MOLECULAR CLONING OF A HUMAN PUTATIVE 7-TRANSMEMBRANE SEGMENT (7TMS) RECEPTOR

by

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We accept this thesis as conforming
to the required standard

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I. Abstract:

Seven transmembrane-segment (7TMS) receptors are highly homologous proteins that mediate a variety of cell functions through the interaction with G-proteins. They are important mediators in the response to neurotransmitters, hormones and cytokines. Moreover, two of these receptors have been found to be proto-oncogenes or to possess transforming activity. During the immune response they are involved in inflammation, producing and modulating this process through the interaction with ligands that are primarily chemotactic and activating factors. A family of such ligands has already been characterized and their receptors, when known, were all of the 7TMS type. To further investigate this category of proteins in cells involved in immunity a group of oligonucleotides was patterned on predicted conserved areas of chemotactic 7TMS receptors, and also on divergent non-homologous sequences. To facilitate subcloning procedures restriction sites where engineered at the 5'-end of the primers. Human monocyte mRNA was converted to single strand cDNA by antisense priming and reverse transcription. This cDNA was used as a template for the *in vitro* enzymatic amplification by the polymerase chain reaction (PCR) with upstream and downstream primers. The amplified cDNA (388 bp) was cloned in plasmid Bluescript and sequenced by the chain termination method. Homology searches for the sequence revealed that the fragment belonged to the 7TMS superfamily. The cDNA was radiolabeled and used to screen a human fetal spleen library constructed in the λ-phage system. A clone was isolated that when sequenced revealed a complete open

reading frame for a 352 residue protein. This polypeptide was found to be a human homologue of a neuropeptide receptor: neuropeptide tyrosine (NPY) subtype 3. Apart from having the typical topology of the 7TMS superfamily it also displayed a high degree of homology to chemotactic factor receptors. Northern blot analysis demonstrated that this protein is expressed in locations outside the nervous system like spleen and thymus.

II. Table of contents

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V. List of Abbreviations:

A_{280}	absorbance at 280 nm
A_{260}	absorbance at 260 nm
Amp	ampicillin
bp	base pair(s)
βARK	β-adrenergic receptor kinase
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
DAG	diacylglycerol
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytosine 5'-triphosphate
DEPC	diethylpyrocarbamate
dGTP	2'-deoxyguanosine 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
dH_2O	autoclaved, destilled water
DNA	deoxyribonucleic acid(s)
ddNTP	dideoxynucleotide triphosphate(s)
dNTP	deoxynucleotide triphosphate(s)
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetra-acetic acid
fMLP	N-formyl-Met-Leu-Phe
Gro/MGSA	melanocyte growth stimulating activity
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	kilobases
kDa	kiloDalton

KOAc	potassium acetate
l	litre(s)
M	molar
mA	milliampere
MilliQ	grade of water purity (resistivity $\geq$ 16 MΩ.cm)
min	minute(s)
ml	millilitre(s)
mM	millimolar
MOPS	3-(N-morpholino)-propanesulfonic acid
nm	nanometer(s)
ORF	open reading frame
pfu	plaque forming unit(s)
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
RNA	ribonucleic acid
RNase	ribonuclease
RNAsin	ribonuclease inhibitor
rpm	revolution(s) per minute
RT	room temperature
SDS	sodium dodecyl sulfate
sec	second
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
2-ME	2-mercaptoethanol
v/v	volume to volume ratio
w/v	weight to volume ratio
x g	times gravity

μg   microgram(s)

μl   microliter(s)

°C   degree(s) Celsius

X-Gal   5-bromo-4-chloro-3-indolyl β-D-galactose

VI. Acknowledgments

VII.- INTRODUCTION

1. Inflammation:

Inflammation is the first step in the immune response and in tissue repair activities. It is due to the interaction of a host of different cells. The recruitment and co-ordination required are due to specific mediators most of which are cytokines. These soluble polypeptide mediators are produced by accessory cells (fibroblasts, endothelial cells, keratinocytes, platelets, smooth muscle cells) and by leukocytes. The initial steps usually involves the production of the systemic pro inflammatory cytokines interleukin-1 (IL-1) and/or tumor necrosis factor (TNF).

IL-1 is a pleiotropic cytokine which is produced by an array of accessory cells but mainly by activated macrophages (Oppenheim et al.,1986). IL-1 activates cells in a prothrombotic, pro inflammatory manner. It is not chemotactic per se but elicits the extravasation of leukocytes from the blood by changing the adhesive properties of endothelial cells and inducing the secretion of chemotactic cytokines (Mantovani et al.,1989). TNF produces a set of responses that overlap with those from IL-1 (Old, 1985) but it also augments the expression of the major histocompatibility complex (MHC) class I antigens whereas IL-1 has little effect on MHC expression (Pober et al., 1987). This action is also coincident with the activity of γ-interferon (IFN-γ) but the latter does not induce a pro inflammatory reaction. IFN-γ also induces the expression of MHC class II antigens and the invariant chain, rather priming the cells to act as accessory cells (Pober et al.,1983; Collins et al., 1984).

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Lymphotoxin (or   TNF-β) is a cytokine related to TNF which is produced by B- and T-lymphocytes. It acts through the same receptor and its activities are similar to TNF.

The name "intercrine" has been suggested to separate the structurally related chemotactic and activating cytokines from the exclusively pro inflammatory IL-1 and TNF (Oppenheim et al.,1991). Interleukin-8 (IL-8) is a pro inflammatory intercrine that stimulates chemotaxis on neutrophils as well as the release of lysosomal enzymes and the respiratory burst (Oppenheim et al., 1991). It is produced by several cell lineages and its production is induced by   IL-1, TNF and Lipopolysaccharide (LPS). It has been shown to have chemoattractant properties for neutrophils and basophils (in addition, basophils release histamine upon IL-8 stimulation). The counterpart of IL-8 for monocytes is the monocyte chemotactic and activating factor (MCAF) (Leonard et al.,1990). In contrast with the respiratory burst induced by formyl-peptide chemoattractants MCAF causes no respiratory burst in monocytes. Basophils but not eosinophils respond to MCAF. It is chemotactic and induces the release of lysosomal enzymes in cells of the macrophage lineage. It may be also important in response to acute tissue injury by participating in host defense mechanisms and in tissue repair. MCAF has also been shown to activate cultured monocytes to be cytostatic for several tumor cell lines (Matsushima et al., 1989; Yoshimura et al., 1989).

IL-8 and MCAF are representative of the two subfamilies in which the intercrines are divided. There are four half-cystines in these molecules and they exist as disulfides. The IL-8 subset differs

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from the MCAF subset in that the first two cysteines are separated by a single amino acid residue forming the motif C-X-C (X being any amino acid). In the MCAF subgroup this motif is C-C. Structural analysis have shown that these molecules have two disulfide bridges formed by the first and the third and the second and the fourth cysteines in the sequence. The base of the bridges is therefore in close proximity. Apart from these structural features the other criterion to divide the intercrines in two subfamilies is their chromosomal localization. Thus, the human subset localized in chromosome 4 and presenting the C-X-C motif belongs to the subfamily α (i.e. IL-8). Those containing the C-C motif and located on the chromosome 17 belong to the β-subgroup (i.e. MCAF). Both subsets are in the molecular weight range of 8-10 kD and are basic polypeptides.

There is 20-40% homology between these subfamilies. Both IL-8 and MCAF are produced in a precursor form of 99 residues with a signal sequence that is cleaved by proteolysis. The mature forms are 72 amino acids long and are not glycosylated. IL-8 is active as an hydrogen bonded dimer whose structure has been resolved by x-ray diffraction analysis (Baldwin et al., 1990) and NMR (Clore et al.,1989, 1990). Even though IL-8 and MCAF display only 21% homology their tertiary structure can be superimposed. The tertiary structure of MCAF was predicted having as a base the known structure of IL-8 (see tables I and II, next page).

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Table I:

Relationship of intercrine family members		
	Intercrine α-subfamily	Intercrine β-subfamily
chromosome	4 (q12-21)	17 (q11-32)
structure	C-X-C	C-C
members	IL-8	MCAF
	NAP-2	I-309
	GRO	LD-78
	PF-4	ACT-2
	βTG	RANTES
	IP-10	

Table II

Human diseases characterized by predominant neutrophil and/or monocyte infiltration
Rheumatoid arthritis
Psoriasis
Gout
Immune vasculitis
Glomerulonephritis
Inflammatory bowel disease
Myocardial infarction
Adult respiratory distress syndrome
Emphysema
Asthma
Arthritis associated with Mediterranean fever

2. Seven-transmembrane segment receptors:

Transmembrane signaling systems are the sensors that cells evolved to detect changes in the environment and to adapt to them. The cell membrane is a physical barrier between the extra cellular space and the tightly controlled intracellular matrix. As it is well documented this barrier is far from being inert, but the lipids of the bilayer form an effective hindrance to the movement of proteins and most solutes across the membrane. For any signaling mechanism to induce a productive response this obstacle has to be circumvented. The molecular switches that convey information inside the cell reside in a particular type of membrane proteins generically known as receptors. This function, plus the specific recognition of the ligand, places receptors in a key position within the processes of cell-cell communication mediated by protein, and non-protein factors.

One of the best characterized of such systems is a receptor family that shares significant homology and which overall topology has been established or can be inferred from predictive algorithms. This group is known as the seven-transmembrane segment (7TMS) receptors following the prediction that the seven hydrophobic segments form membrane-spanning α-helices (figure 1). They are present in organisms so divergent as man and yeast and they modulate a host of processes where the initial stimulus can be a neurotransmitter, a neuropeptide, a hormone or a cytokine. They are also the molecular basis for light detection and odor recognition (Khorana, 1992; Buck and Axel, 1991).

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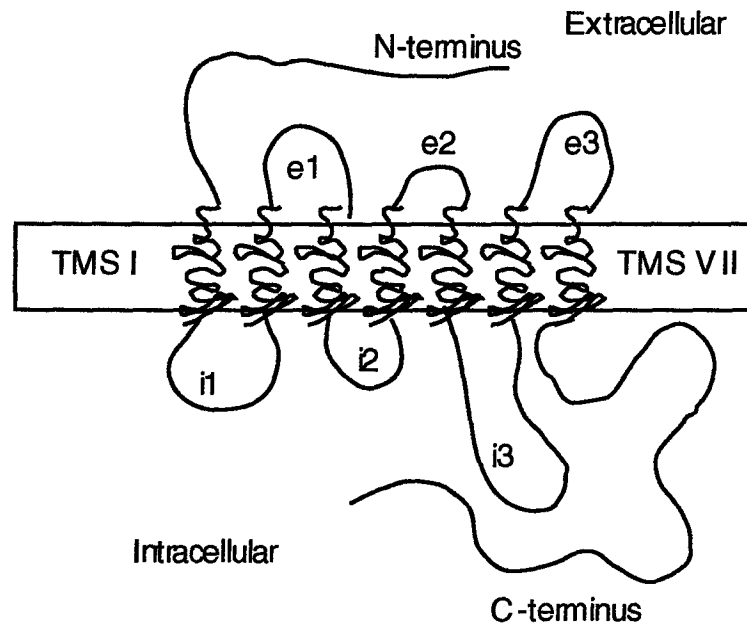
Figure 1: A schematic representation of the topological organization of G-protein coupled receptors in the cell membrane: segments I trough VII are predicted to span the membrane. The N-terminus and the connecting loops e1, e2, and e3 would be located on the extra cellular surface of the bilayer. The C-terminus and connecting loops i1, i2, and i3 would lie in the cytoplasmic side.

These molecular switches consist of a transmembrane protein (the receptor) coupled to an effector protein inside the cytoplasm of the cell. The effector system to which they are coupled is often an enzyme or an ion channel and the coupling is in turn mediated by the system of guanine-nucleotide binding proteins (G-proteins). This allows them not only to transmit a signal toward the cytoplasm, but also to amplify the output response (figure 2). Growth factors, neuropeptides and cytokines can use a common signal transducing

mechanism but it is important to note that the final outcome for any given cell depends on the interaction of different stimuli and on that cell phenotypic expression. The receptor-coupled G-proteins consist of three subunits called α, β and γ (in order of decreasing molecular weight). They are present in the form of heterotrimeric complexes with a stoichiometry of 1:1:1. Upon agonist binding the α-subunit exchanges bound GDP (which forms part of the complex) for GTP. This induces a conformational change and the α-subunit dissociates from the complex and this is the active, effector modulating protein. The $\beta\gamma$ complex enhances the efficiency of GTP/GDP exchange (Bourne et al.,1991) by driving the replacement of GDP for GTP. Amplification results from the multiplying effect of having a single activated receptor molecule interacting with several ternary complexes. There are more than 20 α-subunits in man and only four each β- and γ-subunits. The α-subunit is the GTPase and it is also the substrate for ADP-ribosylation by bacterial toxins. The ligand activated receptor functions as a guanine nucleotide release protein (Bourne et al., 1991). Ligand binding and G-protein activating moiety reside on a single polypeptide. Binding sites for agonists and antagonists have both distinct and shared determinants, and important determinants for G-protein recognition are present in the transmembrane segment V and VI as well as in the interconnecting cytoplasmic loop (Dohlman et al., 1991).

3. Receptors for intercrine ligands:

Scatchard analysis of IL-8 binding data showed that there are 20,000 high affinity sites (Kd= 0.8 nM) per cell in human neutrophils.

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Cross-reactivity has been observed between IL-8 and other members of the α-subfamily, specially Gro and its murine homologue MIP-2.
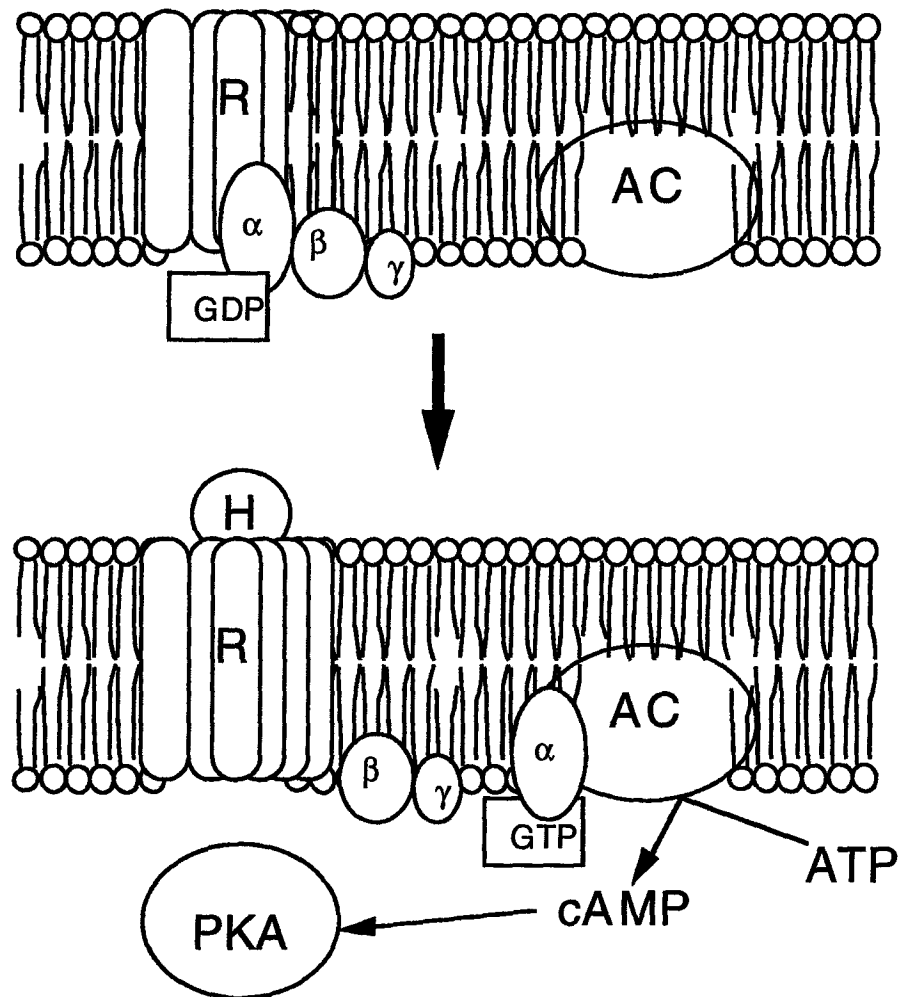
Figure 2: Adenylyl cyclase signal transduction pathway. R: receptor; α, β and γ: the three subunits of a G-protein; AC: adenylyl cyclase; GDP: guanosine diphosphate; GTP: guanosine triphosphate; H: hormone (or other ligand); ATP: adenosine triphosphate; cAMP: cyclic adenosine monophosphate; PKA: protein kinase A.

IL-8 receptor (IL-8 R) expression is dynamically regulated by the ligand itself (Oppenheim et al.,1991). IL-8 causes a rapid

increase in cytosolic free calcium in human neutrophils that is inhibited by *Bordetella pertussis* toxin (Thelen et al., 1988; Dewald et al., 1988). It also causes a respiratory burst that is inhibited by 17-hydroxywortmannin and staurosporine (Thelen et al., 1988). From these findings it was deduced that a G-protein is associated to the IL-8 R and that Protein Kinase C activation is needed for the action of IL-8.

The initial path for signal transduction appears to be the same for all the chemotactic factor receptors which have been characterized up to now: they have the predicted topology of the 7TMS family and they associate with G-proteins. Recently two cDNAs encoding IL-8 binding proteins have been cloned by expression cloning or by hybridization (Holmes et al., 1991; Murphy et al., 1991). One of them is the high affinity site (Holmes et al.,1991) and the other is low affinity. The latter is an IL-8 functional receptor but its specificity is doubtful in light of the existing binding studies and the cross-reactivity observed between some intercrines and their receptors (Moser et al. 1990). SDS-PAGE analysis of cross-linked ^{125}I-IL-8 and receptor complexes showed two polypeptides with receptor moieties estimated at 67 and 59 kD. As the cells from the promyelocytic lineage mature they express more IL-8 R, with mature neutrophils expressing the highest number. By contrast very little is known about MCAF receptors. Current estimation of their numbers in mature monocytes is 1,700 per cell with a Kd= 2 nM.

4. Neuropeptides:

In recent years a large development in the field of neuropeptides took place (Hokfelt et el.,1980). Neuropeptides now outnumber the classical neurotransmitters in both central and peripheral nervous system. It has been thought that the main action of neuropeptides was in the area of neurotransmission where they would mainly mediate the depolarization of the cell. In this context it is important to mention that is a change in membrane potential which modulates the release of TNF in LPS-stimulated macrophages (Haslberger et al., 1992). Neuropeptides act as neurotransmitters through specific cell surface receptors but they can also act as mitogens for a variety of cell types. Even in the case of classical neurotransmitters it has been observed that at least two muscarinic receptor subtypes coupled to inositol metabolism can induce DNA synthesis when stimulated with the stable muscarinic agonist carbachol (Ashkenazi et al., 1989). This was observed in transfected CHO cells and also with cell lines expressing the specific acetylcholine receptor subtypes. Furthermore, the transfection of NIH 3T3 cells with the 5HT1c serotonin receptor results in the induction of a transformed phenotype and tumorigenicity upon serotonin stimulation (Julius et al., 1989). It should be taken into account that even though the neurotransmitter receptors are restricted to neural cells, the signaling pathways that they activate are present in both neural and non-neural cells. It has also been observed that in the case of neuropeptides mitogenic activity is possible. This is clearly the case for the mammalian *mas* oncogene that encodes for an angiotensin receptor (Young et al., 1986; Jackson

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et al., 1988). Growth factors or mitogens act in a diverse array of events including embryogenesis, tissue repair, immune response, and oncogenesis. In several of such cases sensory nerve endings are in close apposition to the target cells.

It has also been observed that platelet derived growth factor (PDGF) and epidermal growth factor (EGF) are able to induce contraction in smooth muscle cells. All these findings strongly suggest that the appropriate response is more determined by phenotypic factors rather than by the initial stimulus itself. Thus, in the well characterized case of norepinephrine and epinephrine which are both neurotransmitters in sympathetic neural cells but epinephrine also acts as a hormone for peripheral tissues.

The tachykinins substance P (SP) and neurokinin A (NKA) also present mitogenic activity on smooth muscle cells and fibroblasts (Nilsson et al., 1985). Comparing the effect on smooth muscle cells of SP and NKA to that of PDGF it was found that PDGF decreases the amplitude of the response to the tachykinins. This suggests a competition for the same intracellular messengers (both calcium and calmodulin antagonists inhibit the mitogenic effect) (Hultgaard-Nilsson et al., 1989). SP stimulates the proliferation of T-lymphocytes and potentiates the response to mitogens (Payan et al., 1983).

Beta-endorphin, vasoactive intestinal polypeptide (VIP) and somatostatin both inhibit and stimulate lymphocytes in a dose dependent manner (Moore, 1984; O'Dorisio et al., 1985; Ottaway et al., 1984; Pawlikowski et al., 1985). It was shown that VIP

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synergizes with EGF in stimulating the growth of keratinocytes but it was inactive by itself.

Lung epithelial cells respond to growth stimulation by bombesin and its mammalian counterpart gastrin releasing peptide (GRP). GRP is an autocrine factor for small cell lung carcinoma (Cuttitta et al., 1985).

Given all these findings it is interesting to speculate over the physiological as well as the pathophysiological role of neuropeptides apart from their usual function as neurotransmitters. Locally released neuropeptides could be associated to the wound healing process. During development bombesin-like immunoreactivity is present in high concentration in fetal and neonatal lung, but it is absent in adults, indicating a possible involvement of bombesin in lung embryogenesis. Some evidence exists pointing to a role for gastrin, enteroglucagon, neuropeptide tyrosine (NPY) and GRP in the control of gastrointestinal epithelial cell proliferation. Neuropeptides could also be involved in the desmoplasia associated with some tumors if the malignant cells produce NKA or SP, or they could be involved in some autocrine loop. SP and NKA are present in neurons surrounding large arteries. SP induces dilatation when acting on endothelial cells but contraction when acting on smooth muscle cells. Thus, SP is more likely to cause contraction when acting on vessels with advanced atherosclerotic lesions.

Sensory neurons may cause vasodilatation and edema (neurogenic inflammation) and this response involves SP (Dalsgaard et al., 1989). SP involvement has also been thought to be involved in

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chronic inflammation and arthritis (Levine et al.,1984). SP also enhances the production of immunoglobulins by B-lymphocytes (Stasnitz et al., 1986).

5. Neuropeptide tyrosine (NPY) and its receptor:

The sympathetic nervous system innervates lymph nodes, spleen, bone marrow and thymus (Besedowski et al., 1979). Its function is related to both vascular cells and parenchymal cells and its has been implicated in the modulation of the immune response and inflammation (Felten et al., 1987). There are adrenergic receptors in bone marrow derived cells such as macrophages but there is no evidence for a specific role for catecholamines in modulating immune function. Sympathetic nerves innervating peripheral organs also possess a neuropeptide which is 36 amino acids long and is called neuropeptide tyrosine (NPY) due to its C-terminal tyrosine amide (Tatemoto et al., 1982). NPY colocalizes with noradrenaline and it is released in the same noradrenergic vesicles in some synapsis. (Fried et al., 1985; Stjarne et al., 1986). It is thought that NPY may potentiate noradrenaline evoked vasoconstriction and that it has a direct vasoconstrictory effect *per se* (Ekblad et al., 1984). There also exist intrinsic NPY-neurons in pancreatic tissue (Sheikh et al., 1988). It is believed that NPY participates in a negative loop at the pre-junctional release of noradrenaline (Lundberg et al., 1984) and it has been suggested that the pre- and post-synaptic effects are due to different types of receptors (Wahlestedt et al., 1986).

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NPY is one of the most abundant neuropeptides in the mammalian nervous system. In the central nervous system it colocalizes with adrenergic and noradrenergic neurons in the brain stem (including the locus coerulus). It also colocalizes with the neurotransmitter γ-amino butyrate and somatostatin in the human brain cortex. In rats it has been observed to induce feeding behavior and to produce hypothermia (Roscoe et al., 1991). It regulates blood pressure, heart rate, respiratory rate and influences hypothalamic-neuroendocrine systems. In relation to the immune system NPY induces the release of histamine from mast cells (Lundberg et al., 1982) therefore having a modulatory activity and it has also been implicated in depressed activity of natural killer cells (Irwin et al.,1991). It has been also found to up regulate the adhesiveness of human neutrophils or U937 cells (a human monocytic cell line) to human umbilical vein endothelial cells (Sung et al., 1991). This event was not associated with intercellular adhesion molecule-1 (ICAM-1) nor through the induction of cytokines such as IL-1 or TNF.

