.

4.3 Human type II GnRHR

Human genome data base searches performed with the sequence of a type II GnRH receptor from reptile, revealed the presence of antisense human type II GnRHR EST cDNA clones in several human tissues, showing that a gene for a type II GnRHR exists in the human genome. Using gene specific primers designed to this sequence, we showed that several human tissues and cell lines expressed intron-containing type II GnRHR RNA. PCR results which support this finding are unlikely to be due to genomic DNA contamination, since for several of the samples, poly A RNA was used as a template for cDNA synthesis. In addition, Northern blot

analysis of RNA from these tissues has revealed the presence of distinct RNA bands containing type II GnRH sequences (R.P. Millar, personal communication). The presence in the date base of antisense cDNA type II clones, together with our results, raises the possibility that antisense type II GnRHR transcripts containing an intron are widely expressed in human tissue. The possible biological function of such transcripts is unknown, but they could have a regulatory function in suppressing transcription of the sense transcript.

We isolated a testes cDNA clone (Section 4.3, Results) containing type II human GnRHR from EC2 (located in exon 2) to the end of TM 7 (located in exon 3). This result shows that a processed RNA transcript (i.e. not containing intron) coding for part of a GnRHR, i.e. exons 2 and 3, is indeed expressed in at least one human tissue. This processed transcript is clearly of much lower abundance than the intron-containing transcript. From our results we are unable to determine whether the processed transcript is the sense or the antisense strand. Using a primer designed to span the exon 2/exon 3 boundary to selectively amplify processed RNA rather than intron-containing RNA, we showed that several other human tissues may also express the processed RNA. However, this result must be interpreted with some caution, as it is possible that the 3'end of the primer annealed to the one exon without the 5'part annealing to the other exon. This is unlikely, as the 3'part of the primer would have to anneal to only 3 bp.

Different strategies were used to attempt to obtain exon 1 of the human type II GnRHR. These include PCR on genomic DNA with degenerate primers designed to regions of exon 1 of the human type I GnRHR that are highly conserved between different mammals, as well as 5'RACE on cDNA from various tissues. 5'RACE reactions, which exploited the use of the exon 2/exon 3 gene specific primer to the human type II GnRHR (JHE2E3) were performed, followed by Southern blotting and probing with an internally nested oligo. 5'RACE performed on testes cDNA produced DNA fragments of 3.5 kb and 1.6 kb (see section 4.4). We were unable to isolate a clone containing the 3.5 kb RACE product, despite extensive efforts that included reamplification of the 3.5 kb fragment with internally nested primers, and extensive colony screening (in excess of 125 colonies). We succeeded in isolating the 1.6 kb 5'RACE product. Figure 35 shows the sequence of the full-length 1.6 kb 5'RACE product. The sequencing data was analysed by homology searches on databases

on both the amino acid level and the DNA level. The sequencing results showed the presence of putative exons 2 and 3, and 5' to exon 2 was an open reading frame (out of frame with the exon 2/exon 3 reading frame) which has no similarity to exon 1 of the human type I GnRHR, or to any other receptor or protein. However, there was a stop codon between this frame and the putative exon 2. The sequences 5' to exon 2 may be part of an intron, or possibly a PCR artifact.

The function of this 1.6 kb cDNA fragment is currently unknown. Whether a functional type II human GnRHR protein is expressed, is not known at this stage. It is possible that a functional protein containing only putative exons 2 and 3 is expressed in human testes. Preliminary investigation to test this possibility, by expressing the cDNA in COS-1 cells, indicate that the exon 2/exon 3 product (i.e. from TM 4 to TM 7) is not functional (J. Hapgood, personal communication).

The failure to isolate an exon 1-containing type II GnRHR cDNA by 5'RACE, might be due to a very low abundance of the type II GnRHR transcript. This is supported by the very weak signals obtained in the PCR reactions using primers 10417 + 10070 to generate the minor ~ 200 bp band of intron-less RNA (see Figure 25). It is possible that an exon 1 containing transcript is expressed in tissues other than those we have examined. It might be expressed in a small population of cells at distinct times in development. In this case it would be extremely difficult to detect. The 3.5 kb 5'RACE product identified in our Southern (see Figure 33) in five tissues and one cell line (testes, fetal brain, thyroid, placenta, daudi, and lymphnode) may be the full-length exon 1-containing product. Unfortunately we were unable to clone this DNA, most likely due to its extremely low abundance.