NPY, peptide YY (PYY) and pancreatic polypeptide (PP), all belong to the PP-fold family because of their characteristic and highly conserved structural features. PYY and PP are intestinal hormones, PYY being present in endocrine cells of the gut and PP in pancreas. In contrast to other peptides of the same size (36 residues) this family conserves a compact globular structure in solution (Glover et al., 1983). This structure has been resolved to a resolution of less than 1 Å for avian PP by x-ray diffraction analysis (Wood et al., 1977). The structure consist of two anti parallel α-helices: a N-terminal Pro rich helix and an amphipathic helix

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joined by a β-turn. The structure is stabilized by interdigitating hydrophobic residues.

High affinity NPY-binding sites (0.1 to 1 nM) had been observed in central nervous system, spleen and enterocytes, and in a rat pheochromocytoma cell line. It has been suggested, based on binding characteristics, that there exist three NPY receptor subtypes termed Y1, Y2 and Y3. Apparently post-synaptic receptors are of the Y1 and Y2 subtypes while the pre-synaptic site belongs to the Y2 subtype. The Y2 receptor is the major one in central nervous system (Fuhlendorff et al., 1990). The Y1 receptor binds NPY and PYY with similar affinity but a "long" C-terminal fragment (e.g. NPY 13-36) does not bind. In contrast this fragment does bind to the Y2 subtype. The Y3 receptors are characterized because they bind NPY with much higher affinity than PYY. Recently three cDNAs have been cloned that code for NPY receptors. A human cDNA belongs to the putative Y1 subclass. A Drosophila NPY receptor has been tentatively equated to the Y2 subtype and a bovine protein which cDNA was cloned from locus coerulus appears to belong to the Y3 sub category (Larhamar et al., 1992; Li et al., 1992; Rimland et al., 1991).

6. Project purpose:

The identification and characterization of receptor proteins for two of the chemotactic and activating factors was the initial scope of this work. The factors in question were Gro/MGSA and MCAF. The biological activities of Gro are not well defined but it is induced by IL-1 and TNF. It presents growth stimulatory activity on fibroblasts and is an autocrine growth factor for melanoma cells. It

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also compete with IL-8 for the binding to the IL-8 R. MCAF is the monocytic chemotactic and activating factor. There are several pathological processes where its involvement is suspected (see Table II). During the course of this work a clone was isolated from a human spleen cDNA library. It was sequenced and analyzed, resulting in the identification of a human homologue of a bovine protein isolated from locus coerulus that is a putative NPY R (subtype Y3). The cloning was done by hybridization with a probe obtained by the reverse transcriptase/polymerase chain reaction from human monocyte RNA. To prime both the reverse transcription and the polymerase reaction a set of primers was designed on the basis of homology between conserved IL-8 R sequences. This neuropeptide receptor is closely related to some of the intercrines receptors and appears to be expressed in cells of bone marrow origin.

VIII. MATERIAL AND METHODS:

1. Materials:

All chemicals were analytical or reagent grade. They were either from Sigma Chemical Co., BDH Inc., or Fisher Scientific. Water was processed with a Zenopure Laboratory water system (Mega 90) to $\geq$ 16 MΩ.cm. It was sterilized by autoclaving.

Bacto-tryptone, yeast extract and bacto-agar were from Difco Laboratories. Penbritin-1000 (ampicillin) was from Ayerst Laboratories. Hybridization membranes were Hybond-N (0.45 μm) from Amersham. Enzymes were either from Boehringer-Mannhein or from New England Biolabs. DNA polymerase for sequencing was Sequenase®* Version 2.0 from United States Biochemical. Agarose was from GIBCO Bethesda Research Laboratories. Films for autoradiography were from Kodak. For the radioactive labeling of DNA two isotopes were used: ^{35}S and ^{32}P. Deoxyadenosine 5'(α^{32}P) triphosphate, triethylammonium salt, in aqueous solution containing 5 mM 2-mercaptoethanol and at a concentration of 370 MBq/ml (10 mCi/ml; >3,000 Ci/mmole)(by the reference due date), and deoxycytosine 5'(α–^{35}S) triphosphate, triethylammonium salt, in aqueous solution containing 5 mM 2-mercaptoethanol and at a concentration of 370 MBq/ml (10 mCi/ml; >600 Ci/ml) (by the reference due date). Both were purchased from Amersham.

Deoxynucleotide triphosphates, Ficoll-Paque and chromatographic gels (Sephadex; Sephacryl) were from Pharmacia P-L Biochemicals.

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For sequencing, the LKB (Bromma), 2010 Macrophor Electrophoretic Unit was used.

2. Plasmids:

The pBluescript® II vector (Stratagene) is a phagemid derived from pUC19 by replacing the polylinker with a synthetic multicloning site containing 21 unique restriction sites. Its size is 2.95 kb. It allows for blue/white selection because it contains the portion of the *lacZ* gene that provides α-complementation when propagated in cells containing the lacI^{q}ZΔM15 mutation on the F‘. There are two possible orientations of the polylinker according to the direction in which the β-galactosidase transcription occurs. The phagemid used was the SK in which the transcription proceeds from *Sac I* to *Kpn I*. The presence of a f1 origin of replication allows for single stranded DNA rescue upon co-infection with a helper phage (see figure 3 and 4).
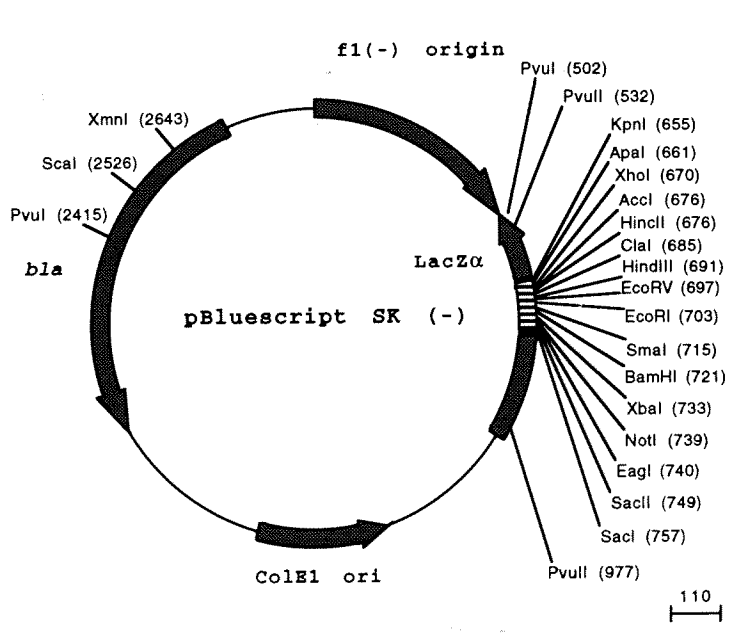
Figure 3: Scheme of the plasmid Bluescript.

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3. Bacteria:

A *E.Coli* derivative, XL1-Blue strain was used trough this work (Bullock et al., 1987). Its genotype is: ndA1, hsdR17 (r_{k-},m_{k+}), supE44, thi-1, λ-, recA1, gyrA96, relA1,(lac-), [F',proAB,lacl^{q}ZΔM15,Tn10, (tet^r)]. This strain was propagated in 2x YT medium (16 g/l bacto-tryptone, 10 g/l yeast extract, and 5 g/l NaCl) at 37°C with vigorous shaking, or on YT plates (YT medium plus 15 g/l bacto-agar) incubated 16 hours at 37°C. Other media used were NYT (16 g/l casein hydrolysate, 2 g/l MgSO$_4$.7H$_2$O, 5 g/l yeast extract) and LB (bacto-tryptone 10 g/l, yeast extract 5 g/l, NaCl 10 g/l).

The lacl^{q}ZΔM15 mutation contained in the F' episome allows for blue/white color selection and the episome bearing cells can be selected with tetracycline. These cells are restriction negative, and both endonuclease and recombination deficient. After transformation, cells bearing the pBluescript were selected by adding ampicillin to the plates or to the media (at a concentration of 50 to 100 µg/ml).

4. The human Fetal Spleen cDNA library:

This premade library was purchased from Stratagene. It was constructed in a λ-vector (Uni-ZAP™ XR). It was derived from poly(A)$^+$ enriched RNA using oligo(dT) primers. The origin of the RNA was pooled tissue.

All inserts were cloned with the 5'-end closest to the *LacZ* promoter allowing the expression of β-galactosidase fusion proteins (the cDNA is unidirectionally inserted in the sense orientation). Each

insert is flanked by specific T3 and T7 promoters that can be used to generate end-specific RNA transcripts. (see figure 4)
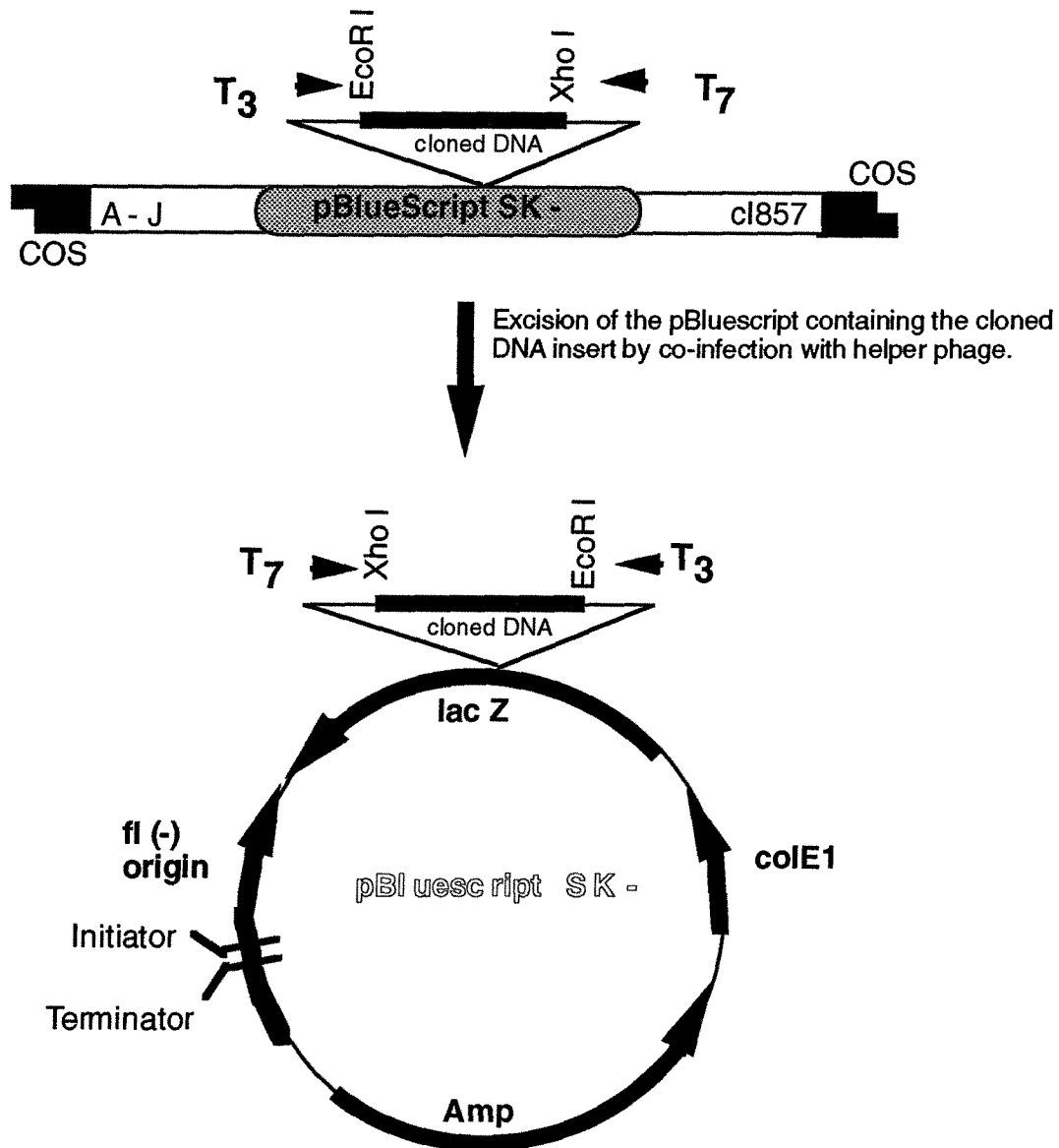
Figure 4: The Uni-Zap vector arms have been digested with EcoR I and Xho I and dephosphorylated, therefore, it allows construction of unidirectional cDNA libraries.

The average insert size was 1.0 kb. The possibility of *in vivo* excision of the plasmid Bluescript from the UniZap vector using a helper phage is convenient because a plasmid system containing the insert of interest for characterization purposes can be obtained without having to subclone. The cloning sites are produced in the UniZap vector by *Xho I* and *EcoR I* double digestion. The insert size can be anywhere from 0 to 10 kb.

5. Synthesis of oligonucleotides:

Oligonucleotides were synthesized with an automatic ABI model 391B DNA synthesizer by John Babcook (Biomedical Research Centre, University of British Columbia) following the phosphoramidite protocol according to the instructions of the manufacturer. After synthesis the resin was treated with concentrated ammonia (fresh solution) to release and deprotect the bound oligonucleotides. Briefly, the cartridge containing the resin and the bound oligonucleotide was incubated for 15 min at room temperature with approximately 0.5 ml of ammonia. After this incubation the aliquot was eluted in an screw cap eppendorf tube. The treatment was repeated three times to increase the yield and the aliquots were pooled. The tubes were incubated at 55 °C overnight for deprotection. Then the samples were concentrated in a speed-vacuum for 3-4 hours (until total dryness). Dried oligonucleotides were redissolved in 0.5 ml of destilled water and further purified by desalting on a Sephadex G50 spin column (2 ml of packed beads equilibrated against destilled water). The concentration of the purified oligonucleotides was estimated by

measuring the A at 260 nm. The usual concentration obtained was 100 mM. The aqueous solution was aliquoted and stored at - 80°C until needed.

6. Purification of rabbit or human leukocytes:

Anticoagulant-treated blood (200 ml) was centrifuged for 10 min at 3,500 rpm (2,000 xg) at room temperature. The interphase between the plasma and the red blood cells contains the leukocytes and the platelets ("buffy" coat). This fraction was recovered with a Pasteur pipette, together with some of the plasma but avoiding the erythrocytes, and recentrifuged. The upper layer containing plasma was discarded and the leukocyte pellets, still contaminated with red blood cells, were resuspended in red blood cells (RBC)-lysis buffer (NH_4Cl 6.57 g/l and Tris base 2.59 g/l adjusted to pH 7.2 with HCl) and then incubated for 7 $^1/_2$ min at 37°C in a water bath. The osmolarity was normalized by adding an equal volume of ice-cold PBS (NaCl 8 g/l, KCl 0.2 g/l , Na_2HPO4 1.44 g/l, KH_2PO_4 0.24 g/l , adjusted to pH 7.4 with HCl) and the leukocytes were recovered by centrifugation.   Cell pellets were resuspended in GT buffer immediately to isolate the RNA (see below).

7. Purification of Monocytes:

200 ml of heparinized blood were collected from a single human volunteer donor. After centrifuging for 5 min at 2,000 xg the "buffy" coats containing mainly leukocytes and platelets were pooled and recentrifuged. The pooled leukocytes were transferred to a new tube and underlayered with 10 ml of Ficoll-Paque. (Boyum, 1968).

The tube was then centrifuged for 20 min at 650 x g. The interphase containing monocytes and lymphocytes was collected and spun down. The supernatant was discarded and the cells were resuspended in 100 ml of RPMI-1640 containing 2-mercaptoethanol (2ME) and supplemented with Gln (2 mM) and 10% FCS. The suspension was transferred into a 200 ml Falcon tissue culture flask and incubated overnight at 37 °C in a tissue culture incubator. After this incubation the flask was gently shaken to remove non-adherent cells and the supernatant was discarded. The remaining adherent cells were washed 10 times with 50 ml aliquots of ice-cold PBS. The cells were lysed *in situ* with 2 ml of GT solution (25 mM sodium citrate buffer pH 7.5 containing 4 M guanidine thiocyanate, 0.5% sarcosyl and 0.1 M 2-ME added just before using). This solution was prepared with diethylpyrocarbamate (DEPC)-treated water. An extra 2 ml were used to rinse the flask and this wash was pooled with the lysis solution.

8. Extraction of total cell RNA:

The basic molecular cloning techniques used here can be found in Sambrook et al. (1989). All the modifications are indicated in the respective protocols.

This procedure was performed on ice unless otherwise indicated and all the material was treated with DEPC to minimize the contamination with ribonucleases. The lysed samples were homogenized by 25 passages through a 22G1-bore needle to shear the genomic DNA (avoiding foaming). Per 500 μl aliquots 50 μl of 3 M sodium acetate pH 4.0 were added. After a short vortexing to mix

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500 µl of phenol/water (50:50) were added and the tubes were vortexed again. 100 µl of chloroform/isoamyl alcohol (24:1) were added and mixed by inverting the tubes; then, they were incubated on ice for 20 min. The samples were centrifuged for 20 min at 14,000 rpm at 4°C in an Eppendorf microcentrifuge. The upper layer was transferred to a new tube and 600 µl of ice-cold isopropanol were added and mixed by inverting the tubes. After incubating at -20°C for 1 hour the precipitate was collected by centrifugation at 14,000 rpm for 20 min at 4°C. The supernatant was aspirated and the pellet was resuspended in 200 µl of GT solution. Three of such aliquots were pooled and re-precipitated with 600 µl of ice-cold isopropanol. The resulting pellets were resuspended in 200 µl of DEPC-treated double destilled water and 20 µl of 3 M sodium acetate pH 4 were added followed by 500 µl of 100% ethanol cooled at -20 °C. After 1 hour incubation at -20 °C, the precipitated RNA was recovered by centrifugation for 20 min at 14,000 rpm (at 4°C). The supernatant was aspirated and the pellet was dried in a dessicator for 10 min under vacuum. Each pellet was resuspended in 50 µl of DEPC-treated water. The $A_{260\ nm}$ and $A_{280\ nm}$ were measured for a 1/250 dilution and the remaining sample was stored at -80°C.

Normally, 0.5 to 1 µg of total cell RNA is enough to amplify by PCR even rare mRNA sequences. Considering that the RNA content per cell is approximately 10 pg, 1 µg is the amount obtained from 1 x 10^5 cells. Thus, the number of messenger copies per 1 µg RNA is at least the same as the number of cells. Therefore preparation of poly[A]+RNA is usually not required.

The A_{260nm}/A_{280nm} ratio (which is indicative of the quality of the preparation with respect to protein contamination) was consistently close to 2.0 with this technique.

9. Extraction of RNA from tissues:

The tissue was removed, quickly frozen in liquid nitrogen and grinded into pieces of about 2 g. For each 2 g of tissue 20 ml of guanidine solution (without sarkosyl) were added. The tissues were homogenized by three burst of 10 seconds each with an Ultraturrax. After centrifuging for 10 min. at 10,000 rpm in a Sorvall SS34 rotor (at 4°C), the supernatant was decanted and 1/10 volume of 20 % sarkosyl was added. Total RNA was isolated using the guanidine thiocyanate-acid phenol method with the addition of a cesium chloride ultracentrifugation step to purify the RNA.

10. Reverse Transcription:

All the materials and labware were either treated with DEPC or free of ribonuclease contamination. The following reagents were combined in a 20 µl final volume: reaction buffer: 50 mM Tris.HCl (HCl-neutralized Tris[hydroxymethyl]aminomethane) pH 8.3 at room temperature containing 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT (the DTT solution was stored at -20°C as a 10x solution and it was added just before starting the reaction), 1 mM of each deoxynucleotide triphosphate (dNTPs) (from a stock solution at neutral pH), 1 unit/µl RNAsin (Promega), 100 pmole of reverse primer oligonucleotide, 0.5 µg of RNA and 200 units of Moloney Murine Leukemia Virus RNAse H⁻ reverse transcriptase (GIBCO BRL)

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(Kotewics et al., 1988). The RNA sample and the primer were first mixed together and the reaction tube was heated at 65°C for 5 min. After a short spin the mixture was incubated on ice for 3 min. This was intended to disrupt secondary structure elements that could hinder the cDNA synthesis. After this, the reaction mixture was completed as indicated and the reverse transcription was allowed to proceed for 1 hour at 42 °C. At the end of this incubation the reaction was heated at 95°C for 5 min in a water bath, briefly spun to collect condensed water from the tube walls, and then quick-chilled on ice. This treatment denatures RNA-cDNA hybrids and inactivates the enzyme (Veres et al., 1987)

11. Polymerase Chain Reaction (PCR) amplification:

The heat-treated reverse transcriptase reaction was scaled up to 100 μl by adding 100 pmole of direct primer oligonucleotide, 10 μl of 10x PCR buffer (250 mM Tris.HCl pH 8.3 at 25 °C containing 20 mM $MgCl_2$, 500 mM KCl and 10 mM DTT in MilliQ grade water) 5 units of *Thermus aquaticus* (Taq) DNA polymerase and MilliQ water. After mixing by vortexing and a short spin to collect the sample, 60 μl of liquid paraffin were layered on top of the PCR reaction mix to prevent liquid evaporation during the thermal cycling. The number of cycles for amplification was 35 and the thermal cycle profile was as follows: 1) denaturing for 30 seconds at 96°C, 2) cooling over 1 min to 55°C, 3) annealing primers for 30 seconds at 55°C, 4) heating over 30 seconds at 72°C, 5) primer extension for 90 seconds at 72°C. After the number of cycles was completed the reaction mix was kept

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at 4°C until further analysis or stored at -20°C for prolonged periods of time.

12. Electrophoretic analysis of Nucleic Acids:

The electrophoresis was performed in horizontal submarine gels. The desired agarose (w/v) concentration was added to the appropriate volume of TAE buffer (40 mM Tris.HCl pH 7.5 containing 1 mM EDTA and 5 mM Sodium acetate) and dissolved by microwave radiation. The gel was poured and allowed to cool down either at room temperature or at 4°C to accelerate the solidification process. Two types of agarose were employed, either low melting point agarose for preparative or electrophoretic grade agarose for analytical electrophoresis. The samples were applied in a loading buffer mix (25% glycerol, 0.25% xylene cyanol FF, 0.25% bromophenol blue) diluted 1/10 and electrophoresed at 100 mA constant current, until the marker dyes reached the desired reference points. A 1 kb DNA-ladder from BRL was used as size marker (1 μg/lane).

After electrophoresis the gel was stained by soaking in a ethidium bromide solution (10 μg/ml freshly made in tap water) for at least 10 min. Photographs were taken on a short wave UV (254 nm) transilluminator using an orange photographic filter and a high speed polaroid film with the camera set at f=5.6 and an exposure time of 1/2 second.

13. Purification of DNA from agarose gels:

After electrophoresis and staining as described the gel was transilluminated with a low energy UV lamp and the bands of the

desired size were excised using new surgical blades. Excess agarose was trimmed and the agarose plug was transferred to pre-weighted microfuge tubes. The Sephaglas™ BandPrep kit from Pharmacia was employed according to the manufacturer instructions. Briefly, for each 250 mg of agarose plug 250 µl of gel solubilizer were added and vortexed for 1 min. The tube was heated at 67°C for 5 min. Then 5 µl per µg of DNA of a uniform suspension of Sephaglas BP were added to the dissolved gel slice and the tube was vortexed gently. The suspension was centrifuged at 14,000 rpm in a microcentrifuge and the supernatant was removed by aspiration. The pellet was washed with a buffer containing ethanol (8x times the volume of Sephaglas added). After a spin at high speed for 1 min the supernatant was removed. This wash was repeated three times. The tube was inverted on a paper towel on the bench top and left to dry for 10 min. The adsorbed DNA was eluted with a minimum of 10 µl of elution buffer (after vortexing gently to resuspend the pellet) and incubated for 5 min at room temperature. After a high speed centrifugation the supernatant was recovered (with care not to resuspend the glass pellet). This step was repeated once more to improve the final yield.

14. Restriction enzyme digestion of plasmid DNA and amplified cDNA:

To obtain a recombinant plasmid the amplified cDNA was either restricted using the *EcoR I* sites engineered in the primers (for cohesive end ligation) or was left uncut (for blunt-end ligation). The phagemid plasmid Bluescript SK (-) was restricted accordingly. 10 µl containing approximately 1 µg of purified cDNA were mixed

with 2 µl of 10x buffer (1x = 50 mM NaCl,100mM Tris.HCl pH 7.5, 5 mM MgCl$_2$, 100 µg/ml BSA) and 1 µl (5 U) of *EcoR I* . The volume was brought up to 20 µl with MilliQ water and the reaction was allowed to proceed at 37°C overnight. Plasmid DNA (10 µg) was digested in 50 µl of a mixture containing 5 µl of 10x buffer and 25 units of *EcoR I* in MilliQ water for complementary ends or in 50 µl of a mixture containing 5 µl of 10x buffer (1x = 25 mM Tris.HCl pH 7.7, 10 mM MgCl$_2$, 1 mM DTT, 100 µg/ml BSA) and 25 units of *Sma I* for blunt ends. In both cases the reaction was allowed to proceed at 37°C overnight.

15.   Ligation:

For complementary-ends ligation 0.5 µg of amplified cDNA (*EcoR I* restricted) were mixed with 1 µg of *EcoR I* restricted pBluescript in a final volume of 10 µl containing ligase buffer (10x concentrate: 0.5 M Tris.HCl pH 7.6, 100 mM MgCl$_2$, 100 mM DTT, 500 µg/ml BSA Fraction V) and 400 units of T$_4$ DNA-ligase (NEB). The reaction mix was incubated overnight at 16 °C.

For blunt ends ligation the same amount of amplified cDNA (unrestricted) was admixed with 1 µg of *Sma I* restricted pBluescript in a final volume of 10 µl containing ligase buffer and 400 units of T$_4$ ligase (NEB). This reaction was incubated overnight at room temperature.

Both reactions were heat inactivated by incubating the tubes at 70 °C for 15 min and briefly spun to collect condensed water. The ligation volume was brought up to 80 µl with 10.10.10 buffer (10 mM

Tris.HCl pH 7.5, 10 mM EDTA, 10 mM NaCl). 40 μl were used for transformation and the remaining was stored at -80°C.

16. Preparing competent cells:

A single colony of XL1-blue was chosen from a fresh agar plate and used to inoculate 2 ml of 2x YT medium. The cell suspension was incubated at 37°C in a shaker for approximately 2 hours (until the cells reached log phase). Then the culture was expanded into 200 ml of 2x YT medium and incubated for a further 2 hours. After reaching confluence it was transferred into an ice bucket, aliquoted in 50 ml conical Falcon tubes and centrifuged for 10 min at 3,000 rpm (1500 xg) at 4 °C. The supernatant was discarded and the pellets were resuspended in 20 ml of ice-cold, sterile 100 mM $CaCl_2$. The resulting suspension was incubated on ice for 30 min. and then centrifuged as described. The supernatant was poured off and the pellets were resuspended in 5 ml of 15% glycerol in 100 mM $CaCl_2$. Aliquots of 200 μl were transferred to pre-chilled eppendorf tubes and the tubes were quickly frozen in a dry ice-methanol bath and stored at -80°C.

17. Transformation:

To a 200 μl aliquot of competent cells that was quickly thawed at 37 °C and transferred into ice, 40 μl of the ligation mix (in 10.10.10 buffer) were added. After mixing by gently inverting the tubes the reaction mixture was incubated on ice for 15-30 min. The cells were then heat-shocked for 2 min at 42 °C. After this the tubes were incubated for 5 min on ice. 750 μl of NZY medium were

added and the bacterial suspension was incubated at 37 °C for 20 min. without shaking.

For blue-white selection 2x YT agar plates containing ampicillin (100 μg/ml) were spread till dryness first with 40 μl of X-gal (20 mg/ml in dimethylformamide), and then with 10 μl of IPTG (1 M in dH$_2$O). The cells were plated at two concentrations: 200 μl of transformed cells were spread on the surface of the agar and the remaining cells were spun down resuspended in 200 μl of medium and then plated as above. In both cases the plates were incubated overnight at 37 °C in an incubator. After overnight incubation the plates were stored for at least 2 hours at 4°C to increase the blue color.

18. Minipreps for plasmid DNA:

To purify plasmid DNA for further characterization 3 ml of 2x YT medium containing 100 μg/ml ampicillin were inoculated with a sterile toothpick containing a single white colony. The cultures were incubated for 6 hours in a shaker at 37 °C and the tubes were placed on ice at the end of this incubation. Two aliquots of 750 μl for each culture were transferred to pre-chilled eppendorf tubes and centrifuged at high speed for 30 seconds in a microcentrifuge. The supernatant was aspirated carefully and the pellets were resuspended by up and down pipetting in 110 μl of STET buffer (0.1 M NaCl, 10 mM Tris.HCl pH 8.0, 1 mM EDTA pH 8.0, 5% Triton X-100). The tubes were incubated for 5-10 min at room temperature. Samples were heated at 95 °C for precisely 3 min to burst open the cells, and the cell debris was collected by centrifugation at 14,000

rpm for 5 min. Pellets were pulled out with sterile toothpicks and discarded. Tubes were allowed to reach room temperature and 110 µl of isopropanol at room temperature were added and mixed. Plasmid DNA was pelleted and the supernatant was aspirated (exercising care not to aspirate the small pellets) and the samples were dried under vacuum for 3 min. Plasmid DNA was resuspended in 40 µl of TE buffer (10 mM Tris.HCl pH 8.0, 1 mM EDTA pH 8.0) and centrifugated at high speed for 10 min. Pellets were discarded and the samples were stored frozen at - 20°C until further processing.

19. Mapping of recombinant plasmids:

To confirm the presence of the insert in the recombinant plasmids an aliquot of 5 µl of plasmid DNA (300 ng) was incubated in a final volume of 20 µl containing 0.5 µl of RNAse A (20 mg/ml), 1 µl of Eco RI and 2 µl of 10x buffer (1x = 50 mM NaCl, 100 mM Tris.HCl pH 7.5, 5 mM $MgCl_2$, 100 µg/ml BSA) for 90 min at 37 °C. To analyze the composition of the plasmids the digested samples were electrophoresed in 1.5% agarose as described.

20. Plasmid DNA purification for sequencing insert:

Recombinant cultures (as analyzed by restriction digestion and electrophoresis) were plated on ampicillin/2x YT agar plates and incubated overnight to obtain single colonies. These single colonies were used to start 20 ml cultures in NZY containing 100 µg/ml ampicillin (50 ml Falcon conical tubes with secured but loose caps were used for this purpose). After 16 hours incubation in the shaker at 37°C the cells were collected by centrifugation at 2,800 rpm

(1,400 xg) for 10 min. at 4°C. The supernatant was decanted and the resulting cell pellets were thoroughly resuspended in 1 ml of Glucose solution (50 mM glucose, 25 mM Tris.HCl pH 8.0, 10 mM EDTA pH 8.0) at 4°C. Then, 2 ml of a solution containing 0.2N NaOH and 1% SDS were added to each tube and the contents were mixed by swirling. After incubating for 5 min on ice, 1 ml of 3M potassium acetate buffer, pH 5.7, was added and mixed. The precipitate was collected by centrifugation at 3,500 rpm (2,000 xg) for 10 min (at 4°C). The supernatant was saved by decanting into Falcon 2059 tubes to which one volume (approximately 3.5 to 4 ml) of phenol/chloroform were added. The two phases were thoroughly mixed by shaking and then separated by centrifugation at 3,500 rpm (2,000 xg) for 10 min at room temperature. The aqueous (upper) layer was transferred to new tubes. One volume (3.5 ml) of isopropanol at -20°C was added and, after mixing by gentle inversion of the tubes, the plasmid DNA was precipitated at -20°C for 1 hour. The precipitate was collected by centrifugation at 3,500 rpm (2,000 xg) for 10 min (at 4°C), the supernatant was poured off and the pellet let to dry (but not completely). The white, gelatinous pellet was resuspended in 200 µl of STE (0.1 M NaCl, 10 mM Tris.HCl pH 8.0, 1 mM EDTA pH 8.0) by vortexing after which 200 µl more of STE were added. On occasion the pellet did not solubilize, in this case the samples were incubated for 5 min at 55°C. To eliminate contaminating RNAs the solubilized samples were treated with 200 µg of RNase A at 37°C for 20 min to 1 hour. The sample was extracted with 1 volume of phenol/chloroform. The aqueous phase was transferred to clean tubes and further purified by desalting on

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Sephacryl S300 spin columns (3 ml of packed beads equilibrated against TAE buffer). The excluded volume was spun off by centrifuging the columns for 1 min at 1100 rpm (250 xg), the samples were then applied and the DNA recovered by centrifugation for 2 min at 1100 rpm (250 xg). The salt content was increased by adding 40 µl of 3 M potassium acetate, pH 5.7, and the DNA was precipitated with 2.5 volumes of 100% ethanol (at -20°C) for 1 hour at -20°C. Precipitated material was recovered by high speed centrifugation at 4°C in a microcentrifuge. The supernatant was aspirated, the plasmid DNA was dried under vacuum in a dessicator for 10 min., and then resuspended in 50 µl of TE buffer. The purity and concentration were estimated from electrophoresis and ethidium bromide staining.

21. Sequencing reactions:

The chain termination method involves the synthesis of DNA by DNA polymerase. It occurs only at the site where a primer oligonucleotide is annealed to the template. The synthesis proceeds until a nucleotide analog, that does not allows further elongation of the DNA strand, is incorporated (Sanger et al.,1977). These analogs are the 2',3'-dideoxynucleoside 5'-triphosphates (ddNTPs) that lack the 3'-OH group necessary for the synthetic reaction to continue. When mixtures of dNTPs with one of the ddNTPs are employed elongation will be terminated in a fraction of the DNA strands at each site where the ddNTP could be incorporated. Hence, four separate reactions each with one of the four ddNTPs will render

the complete sequence information. Pyrophosphatase was used in conjunction with Sequenase®Version 2.0 (USB). The former is used to avoid the slow, sequence dependent reversal of the DNA polymerase reaction (Tabor and Richardson, 1990). The latter is characterized by high processivity, lack of 3' to 5' exonuclease activity and the efficient use of the nucleotide analogs.

The reaction is carried out in two steps. First, the primer is extended in the presence of limiting concentrations of dNTPs and radioactive labeled dATP. The labeled chains synthesized in this step have a random length distribution. Then, the concentration of the dNTPs is increased and one of the four ddNTPs is added. This is the actual chain-termination step. Finally the reactions are halted by adding EDTA and formamide and heating up to 85°C for two minutes. The samples are immediately applied to the gel and electrophoresed at 55°C and 35 W (constant power). The gel is dried as described and the sequence pattern is revealed by autoradiography. The use of the low energy [α-^{35}S]-dATP (instead of ^{32}P) increases the resolution at this step. Double stranded templates work well provided that the plasmid DNA is free of RNA and salts. As described above, the alkaline lysis method, if combined with a RNase treatment step followed by desalting on a molecular sieve (e.g. with spin columns), gives templates of enough purity for sequencing. The templates are then alkali denatured , and after neutralization and ethanol precipitation, the annealing to the primers can be performed as it would with single stranded DNA.

21.1. Alkaline denaturation of double stranded DNA:

To 8 μl of DNA containing 1-3 μg of plasmid 2 μl of 2 M NaOH were added (when a smaller volume of DNA was used the volume was completed up to 10 μl with MilliQ water). After mixing by vortexing and a short spin to collect the solution from the tube walls the samples were incubated for 10 min at room temperature. The mixture was neutralized by adding 3 μl of 3 M sodium acetate pH 4.8 and the balance up to 20 μl was made up with MilliQ water. The DNA was precipitated with 3 volumes of 100% ethanol (at -20°C) by incubation at -20°C for 1 hour, and recovered by high speed centrifugation in a microcentrifuge. The supernatant was discarded and the pellet was washed with ice-cold 70% ethanol. After centrifuging and discarding the washing solution, the final pellet was dried briefly and resuspended in 10 μl of MilliQ water.

21.2. Annealing reaction:

For every four sequencing lanes a single annealing and labeling reaction was performed. To 10 μl of template DNA 2 μl of annealing buffer (5x concentrate: 200 mM Tris.HCl pH 7.5 containing 100 mM $MgCl_2$ and 250 mM NaCl) and 2 μl (0.5 pmole/μl) of either T_7 or T_3 primer were added before mixing and incubating at 37°C for 20 min. This gives a primer:template molar stoichiometry close to 1:1. The annealing reaction was allowed to proceed for 10 min at room temperature. After a short spin the samples were either stored at -20°C or used immediately for the labeling reaction.

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21.3. Labeling reaction:

For each template set the following was added: 3 µl of labeling mix diluted 1/3 in water (5x concentrate containing 7.5 µM dGTP, 7.5 µM dCTP and 7.5 µM dTTP), 0.5 µl of 0.1 M DTT and 0.5 µl of pyrophosphatase (USB). The DNA-polymerase (Sequenase version 2.0, USB) was diluted 1/8 in 10 mM Tris.HCl, pH 7.5, containing 5 mM DTT and 0.5 mg/ml BSA. Per template, 0.5 µl (5 µCi) of ^{35}S-dATP were added immediately followed by 2 µl of diluted enzyme. Tubes were quickly vortexed and spinned and incubated for 10 min at room temperature.

21.4. Termination reactions:

The composition of the termination mixes was as follows: 80 µM each of dGTP, dATP, dCTP, dTTP and 8 µM of the corresponding ddNTP and 50 mM NaCl. 2.5 µl of the appropriate termination mix were placed in the wells of microtitration plates (the plates were warmed at 37°C just prior to initiate the reaction). When the labeling incubation was complete, 4.5 µl of labeled template (per termination reaction) were mixed by up and down pipetting resulting in four sets (G,A,T, and C) of reactions per template. The incubations were continued for 5 min at 37°C. At the end of this incubation 4 µl of stop solution (98% formamide, 20 mM EDTA pH 8.0, 0.05% Bromophenol Blue and 0.05% Xylene Cyanol FF) per reaction were added. Just before loading the samples in the gel they were heated at ≥ 85°C. If the samples were stored at -20°C the final heating step was repeated before the electrophoresis.

21.5. Casting of polyacrylamide gels for sequencing:

The acrylamide stock solution was prepared by dissolving 100 g of ultrapure acrylamide (ICN) and 5 g of N,N'bismethylene acrylamide in 150 ml of MilliQ water. Once dissolved the volume was brought up to 250 ml and the solution was deionized by stirring with 5 g of mixed-bed resin (Dowex XG8) for 2 hours in the cold room . The resin was filtered out through Whatman #2 and the resulting solution was stored wrapped in tin foil at 4°C .

The LKB system (2010 Macrophor - Electrophoresis Unit) was used for sequencing. The thermostatic plate was siliconized by thoroughly wiping 6 ml of Repel-silane (LKB) until dryness two times. The front glass plate was siliconized with 5 ml of a solution containing 20 µl of Bind-silane (LKB A-174), 5 ml of 95% ethanol and 2.5 ml of 10% acetic acid. The solution was wiped on the surface until dryness followed by 2 quick rinses with 95% ethanol. When the plates were ready, a polymerizing solution was prepared by mixing 7 ml of acrylamide stock solution with 20 ml of MilliQ water, 5 ml of 10x TBE (45 mM Tris.borate pH 8.0, 1 mM EDTA H 8.0) and 21 g of urea. After stirring to dissolve the urea the volume was brought up to 50 ml with water and the solution was filtered through a 0.45 µm filter. To initiate the polymerization 400 µl of 10% ammonium persulfate freshly made, and 40 µl of TEMED, were added. After mixing thoroughly, the gel (0.2 mm thickness) was poured according to the instructions for this particular apparatus.

21.6. Denaturing gel electrophoresis:

Before applying the samples the gel was pre-run for 30 min to 1 hour at 55 °C and 45 W (constant power). 3 µl for each termination reaction were loaded (after heat denaturing them for 2 min. at ≥ 85°C; see above). The electrophoresis was allowed to proceed using the dyes as references. After the run was completed the gel was soaked for two periods of 15 min with 1 liter each of 10% acetic acid (in a shaker). The gel was dried with a hair drier and exposed with a fast film (Kodak XAR-5) overnight at room temperature.

22. Large scale preparation of plasmid DNA:

This protocol was performed at 4°C or on ice unless otherwise indicated. A fresh overnight culture of transformed bacteria in 2 aliquots of 500 ml NZY containing 100 µg ampicillin per ml was prepared. Cells were pelleted by centrifuging for 10 min at 5,000 rpm in a Sorvall GS3 rotor. The supernatant was discarded and the walls were dried with a Kimwipe. The pellets were vortexed and flicked till they formed a thick slurry. To each pellet 7 ml of cold glucose solution (50 mM glucose, 25 mM Tris.HCl pH 8.0, 10 mM EDTA pH 8.0) were added and mixed thoroughly. Each slurry was divided in two pre-chilled 50 ml conical tubes and 14 ml of a 0.2N NaOH/1% SDS solution were added to lyse the cells. After mixing by inversion, tubes were incubated for 10 min on ice (with occasional gently shaking). 7 ml of potassium acetate, pH 5.7, were added and the mix was transferred to pre-chilled 50 ml Sorvall tubes and spun for 10 min at 10,000 rpm in a SS34 Sorvall rotor. The supernatants were

then transferred to pre-chilled 50 ml conical tubes and extracted with an equal volume of phenol/chloroform. The phases were separated by centrifugation in a benchtop centrifuge for 10 min at 3,000 rpm (at room temperature). The top layer was carefully removed and transferred to new tubes and the nucleic acids were precipitated with 1 volume of isopropanol (at -20°C) for 1 hour at -20°C. After centrifuging for 15 min at 10,000 rpm in the SS34 rotor the supernatant was discarded and the pellets were dried for 10 min in a dessicator. They were resuspended and pooled in 800 µl STE buffer, and treated with 200 µg of RNAse A for 1 hour at 37°C. The reaction mix was extracted with 1 volume of phenol/chloroform and the top aqueous layer was precipitated with 2.5 volumes of 100% ethanol (at -20°C) for 1 hour at -20°C. The DNA was pelleted by spinning at 14,000 rpm for 20 min in a microcentrifuge, the supernatant was removed and the pellet was dried in a dessicator for 10 min. The DNA was resuspended in 500 µl of TE buffer and either stored at -20°C or processed immediately.

23. Purification of insert cDNA from recombinant plasmids:

Plasmid DNA obtained through the large prep procedure was digested with *EcoR I* and purified by preparative agarose electrophoresis as described above and stored in TE buffer at a concentration of 100 ng/µl.

24. Screening of the cDNA library:

A premade human fetal spleen cDNA library was purchased from Stratagene (Vector Uni-ZAP™XR). Upon arrival the library was

thawed, diluted 1/4 in SM buffer (5.8 g/l NaCl, 2 g/l $MgSO_4$, 50 ml/l Tris.HCl, pH 7.5, 5 ml/l 2% ($^w/_v$) gelatin) and 40 μl of chloroform/ml were added (250 μl aliquots were stored at -80°C until needed).

24.1. Preparation of host cells:

An isolated single colony of XL1-Blue cells was used to inoculate 50 ml of NZY medium containing 500 μl of 20% maltose and 500 μl of 1 M $MgSO_4$. After 16 hours in the shaker at 37°C the cells were collected by centrifugation at 2,000 rpm (750 xg) for 10 min. The supernatant was discarded and the resulting pellet was resuspended as a slurry in 15 ml of 10 mM $MgSO_4$. This suspension was stored at 4°C and made fresh every week.

24.2. Titration procedure:

An initial 1/1,000 dilution of the library was prepared in SM buffer. Different aliquots of this dilution were used to inoculate 500 μl of host cells. Two extra dilutions (1/10,000 and 1/100,000) were also tested. After a brief incubation of 20 min at 37°C in the shaker to initiate the infection cycle, the cells were mixed with 6.5-8 ml of melted top agar at 45°C and poured on top of pre-warmed 2x YT agar plates. The plates were maintained for 10 minutes at room temperature and then incubated overnight at 37°C. The plaques from the best dilution were counted and the titer expressed as number of plaque-forming units (pfu) per ml.

24.3. Screening procedure:

A dilution corresponding either to 5,000 pfu/150 mm plate was employed for overnight or 10,000 pfu/150 mm plate for 6-8 hours incubation. After the incubation period was completed the plates were refrigerated for 2 hours at 4°C to prevent the top agar from sticking to the nylon filters (Hybond-N). The filters were set on top of the plates for transfer during 2 min, and marked for orientation purposes. Filters were denatured after lifting by setting them on a Whatman 3 MM soaked in 1.5M NaCl, 0.5M NaOH for 2 min. Excess agar was removed at this stage. Then, the filters were neutralized by incubating on a Whatman 3 MM soaked in 1.5M NaCl, 0.5M Tris.HCl pH 8.0 for 5 min. After that, they were rinsed for 30 sec. in 0.2M Tris.HCL pH 7.5 prepared in 2x SSC (20x concentrate: 175.3 g/l NaCl, 88.2 g/l sodium citrate pH 8.0, adjusted with 10N NaOH) and blotted on Whatman 3 MM to absorb excess humidity but without let them dry completely. To cross-link the DNA to the filters the Stratalinker™ UV crosslinker with a setting of 1,200 W in 60 sec. was employed. A duplicate filter was obtained in which the transfer time was extended to 7 min., the remaining steps were kept the same.

24.4. DNA labeling reaction:

To radioactive label the fragment the method of Feinberg and Vogelstein (1983; 1984) was used. It is based on the hybridization of a mixture of all possible hexanucleotides to the DNA to be labeled. The synthesis of the second strand proceeds from the 3' end of the random primer. The large fragment of DNA polymerase (Klenow

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enzyme) was used for this step. Analogs of the dNTPs present in the mixture are going to be incorporated. Thus radioisotopically or otherwise labeled dNTPs form part or the replicated strands.

After purification as described, insert cDNA (388 bp) was radioisotopically labeled with ^{32}P. Briefly, 100 ng cDNA in a final 10 μl (in water) were heat denatured by boiling for 5 min and incubated on ice for ≥ 3 min after a short spin. To this were added in the following sequence: 2 μl of 10x Klenow buffer (400 mM Tris.HCl pH 7.5, 66 mM MgCl$_2$ and 10 mM 2-mercaptoethanol), 2 μl of ATG mix (containing 25 mM each of dATP, dTTP, dGTP), 1 μl of random hexamers (equivalent to 100 pmole), 1 μl of the Klenow fragment of DNA polymerase and quickly afterwards 5 μl of ^{32}P-dCTP. The mixture was incubated for 60 min. at 37°C. The reaction was halted by adding 60 μl of 10 mM Tris.HCl pH 7.5 containing 10 mM EDTA and 0.5% SDS, mixing and adding 80 μl of 5 M ammonium acetate followed by 10 μl of a solution containing 10 μg/μl tRNA (as a carrier). The resulting radiolabeled probe was precipitated with 400 μl of 100% ethanol and collected by centrifugation for 15 min at 14,000 rpm in a microcentrifuge. The supernatant was discarded and the pellet dissolved in 100 μl of dH$_2$O. The solution was boiled for 5 min and after a short spin transferred onto ice. The random-primed labeled cDNA was prepared always shortly before use.