We used an alternative genomic DNA approach to attempt to isolate putative exon 1 of the type II human GnRHR. P1 genomic clones containing the putative exon 2 and exon 3 human type II GnRHR sequences were purchased from Clonetech. The presence of putative exons 2 and 3 of the human type II GnRHR in these genomic clones was confirmed by Southern blotting (Section 5, Results). Although PCR was performed with different combinations of degenerate primers designed to human type I GnRHR sequences that were highly conserved between different mammals, we found no indication of the presence of a putative exon 1.

Our inability to obtain any indication of exon 1-like sequences could be due to the absence of exon 1 in our genomic clones. However, since the clones contain inserts of 70 kb, we would expect exon 1 to be present. Another possibility is that exon 1 of the type II GnRHR differs so much from that of the human type I GnRHR, that PCR with degenerate primers did not amplify exon 1. One other possibility is that the human type II GnRHR does not contain any exon 1-like sequence, but that is very unlikely, since the putative exon2/exon3 sequence is so similar to that of the human type I GnRHR. It is interesting to note that my colleagues working on the type II GnRHR have also not been successful in isolating a putative exon 1 from toad (*Xenopus*) cDNA using a similar strategy of 5' RACE (Brigitte Troskie, personal communication).

The type II GnRHR sequence isolated is 67% homologous to the human type I GnRHR, with the greatest homology in the carboxyl terminal halves of IC 3, TM 6 and TM 7. The most notable feature of the human type II GnRHR is the presence of an intracellular C-terminal tail, which is absent in all of the mammalian type I GnRHRs, but present in all other G-protein coupled receptors (GPCRs) (Millar *et al.*, 1993, Stojilkovic *et al.*, 1994). Another interesting feature of the human type II GnRHR is the uncharacteristically long IC3. The human type II GnRHR IC3 is 5 amino acids longer than that of the human type I GnRHR. The significance of the long IC3 is not known.

Whether this type II human GnRHR is actually ever expressed as a functional protein, and in which tissues, is not known at this stage. It may be that the type II receptor is the receptor for the CII GnRH ligand in humans. If this is the case, then the differences in the amino acids of the type I compared to the type II human GnRHR would be likely to confer specificity for the mGnRH and CII GnRH ligands, respectively. Further investigations are necessary to answer these important questions.

4.4 Perspectives on PCR as a tool

In this thesis, PCR has been extensively exploited to investigate several issues, under a variety of different applications. Degenerate PCR primers have been used to amplify GnRHRs from genomic DNA (gDNA) of different species. In some instances cDNA has been used as

template. Gene specific primers (GSPs) have also been exploited to amplify specific cDNAs from total cDNA, and to amplify selected regions from genomic clones. A primer designed across an exon-exon boundary (in other words, spanning an intron) was used to selectively amplify "processed" intron-less GnRHR cDNA from total cDNA. It is thus possible to change PCR primers to suit the specificity (GSP vs degenerate primers) of your reactions, as well as changing the conditions. Another use of PCR in this thesis was to verify cloned inserts directly from colonies, without having to do plasmid preparations. Not only is it much less time-consuming than traditional analysing methods, such as plasmid preparations and restriction enzyme digestions, but it is also more convenient and inexpensive. It also enables one to screen a much greater amount of colonies at the same time, thus in the end permitting one to analyze more colonies than with traditional analysis methods.

In all the PCR applications, reactions were always optimized by choosing the appropriate conditions, such as annealing temperature, ionic strength, divalent cation, primer concentration, type of enzyme, and cycling conditions. Where necessary, such as in the 5'RACE reactions, hotstarts were achieved by using Taq antibody. The results of this thesis clearly show that by careful choice of primers and optimal conditions, PCR can be an invaluable tool.