24.5. Hybridization:

The excess of binding sites on the filters were quenched by incubating them in 20 ml of 0.33M phosphate buffer, pH 7.5,

containing 1% (^{w}/$_v$) BSA, 30% (^{v}/$_v$) deionized formamide and 7% (^{w}/$_v$) SDS. The filters were incubated in this solution from a minimum of 1 hour at 55°C to overnight at room temperature. After this the hybridization bag was opened and the contents replaced with 10 ml of fresh hybridization solution. To this, 100 µl of ^{32}P-labeled probe were added (see above), and the incubation was allowed to proceed for 12-16 hours at 55°C in a water bath with agitation. After discarding the hybridization solution the filters were taken out of the bag, and transferred into 500 ml of washing solution (150 mM phosphate buffer, pH 7.5, containing 0.1% SDS) at room temperature. After a short wash this solution was discarded and replaced with 500 ml of fresh buffer warmed up to 60°C and incubated in the shaker for 10 min. Filters were blotted dry on Whatman 3 MM paper and the radioactivity was measured with a Geiger-Mueller tube. This was repeated every 10 min until the measured back ground was low. Then the wash was interrupted, the excess of humidity was blotted on Whatman 3 MM paper and the filters transferred, one at a time, onto Saran wrap, wrapped, positioned onto used film and secured with tape. For orientation purposes the film was marked with ^{35}S-ink. Autoradiographic replicas were obtained by overnight exposure to Kodak fast film (XAR-5) at -80°C. Usually 20 filter at a time were processed using this protocol. All manipulations were performed with gloved hands and forceps.

24.6. Rescue protocol:

The positive plaques were cored from the master agar plate and transferred into a sterile eppendorf tube containing 500 µl of

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SM buffer and 20 µl of chloroform. The tube was vortexed to release the phage particles and incubated overnight at 4°C. Assuming a phage concentration of 0.5 to 1 x 10^6 phage particles per core, three different dilutions were tested around a desired titer of 5 x 10^2 plaques per plate. Incubation, lifting and hybridization were performed as described. Positive plaques in this secondary screening that were well isolated from neighboring plaques were cored and the resulting phage solution was used in the subsequent rescue procedure.

An aliquot of 200 µl of competent XL1-Blue were infected with an aliquot of 1 x 10^5 phage particles (from the stock obtained after secondary screening of the plaques of interest) and 1 µl of R408 helper phage (1 x 10^{11} pfu/ml). Negative controls were performed with helper phage alone. This mixture was incubated at 37°C for 15 min after which 5 ml of 2x YT medium were added and the incubation was allowed to proceed for 3 hours with shaking. Then, the cultures were heated at 70°C for 20 min.; unlysed cells and cell debris were removed by centrifugation for 5 min at 1,200 rpm (4,000 xg). The supernatant containing the phagemid particles was decanted on a sterile tube and stored at 4°C (for up to one month) after use. Different aliquots of this stock were used to infect 200 µl aliquots of competent XL1-Blue. These cultures were incubated at 37°C for 15 min.; 10 µl were then plated on LB/ampicillin plates and incubated overnight at 42°C. Colonies that grew under ampicillin selection were streaked on new plates and/or expanded for further analysis.

24.7. Mapping of phagemids:

The rescued pBluescript phagemids containing the hybridizing cDNA insert were expanded in 20 ml cultures and plasmid DNA was isolated by the alkaline-lysis method as described above. To confirm the rescue of the recombinant phagemid, an excision of the cloned insert was performed with a combination of *Xho I/ Not I.*

To map restriction sites in the insert different enzymes were employed to digest the recombinant plasmid (see results).

25. Northern blot analysis of tissue and cell RNAs:

A hybridization membrane containing total RNA from various *Rhesus* monkey tissues was purchased from BIOS. Cell lines and mice tissues RNA were prepared as described (see Methods 8 and 9) and processed according to the following protocol.

The gel was prepared by first melting the agarose in DEPC treated MilliQ water. To 1 volume of this solution (when reached approximately 60°C) 1/10 volume of 10x MOPS (200 mM MOPS pH 7.0 containing 50 mM NaOAC, 10 mM EDTA and 0.1% DEPC; autoclaved) and 1/10 volume of 37% formaldehyde were added. The components were quickly mixed and the gel was poured while this solution was still hot (in a fume hood). In the meanwhile, 20 μg of RNA were ethanol precipitated. Briefly, to 1 volume of RNA sample 1/10 volume of 3 M NaOAc, pH 5.0, (DEPC treated) and 2.5 volumes of 100% ethanol (at -20°C) were added and these mixtures were incubated for ≥ 20 min. at -20°C. The RNA precipitate was collected by centrifugation at 14,000 rpm for 15 min. in a microcentrifuge. The excess of ethanol was aspirated and the pellet was dried in a

dessicator for 5 min. The RNA was redissolved in 25 μl of DEPC water to which an equal volume of loading buffer (1,000 μl of deionized formamide plus 250 μl of 10x MOPS and 250 μl of 37% formaldehyde) was added. The RNA was denatured by heating the sample at 70°C in a water bath for 10 min. Ideally the gel should be allowed to polymerize for 1 hour. The buffer chambers in the electrophoresis system were filled with running buffer (MOPS buffer containing 6% formaldehyde) but without flooding the gel. To the samples, 10 μl of formaldehyde loading buffer were added, mixed, spinned for a few seconds, and loaded onto the gel. Samples were applied in duplicates to make a replica for staining. The gel was run at a constant voltage of 5 V/cm for 3 hours or until the Bromophenol Blue had migrated halfway down. The section to be transferred was cut and rinsed several times with dH_2O to wash out the formaldehyde. The gel was incubated in 0.5 l of 10x SSC in a rotating platform for 45 min. Transfer to Hybond-N membranes was done overnight in 20x SSC using a stack of paper over the membrane to promote the movement of buffer plus solutes upward, and through the gel and the filter. The duplicate gel was stained with ethidium bromide. Prehybridizing, hybridizing and washing of the filters was done as described for library screening.

26. Chromosomal Localization:

These experiments were performed in the laboratory of Dr. Alessandra M.V. Duncan, Department of Pathology, Queens University, Kingston, Ontario K7L 2V7. The recombinant phagemid containing the insert DNA (388 bp) was labeled to a specific activity of 7.8 x

10^7 cpm/mg of DNA with [^{3}H]dTTP and [^{3}H]dATP using a multiprime DNA labeling kit (Amersham). *In situ* hybridization to BrdU-synchronized peripheral blood lymphocytes was performed according to Jirik et al. (1992). After washing and dehydration, the slides were coated with a Kodak NTB/2 emulsion, exposed, and developed. The staining of chromosomes was performed by a modified fluorescence, 0.25% Wright's stain procedure (Lin et al., 1985).

IX. RESULTS:

1. Experimental strategy:

The experimental design employed to isolate cDNAs encoding chemotactic factor receptors was based on the fact that these proteins are likely to belong to a subclass of the 7 TMS superfamily, thereby transducing intracellular signals by coupling to G-proteins. Given the variety of ligands that act as chemotactic and activating factors, this subclass should exhibit significant diversity. On the other hand, because of the overlap in the activities of the factors, the structural relatedness for some of them, and the cross-competition for binding to the receptors observed in others, the receptors should also exhibit more or less extensive areas of identity.

To identify, in peripheral-blood leukocytes, proteins that belong to the family of receptors for chemotactic factors, primers designed either on conserved areas of these molecules or primers patterned in specific sequences were used in the amplification of white blood cell cDNA by PCR. To ascertain whether the PCR products consisted of a mixture or a single species the isolated fragments were cloned and sequenced. All the clones analyzed were the amplification of the IL-8 R (high affinity). This was so either in the rabbit or in the human. Neutrophils are the predominant white cells in unfractionated blood and the sets of primers tested were preferentially amplifying the IL-8 R mRNA. The possibility that the primers would solely target the IL-8 R message could not be discarded at this stage.

RNA from highly purified cell fractions was then used as the starting material. When monocyte RNA was tested and the PCR products were sequenced it was found that the IL-8 R and the material amplified from monocytes presented only partial homology, likely being closely related but divergent 7 TMS receptors. To clarify whether this protein was a novel chemotactic receptor, a cross-hybridizing, full-length cDNA was isolated from a spleen library, subcloned and sequenced. Alignments from this sequence with known sequences deposited in data banks established that it is a human homologue of the putative bovine neuropeptide Y receptor (Y3 subtype; bovnyr). It also presents a high degree of homology with the human IL-8 R (low affinity).

2. Primer oligonucleotides:

When this work was initiated only one of the chemotactic factor receptors had been isolated: the rabbit interleukin-8 receptor. This protein had some homology with two of the tachykinins (the substance P and substance K) receptors. Only a few long clusters appeared conserved between these proteins with the intervening sequences presenting higher variability (see figure 5). These clusters were also partially conserved in other 7 TMS proteins, the less divergent were the proteins the more extended the sequence homology around the cluster area.   They were targeted for oligonucleotide primers design because of the possibility that the same conserved pattern could be present in other members of the chemotactic factor family of receptors. Given the high homology of the intercrines themselves this could be somewhat mirrored in the

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structure of the receptors (this assumption proved to be correct when more of these sequences became available). According to the predicted topology the chosen fragment encompasses the second cytoplasmic loop, transmembrane domains IV, V and part of the VI, and the second extracytoplasmic loop. The forward primer is in an area of the molecule that is thought critical for interaction with G-proteins and therefore tends to be conserved. The reverse primer is in one of the transmembrane domains around a conserved Cys. In both cases the selection was biased toward those clusters which residues could indicate important structural determinants for the protein.

```
MEV-NVWNMTDLWTWFEDEFANATGMPPVEKDYSPCLVVTQTLNKYVVVVIYALVFLLSLLGNSLVMLVI    69   IL-8 R
MDN-VLPVDSDLSPNISTNTSEPNQFVQPAWQIV--LWAAAYTVIVVTSVVGNVVVMWIILAHKRMRTVT    67   Substance P R
MGTRAIVSDANILSGLESNATGVTAFSMPGWQLA--LWATAYLALVLVAVTGNATVIWIILAHERMRTVT    68   Substance K R
*                                   *         *          *         *
                                              TMS I


LYSRSNRSVTDVYLLNLAMADLLFALTMPIWAVSKEKGWIFGTPLCKVVSLVKEVNFYSGILLLACISVD   139   3
NYFLVNLAFAE---ASMAAFN-----TVVNFTYAVHNEWYYGLFYCKFHNFFPIAAVFASIYSMTAVAFD   129   1
NYFIINLALAD---LCMAAFN-----ATFNFIYASHNIWYFGRAFCYFONLFPITAMFVSIYSMTAIAAD   130   2
   *     *        *              *     *    *         *           *    *
          TMS II                             TMS III


RYLAIVHATRTLTQKRHLVKFICLGIWALSLILSLPFFLFRQVFSPNNSSPVCYEDLGHNTAKWRMVLRI   209   3
RYMAIIHPLQPRLSATATKVVIC-VIWVLALLLAFPQGYYSTTETMPSRVVCMIEWPEHPNKIYEKVYHI   198   1
RYMAIVHPFQPRLSAPSTKAIIA-GIWLVALALASPQCFYSTITVDEGATKCVVAWPNDNGGKMLLLYHL   199   2
** ** *               *   **    * *   *
          TMS IV


LPHTFGFILPLLVMLFCYGFTLRTL-------------FQAHMGQKHRAMRVIFAVVLIFLLCWLPYNLV   266   3
CVTVLIYFLPLLVIGYAYTVVGITLWASEIPG-DSSDRYHEQVSAKRKVVKMMIVVVCTFAICWLPFHIF   267   1
VVFVLIYFLPLLVMFGAYSVIGLTLWKRAVPRHQAHGANLRHLQAKKKFVKAMVLVVLTFAICWLPYHLY   269   2
       * * * * *     *      * *                  *           * *  * * * * *
     TMS V                                               TMS VI


LLADTLMRTHVIQETCQRRNDIDRALDATEILGFLHSCLNPIIYAFIGQNFRNGF--------------   321   3
FLLPY------INPDLYLKKFIQQVYLAIMWLAMSSTMYNPIIYCCLNDRFRLGFKHAFRCCPFISAGDY   331   1
FILGT------FQEDIYYHKFIQQVYLALFWLAMSSTMYNPIIYCCLNHRFRSGFRLAFRCCPWVTPTEE   333   2
             *      *      *       * * * * *       ** **
                             TMS VII
```

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--LKMLAARGLISKEF---LTRHRVTSYTSSSTNVP-------------------------------  352  3
EGLEMKSTRYLQTQGSVYKVSRLETTISTVVGAHEEEPEDGPKATPSSLDLTSNCSSRSDSKTMTESFSF  401  1
DRLELTHTPSLSRRVN---RCHTKETLFMTGDMTHSEATNGQVGSPQD----------------GEPAGP  384  2
         *        *                *


---SNL  355  3
SSNVLS  407  1
ICKAQA  390  2
```

Figure 5: Alignment of the amino acids sequences from the human substance P receptor, rabbit IL-8 receptor and the mouse substance K receptor. The underlined sequences are the transmembrane domains as defined by hydrophobicity plots. The alignment was produced by the HEIN program (Hein, 1990). The bold residues indicate the position of the primers used in the cloning of the human neuropeptide Y receptor.

A survey of 12 proteins belonging to the 7TMS superfamily demonstrate that the combination for the upstream primer is present only in the β1-adrenergic receptor and the downstream primer is absent in 7 of them (see appendix I). The proteins involved were some of the receptors for the classical neurotransmitters in all their different subtypes: dopamine, acetylcholine (muscarinic), α- and β-adrenergic.

To facilitate subcloning procedures an *EcoR I* site was created upstream in each of the primers. A (G+C) content ≥ 50% was reached by manipulating the codons when necessary but taking into account the codon usage. The complete sequence for the forward primer was 5'-CGGAATTCGACCGCTACCTGGCCATT-3', which translate as Asp-Arg-Tyr-Leu-Ala-Ile (DRYLAI), and for the antisense primer 5'-ACGAATTCGTTGTAGGGCAGCCAGCA-3', which translate as Cys-Trp-

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Leu-Pro-Tyr-Asn (CWLPYN). Both contain an extra complement of 8 nucleotides to accommodate the restriction site.

The average yield for the production and purification of the 26-mers was 35 μmole/batch.

3. The Polymerase Chain Reaction:

The amplified products of the PCR reaction were analyzed by agarose gel electrophoresis. A single band was seen with the described primers combination and it was within the expected size range (figure 6). The sequencing of the monocytes cDNA, and of the fragments obtained by PCR of either human or rabbit leukocytes, proved that the primers were targeting legitimate 7TMS sequences.

Figure 6: PCR amplification of rabbit and human cDNA: 2% agarose gel electrophoresis;(A) lanes 1,4,5, and 8: 1 kb DNA ladder (1 μg/lane), lanes 2 and 3: human white blood cells RNA to cDNA (0.4 μg/lane), lanes 6 and 7: rabbit white blood cells RNA to cDNA (0.4 μg/lane). (B) The same gel as in (A) after excising the bands for cloning.

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The possibility of using the thermostable enzyme *Taq* DNA-polymerase over the Klenow fragment of *E.coli* has improved the specificity and the yield , on top of simplifying the whole PCR cycle. The fact that high temperatures can be used has a direct bearing on the elimination of non-specific amplification. Furthermore, long and short PCR products can be amplified from genomic DNA due to the disruption of secondary structure obtained at high temperature.

Taq DNA-polymerase does not have proof reading capabilities (no 3' to 5' exonuclease activity) which has a bearing on the misincorporation rate. The appropriate PCR conditions, such as the right concentration of dNTPs and Mg^{2+}, high annealing temperatures and short extension times have brought the misincorporation rate below than 10^{-5} per cycle (Eckert and Kunkel, 1990; Gelfand and White, 1990). For library screening it is the whole of the amplified products that counts and rare misincorporation go unnoticed. The amplified 388 bp of cDNA used as the probe for library screening corresponded unequivocally to the sequence between bases 486 and 858 for the full length sequence (see figure 10). In vitro recombination or template switching is a problem that only occurs with genomic amplifications and therefore being an unlikely event here.

The strategy of modeling the primers on areas of high homology to search for cDNAs encoding related but novel proteins has its rationale in the highly conserved structural and functional features among the subfamilies in this particular group. There is some intrinsic flexibility inherent to the system : note that the antisense primer matches only five codons from the original six

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intended but obviously it did not hinder the annealing of this 26-mer to only 15 bases in the template. The destabilizing effect that non-annealed termini have was probably compensated by the high (G+C) content. The 3'-end of the primer was the properly annealed side and thereby synthesis could progress unhindered.

4. Purification of DNA from agarose gels:

The Sephaglas™ BandPrep kit consistently gives a high yield of purified DNA. It is based on the specific adsorption of DNA to glass matrices in the presence of sodium iodide that also functions as gel solubilizer. The matrix is then washed from contaminants with buffered ethanol that promotes the binding of DNA to the glass powder. The DNA is eluted in low ionic strength buffer and further processed (see figure 7).
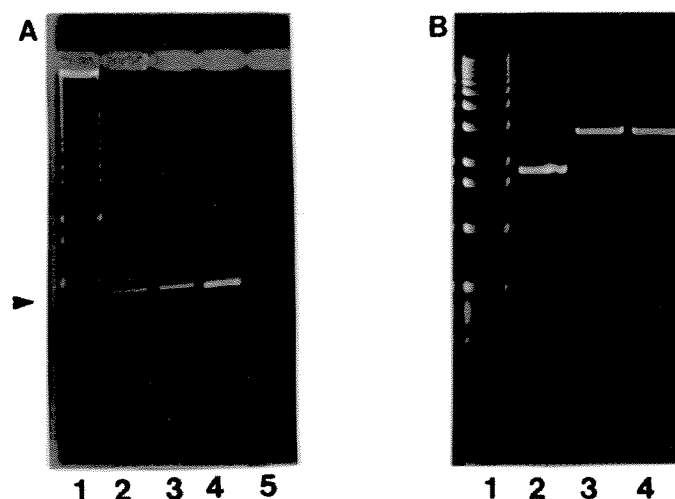
Figure 7:2% agarose gel electrophoresis.(A) lane 1: 1 kb DNA ladder (1µg). Lanes 2 and 3: Sephaglas purified cDNA, 80 ng/lane. Lanes 4 and 5: EcoR I digested cDNA, Sephaglass purified, 80 ng/lane. (B) Lane 1: 1 kb DNA ladder (1 µg). Lane 2: Supercoiled pBluescript (80 ng). Lane 3: pBluescript linearized with Sma I (80 ng). Lane 4: pBluescript linearized with EcoR I (80 ng).

5. Insertion of cDNA into plasmid Bluescript:

To create the sequencing and cloning vector two strategies were attempted: cohesive ends ligation and blunt ends ligation. Digestion of pBluescript with *EcoR I* produces a linear duplex with short single stranded tails (this site occurs only once and solely in the multicloning site). The same result is obtained when digesting the cDNA with this enzyme because of the *EcoR I* sites engineered upstream of the primers used in this amplification (and provided that it lacks internal *EcoR I* sites). Because of the possibility that a small fragment of the cDNA could be actually digested without being detected by size fractionation on agarose gels, blunt ends ligation was also performed using *Sma I* to digest pBluescript. This enzyme creates blunt termini that can associate with the undigested cDNA. The ligation reaction was performed in both cases using T4 DNA-ligase and for blunt ends was carried out at 25 °C overnight. This is a compromise between the rate of enzymatic action and stability of association of the termini. To favor the creation of recombinants the reaction was performed at high cDNA concentration thereby increasing the probability of recombinants to form, and simultaneously decreasing the chances of recircularization for the plasmid DNA. The T4-DNA ligase does catalyze blunt end ligation.

After ligation the mixtures were used to transform XL-1 Blue cells. The selection was done with ampicillin, the chromogenic substrate X-gal and IPTG. When recombination took place there is insertional inactivation of the *lacZ* α-peptide and α-complementation does not occur. Therefore the colonies of cells bearing recombinants appear white. These colonies were expanded in

liquid cultures containing amp and processed to isolate recombinant plasmid DNA. An aliquot of these preparations was digested with *EcoR I* to verify the insertion (see figure 8).
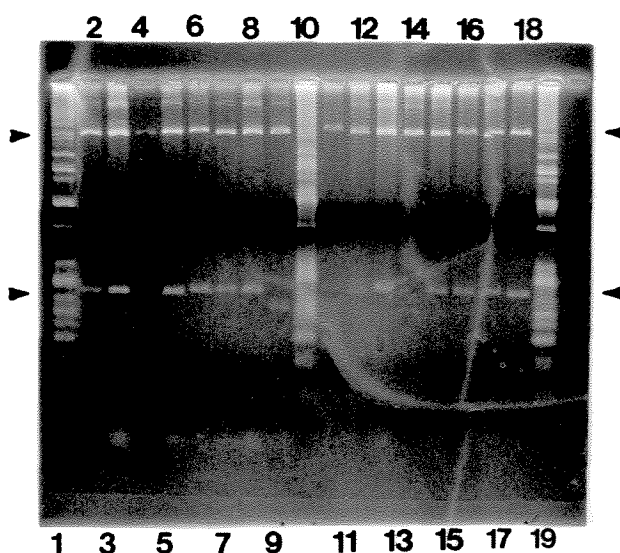
Figure 8: 2% agarose gel electrophoresis. Lanes 1, 10 and 19: 1 kb DNA ladder (1μg) Lanes 2 to 9: recombinant plasmid DNA/EcoR I digested, obtained from cohesive ends ligation (240 ng). Lane 11 to 18: recombinant plasmid DNA/EcoR I digested, obtained by blunt ends ligation (240 ng). Lanes 9 and 18 are originated in rabbit cDNA. The remaining recombinants contain a human insert.

6. Results from total white blood cell cDNA:

6.1. Primers specificity:

Total cell RNA was prepared from rabbit and human white blood cells and reverse transcribed using the antisense oligonucleotide to prime the reaction. The cDNA so obtained was PCR amplified after adding the sense primer (Methods, 10 and 11). A fragment of the expected size was amplified by the polymerase.

To screen the clones for putative members of the chemotactic factor receptor group plasmid DNA was isolated from several clones and used as a template to sequence the insert. In all the cases analyzed the sequence corresponded to the human IL-8 R (high affinity) (figure 9).

```
t3se7pcr      ------------------VHATRTLTQKRHLVKFVCLGCWGLSMNLSLPFFL-------
t3be3pcr      ------------------VHATRTLTQKRHLVKFVCLGCWGLSMNLSLPFF--------
t7se3pcr_p    ------------------VHATRTLTQKRHLVKFVCLGCWGLSMNLSLPFFLFRQAYHP
t3se2pcr      ------------------VHATRTLTQKRHKVKFVCLGCWGLSMNLSLPFF--------
t3se2lr       ---------------------------------------------------------AYHP
humil8ra      FYSGILLLACISVDRYLAIVHATRTLTQKRHLVKFVCLGCWGLSMNLSLPFFLFRQAYHP


t3se7pcr      ----------------------------------------------------------
t3be3pcr      ----------------------------------------------------------
t7se3pcr_p    NNSSPVCYEVLGNDTAKWRM--------------------------------------
t3se2pcr      ----------------------------------------------------------
t3se2lr       NNSSPVCYEVLGNDTAKWRMVLRILPHTFGFIVPLFVMLFCYGFTLRTLFKAH------
humil8ra      NNSSPVCYEVLGNDTAKWRMVLRILPHTFGFIVPLFVMLFCYGFTLRTLFKAHMGQKHRA
```

Figure 9: Alignment of some of the plasmid sequences obtained from human white blood cells. Humil8ra is the human interleukin-8 receptor (high affinity), the remaining sequences are some of the PCR-amplified cDNA clones.

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7. Results from Monocytes cDNA:

7.1 Amplification of cDNA and genomic DNA:

Genomic DNA was also used as template in PCR. The resulting amplifications were clean and appear specific (see figure 10). The band size is identical to that obtained by amplification of cDNA. The explanation for this could be that the whole fragment is located in a single exon. It is more likely that the gene encoding for this protein is intronless. The genes of the α2- and β2-adrenergic receptors (Kobilka et al., 1987a and b respectively), as well as each of the muscarinic cholinergic receptors (Bonner et al., 1987) do not possess introns, be it in the coding or in the 3'-untranslated region. Some of them do but in the 5'-unstranslated region. The more likely explanation for this is that this protein is also intronless in the coding region. It has been suggested that this superfamily has evolved by a gene-processing event from an ancestral gene (O'Dowd et al., 1989).
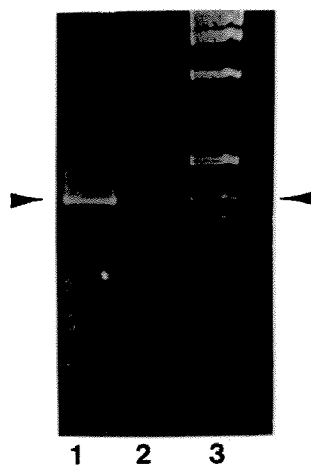
Figure 10: Ethidium bromide stained 2% agarose gel showing the PCR amplification of human monocyte cDNA (lane 1), human monocyte genomic DNA (lane 2) and the size standard, 1 kb DNA ladder (lane 3)

7.2. Library Screening:

The library was plated at 5,000 to 10,000 plaques per plate. This dilution gave a convenient plaque size to facilitate the transfer of the DNA to the filters in spots that could be clearly individualized at the end of the procedure. Positives plaques were replated for a second and even a third time to ensure that the proper purity was obtained. The clone on which this work is based (s9a) was present at a frequency of 1/200,000 plaques and a total of 400,000 plaques were screened. The library is expected to have a minimum of 1×10^6 different clones.

7.3. *In vivo* excision of the recombinant s9a:

The UniZAP vector has been constructed to allow the *in vivo* excision of the cloned insert contained within the pBluescript which is in turn contained within the λ-vector. pBluescript contains sequences that belong to the filamentous phage f1 origin of replication and termination signals. Bacteriophage f1 (or M13) encode proteins that recognize the origin of replication for positive strand synthesis. For this to happen a single cell has to be simultaneously infected with the helper filamentous phage and the λ-vector. Inside the cell the proteins of f1 will bind to the f1 initiation sequences which are present in the pBluescript. These proteins then nick one of the strands and synthesis begins, duplicating all the sequences that are down-stream from the nicking site. Once the termination signal is reached duplication stops and the single strand DNA is circularized by a protein that is a gene II product from the phage f1. This includes all the sequences of the

pBluescript phagemid and the insert that is contained in it. In the terminator sequences are also signals for packaging the phagemid. The cells can be killed by heat treatment and removed by centrifugation. The supernatant containing the packed single stranded phage is used to infect new cells. In this step the phage is converted to the double stranded form. The phage used in this work was the R408 interference-resistant helper phage. It does not use a selectable marker, thus, bacteria infected with helper phage alone will not grow under ampicillin selection. Single-stranded R408 is approximately 4 kb. It was developed by Russel et al. (1986) (see figure 11).

(A) *Xho I/Not I* double digestion of several isolates from clone s9a
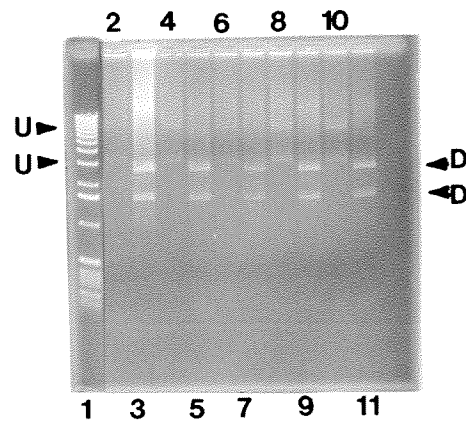
Figure 11 (A): Agarose gel electrophoresis of 200 ng of recombinant s9a. Lane 1: 1 kb DNA ladder; odd lanes are the undigested controls, with supercoiled and nicked plasmid DNAs; even lanes are the digested samples. A fragment of approximately 1.6 kb was excised with this enzyme combination and the linearized plasmid is 2.95 kb (arrowheads).

(B)Mapping of clone s9a with various restriction enzymes:
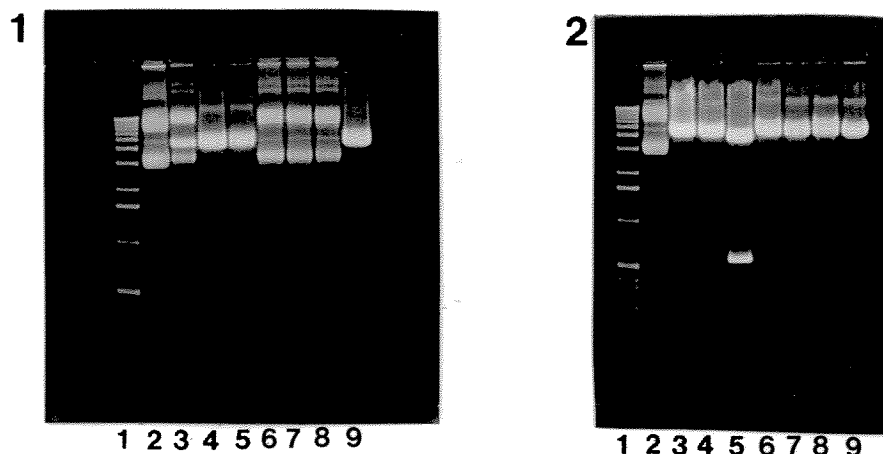
Figure 11 (B):Gel 1; lane 1: 1 kb DNA ladder (1 µg), lane 2: clone s9a supercoiled and nicked circular (0.4 Mg);the same amount of plasmid DNA was digested with the following enzymes (Methods 24.7): lane 3: Kpn I; lane 4: Apa I; lane 5: Xho I; lane 6: Sal I; lane 7: Hind III; lane 8: EcoR V; lane 9: EcoR I. In gel 2: lanes 1 and 2 were the same as in gel 1. Remaining lanes were as follows: lane 3: Pst I; lane 4: Sma I; lane 5: BamH I; lane 6: Spe I; lane 7: Xba I; lane 8: Not I; and lane 9: Sac I. Those enzymes that did not cut were tested again to confirm.

(C)Schematic of the recombinant clone s9a:
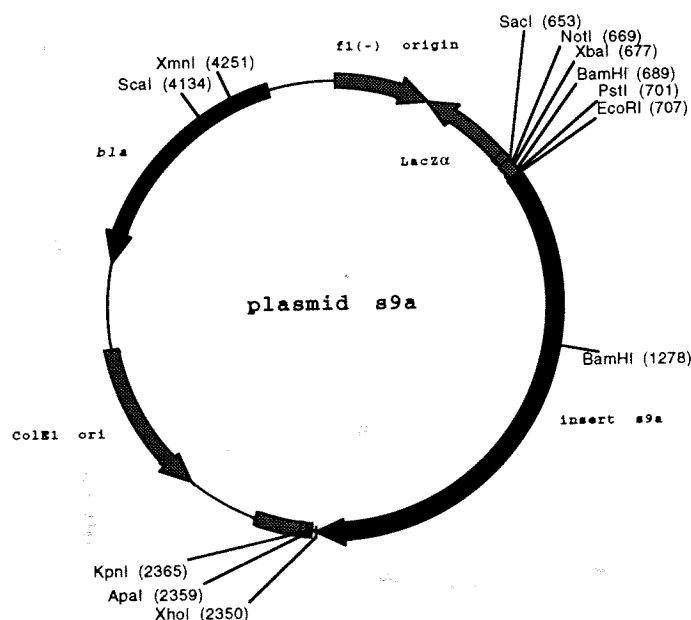
7.4. Sequence information of the clone s9a:

The clone s9a was excised in vivo by co-infection with the phage R408. Cells containing pBluescript carrying the insert were selected with ampicillin. Plasmid s9a was prepared for sequencing as described in methods (figure 12).

```
                    --- --- GGC ACG AGC GGC ACA GGG TAG     27
CAA AGT GAC GCC GAG GGC CTG AGT GCT CCA GTA GCC ACC GCA TCT    72
GGA GAA CCA GCG GTT ACC ATG GAG GGG ATC AGT ATA TAC ACT TCA   117
                        M   E   G   I   S   I   Y   T   S


GAT AAC TAC ACC GAG GAA ATG GGC TCA GGG GAC TAT GAC TCC ATG   162
 D   N   Y   T   E   E   M   G   S   G   D   Y   D   S   M


AAG GAA CCC TGT TTC CGT GAA GAA AAT GCT AAT TTC AAT AAA ATC   207
 K   E   P   C   F   R   E   E   N   A   N   F   N   K   I


TTC CTG CCC                                                   216
 F   L   P
```

Figure 12: Partial sequence with best predicted translation of the insert in s9a.

Homology searches of this fragment revealed that it was a novel protein possibly related to the chemotactic factor receptors. XL-1 Blue cells carrying the plasmid s9a were streaked on ampicillin/2x YT-agar plates, and this material was sent for full length sequencing to the DNA sequencing core facility of the Canadian genetic diseases network (Dr. Keith Schappert) (Figure 13). The 352 residue predicted protein was called humnyr3 after the high homology found with the bovine neuropeptide Y receptor (subtype 3) (see figure 13 C).

(A) cDNA sequence and translation of the ORF for clone s9a:

```
                              GAATTCGGCACGAGCGGCACAGGGTAG
CAAAGTGACGCCGAGGGCCTGAGTGCTCCAGTACCGCATCTGGAGAACCAGCGGTTACC      90
ATG GAG GGG ATC AGT ATA TAC ACT TCA GAT AAC TAC ACC GAG GAA     135
 M   E   G   I   S   I   Y   T   S   D   N   Y   T   E   E
                                       *

ATG GGC TCA GGG GAC TAT GAC TCC ATG AAG GAA CCC TGT TTC CGT     180
 M   G   S   G   D   Y   D   S   M   K   E   P   C   F   R

GAA GAA AAT GCT AAT TTC AAT AAA ATC TTC CTG CCC ACC ATC TAC     225
 E   E   N   A   N   F   N   K   I   F   L   P   T   I   Y

TCC ATC ATC TTC TTA ACT GGC ATT GTG GGC AAT GGA TTG GTC ATC     270
 S   I   I   F   L   T   G   I   V   G   N   G   L   V   I

CTG GTC ATG GGT TAC CAG AAG AAA CTG AGA AGC ATG ACG GAC AAG     315
 L   V   M   G   Y   Q   K   K   L   R   S   M   T   D   K

TAC AGG CTG CAC CTG TCA GTG GCC GAC CTC CTC TTT GTC ATC ACG     360
 Y   R   L   H   L   S   V   A   D   L   L   F   V   I   T

CTT CCC TTC TGG GCA GTT GAT GCC GTG GCA AAC TGG TAC TTT GGG     405
 L   P   F   W   A   V   D   A   V   A   N   W   Y   F   G

AAC TTC CTA TGC AAG GCA GTC CAT GTC ATC TAC ACA GTC AAC CTC     450
 N   F   L   C   K   A   V   H   V   I   Y   T   V   N   L

TAC AGC AGT GTC CTC ATC CTG GCC TTC ATC AGT CTG GAC CGC TAC     495
 Y   S   S   V   L   I   L   A   F   I   S   L   D   R   Y

CTG GCC ATC GTC CAC GCC ACC AAC AGT CAG AGG CCA AGG AAG CTG     540
 L   A   I   V   H   A   T   N   S   Q   R   P   R   K   L

TTG GCT GAA AAG GTG GTC TAT GTT GGC GTC TGG ATC CCT GCC CTC     585
 L   A   E   K   V   V   Y   V   G   V   W   I   P   A   L

CTG CTG ACT ATT CCC GAC TTC ATC TTT GCC AAC GTC AGT GAG GCA     630
 L   L   T   I   P   D   F   I   F   A   N   V   S   E   A

GAT GAC AGA TAT ATC TGT GAC CGC TTC TAC CCC AAT GAC TTG TGG     675
 D   D   R   Y   I   C   D   R   F   Y   P   N   D   L   W

GTG GTT GTG TTC CAG TTT CAG CAC ATC ATG GTT GGC CTT ATC CTG     720
 V   V   V   F   Q   F   Q   H   I   M   V   G   L   I   L

CCT GGT ATT GTC ATC CTG TCC TGC TAT TGC ATT ATC ATC TCC AAG     765
 P   G   I   V   I   L   S   C   Y   C   I   I   I   S   K

CTG TCA CAC TCC AAG GGC CAC CAG AAG CGC AAG GCC CTC AAG ACC     810
 L   S   H   S   K   G   H   Q   K   R   K   A   L   K   T

ACA GTC ATC CTC ATC CTG GCT TTC TTC GCC TGT TGG CTG CCT TAC     855
 T   V   I   L   I   L   A   F   F   A   C   W   L   P   Y

TAC ATT GGG ATC AGC ATC GAC TCC TTC ATC CTC CTG GAA ATC ATC     900
 Y   I   G   I   S   I   D   S   F   I   L   L   E   I   I

AAG CAA GGG TGT GAG TTT GAG AAC ACT GTG CAC AAG TGG ATT TCC     945
 K   Q   G   C   E   F   E   N   T   V   H   K   W   I   S
```

64

```
ATC ACC GAG GCC CTA GCT TTC TTC CAC TGT TGT CTG AAC CCC ATC     990
 I   T   E   A   L   A   F   F   H   C   C   L   N   P   I

CTC TAT GCT TTC CTT GGA GCC AAA TTT AAA ACC TCT GCC CAG CAC     1035
 L   Y   A   F   L   G   A   K   F   K   T   S   A   Q   H

GCA CTC ACC TCT GTG AGC AGA GGG TCC AGC CTC AAG ATC CTC TCC     1080
 A   L   T   S   V   S   R   G   S   S   L   K   I   L   S

AAA GGA AAG CGA GGT GGA CAT TCA TCT GTT TCC ACT GAG TCT GAG     1125
 K   G   K   R   G   G   H   S   S   V   S   T   E   S   E

TCT TCA AGT TTT CAC TCC AGC TAA CAC AGA TGT AAA AGA CTT TTT     1170
 S   S   S   F   H   S   S   #
                TTTATACGATAAATAACTTTTTTTTAAGTTACACATTTTTCAGAT     1215
                ATAAAAGACTGACCAATATTGTACAGCTTTTATTGCTTGTTGGAT     1260
                TTTTGTCTTGTGTTTCTTTAGTTATTGTGAAGTTTAATTGACTTG     1305
                ATTTATATAAATTTTTTTTGTTTCATATTGACGTGTGTCTAGGCA     1350
                GGACCTGTGGCCAAGTTCTTGATGCGGATGCTCTGTGGTAGGACT     1395
                GTAGAAAGGGAACTGAACATTCCAGAGCTGTAGTGAATCACGTAA     1440
                AGCTAGAAATGATCCCCAGCTGTTTATGCATAGATAATCTCTCCA     1485
                TTCCCGTGGAACGTTTTTCCTGTTCTTAAGACGTGATTTTGCTGT     1530
                AGAAGATGGCACTTATAACCAAAGCCCAAAGTGGTATAGAAATGC     1575
                TGGTTTTTCAGTTTTCAGGAGTGGGTTGATTTCAGCACCTACAGT     1620
                GTACAGTCTGTATTAAGTTG                             1641
```

(B) Hydrophobicity plot of the predicted humnyr3 protein.

residue number

65
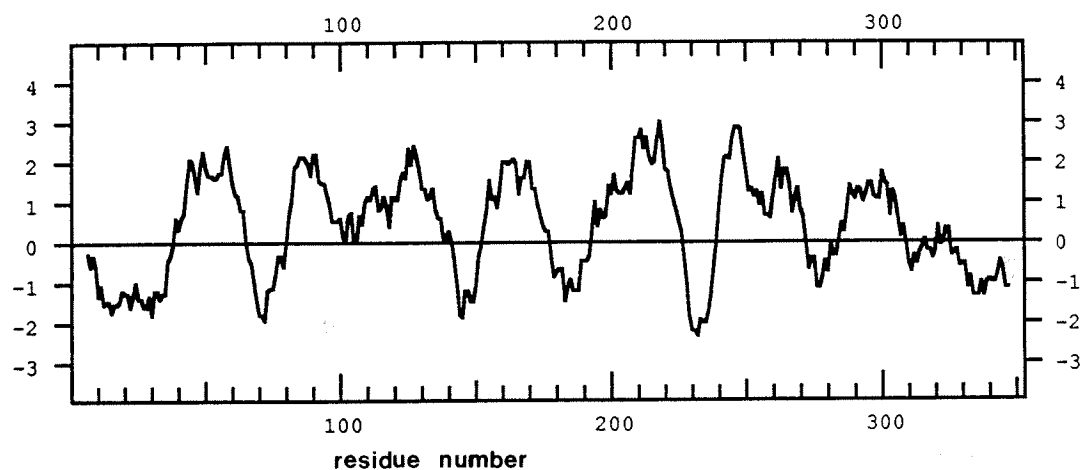
(C) Alignment of the bovine and human homologues of the NPY R (type Y3)

```
bovnyr    MEGIRIFTSDNYTEDDLGSGDYDSMKEPCFREENAHFNRIFLPTVYSIIFLTGIVGNGLV    60
humnyr3   MEGISIYTSDNYTEE-MGSGDYDSMKEPCFREENANFNKIFLPTIYSIIFLTGIVGNGLV
          ****.*.*******. .*****************.**.*****.***************

bovnyr    ILVMGYQKKLRSMTDKYRLHLSVADLLFVLTLPFWAVDAVANWYFGKFLCKAVHVIYTVN   120
humnyr3   ILVMGYQKKLRSMTDKYRLHLSVADLLFVITLPFWAVDAVANWYFGNFLCKAVHVIYTVN
          *****************************.****************.*************

bovnyr    LYSSVLILAFISLDRYLAIVHATNSQKPRKLLAEKVVYVGVWLPAVLLTIPDLIFADIKE   180
humnyr3   LYSSVLILAFISLDRYLAIVHATNSQRPRKLLAEKVVYVGVWIPALLLTIPDFIFANVSE
          **************************.***************.**.******.***.. *

bovnyr    VDERYICDRFYPSDLWLVVFQFQHIVVGLLLPGIVILSCYCIIISKLSHSKGYQKRKALK   240
humnyr3   ADDRYICDRFYPNDLWVVVFQFQHIMVGLILPGIVILSCYCIIISKLSHSKGHQKRKALK
          *.*********.***.********.***.**********************.******

bovnyr    TTVILILTPFACWLPYYIGISIDSFILLEIIQQGCEFESTVHKWISITEALAFFHCCLNP   300
humnyr3   TTVILILAFFACWLPYYIGISIDSFILLEIIKQGCEFENTVHKWISITEALAFFHCCLNP
          *******.*********************.******.**********************

bovnyr    ILYAFLGAKFKTSAQHALTSVSRGSSLKILSKGKRGGHSSVSTESESSSFHSS        352
humnyr3   ILYAFLGAKFKTSAQHALTSVSRGSSLKILSKGKRGGHSSVSTESESSSFHSS
          *****************************************************
```

Figure 13:
(A) Sequence of s9a and translation of its major open reading frame. DNA sequencing was done on both sense and anti-sense strands. Positions discussed in the text (see below) are in bold. Putative hydrophobic transmembrane domains are underlined. Potential N-glycosylation site is marked with a star. The sequence has been deposited in GenBank under the accession #M99293.
(B) Average hydrophobicity values were determined for spans of 9 residues using the method and values of Kyte and Doolittle (1982).
(C) Alignment obtained by comparing the protein sequence of the protein encoded by s9a (humnyr3) and a NPY receptor isolated from bovine brain (Rimland et al, 1991). The homology is 93%. Positions differing in both sequences were verified.

7.5. ORF for the protein humnyr3:

Previous analysis of eukaryotic 5'-noncoding sequences revealed that those sequences surrounding the initiation codon are not random. These analysis have permitted to identify the expanded consensus sequence for initiation: (GCC)GCC^A/GCC<u>ATG</u>G (Kozak,

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1987). Site-directed mutagenesis experiments have demonstrated that the position -3 and +4 have the strongest influence; hence, an initiator codon can be considered weak or strong according to these two positions. In the s9a sequence the first in frame Met has these characteristics. It has been theorized that the initiator methionine and flanking sequences function as a stop signal for the 40s ribosomal subunit. Therefore, efficient translation is only achieved with an ATG triplet in the right context (Kozak, 1983). The repetition of G at the positions -3, -6 and -9 have been signaled as playing a role by helping the ribosomes to stay in frame during translation. This is also the case with the first ATG codon in s9a.

Upstream initiator codons are uncommon in vertebrate RNA, except for the case of oncogenes, but they are an observed feature in adrenergic and muscarinic receptors (Kobilka et al., 1987c). In this respect s9a and the chemotactic factor receptors follow the general rule. The length of the s9a leader sequence falls in the communicated range for vertebrate mRNA (20 to 100 bp).

At the 3'-end there is a long stretch of about 500 bp after the first termination codon that interrupts the ORF. The mammalian consensus for addition of the poly(A) tail is either AAUAAA or AUUAAA (Wickens, 1990). No consensus sequence for polyadenylation was observed in s9a. Neither was a poly(A) tail detected. This could indicate that even if the cDNA contains the full length information for the ORF, it actually is an incomplete fragment of the original mRNA. A strong argument for this is that the mRNAs of other members of the 7 TMS family of proteins have long, sometimes up to

1 kb, 3'-untranslated sequences. Also, the band detected by Northern blots have a size of 1.8 kb to 2.0 kb, while the insert in s9a is 1.6 kb.

The alignment of the human and bovine proteins confirms the assumptions made about the initiation codon, and the extended reading frame has a termination codon that coincides in these two highly homologous proteins.

8. Chromosomal localization:

In situ hybridization localized the gene for humnyr3 in the 2q21 region of chromosome 2 (human). The loci that have been given the same regional assignment do not seem particularly relevant (in the context of this superfamily). The fact that there is a unique site shows that there is only one copy of the gene per haploid genome and that there are not pseudogenes or highly homologous sequences for this receptor (figure 14).

9. Northern blots:

Primate (*Rhesus*) tissues RNA northern blot showed different levels of an approximately 2 kb hybridizing band (figure 15 A and B). The pattern of expression is similar to that from the bovine homologue (Rimland et al., 1991) on bovine and rat tissues with the exception of kidney. The intensity of the signal in brain and heart is not surprising: NPY itself is highly expressed in both tissues. The low expression on spleen contrast with the results in the mice where spleen and thymus gave prominent signals; it might reflect species differences. NPY has been identified in spleen innervation by *in situ* hybridization. NPY-like immunoreactivity has been

demonstrated in various areas of the heart and the gastrointestinal tract (Dumont et al., 1992).

Transcripts have also been detected in the human Burkitt lymphoma cell lines (Ramos, Daudi and Raji), where humnyr3 appears to be highly expressed, and in the promyelocytic leukemia cell line HL-60 (without inducing differentiation) (figure 15 B).
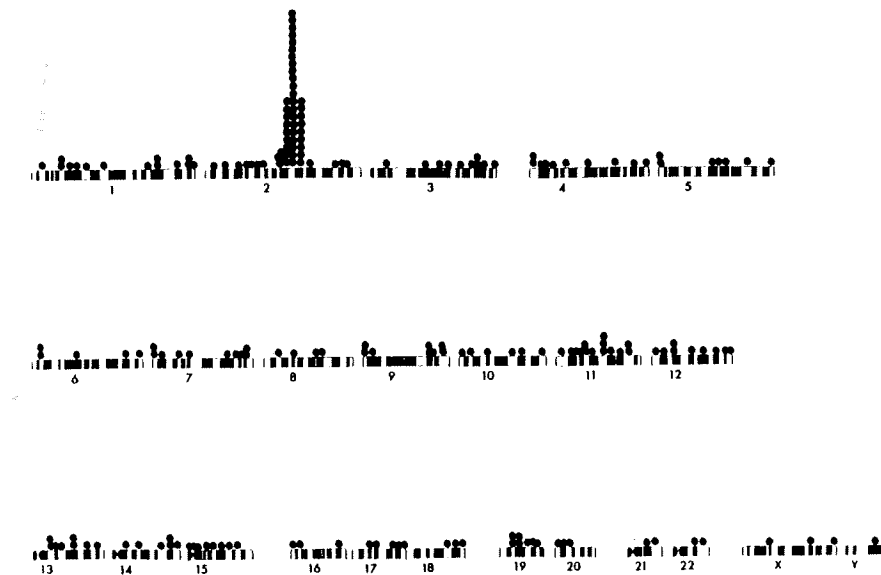
Figure 14: The position of grains on metaphase chromosomes were mapped to an ideogram. Analysis of the distribution of silver grains revealed a cluster (p<0.0001) in the 2q21 region of chromosome 2.
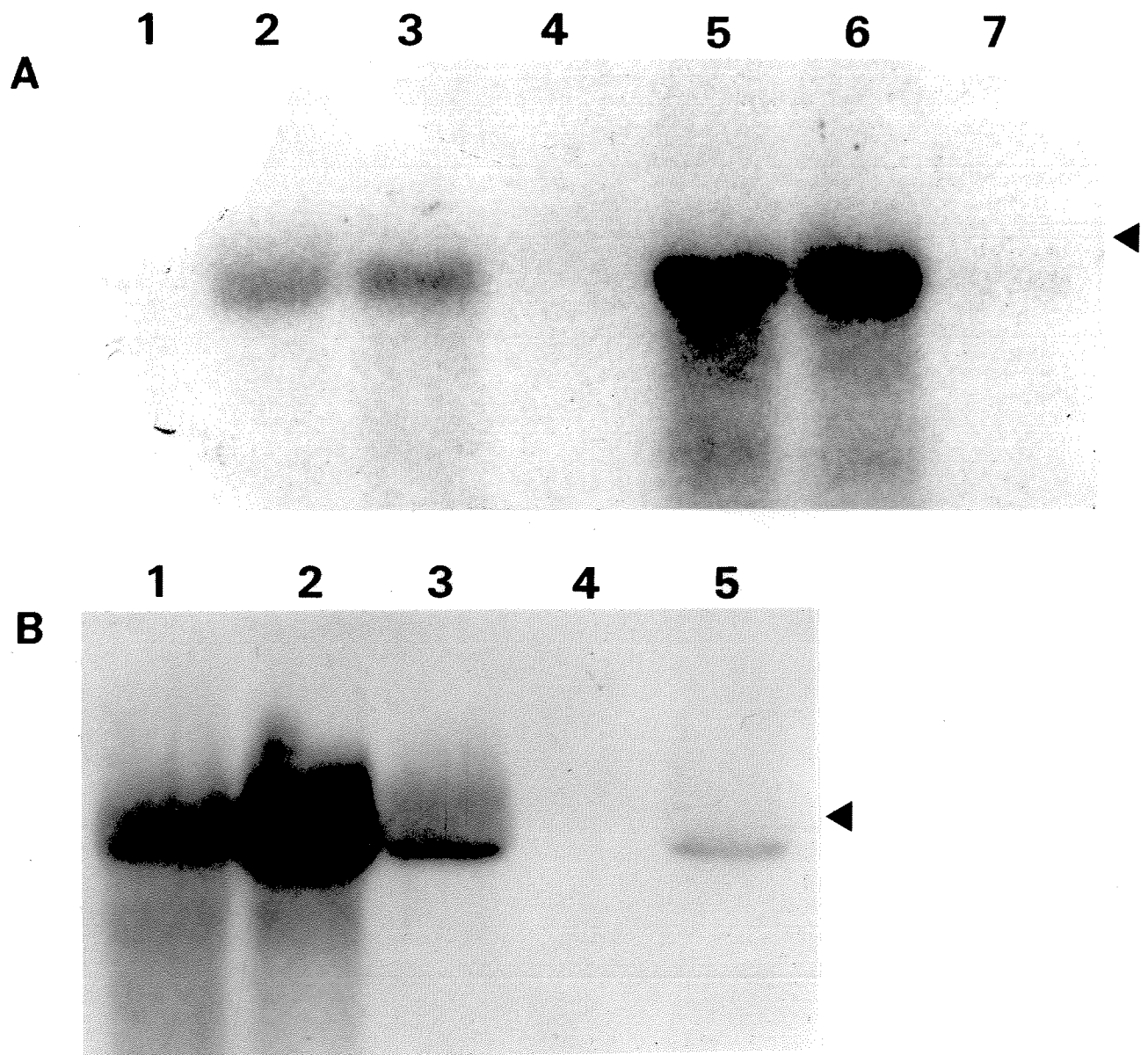
Figure 15: Autoradiography of humnyr3 expression from various sources. Arrowhead indicates the position of the 28S ribosomal band.(A)*Rhesus* total RNA; 1) skeletal muscle, 2) liver, 3) colon, 4) kidney, 5) brain, 6) heart, and 7) spleen. Panel (B) lanes: 1)Ramos, 2) Daudi, and 3) Raji human Burkitt lymphoma cell lines; 4) murine 3T3 fibroblasts, and 5) HL-60 human promyelocytic leukemia cell line.

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X. DISCUSSION:

1. Structural and functional determinants of the 7 TMS superfamily:

The adrenergic and related receptors, together with the visual pigment rhodopsin, have been the best characterized of the 7 TMS superfamily and they became the model for understanding the molecular basis of functioning in G-protein coupled receptors. To study the structural determinants of bioactivity several approaches have been attempted. The $\alpha 2$- and $\beta 2$-adrenergic receptors are specially well suited to be studied by the construction of chimeras. They have a high degree of similarity and have ligand binding characteristics that can be differentiated. Also, they are coupled to distinct G-proteins. It was observed that the binding of the antagonists was dependent on the origin of the TMS VII (Kobilka et al., 1988). This was also the case for chimeras of two α-factor receptors for two different but related yeast species: the TMS VII was determinant of the pheromone specificity (Marsh and Herkowitz, 1988) Homology analysis of this TMS for the humnyr3 protein shows that there exist a high degree either of identity or conservative substitutions when this area is compared with both the receptors for IL-8 (see Figure 16).

Following the same approach it was found that TMS V, TMS VI and the interconnecting loop seem to specify the binding to a particular type of G-protein (Cotecchia et al., 1990; Kubo et al., 1988). The TMS VI in particular contains a motif that is conserved in several members of this superfamily: Cys-Trp-Leu-Pro (CWLP) and that is prominent in the chemotactic, neuropeptide, and some of the adrenergic and

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dopaminergic receptors. On the extended motif Cys-Trp-Leu-Pro-Tyr-Asn (CWLPYN) was designed the 3'-end primer used in the cloning of humnyr3.