One of the major drawbacks of PCR is also illustrated in this thesis. The ability of the Polymerase Chain Reaction (PCR) to amplify a single molecule means that trace amounts of DNA contaminants, especially plasmids and PCR products which contain many copies of the same DNA molecule, could serve as templates, resulting in amplification of the wrong template (false positives). In order to minimize any contamination, various precautions were taken when performing PCR (see section 12, Materials and Methods). However, despite all these precautions, I still had some contamination (see section 2, Results). Since PCR has the ability to amplify minute amounts of template, contamination is always a potential problem when performing PCR, especially when there is more than one person in a laboratory working on the same strategy. It is therefore absolutely critical to include control reactions in all PCR reactions performed, and also to confirm any possible positive results obtained.

The GnRHR has been cloned in a variety of mammalian species, including human (Chi *et*

al., 1993; Kakar *et al.*, 1992), mouse (Reinhart *et al.*, 1992), rat (Eidne *et al.*, 1992; Kaiser *et al.*, 1992), cattle (Kakar *et al.*, 1993), pig (Weesner and Matteri, 1994), and sheep (Brooks *et al.*, 1993; Illing *et al.*, 1993). The first GnRHR to be cloned was the mouse GnRHR, using a method based on transfection of cDNAs into cells and screening for GnRH ligand binding. Subsequently, the other receptors were all cloned by cDNA library screening, probing with cDNAs of mouse GnRHR or other previously identified GnRHRs. From 1994, by which time most of the interesting mammalian GnRHRs had been cloned, until 1997, there had been no reports in the literature of non-mammalian GnRHRs having been cloned. This is most likely due to the low degree of homology at the nucleic acid level between mammalian and non-mammalian GnRHRs. Attempts in our laboratory to clone toad and chicken GnRHRs prior