```
humnyr3                                    FQHIMVGLILPGIVILSCYCIIISKLSH
humintleu8                                 ILPOSFGFIVPLLIMLFCYGFTLRTLFK
                                            .  *.*.*  ...* **  .  .  *
                                              TMS V              i3

          SKGHQKRKALKTTVILILAFFACWLPYYIGISIDSFILLEIIKQGCEFENTVHKWISITE
          AHMGQKHRAMRVIFAVVLIFLLCWLPYNLVLLADTLMRTQVIQETCERRNHIDRALDATE
           .  **..*..    ..* *. *****  . .  *...  ..*.. **  *  ... .  **
              i3              TMS VI              e3

          ALAFFHCCLNPILYAFLGAKFKTSAQHALTSVSRGSSLKILSKGKRGGHSSVSTESESSS
          ILGILHSCLNPLIYAFIGQKFRHGLLKILA-IHGLISKDSLPKDSRPSFVGSSSGHTSTT
          *...* ****..***.* **.  .    *. .   *   *.*. *  . . *.    *..
              TMS VII                        C-terminus

          FHSS  putative human NPY R (Y3)
          L--- Human IL-8 R (low affinity)
          .
```

Figure 16: Alignment of humnyr3 and humintleu8: identical residues are marked by stars and conservative substitutions by dots. Note the high degree of overall homology in areas thought to participate in the interaction with G-proteins as well as in ligand binding. The program used was CLUSTAL (Higgins and Sharp, 1989).

In the intercrine receptors and in humnyr3 there is another feature conserved in TMS V and that is likely to have a strong effect on the helical structure: a Pro (P) in a very hydrophobic context (likely to induce a bend in the α-helix) and a Cys-Tyr (CY) pair at the end of the membrane spanning domain, facing the cytoplasmic side.

The intracytoplasmic loop (i3) is very divergent amongst different subgroups: again, the subfamily of adrenergic and muscarinic receptors appears unrelated to humnyr3 (because of both the lack of identity and the differences in length) (see Appendix 1). Nonetheless, there is a motif composed of conservative substitutions plus identities located at the C-terminal side of i3, where the TMS VI begins. It is

composed of (+)(A/P)(hydrophobic)(+)(hydrophobic/T). This motif, or the variations thereof, are present in most of the proteins analyzed and may be important for the interaction with some well conserved feature of G-proteins. Specificity determinants for the interaction with G-proteins in the i3 have been also demonstrated with chimeras. This is the longest loop and the more variable in length and together with both the N- and C-termini shows the least sequence homology. It is in general the ends of the loop which renders its specificity (Strader et al., 1987) but at least in one case (the α1-adrenergic receptor) the middle of the loop is also important for G-protein binding specificity.

The second intracytoplasmic loop, i2, also appears to be involved in maintaining the appropriate surface for G-protein binding (see figure 17).

```
humil8ra    LSLLGNSLVM-LVILYSRVGRSVTDVYLLN-LALADLLFALTLPIW--AASKVNGWI-FGTFL
humintleu8  LSLLGNSLVM-LVILYSRVGRSVTDVYLLN-LALADLLFALTLPIW--AASKVNGWI-FGTFL
rabil8c     LSLLGNSLVM-LVILYSRSNRSVTDVYLLN-LAMADLLFALTMPIW--AVSKEKGWI-FGTPL
humc5aar    VGVLGNALVV-WVTAF-EAKRTINAIWFLN-LAVADFLSCLALPILFTSIVQHHHWP-FGGAA
rabfmlp     LSLLGNSLVM-LVILYSRSNRSVTDVYLLN-LAMAPA-FCPDHAYL--GRLQGKRLD-FRTPL
humfmlpy    FGVLGNGLVI-WVAGF-RMTRTVNTICYLN-LALADFSFSAILPFRMVSVAMREKWP-FASFL
humfmlpx    LGVLGNGLVI-WVAGF-RMTRTVTTICYLN-LALADFSFTATLPFLIVSMAMGEKWP-FGWFL
            ...**.**.  *  .   *..  . *. * .*        .    .       *
humnyr3     GIVGNGLVI-LVMGYQKKLRSMTDKYRLH-LSVADLLFVITLPFWAVDAVANWYFGNFLCKA
```

i1

```
humil8ra    CKVVSLLKEVNFYSGILLLACISV-DRYLAIVHATRTLTQKRH-LVK-FVCLGC-WGLSMNLS
humintleu8  CKVVSLLKEVNFYSGILLLACISV-DRYLAIVHATRTLTQKRY-LVK-FICLSI-WGLSLLLA
rabil8c     CKVVSLVKEVNFYSGILLLACISV-DRYLAIVHATRTLTQKRH-LVK-FICLGI-WALSLILS
humc5aar    CSILPSLILLNMYASILLLATISA-DRFLLVFKPIWCQNFRGAGLAW-IACAVA-WGLALLLT
rabfmlp     CKVVSLVKEVNFYSGILLLACISV-DRYLAIVQSTRTLTQKRH-LVK-FICLGI-WALSLILS
humfmlpy    CKLVHVMIDINLFVSVYLITIIAL-DRCICVLHPAWAQNHRTMSLAK-RVMTGL-WIFTIVLT
humfmlpx    CKLIHIVVDINLFGSVFLIGFIAL-DRCICVLHPVWAQNHRTVSLAM-KVIVGP-WILALVLT
            ..  .   .*  ... *. *. **  . .. . . . . **        * ... *.
humnyr3     VHVIYTVNLYSSVLILAFISL-DRYLAIVHATNSORPRKL-LAEK-VVYVGVWIPALLLT
```

i2

Figure 17: Comparison between the intracytoplasmic loops i1 and i2 for chemotactic factor receptors and humnyr3. Stars show identity and dots conservative substitutions. See name code in figure 18.

Where i2 joins TMS III there is always the sequence Asp-Arg (DR) that is highly conserved between 7TMS receptors and that seems to play an important role in creating the G-protein binding domain. Variations such as Glu-Arg (ER) have been observed in the thyrotropin receptor subfamily. It may also be totally absent, as in the human Platelet Activating Factor receptor. In some cases also the remaining of loop i2 (and loop i4, only when there is a Cys modified by palmitoylation, see below) have been found to be determinants of the coupling specificity. There is a dissociation between the binding and the activation of the specific G-protein: some amino acids in loops i2 and i3 are necessary for activation but do not affect binding. Synthetic peptides corresponding to loops i1 and i2 inhibit the coupling of G-proteins to the receptors. For the chemotactic receptors and humnyr3 these two loops are fairly short and have a high degree of conservation among related proteins (see figure 17 and appendixes)

Another β1-adrenergic receptor peptide (C-terminal segment of loop i3) activates adenylyl cyclase as it were the ligand occupied receptor (Palm et al., 1990). This region is also conserved between closely related proteins. In humnyr3 this area appears as a blend between the chemotactic receptors and some of the catecholamine receptors.

For rhodopsin and other 7 TMS receptors for small molecules it has been proposed that the TMSs assemble in a ring structure defining in this way a ligand binding pocket (Dohlman et al., 1987). Therefore several determinants contribute to the ligand binding site. Usually the membrane spanning domains display the highest degree of homology. Those amino acids conserved among the adrenergic and muscarinic

receptors are located in the half cytoplasmic side of the transmembrane α-helices. On the other hand, the extracytoplasmic half contain those residues showing the more variation. This is in agreement with the fact that G-proteins, that couple to the receptor by binding to its cytoplasmic surface, have highly conserved primary structures. The external halves contain the determinants accounting for ligand binding specificity (O'Dowd et al., 1989). This is also true for some of the chemotactic receptors and humnyr3 (see figure 18).

```
                          N-terminus

humfmlpx      MET-N------------FSTP----------LNEYEEVSYESAGYTVLRILPLVVLGVTF
humfmlpy      MET-N------------FSIP----------LNETEEVLPEPAGHTVLWIFSLLVHGVTF
rabfmlp       M----EVNVWNMTDLWTWFEDEFANATGMPPVEKDYSPCLVV-TQTLNKYVVVVIYALVF
humc5aar      MNSFN-----------YTTPDYGHYDDKDTLDLNTPVDKTSNTLRVPDILALVIFAVVF
rabil8c       M----EVNVWNMTDLWTWFEDEFANATGMPPVEKDYSPCLVV-TQTLNKYVVVVIYALVF
humintleu8    MESDSFEDFWKGEDL-----SNYSYSSTLPPFLLDAAPCEPE-SLEINKYFVVIIYALVF
humil8ra      MSNITDPQMWDFDDL------NF---TGMPPADEDYSPCMLE-TETLNKYVVIIAYALVF
humnyr3       MEGIS-----------IYTSDNYTEEMGSGDYDSMKEPCFREENANFNKIFLPTIYSIIF
              *                                                    .. *
                                                       TMS I
humfmlpx      VLGVLGNGLVIWVAGF-RMTRTVTTICYLNLALADFSFTATLPFLIVSMAMGEKWPFGWF
humfmlpy      VFGVLGNGLVIWVAGF-RMTRTVNTICYLNLALADFSFSAILPFRMVSVAMREKWPFASF
rabfmlp       LLSLLGNSLVMLVILYSRSNRSVTDVYLLNLAMAPA-FCPDHAYL--GRLQGKRLDFRTP
humc5aar      LVGVLGNALVVWVTAF-EAKRTINAIWFLNLAVADFLSCLALPILFTSIVQHHHWPFGGA
rabil8c       LLSLLGNSLVMLVILYSRSNRSVTDVYLLNLAMADLLFALTMPIW--AVSKEKGWIFGTP
humintleu8    LLSLLGNSLVMLVILYSRVGRSVTDVYLLNLALADLLFALTLPIW--AASKVNGWIFGTF
humil8ra      LLSLLGNSLVMLVILYSRVGRSVTDVYLLNLALADLLFALTLPIW--AASKVNGWIFGTF
humnyr3       LTGIVGNGLVILVMGYQKKLRSMTDKYRLHLSVADLLFVITLPFW--AVDAVANWYFGNF
              . ...**.**. *   .   *..    *.*..*       .   .        *
                                          TMS II
humfmlpx      LCKLIHIVVDINLFGSVFLIGFIALDRCICVLHPVWAQNHRTVSLAMKVIVGPWILALVL
humfmlpy      LCKLVHVMIDINLFVSVYLITIIALDRCICVLHPAWAQNHRTMSLAKRVMTGLWIFTIVL
rabfmlp       LCKVVSLVKEVNFYSGILLLACISVDRYLAIVQSTRTLTQKRHLV-KFICLGIWALSLIL
humc5aar      ACSILPSLILLNMYASILLLATISADRFLLVFKPIWCQNFRGAGLAWIACAVAWGLALLL
rabil8c       LCKVVSLVKEVNFYSGILLLACISVDRYLAIVHATRTLTQKRHLV-KFICLGIWALSLIL
humintleu8    LCKVVSLLKEVNFYSGILLLACISVDRYLAIVHATRTLTQKRYLV-KFICLSIWGLSLLL
humil8ra      LCKVVSLLKEVNFYSGILLLACISVDRYLAIVHATRTLTQKRHLV-KFVCLGCWGLSMNL
humnyr3       LCKAVHVIYTVNLYSSVLILAFISLDRYLAIVHATNSQRPRKLLAEKVVYVGVWIPALLL
              *   .   .*  .  .. ..  *. **  .  .      .        *  .. *
              TMS III                                       TMS IV
```

```
humfmlpx    TLPVFLFLTTVTIP-NGDTYCTFNFASWGGTPEERLKVAITMLTARGIIRFVIGFSLPMS
humfmlpy    TLPNFIFWTTISTT-NGDTYCIFNFAFWGDTAVERLNVFITMAKVFLILHFIIGFTVPMS
rabfmlp     SLPFFLFRQVFSPN-NSSPVC---YEDLGHNTAKWCMVL------RILPHTFGFILPLL
humc5aar    TIPSFLYRVVREEYFPPKVLCGVDYSH--DKRRER--------AVAIVRLVLGFLWPLL
rabil8c     SLPFFLFRQVFSPN-NSSPVC---YEDLGHNTAKWRMVL------RILPHTFGFILPLL
humintleu8  ALPVLLFRRTVYSS-NVSPAC---YEDMGNNTANWRMLL------RILPQSFGFIVPLL
humil8ra    SLPFFLFRQAYHPN-NSSPVC---YEVLGNDTAKWRMVL------RILPHTFGFIVPLF
humnyr3     TIPDFIFANVSEAD-DRY-IC---DRFYPNDL--WVVVF-------QFQHIMVGLILPGI
            ..* ...              *             .         *.  *
                                                         TMS V


humfmlpx    IVAICYGLIAAKIHKKGMIKSSRPLRVLTAVVASFFICWFPFQLVALLGTVWLKEMLFYG
humfmlpy    IITVCYGIIAAKIHRNHMIKSSRPLRVFAAVVASFFICWFPYELIGILMAVWLKEMLLNG
rabfmlp     VMLFCYGFTLRTLFQAHMGQKHRAMRVIFAVVLIFLLCWLPYNLV-LLADTLMRTHVIQE
humc5aar    TLTICYTFILLRTWSRRATRSTKTLKVVVAVVASFFIFWLPYQVTGIMMS-FLEPS--SP
rabil8c     VMLFCYGFTLRTLFQAHMGQKHRAMRVIFAVVLIFLLCWLPYNLV-LLADTLMRTHVIQE
humintleu8  IMLFCYGFTLRTLFKAHMGQKHRAMRVIFAVVLIFLLCWLPYNLV-LLADTLMRTQVIQE
humil8ra    VMLFCYGFTLRTLFKAHMGQKHRAMRVIFAVVLIFLLCWLPYNLV-LLADTLMRTQVIQE
humnyr3     VILSCYCIIISKLSHSKGHQKRKALKTTVILILAFFACWLPYYIG-ISIDSFILLEIIKQ
            .  **  .          .  ..    ..  *.  *.*.  .  .         .
                                                 TMS VI


humfmlpx    KYKI---IDILVNPTSSLAFFNSCLNPMLYVFVGQDFRERLIHSLPTSLERALSE--DSA
humfmlpy    KYKI---ILVLINPTSSLAFFNSCLNPILYVFMGRNFQERLIRSLPTSLERALTEVPDSA
rabfmlp     TCQRRNELDRALDATEILGFLHSCLNPIIYAFIGQNFRNGFLKMLAA--RGLISKEFLTR
humc5aar    TFLL---LNKLDSLCVSFAYINCCINPIIYVVAGQGFQGRLRKSLPSLLRNVLTE-ESVV
rabil8c     TCQRRNDIDRALDATEILGFLHSCLNPIIYAFIGQNFRNGFLKMLAA--RGLISKEFLTR
humintleu8  TCERRNHIDRALDATEILGILHSCLNPLIYAFIGQKFRHGLLKILAI--HGLISKDSLPK
humil8ra    TCERRNNIGRALDATEILGFLHSCLNPIIYAFIGQNFRHGFL----------------
humnyr3     GCEFENTVHKWISITEALAFFHCCLNPILYAFLGAKFKTSAQHALTSVSRGSSLK-ILSK
               .      .. .. *.**..*   *  *.

                         TMS VII



humfmlpx    PTNDTAANCASPPAETELQAM   human fMLP-related receptor II
humfmlpy    QTSNTHTTSASPPEETELQAM   human RMLP-related receptor I
rabfmlp     HRVTSYTSSST-NVPSNL---   rabbit fMLP receptor
humc5aar    RESKSFTRSTVDTMAQKTQAV   human C5a anaphylatoxin receptor
rabil8c     HRVTSYTSSST-NVPSNL---   rabbit IL-8 receptor
humintleu8  DSRPSFVGSSSGHTSTTL---   human IL-8 receptor (low affinity)
humil8ra    --------------------   human IL-8 receptor (high affinity)
humnyr3     GKRGGHSSVSTESESSSFHSS   putative human NPY Y3 receptor
```

C-terminus

Figure 18: Clustal alignment of the chemotactic factor receptors and humnyr3. Identities and conservative substitutions are labeled with stars and dots respectively. Predicted transmembrane segments are underlined and labeled.

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A possible functional role for cysteine palmitoylation at the C-terminal region of the protein has been indicated (O'Dowd et al.,1989). Cys palmitoylation in this area has been observed in both the $\beta2$-adrenergic receptor and rhodopsin. The C-terminus of humnyr3 lacks Cys eliminating the possibility that such a modification could occur.

Four cysteines important for both ligand binding and cell surface expression have been identified in the $\beta2$-adrenergic receptor, indicating that disulfides are an important structural feature. The prediction is that the two disulfides are extracellular and may stabilize the hydrophilic extracytoplasmic loops (Dohlman et al., 1990). This does not appear to be a universal requisite for these receptors: the only two cysteines in yeast α-factor receptor can be replaced by site-directed mutagenesis without affecting the function of the receptor. Intramolecular disulfides bridges have been also thought to take part in receptor activation processes (Malbon et al., 1987). In humnyr3 there is a Cys in position 109 (in e1) and another in position 186 (in e2). Both are conserved in adrenergic and chemotactic receptors and could fulfill the role of forming a stabilizing disulfide. It is noteworthy that e1, which is a short loop, has two conserved residues (Trp and Cys) in almost all the adrenergic, dopaminergic, muscarinic and the more distant chemotactic receptors. It may contain structural determinant that affect the overall alignment of the TMSs in the membrane and therefore the effect on binding specificity would be indirect (see Figure 19). This does not appear to be the case for those pituitary hormone receptors in which the ligand is of appreciable size (see Appendix II).