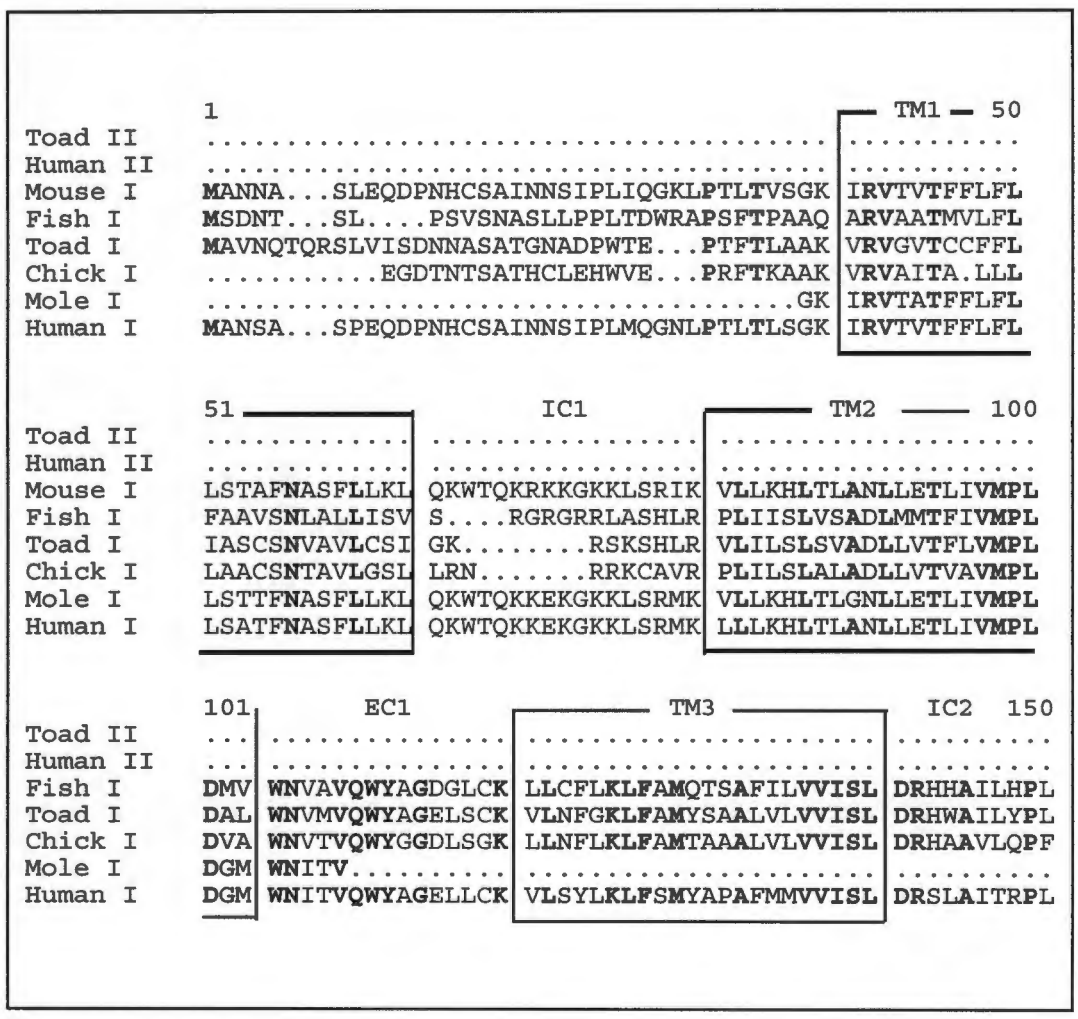


Figure 45 Amino acid alignment of GnRHRs cloned from various different species. Conserved amino acids are printed in bold. Transmembrane (TM) helices are indicated with boxes. Extracellular (EC) and intracellular (IC) loops are also marked.