```
humil8ra      LLSLLGNSLVMLVILYSRVGRSVTDVYLLNLALADLLFALTLPIW--AAS--KVNGWIFGTFLCK-V
humintleu8    LLSLLGNSLVMLVILYSRVGRSVTDVYLLNLALADLLFALTLPIW--AAS--KVNGWIFGTFLCK-V
rabil8c       LLSLLGNSLVMLVILYSRSNRSVTDVYLLNLAMADLLFALTMPIW--AVS--KEKGWIFGTPLCK-V
rabfmlp       LLSLLGNSLVMLVILYSRSNRSVTDVYLLNLAMAPA-FCPDHAYL--GRLQG--KRLDFRTPLCK-V
humfmlpy      VFGVLGNGLVIWVAGF-RMTRTVNTICYLNLALADFSFSAILPFRMVSVAMRE--KWPFASFLCK-L
humfmlpx      VLGVLGNGLVIWVAGF-RMTRTVTTICYLNLALADFSFTATLPFLIVSMAMGE--KWPFGWFLCK-L
humnyr3       LTGIVGNGLVILVMGYQKKLRSMTDKYRLHLSVADLLFVITLPFW--AVDAV--ANWYFGNFLCK-A
              . ...**.**. *   . .  *..      *.*..*   *    .   .        *   ***
                                                                             e1
```

```
acm2_human    TV----IGYWPLGPVVCD--LWLALDYVVSNASVMNLLIISFDRYFCVTKPLTYPVKRTTKMAG-
b1ar_human    VV----WGRWEYGSFFCE--LWTSVDVLCVTASIETLCVIALDRYLAITSPFRYQSLLT-RARAR
d4dr_human    EVQ---GGAWLLSPRLCD--ALMAMDVMLCTASIFNLCAISVDRFVAVAVPLRYN-RQGGSRRQL
a2aa_human    EV----MGYWYFGKAWCE--IYLALDVLFCTSSIVHLCAISLDRYWSITQAIEYNLKRTP-RRIK
a2ab_human    EL----MAYWYFGQVWCG--VYLALDVLFCTSSIVHLCAISLDRYWSVTQAVEYNLKRTP-RRVK
acm5_human    IL----MGRWALGSLACD--LWLALDYVASNASVMNLLVISFDRYFSITRPLTYRAKRTPKRAG-
b2ar_human    IL----MKMWTFGNFWCE--FWTSIDVLCVTASIETLCVIAVDRYFAITSPFKYQSLLT-KNKAR
d5dr_human    EV----AGYWPFGAF-CD--VWVAFDIMCSTASILNLCVISVDRYWAISRPFRYKRKMT-QRMAL
a2ac_human    EL----LGYWYFRRTWCE--VYLALDVLFCTSSIVHLCAISLDRYWAVSRALEYNSKRTP-RRIK
b3ar_human    AL----TGHWPLGATGCE--LWTSVDVLCVTASIETLCALAVDRYLAVTNPLRYGALVT-KRCAR
d2dr_human    EV----VGEWKFSRIHCD--IFVTLDVMMCTASILNLCAISIDRYTAVAMPMLYNTRYSSKRRVT
d1dr_human    EI----AGFWPFGSF-CN--IWVAFDIMCSTASILNLCVISVDRYWAISSPFRYERKMT-PKAAF
              .    *      *      . * .   .*.  *  .. **.  ... .  *
                    e1
```

```
mussubkrec    HERMRTVTNYFIINLALADLCMAAFNATFNFIYASH-NIWYFGSTFCY-FQNLFPVTAMFVS
ratskr        HERMRTVTNYFIINLALADLCMAAFNATFNFIYASH-NIWYFGRAFCY-FQNLFPITAMFVS
bosskr        HQRMRTVTNYFIVNLALADLCMAAFNAAFNFVYASH-NIWYFGRAFCY-FQNLFPITAMFVS
guipigspre    HKRMRTVTNYFLVNLAFAEASMAAFNTVVNFTYAVH-NEWYYGLFYCK-FHNFFPIAAVFAS
humsubpra     HKRMRTVTNYFLVNLAFAEASMAAFNTVVNFTYAVH-NEWYYGLFYCK-FHNFFPIAAVFAS
gpisprec      HKRMRTVTNYFLVNLAFAEASMAAFNTVVNFTYAVH-NEWYYGLFYCK-FHNFFPIAAVFAS
              * ********..***.*. *****. ** ** * * **.*  .*  *.*.**..*.* *
                                                              e1
```

Figure 19: Comparison of extracellular loop e1 in humnyr3, chemotactic factor, classical neurotransmitter and neuropeptide receptors. Convention to mark homology positions is as described previously. The name code is in the appendices an figure 18.

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Analyzing the phenomenon of desensitization for β-adrenergic receptors it was found that phosphorylation is a transient covalent modification that modulates receptor activity (Bouvier et al., 1988). It appears to cause the uncoupling or attenuated coupling to G-proteins. Therefore the presence of consensus sequences for different kinases is a very important feature (Protein Kinase cAMP dependent, PKA, for heterologous desensitization; PKA and β–Adrenergic Receptor Kinase, βARK, for homologous desensitization). Clusters of Ser and Thr are common at the C-terminus of the 7TMS receptors. In mutants of the β2-adrenergic receptor where the hydroxy-amino acids had been replaced or the C-terminus has been truncated by deletion there exist a significant attenuation of receptor desensitization and of ligand-induced phosphorylation. The findings implicate βARK and because they are only observed at high agonist concentration they point to the fact that PKA is active on the receptor even at low agonist concentration (Hausdorff et al., 1989). βARK appears to have a broad substrate specificity, phosphorylating *in vitro* other 7TMS proteins (Kwatra et al., 1989). It has been demonstrated that for maximal desensitization at high agonist concentration both kinases are needed (Lohse et al.,1990). Because the action of βARK is manifest at high agonist concentration it is currently thought that this enzyme is important in highly innervated tissues at the synaptic level (Benovic et al.,1990).

Dephosphorylation seems to play a role in regenerating active receptors but it needs more investigation. The action of the phosphatases has been linked rather to receptor sequestration.

In the C-terminus of humnyr3 there are 15 Ser and 3 Thr over 46 residues, suggesting that modulation by phosphorylation is also

important for this protein. Some of these hydroxy-amino acids are within a consensus frame for PKA phophorylation (Kennelly et al., 1991).

From the different studies performed with adrenergic and muscarinic receptors it was concluded that: 1) the same ligand may bind and activate two functionally distinct receptors, 2) Different G-proteins with similar receptor and effector binding characteristics exist, 3) receptors can couple to different G-proteins, mainly when expressed at high levels, and 4) individual G-proteins can activate different effector systems. All this complexity is further increased by the existence of receptor subtypes. It has been speculated that subtypes reflect different expression pattern in different tissues or during development or that different patterns of desensitization are involved (Dohlman et al., 1991).

The *Drosophila* mutant *ninaA* displays a marked reduction in the level of its rhodopsin. This mutant is defective in a cis-trans prolylisomerase. (Shieh et al., 1989). In these mutants as well as in one form of the degenerative disease retinitis pigmentosa (with the mutation Pro23His) rhodopsin accumulates in the endoplasmic reticulum. The equivalent Pro in humnyr3 is located in position 27, 13 residues downstream of the sole N-glycosylation consensus site in the protein. In humnyr3 and the intercrine receptors (but not in the catecholamine receptors) this Pro is associated to a Cys forming the pair Pro-Cys (see figure 20).

```
(A)
humil8ra      MSNITDPQMWDFDDL------NF---TGMPPADEDYS--PCM-LE-TETLNKYVVIIAYALVF
humintleu8    MESDSFEDFWKGEDL-----SNYSYSSTLPPFLLDAA--PCE-PE-SLEINKYFVVIIYALVF
rabil8c       M----EVNVWNMTDLWTWFEDEFANATGMPPVEKDYS--PCL-VV-TQTLNKYVVVVIYALVF
rabfmlp       M----EVNVWNMTDLWTWFEDEFANATGMPPVEKDYS--PCL-VV-TQTLNKYVVVVIYALVF
humfmlpy      MET----------------NFSI---------PLNETEEVLPEPAGHTVLWIFSLLVHGVTF
humfmlpx      MET----------------NFST--------PLNEYEEVSYESAGYTVLRILPLVVLGVTF
humnyr3       MEGIS----------IYTSDNYTEEMGSGDYDSMKE--PCF-REENANFNKIFLPTIYSIIF
              *                      ..                              .. *
```

N-terminus

```
(B)
acm2_human    MNNSTNSSNNSLAL-----------------------------TS-------PYKTFEVV
b1ar_human    MGAGVLVLGASEPGNLSSAAPLPDGAATAARLLVPASPPASLLPPASESPEPLSQQWTAG
d4dr_human    MGNRSTA------------------DADGLLAGRGPAAGASAGASAGLAGQGAAALVG
a2aa_human    M---------------------GSLQPDAGNASWNGTEAPGGGARATP---YSLQVTL
a2ab_human    MASPALAA--------ALAVAAAAGPNASGAGERGSGGVANASGASWGPPRGQYSAGAVA
acm5_human    MEGDSYHNATTVNG--------------------------TPVNHQPLERHRLWEVI
b2ar_human    MG--------QPGN-----------GSAFLLAPNRSHA----PDHDVTQQRDEVWVVG
d5dr_human    MLPPG-SNGTAYPGQFALYQQLAQGNAVGGSAGAPPLGPSQV----------------
a2ac_human    MD-------------------------------------------HQDPYSVQATA
b3ar_human    M----------------APWPHENSS----LAPWPDLPTLAPNTANTSGLPGVPWEAA
d2dr_human    MDPLNLS----------------WYDDDLERQNWSRPFNGSDGKADRPHYNYYATLLT
d1dr_human    M----------------RTLNTSAMDGTGLVVERDFSVRI----------------
              *
```

N-terminus

```
(C)
ratskr        MGTRAIVSDANILSGLESNATGVTAFSMPGWQLA---LWATAYLALVLVAVTGNATVIWIILA
humsubprec    MDN-VLPVDSDLSPNISTNTSEPNQFVQPAWQIV---LWAAAYTVIVVTSVVGNVVVMWIILA
mussubkrec    MGAHASVTDTNILSGLESNATGVTAFSMPGWQLA---LWATAYLALVLVAVTGNATVIWIILA
guipigspre    MDN-VLPVDSDLFPNISTNTSEPNQFVQPAWQIV---LWAAAYTVIVVTSVVGNVVVMWIILA
humsubpra     MDN-VLPVDSDLSPNISTNTSEPNQFVQPAWQIV---LWAAAYTVIVVTSVVGNVVVMWIILA
gpisprec      MDN-VLPVDSDLFPNISTNTSEPNQFVQPAWQIV---LWAAAYTVIVVTSVVGNVVVMWIILA
humsprlong    MDN-VLPVDSDLSPNISTNTSEPNQFVQPAWQIV---LWAAAYTVIVVTSVVGNVVVMWIILA
bosskr        MGACVVMTDINISSGLDSNATGITAFSMPGWQLA---LWTAAYLALVLVAVMGNATVIWIILA
humsubprec    MDN-VLPVDSDLSPNISTNTSEPNQFVQPAWQIV---LWAAAYTVIVVTSVVGNVVVMWIILA
              *.        *  .. . . .*..     *  *.**.     **..**  .*. .* **  *.*****
```

N-terminus   **TMS I**

Figure 20: Comparison of the N-terminal region in humnyr3 and
the chemotactic (A), classical neurotransmitter (B), and
neuropeptide receptors (C). The N-glycosylation site in humnyr3
and the TMS I in neuropeptide receptors are underlined. For the
name code see appendices and figure 18.

Mutations at the N-glycosylation sites on the N-terminus have a more deleterious effect: in some cases no perturbation was observed but in *Drosophila* rhodopsin, and in another form of retinitis pigmentosa, mutations at these sites proved to be impairing.

In some tumors a constitutive activation of adenylyl cyclase is linked to a defect in the G_S component (Landis et al., 1989). Proliferative effects are described for the serotonin (5HT1c), angiotensin (*mas* oncogene) and adrenergic receptors. They stimulate the phosphatidyl inositol and the cAMP pathways.

Until now most of the work performed in the area of structure-function relationship has been with receptors for the classical neurotransmitters, receptors for yeast pheromones and the visual pigment proteins, rhodopsins.

2. Virally encoded 7TMS proteins:

It has been found that there are virally-encoded G-protein coupled receptors. The genome of human cytomegalovirus (HCMV) has three ORFs that encode putative 7TMS receptors (US27 ,US28 and UL33). Two of these genes are arranged consecutively suggesting gene duplication; the third is divergent and is separated from the other two by 180 bp. Features of gene organization seem to indicate that these genes are transcribed. No specific ligand for these HMCV proteins is known but they lack an Asp residue in helix III that is characteristic of the cationic amine receptors. By the other hand US27 and US28 have an Asp in position 96 which is important for ligand binding in the β-adrenergic receptor. All three HCMV sequences have a Lys residue in i3 in a position that is critical for the binding to G-proteins. This Lys is

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conserved in the intercrine receptors and humnyr3. This loop (i3) is short in the viral sequences as it is in the *mas* oncogene, in humnyr3 and in the chemotactic receptors. The viral proteins also contain phosphorylation consensus sequences in the C-termini (Chee et al., 1990). Some of the primary metabolic changes in HCMV-infected cells are typical of G-protein mediated signaling, such as increased Ca^{2+} intracellular and elevation of cAMP and DAG levels. All US27, US28 and UL33 have ORFs with TATA-like promoter elements and stop codons in the appropriate frame but only US28 has a polyadenylation signal. When mRNA was prepared from HCMV infected fibroblasts and was probed with specific oligonucleotides (29- or 30-mers) it was found that US27 and US28 are expressed as a long cotranscript and that US28 alone is also expressed. The same was observed with UL33 and other protein of the unique long repeat UL34, the latter having a nearby polyadenylation signal. This was shown to occur late during the infection (Welch et al., 1991).

Human homologues have not been identified yet. It is not known which role they have in the virus life cycle. Similarity between US 28 and humnyr3 exist and it is spread out over the entire sequence, but is most noticeable in the TMSs (see figure 21).

```
MTPTT--TTAELTTEF---DYDEDATPCVFTDVLNQSKPVTLFLYGVVFLFGSIGNFLVIFTITWRRRIQ   65 US28
MEGISIYTSDNYTEEMGSGDYDSMKEPCFREENANFNKIFLPTIYSIIFLTGIVGNGLVILVMGYQKKLR   70 humnyr3
*      *   * *    ***    **     *   *      *   ** *  ** ***
                                                  TMS I

CSGDVYFINLAAADLLFVCTLPLWMQYLLDHNSLASVPCTLLTACFYVAMFASLCFITEIALDRYYAIVY  135  1
SMTDKYRLHLSVADLLFVITLPFWAVDAVANWYFGNFLCKAVHVIYTVNLYSSVLILAFISLDRYLAIVH  140  2
     * *   *  ****** *** *            *        *     *    * **** ***
         TMS II                            TMS III
```

```
MRYRPVKQACLFSIFW----WIFAVIIAIPHFMV--VTKKDNQCMTDYDYLEVSYPIILNVELMLGAFVI  199  1
ATNSQRPRKLLAEKVVYVGVWIPALLLTIPDFIFANVSEADDRYICDRFYPNDLWVVVFQFQHIMVGLIL  210  2
              *      ** *    ** *    *     *      *   *
                      TMS IV                              TMS V


PLSVISYCYYRISRIVAVSQSRHKGRIVRVLIAVVLVFIIFWLPYHLTLFVDTLKLLKWISSSCEFERSL  269  1
PGIVILSCYCIIISKLSHSKGHQKRKALKTTVILILAFFACWLPYYIGISIDSFILLEIIKQGCEFENTV  280  2
*  **  **  *      *     *          * *    ****     *    **   *   ****
                              TMS VI


KRALILTESLAFCHCCLNPLLYVFVGTKFRKNYTVCWPSFASDSFPAMYPGT----------------  321  1
HKWISITEALAFFHCCLNPILYAFLGAKFKTSAQHALTSVSRGSSLKILSKGKRGGHSSVSTESESSSFH  350  2
     ** *** ****** ** * * **         *     *
            TMS VII


TA  323  1
SS  352  2
```

Figure 21: Clustal alignment of the human cytomegalovirus encoded US28(1) and of humnyr3 (2). Identities are indicated by stars.

Most of these traits are present in the majority of the members of this superfamily, except perhaps the pairs Pro-Cys (PC) in the N-terminus, Cys-Glu (CG) in the third external loop (e3), and Cys-Tyr (CY) in the TMS V that are conserved only in some of the chemotactic factor receptors and humnyr3. The N-terminus and external loops are poorly conserved which speaks against a similar ligand for both, the viral and the human receptors. The homology in internal loops is also poor, therefore, they likely bind to different types of G-proteins.

3. 7TMS receptors for large proteins:

The receptors for pituitary and placental glycoprotein hormones differ from other members of the 7TMS superfamily in having large extracellular domains (see Appendix II). This is consistent with the larger size of their cognate ligands. Site-directed mutagenesis of the thyrotropin receptor involving substitutions and deletions have been performed. This receptor upon activation induces a rise in cAMP levels.

Mutations in i1 anulate the ability to activate adenylate cyclase but did not alter the binding affinity for TSH. When the second loop i2 was targeted a different pattern was observed depending on which region the mutations were clustered. For those mutants with altered N-terminal region within the loop, again the ligand binding was unaffected but the receptor was no longer functional. This confirms the role of the Asp-Arg pair in creating a generic G-protein binding motif. This is the basis for delineating an extended consensus in this area for related proteins. So, in both human IL-8 Rs , the rabbit IL-8 R, the rabbit formyl-peptide receptor and humnyr3 the motif is Asp-Arg-Tyr-Leu-Ala-Ile (DRYLAI) (that was precisely the forward primer in the cloning of humnyr3).

For mutations clustering in the C-terminal region of this loop both the affinity for the ligand and the coupling to G-proteins were abolished. As the N-terminal portion of i3 was mutated, both affinity and coupling were impaired. However. when the C-terminal region was modified no difference between this mutant and the wild type was found. This loop is the more heterogeneous across the whole superfamily, presenting variation in both length and composition. It likely imparts specificity for the binding to G-proteins.

For cytoplasmic tail mutants total truncation affected both the binding affinity and the coupling. When two thirds of the C-terminus proximal sequence were deleted leaving a short tail which is homologue to the C-terminus in the TSH, and lutropin/chorionic gonadotropin receptors both parameters remained unchanged (Chazenbalk et al., 1990). Again, this region presents more variation and tends not to be conserved even in related proteins. One prediction is that the

desensitization mechanisms should be affected because truncation removes most of the phosphorylation sites. These receptors are double in size of the remaining 7TMDS proteins. This difference is mainly due to the N-terminus which is 412 residues long in the case of the thyrotropin receptor.

4. Chemotactic Receptors:

Recently, several cDNAs encoding chemotactic and activating factor receptors have been cloned. No studies of structure-function relationship have been performed on these proteins up to now. The translated sequence follows the predicted topology for the 7TMS receptors: they all contain seven stretches of 20-28 hydrophobic amino acids that likely form membrane spanning domains. Identical residues or conservative substitutions are clustered in these regions. Other regions are less, but still to a significant extent, conserved. These are the two first intracytoplasmic loops. The extracellular domains and the C-terminal tail are quite divergent. Not surprisingly, the greatest similarity is found in those proteins that bind to closely related ligands.

The high-affinity IL-8 R (humil8ra) was isolated by expression cloning from human neutrophils mRNA (Holmes et al., 1991). Cross-reactivity with Gro/MGSA, MIP-1 and NAP 2 but not with fMLP or with other chemotactic factors had been observed (both, by binding assays and by functional studies in human neutrophils) (Moser et al., 1991). There are two glycosylation sites at the N-terminus and this region contains several acidic residues. The third cytoplasmic loop is shorter than in the adrenergic or muscarinic receptors and is thought to contain

determinants for G-protein binding (see above). The C-terminus contains several Ser (S) and Thr (T) residues that could be substrates for phosphorylation.

A second IL-8 binding protein (humintleu8) which displays 74% identity with the previous one was obtained from a cDNA isolated of a promyelocytic leukemia cell line (HL-60) that was induced to differentiate (Murphy et al., 1991). The affinity with which this receptor binds IL-8 is 20 times lower than humil8ra. The protein belongs to the 7TMS superfamily. The C-terminal region contains 11 S or T that could be phosphorylation sites for kinases. It has a single N-glycosylation site in the N-terminus and two in the second external loop. The former is also enriched in acidic residues.

The anaphylatoxin C5a is a 74 amino acids glycoprotein derived from C5 during activation of the complement cascade. It is a potent activator of neutrophils and monocytes apart from inducing spasms in various tissues, stimulating smooth muscle contraction, inducing histamine release from basophils, serotonin from platelets and also inducing vascular permeability. All these activities are due to the interaction of C5a with high affinity receptors in the cell membrane. These receptors are of the 7TMS type and bind G-proteins, and they are related to other chemotactic factor receptors (Boulay et al.,1991). As in the two previous cases the N-terminal domain is enriched in acidic residues, it contains one N-glycosylation site at the N-terminal domain, and the C-terminus is rich in S and T. They have even more similarity with the human N-formylpeptide receptors.

Another trigger for the locomotion and activation of phagocytic cells are the N-formyl peptides, which are thought to be derived from bacterial degradation or injured tissue mitochondria degradation. This group also includes the platelet activating factor (PAF), and the arachidonate metabolite leukotriene B4. The human N-formyl peptides receptor has been characterized. A cDNA has been cloned and sequenced (Boulay et al., 1990). The hydrophobicity profile indicates a 7TMS topology in agreement with previous functional studies that revealed the coupling to G-proteins. There are three putative sites for N-glycosylation but previous studies with endoglycosidase F have showed that only 2 are actually glycosylated. The third intracytoplasmic loop is short and contain a site for PKA phosphorylation (KSSSR). Also, there are several hydroxy-amino acids in the C-terminus that could function as phosphate acceptors.

From all these proteins the humintleu8 (IL-8 R, low affinity) first and then the humil8ra (IL-8 R, high affinity) are the ones with the highest degree of homology with the humnyr3 receptor. Identical residues or conservative substitution are found all over the length of these proteins (see figure 22).

```
humnyr3      MEGISIYTSDNY--TEEMGSGDYDSM------KEPCFREENANFNKIFLPTIYSIIFLT
humintleu8   MESDSF---EDFWKGEDLSNYSYSSTLPPFLLDAAPC-EPESLEINKYFVVIIYALVFLL
             **. *.     ...  *.... * *          **   *. ..** *. **...**
                   N-terminus                          TMS I


             GIVGNGLVILVMGYQKKLRSMTDKYRLHLSVADLLFVITLPFWAVDAVANWYFGNFLCK
             SLLGNSLVMLVILYSRVGRSVTDVYLLNLALADLLFALTLPIWAASKVNGWIFGTFLCK
             ...**.**.**. *  . **.** * *.*..***** .***.**   * * ** ****
                         i1                 TMS II        e1
```

88

```
AVHVIYTVNLYSSVLILAFISLDRYLAIVHATNSQRPRKLLAEKVVYVGVWIPALLLTIPD
VVSLLKEVNFYSGILLLACISVDRYLAIVHATRTLTQKRYLV-KFICLSIWGLSLLLALPV
 *  ..   **.**..*.**  **.*********  .    .. *  * . ...*  .***..*
     TMS III              i2              TMS IV


FIFAN-VSEADDRYIC--DRFYPNDLWVVVFQFQHIMVGLILPGIVILSCYCIIISKLSH
LLFRRTVYSSNVSPACYEDMGNNTANWRMLLRILPQSFGFIVPLLIMLFCYGFTLRTLFK
..*   *  ..   *  *      *  .....    *.*.* ...* **  . .  *
         e2              TMS V          i3


SKGHQKRKALKTTVILILAFFACWLPYYIGISIDSFILLEIIKQGCEFENTVHKWISITE
AHMGQKHRAMRVIFAVVLIFLLCWLPYNLVLLADTLMRTQVIQETCERRNHIDRALDATE
 .   **..*..     ..* *. *****  . .  *...  ..*.. **  * ... .  **
 i3           TMS VI                    e3


ALAFFHCCLNPILYAFLGAKFKTSAQHALTSVSRGSSLKILSKGKRGGHSSVSTESESSS
ILGILHSCLNPLIYAFIGQKFRHGLLKILA-IHGLISKDSLPKDSRPSFVGSSSGHTSTT
*...* ****..***.* **. .   *. .   *  *.*. *  . . *.   *..
     TMS VII               C-terminus


FHSS
L---

•
```

Figure 22: Alignment of humnyr3 and humintleu8 (the low affinity IL-8 R) Notice the high degree of homology, extended over the overall length, between both molecules

This would indicate a rather close relationship in both function and structure but until the structural determinants of bioactivity start to emerge nothing definitive to that respect can be said Both, humnyr3 and chemotactic receptors N-termini are enriched in acidic residues. In the case of the intercrine receptors it was thought that their function was to participate in the binding to the chemotactic factors which are basic polypeptides. But NPY is a rather neutral molecule. The C-terminal side of the loop i3 is conserved but the N-terminal side is not. It would appear that both receptors interact with the same or a similar type of G-protein. The external loop e2 is the least conserved of them all. The high degree of similarity between both proteins (35% identity;

64% similarity counting the conservative substitutions) is suggestive. The affinity of humintleu8 for IL-8 is 20 times lower than the affinity of humil8ra for IL-8. This cast doubts about the specificity of the former. Perhaps, once the specific ligand of humintleu8 is defined the relationship with humnyr3 will also be clarified.

Other chemotactic factor receptors (with the exception of humil8ra, and the rabbit IL-8 R and fMLP receptors) share less homology with humnyr3. This is also true among the intercrine receptors themselves.

5. Neuropeptide receptors:

When the sequence of the rat substance K receptor, mouse substance K receptor and human substance P receptor are compared to humnyr3 the homology is restricted to a few basic features of G-proteins coupled receptors (figure 23 and appendix 3).