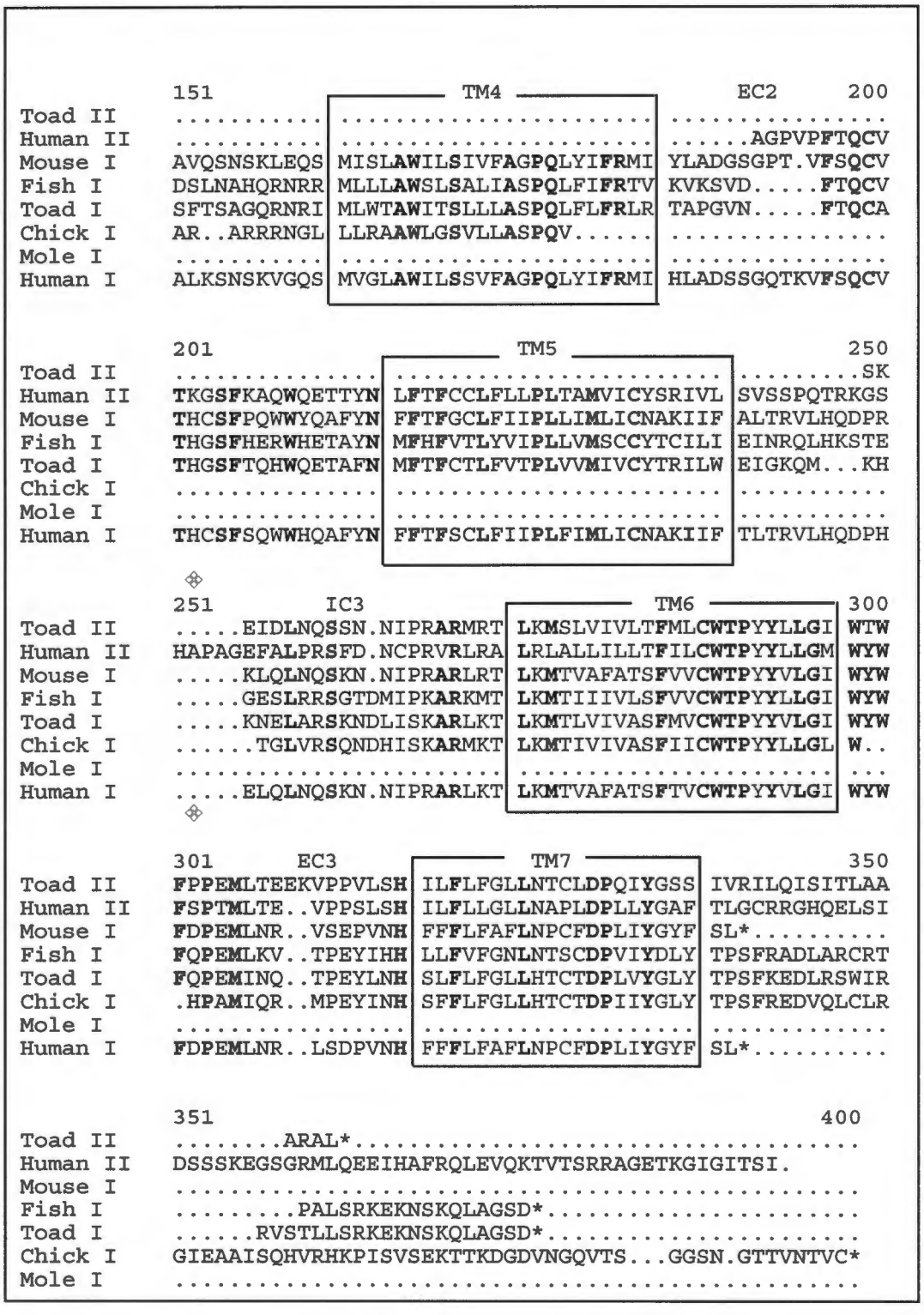


Figure 45 (continued) Amino acid alignment of GnRHRs cloned from various different species. Conserved amino acids are printed in bold. Transmembrane (TM) helices are indicated with boxes. Extracellular (EC) and intracellular (IC) loops are also marked. The position of the exon 2/exon 3 boundary determined for the human testes type II GnRHR is marked by ⊕. Stopcodons are indicated by *.

to the PCR strategy, but based on screening of cDNA libraries with mammalian GnRHR cDNAs, had been unsuccessful. The PCR strategy using degenerate primers to regions of the mammalian GnRHR which were predicted to be conserved, presented a new approach.

Looking at Figure 45 it is quite obvious that there is considerable homology between these GnRHRs from different species. There is a notably higher conservation of sequence in the transmembrane helices, as opposed to the intracellular and extracellular loops, as one would expect. Another interesting observation, is that the non-mammalian GnRHRs differ in length and sequence in the loops quite strikingly from the mammalian GnRHRs.

It is clear that the success of this PCR strategy lies in the occurrence of at least some highly conserved regions in the GnRHRs, and the fortuitous choice of degenerate primers being used to these regions. In additions, these conserved regions probably need to contain codons of low degeneracy to enable the correct primers to anneal specifically in the presence of an excess of non-specific primers in the degenerate mix of primers. This is evident from the lack of success of some of the degenerate primers in amplifying the mole GnRHR (see section 3, Results). Despite a predicted high degree of conservation of the full-length GnRHR of the mole compared to other mammalian GnRHRs, as shown by the results with exon 1, only one pair of the PCR degenerate primers was successful in amplifying the mole GnRHR from gDNA. Similar results were obtained with the non-mammalian GnRHRs from toad (Brigitte Troskie, personal communication) and chicken (Yuh-man Sun, personal communication), although in this case the lack of success of most of the primers may have been due to a lack of conservation of sequence within the transmembrane domains. Thus, while the PCR strategy with degenerate primers is a powerful tool in obtaining GnRHR DNA sequences from different species, its success is by no means guaranteed, even if the full-length receptor, or regions of the receptors are highly conserved with the sequence to which the primers are designed.

APPENDIX A

LIST OF OLIGO'S USED FOR PCR

JH1S:

23 mer

```
5' CTC GAA TTC GGN AAG ATC CGN GT 3'
                        A   A A
                            T
```

JH2S:

29 mer

```
5' CTC GAA TTC GAT GGN ATG TGG AAC ATA AC 3'
                   C                T   T
                                        C
```

JH2A:

29 mer

```
5' ACA CTC GAG TG NAC NGT AAT GTT CCA CAT 3'
                            T   A
                            G
```

JH3A:

30 mer

```
5' ACA CTC GAG CAT GAA NGC NGG NGC GTA CAT 3'
                     A                A
```

JH4S:

28 mer

```
5' CTC GAA TTC AA ATG ACN GTN GCN TTT GC 3'
                  G                    C
```

JH5S:

26 mer

```
5'  CTC GAA TTC GGN ATT TGG TAT TGG TT 3'
                        A       C
                        C
```

JH5A:

26 mer

```
5′ ACA CTC GAG AA CCA ATA CCA AAT NCC 3′
                      G       T
                              G
```

JH6A1:

26 mer

```
5′ ACA CTC GAG GG ATC AAA ACA NGG GTT 3′
                  G   G   G       A
```

JH6A2:

26 mer

```
5′ ACA CTC GAG CC ATA GAT NTG NGG ATC 3′
                  G   T     A     G
                      A
```

JHM1S:

26 mer

```
5′ CCN CCN ACN ATG CTN ACN GAA GTN CC 3′
                                G
```

JHM1A:

20 mer

```
5′ AA NGC GTG TAT TTC TTC TTG 3′
          A   G   C   C   C
              A
```

AP2:

23 mer

```
5′ ACT CAC TAT AGG GCT CGA GCG GC 3′
```

AP1:

27 mer

```
5′ CCA TCC TAA TAC GAC TCA CTA TAG GGC 3′
```

AP:

48 mer

5′ CUA CUA CUA CUA GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG 3′

UAP:

32 mer

5′ CUA CUA CUA CUA GGC CAC GCG TCG ACT AGT AC 3′

JHE1S3:

17 mer

5′ GCA GGA CCA ATT TAC TG 3′

JHE1A2:

20 mer

5′ GGG TTA ACA ATC TGA CTT GC 3′

JHE1S:

22 mer

5′ GAA AGC ACC TGA GTC ATT GGA G 3′

JHE1A:

22 mer

5′ CTC CAA TGA CTC AGG TGC TTT C 3′

JHE2E3A:

25 mer

5′ AAA TTC ACC AGC AGG GGC ATG GCT C 3′

JHE2E3S:

27 mer

5′ CAA GGA AGG GGA GCC ATG CCC CTG CTG 3′

JHE2:

24 mer

5′ CCT TGA AGC TGC CTT TGG TGA CAC 3′

10242:

20 mer

5′ GGT TAT AGG TGG TCT CTT GC 3′

10417:

21 mer

5′ GCA AGA GAC CAC CTA TAA CCT 3′

10063:

26 mer

5′ CTG ACC TTC ATC CTC TGC TGG ACG CC 3′

10068:

27 mer

5′ GAG CAT TTC TCC CAG ACC CTT CTT TAG 3′

10351:

23 mer

5′ GGT GTC CCC GTG TTC GTC TCC GG 3′

10350:

23 mer

5′ GGC CGG AGA CGA ACA CGG GGA CA 3′

10070:

26 mer

5′ GGT GTC CAG CAG AGG ATG AAG GTC AG 3′

10071:

22 mer

5′ GGA GAG CAG GAG TAG AAG TGA G 3′

10349:

25 mer

5′ GGA ACC TCT TCA CCT TCT GCT GCC T 3′

10069:

27 mer

5′ CTA AAG AAG GGT CTG GGA GAA ATG CTC 3′

JHi2S:

30 mer

5′ CCT TAA TCC TTA ACC CTA GAA CCT GTT GCC 3′

JHi2A:

30 mer

5′ GAC ACT CAG TCT TCT GAT CTG AGT ATA GGG 3′

T7 PROMOTER PRIMER:

20 mer

5′ ATT ATG CTG AGT GAT ATC CC 3′

U19MER PRIMER:

19 mer

5′ GTT TTC CCA GTC ACG ACG T 3′

APPENDIX B

COMMONLY USED MEDIUM

LB Medium (Luria-Bertani Medium)

Per liter deionized water, add:

bacto-tryptone	10 g
bacto-yeast extract	5 g
NaCl	10 g

Sterilize by autoclaving.

Terrific Broth

Per liter:
To 900 ml deionized H_2O, add:

bacto-tryptone	12 g
bacto-yeast extract	24 g
glycerol	4 ml

Sterilize by autoclaving. Allow to cool to 60^0C, and then add 100 ml of a sterile solution of 0.17 M KH_2PO_4, 0.72 M K_2HPO_4.

SOC Medium

Per liter:

bacto-tryptone	20 g
bacto-yeast extract	5 g
NaCl	0.5 g

Add 10 mol of a 250 mM solution of KCl. Adjust pH to 7.0 with 5 N NaOH. Adjust volume to 1 liter, and sterilize by autoclaving. Just before use add 5 ml of a sterile solution of 2 M $MgCl_2$, and 20 ml of a sterile 1M solution of glucose.

2 x YT Medium

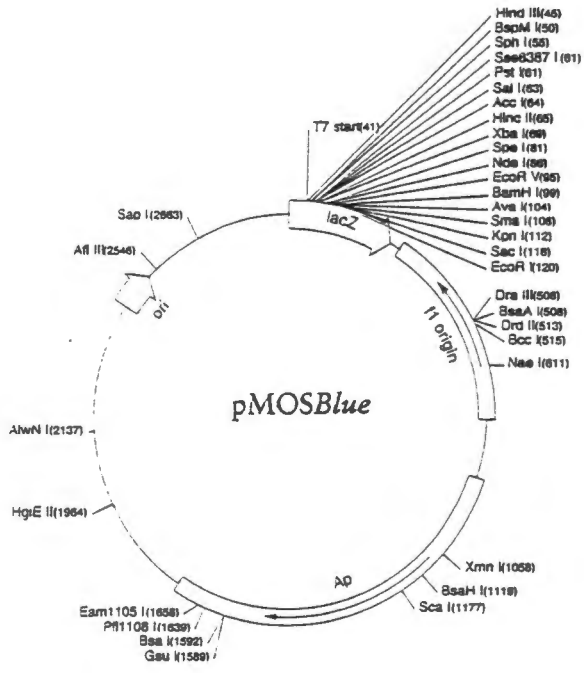
Per liter:

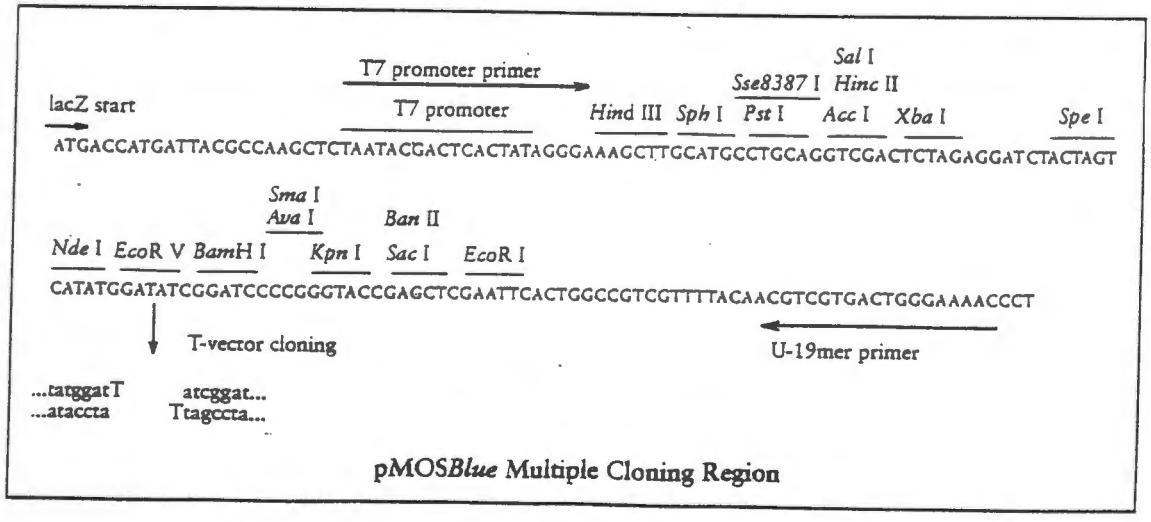
bacto-tryptone	16 g
bacto-yeast extract	10 g
NaCl	5 g

Sterilize by autoclaving.

APPENDIX C

PLASMID MAP OF pMOS*Blue* T-VECTOR





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