```
            *.  *.  *  ..*.  ..*..      *    *.**. **.***   .*. .*.** *.*****
bosskr      MGACVVMTDINISSGLDSNATGITA---FSM--PGWQLALWTAAYLALVLVAVMGNATVIWIILA
humsubpra   MDN-VLPVDSDLSPNISTNTSEPNQ---FVQ--PAWQIVLWAAAYTVIVVTSVVGNVVVMWIILA
humnyr3     MEGISIYTSDNYTEEMGSGDYDSMKEPCFREENANFNKIFLPTIYSIIFLTGIVGNGLVILVMGY
            *     .      .   . .   *     .   .   . *  . ....** *.  ..
                      N-terminus                          TMS I


            *.*********.****.*.  *****.   **  ** ** **.*  .* *.*.***.*.* *
bosskr      HQRMRTVTNYFIVNLALADLCMAAFNAAFNF-VYASHNIWYFGRAFCYFQNLFPITAMFVS
humsubpra   HKRMRTVTNYFLVNLAFAEASMAAFNTVVNF-TYAVHNEWYYGLFYCKFHNFFPIAAVFAS
humnyr3     QKKLRSMTDKYRLHLSVADL---LFVITLPFWAVDAVANWYFGNFLCKAVHVIYTVNLYSS
            ....*...*. . ..*. *.      *      *        **.*   *   .  .   .. *
                i1             TMS II          e1          TMS III


            ******.* ******.**.******      .*. **  **..** ***** .*** *  .
bosskr      IYSMTAIAADRYMAIVHPFQPRLSAP--GTRAVIAGIWLVALALAFPQCFYSTITTDEGATK
humsubpra   IYSMTAVAFDRYMAIIHPLQPRLSAT--ATKVVICVIWVLALLLAFPQGYYSTTETMPSRVV
humnyr3     VLILAFISLDRYLAIVHATNSQRPRKLLAEKVVYVGVWIPALLLTIPDFIFANVSEADDRYI
            .  .. .. ***.**.*. .... .   .. *   .*. ** *..*.   ..
                         i2                        TMS IV
```

```
            *..  ***..         .**. *  *******.*.  **.*.*.***   .**. .. .
bosskr      CVVAWPEDSGGKMLLLYHLIVIALIYFLPLVVMFVAYSVIGLTLWRRSVPGHQAHGANLR
humsubpra   CMIEWPEHPNKIYEKVYHICVTVLIYFLPLLVIGYAYTVVGITLWASEIPGDSSDRYH-E
humnyr3     CDRFYPND---LWVVVFQFQHIMVGLILPGIVILSCYCIIISKLSHS------------
            *    *..         ....  .  .** .*.   * ..    *        *.
            e2                      TMS V                    i3


            .. **.* ** *..** *******.*.          .*.*  .. *.*  ********. ******
bosskr      HLQAKKKFVKTMVLVVVTFAICWLPYHL------YFILGTFQEDIYCHKFIQQVYLALFWLAMSST
humsubpra   QVSAKRKVVKMMIVVVCTFAICWLPFHI------FFLLPYINPDLYLKKFIQQVYLAIMWLAMSST
humnyr3     KGHQKRKALKTTVILILAFFACWLPYYIGISIDSFILLEIIKQGCEFENTVHK------WISITEA
            *.* .*   ....* ****..       ...*  ... .    ....      *..... .
            i3           TMS VI                       e3


            ***********.*** **. ******  ... . .*. *  * *. .       .
bosskr      MYNPIIYCCLNHRFRSGFRLAFRCCPWVTPTEEDKMELTYTPSLSTRVNRCHTKEIFFMS
humsubpra   MYNPIIYCCLNDRFRLGFKHAFRCCPFISAGDYEGLEMKSTRYLQTQGSVYKVSRL----
humnyr3     L--AFFHCCLNPIL-----YAFLGAKFKTSAQHALTSVSRGSSLK----------ILSK
            .  ... ****  .        **        ... .       .         *
                 TMS VII                      C-terminus


            . . *  * .. * *. *  . *
bosskr      GDVAPSEAVNGQAESPQAGVSTEP-----------------------------
humsubpra   -ETTISTVVGAHEEEPEDGPKATPSSLDLTSNCSSRSDSKTMTESFSFSSNVLS
humnyr3     GKRGGHSSVSTESESS----------------------------SFHS---S
            *    .  *  .
```

Figure 23: Alignment of two of the neuropeptide receptors with humnyr3. Identities and conservative substitutions between the two neuropeptide (SP and NKA) receptor proteins are showed by the stars and the dots respectively on top of the sequences. The same convention is used for the comparison among the three sequences, but the homology is marked at the bottom of the sequences

Essentially a five residues cluster at the boundary of TMS III and i3 is conserved. Two more clusters of 4 residues each are conserved in TMS VI and TMS VII. The loop e1 is short and have some conserved features. The overall homology for the four proteins is 13% which is by far below that of the humintleu8 and humnyr3. The putative human NPY receptor subtype Y1 (humneypepy) shows 20% homology with humnyr3 (figure 24).

```
humnyr3     MEGISIYTSDNYTEEMGSGDYDSMKEP--CFREENANF--NKIF-LPTIYSIIFLTGIVG
humneypepy  MNS-TLFS--QVENHSVHSNFSEKNAQLLAFENDDCHLPLAMIFTLALAYGAVIILGVSG
            *.. .... . .. ... . * ... .. ** *. *. ... *. *
                                                        TMS I

humnyr3     NGLVILVMGYQKKLRSMTDKYRLHLSVADLLFVIT-LPF-WAVDAVANWYFGNFLCKAVH
humneypepy  NLALIIIILKQKEMRNVTNILIVNLSFSDLLVAIMCLPFTFVYTLMDHWVFGEAMCKLNP
            * .*... ** .*..*.  ..** .*** * ***   . .* **. .**
                                   TMS II

humnyr3     VIYTVNLYSSVLILAFISLDRYLAIVHATNSQRPRKLLAEKVVYVGV---WIPALLLTIP
humneypepy  FVQCVSITVSIFSLVLIAVERHQLIINPRGW-RPNN----RHAYVGIAVIWVLAVASSLP
            . *.. *.. * .*...*  *... ** . . ***. *. *. ..*
                TMS III                          TMS IV

humnyr3     DFIFANVSEA---------DDRYICDRFYPNDLWVVVFQFQHIMVGLILPGIVILSCYC
humneypepy  FLIYQVMTDEPFQNVTLDAYKDKYVCFDQFPSDSHRLSYTTLLLVLQYFGPLCFIFICYF
            .*. ...              *.*.*   .*.*  . .   ... . *   *. **
                                                        TMS V

humnyr3     IIISKLSHSKG---------HQKRKALKTTVIL---ILAFFACWLPYYIGISIDSFILLE
humneypepy  KIYIRLKRRNNMMDKMRDNKYRSSETKRINIMLLSIVVAFAVCWLP--LTIFNTVFDWNH
            * .* . .     . . . ..*  ..** **** . *   *   .
                                            TMS VI

humnyr3     IIKQGCEFENTVHKWISITEALAFFHC--------CLNPILYAFLGAKFKTSAQ------
humneypepy  QIIATCN----------HNLLFLLCHLTAMISTCVNPIFYGFLNKNFQRDLQFFFNFC
            * *.         . * *. *       *.***.*.**  .*. *
                                   TMS VII

humnyr3     ------HALTSVSRGSSLKILSKG--KRGGHSSVSTESESSSFHSS
humneypepy  DFRSRDDDYETIAMSTMHTDVSKTSLKQASPVAFKKINNNDDNEKI
            . ... .. .** *... . ... .
```

Figure 24: Alignment of the human NPY receptor type 1 and the putative human receptor type 3.

TMS II and e1 are the most conserved regions. In TMS V, VI and VII the homology clusters in the cytoplasmic side of the predicted helices. Both N- and C-termini are highly divergent, and e2 is conserved but e3 is not. These two proteins have distinct binding characteristics: the subtype Y1 binds both NPY and PYY with similar affinities; the subtype Y3 is more specific for NPY, binding PYY very weakly.

6. Humnyr3 and the immune response:

Substance P and substance K induce the release of IL-1, TNF-α and IL-6 from human blood monocytes (Lotz et al.,1988). This effect is specific. Because monocyte-derived cytokines are key mediators in inflammation and immunity, and, neuropeptides can be released from peripheral nerve endings into adjacent tissues, this constitutes a clear mechanism of interaction of the nervous system and cells of the immune response. Neuropeptides are released from unmyelinated axons in response to trauma or inflammation, inducing the production and release of cytokines that mediate host defensive responses in a paracrine and/or endocrine way. Substance P also regulates other monocyte functions such as arachidonic acid metabolism, chemotaxis and respiratory burst (Hartung et al., 1986). IL-1 and TNF initiate cellular and humoral responses and produce systemic changes such as the synthesis of acute-phase proteins and fever. It is likely that a similar, or the same inductive effect, exist in other substance P responsive cells, like fibroblasts and endothelial cells.

Preprotachykinin A is the precursor of both substance P and neurokinin A. It was shown by Northern blot that these two neuropeptides are expressed in the thymic medulla. By the same technique NPY mRNA was detected also in the thymic medulla (Ericsson et al.,1990). Several other neuropeptides have been found in thymus, with some differences according to the species but providing evidence for the intrathymic synthesis of these neuropeptides. It is not clear which is the role of these neurotransmitter/neuromodulators, but it has been suggested that they may act in T-cell differentiation and/or early activation that occurs mainly in the thymus.

By immunocytochemistry for NPY and lymphoid markers the NPY innervation in the rat spleen have been mapped. NPY positive nerves were present along the vasculature, trabeculae, and capsula, and also were found associated with specific lymphoid compartments in close contact with lymphocytes and macrophages (Romano et al.,1991).

Activated B-cells, like those represented in the Burkitt lymphoma cell lines, express high levels of this protein (figure 15 B). The findings reported here, that mRNA for the type 3 receptor has been detected in both thymus and spleen, complements the findings about the expression of the NPY itself in those organs. At this stage nothing can be said about the meaning of the expression of the Y3 subtype, both because the expression of the other two subtypes is not ruled out and because more pharmacological characterization of humnyr3 is desired. Nonetheless, the presence of mRNA for a NPY receptor in organs where the expression of the factor itself has already been established is in good agreement with the hypothesis that this neuropeptide and its receptor could participate in a neuro-immunomodulatory loop, but to clarify these aspects more investigation is needed. The significant homology of humnyr3 with some of the intercrine receptors is another important question that should be addressed.

7. Conclusion:

NPY can induce a variety of physiological responses through the activation of specific pre- and post-synaptic receptors. Different binding characteristics for agonists in several model systems indicate that there are multiple types of NPY receptors. This is being confirmed by the cloning studies. The receptor isolated here could be the putative

Y3 subtype based on the high homology to the characterized bovine Y3 receptor. To complete its pharmacological definition more experiments are needed. The coupling of NPY receptors to specific signal transduction pathways (like inhibition of adenylyl cyclase and stimulation or inhibition of intracellular Ca^{2+} increases) has not been established. The pathophysiological role of NPY and its receptors is not known. The availability of a human cDNA for the Y3 subtype is a tool for different lines of experimentation from *in situ* hybridization to expression of the receptor in stable cell lines.

NPY is one of the most abundant peptides in the human brain and appears as an integrator of endocrine, metabolic and behavioral processes (Leibowitz, 1991). It could be also intervening in the immune response in a more direct way and not only through its actions on endocrine hormones or metabolic processes.

The high degree of homology between this receptor subtype and the chemotactic factor receptors is of significance, and deserves more investigation, as well as its expression in cells that mediate immune responses.

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Cell (1986) **45**: 711-719.

XII. Appendices:

Appendix 1: Clustal alignment of 12 classical neurotransmitter receptors. The code for the names is at the end of the alignment. TMSs are underlined.

```
acm2_human    MNNSTNSSNNSLAL-----------------------------TS-------PYKTFEVV
b1ar_human    MGAGVLVLGASEPGNLSSAAPLPDGAATAARLLVPASPPASLLPPASESPEPLSQQWTAG
d4dr_human    MGNRSTA------------------DADGLLAGRGPAAGASAGASAGLAGQGAAALVG
a2aa_human    M--------------------GSLQPDAGNASWNGTEAPGGGARATP---YSLQVTL
a2ab_human    MASPALAA--------ALAVAAAAGPNASGAGERGSGGVANASGASWGPPRGQYSAGAVA
acm5_human    MEGDSYHNATTVNG--------------------------TPVNHQPLERHRLWEVI
b2ar_human    MG---------QPGN-----------GSAFLLAPNRSHA----PDHDVTQQRDEVWVVG
d5dr_human    MLPPG-SNGTAYPGQFALYQQLAQGNAVGGSAGAPPLGPSQV-----------------
a2ac_human    MD-----------------------------------------HQDPYSVQATA
b3ar_human    M----------------APWPHENSS----LAPWPDLPTLAPNTANTSGLPGVPWEAA
d2dr_human    MDPLNLS----------------WYDDDLERQNWSRPFNGSDGKADRPHYNYYATLLT
d1dr_human    M----------------RTLNTSAMDGTGLVVERDFSVRI-----------------
                  *
```

```
acm2_human    FIVLVAGSLSLVTIIGNILVMVSIKVNRHLQT-VNNYFLFSLACADLIIGVFSMNLYTLY
b1ar_human    M-GLLMALIVLLIVAGNVLVIVAIAKTPRLQT-LTNLFIMSLASADLVMGLLVVPFGATI
d4dr_human    GV-----LLIGAVLAGNSLVCVSVATERALQTP-TNSFIVSLAAADLLLALLVLPLFVYS
a2aa_human    TLVCLAGLLMLLTVFGNVLVIIAVFTSRALKAP-QNLFLVSLASADILVATLVIPFSLAN
a2ab_human    GLAAVVGFLIVFTVVGNVLVVIAVLTSRALRAP-QNLFLVSLASADILVATLVMPFSLAN
acm5_human    TIAVVTAVVSLITIVGNVLVMISFKVNSQLKT-VNNYYLLSLACADLIIGIFSMNLYTTY
b2ar_human    M-GIVMSLIVLAIVFGNVLVITAIAKFERLQT-VTNYFITSLACADLVMGLAVVPFGAAH
d5dr_human    VTACLLTLLIIWTLLGNVLVCAAIVRSRHLRANMTNVFIVSLAVSDLFVALLVMPWKAVA
a2ac_human    AIAAAITFLILFTIFGNALVILAVLTSRSLRAP-QNLFLVSLAAADILVATLIIPFSLAN
b3ar_human    LAGALLALAVLATVGGNLLVIVAIAWTPRLQT-MTNVFVTSLAAADLVMGLLVVPPAATL
d2dr_human    -------LLIAVIVFGNVLVCMAVSREKALQTT-TNYLIVSLAVADLLVATLVMPWVVYL
d1dr_human    LTACFLSLLILSTLLGNTLVCAAVIRFRHLRSKVTNFFVISLAVSDLLVAVLVMPWKAVA
                .. ** **   .       *..    *   . *** .*.  ..    .
                       TMS I                            TMS II
```

```
acm2_human    TV-IGYWPLGPVVCDLWLALDYVVSNASVMNLLIISFDRYFCVTKPLTYPVKRTTKMAG-
b1ar_human    VV-WGRWEYGSFFCELWTSVDVLCVTASIETLCVIALDRYLAITSPFRYQSLLT-RARAR
d4dr_human    EVQGGAWLLSPRLCDALMAMDVMLCTASIFNLCAISVDRFVAVAVPLRYN-RQGGSRRQL
a2aa_human    EV-MGYWYFGKAWCEIYLALDVLFCTSSIVHLCAISLDRYWSITQAIEYNLKRTP-RRIK
a2ab_human    EL-MAYWYFGQVWCGVYLALDVLFCTSSIVHLCAISLDRYWSVTQAVEYNLKRTP-RRVK
acm5_human    IL-MGRWALGSLACDLWLALDYVASNASVMNLLVISFDRYFSITRPLTYRAKRTPKRAG-
b2ar_human    IL-MKMWTFGNFWCEFWTSIDVLCVTASIETLCVIAVDRYFAITSPFKYQSLLT-KNKAR
d5dr_human    EV-AGYWPFGAF-CDVWVAFDIMCSTASILNLCVISVDRYWAISRPFRYKRKMT-QRMAL
a2ac_human    EL-LGYWYFRRTWCEVYLALDVLFCTSSIVHLCAISLDRYWAVSRALEYNSKRTP-RRIK
b3ar_human    AL-TGHWPLGATGCELWTSVDVLCVTASIETLCALAVDRYLAVTNPLRYGALVT-KRCAR
d2dr_human    EV-VGEWKFSRIHCDIFVTLDVMMCTASILNLCAISIDRYTAVAMPMLYNTRYSSKRRVT
d1dr_human    EI-AGFWPFGSF-CNIWVAFDIMCSTASILNLCVISVDRYWAISSPFRYERKMT-PKAAF
               .    *      *.   . *  . ..*.  *   .. **.   ... .      *   .
                        TMS III                            TMS IV
```

```
acm2_human    MMIAAAWVLS-FILWAPAILFWQFIVGVRTVEDG---------------ECYIQ-----
b1ar_human    GLVCTVWAISALVSFLPILMHWWRAES----------------D-EARRCYNDPKCCD
d4dr_human    LLIGATWLLSAAVAA-PVLCGLNDVRGRDPAV------------------------CR
a2aa_human    AIIITVWVISAVISF-PPLISIEKKGGGGGPQPAEPR------------------CE
a2ab_human    ATIVAVWLISAVISF-PPLVSLYRQPDG----AAYPQ--------------------CG
acm5_human    IMIGLAWLIS-FILWAPAILCWQYLVGKRTVPLD---------------ECQIQ-----
b2ar_human    VIILMVWIVSGLTSFLPIQMHWYRATH----------------Q-EAINCYANETCCD
d5dr_human    VMVGLAWTLSILISFIPVQLNWHRDQAASWGGLDLPNNLANWTPWEEDFWEPDVNAENCD
a2ac_human    CIILTVWLIAAVISL-PPLIY---KGDQGPQPRGRPQ--------------------CK
b3ar_human    TAVVLVWVVSAAVSFAPIMSQWWRVGA----------------DAEAQRCHSNPRCCA
d2dr_human    VMISIVWVLSFTISC-PLLFGLNNA---DQNE------------------------CI
d1dr_human    ILISVAWTLSVLISFIPVQLSWHKAKPTS-------------PSDGNATSLAETIDNCD
               .  .* ..  .     *
```

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```
acm2_human   FFSNAAVTFGTAIAAFYLPVIIMTVLYWHISRASKSRIKK------------DKKEP--
b1ar_human   FVTNRAYAIASSVVSFYVPLCIMAFVYLRVFREAQKQVKKIDSCERR--FLGGPARPPSP
d4dr_human   LEDRD-YVVYSSVCSFFLPCPLMLLLYWATFRGLQRW----EVAR--------------
a2aa_human   INDQKWYVISSCIGSFFAPCLIMILVYVRIYQIAKRRTRVPPSRRGPDAVAAPPGGTERR
a2ab_human   LNDETWYILSSCIGSFFAPCLIMGLVYARIYRVAKRRTRTLSEKRAP---VGPDGASPTT
acm5_human   FLSEPTITFGTAIAAFYIPVSVMTILYCRIYRETEKRTKDLADLQGSDSVTKAEKRKPAH
b2ar_human   FFTNQAYAIASSIVSFYVPLVIMVFVYSRVFQEAKRQLQKIDKSEGR--F---------
d5dr_human   SSLNRTYAISSSLISFYIPVAIMIVTYTRIYRIAQVQIRRISS-------LERAAEHAQS
a2ac_human   LNQEAWYILASSIGSFFAPCLIMILVYLRIYLIAKRSNRRGPRAKGGPGQGESKQPRPDH
b3ar_human   FASNMPYVLLSSSVSFYLPLLVMLFVYARVFVVATRQLRLLRGELGR--F-PPEESPPAP
d2dr_human   IANPA-FVVYSSIVSFYVPFIVTLLVYIKIYIVLRRRRKRVNTKRSSRAF------RAHL
d1dr_human   SSLSRTYAISSVISFYIPVAIMIVTYTRIYRIAQKQIRRIAA-------LERAAVHAKN
                  .   .*.  *  .      *   .
                         TMS  V
acm2_human   --------------VANQDPVSPSLVQGR-----IVKPNNNNMPSSD----DGLE-----
b1ar_human   SP----------------SPVPAPAPP---------------------------------
d4dr_human   RA----------------KLHGR-----------------------AP------
a2aa_human   PN---------------GLGPERSAGPGGAEAEPLPTQLNGAPGEPAPAGPRDT----
a2ab_human   EN---------------GLGAAAGEARTGTARPRPPTWSRTRAAQRPRGGAP------
acm5_human   RALFRSCLRCPRPTLAQRERNQASWSSSRRSTSTTGKPSQATGPSANWAKAEQLTTCSSY
b2ar_human   ------------------------------------------------------------
d5dr_human   CR---------------S------------------------------------------
a2ac_human   GG---------------ALASAKLPALASVASAREVNGHSKSTGEKEEGETPED----
b3ar_human   SR---------------SLAPAPVGT----------------------------------
d2dr_human   RA---------------PLKGNCTHPEDMKLCTVIMKSNGSFPVNRRRVEAA------
d1dr_human   CQ---------------T------------------------------------------


acm2_human   HNKIQNGKAPRDPVTENCVQGEEKESSNDSTSVSAVASNMRDDEITQDE-NTVSTSLGHS
b1ar_human   ------------------------------PGPPRPAAAAATA-----------------PL
d4dr_human   -------RRP--------SGPGPP-----SPTPPAPR-LPQDPCGPDCAPPAPGLPPDPC
a2aa_human   ----DALDLEESSSSDHAERPPGPRRPERGPRGKGKA---------------RASQVKPG
a2ab_human   ----GPLRRGGRRRAGAEGGAGGADGQGAGP---GAA---------------QSGALTAS
acm5_human   PSSEDEDKPATDPVLQVVYKSQGKESPGEEFSAEETEETFVKRETEKSDYDTPNYLLSPA
b2ar_human   ------------------------------------HVQNLSQ---------------VE
d5dr_human   ---------------------------------SAA-----------------------
a2ac_human   ----TGTRALPPSWAALPNSGQGQKEGVCGASPEDEAEEEEEEEEEEEECEPQAVPVSPA
b3ar_human   -------------------------------CAPP------------------------
d2dr_human   -------RRAQELEMEMLSSTSPPERTRYSPIPPSHHQLTLPDPSHHGLHSTPDSPAKPE
d1dr_human   ------------------------------TTGNGK---------------PV


acm2_human   KDENSK-QTCI----RIGTKTPKSDSCTPTNTTVEVVGSS--GQNGDEKQNIVARKIVKM
b1ar_human   ANGRAGK--RRPSRLVAL------------------------------------------
d4dr_human   GSNCAPPDAVRAAALPPQTPPQ-------------------TRRRRRAKITGR------
a2aa_human   DSLRGAGRGRRGS----GRR--------------------LQGRGRSASGLPRRRAGAG
a2ab_human   RSPGPGGRLSRAS----SRSVE----------------FFLSRRRRARSSVCRRKVA--
acm5_human   AAHRPKSQKCVAYKFRLVVKADGNQETNNGCHKVKIMPCPFPVAKEPSTKGLNPNPSHQM
b2ar_human   QDGRTGHGLRRSSKFC-L------------------------------------------
d5dr_human   -CA-PDTSLRASIK---------------------------------------------
a2ac_human   SACSPPLQQPQGSRVLATLRGQ----------------VLLGRGVGAIGGQWWRRRAHV
b3ar_human   -EGVPACG-RRPARLLPL-----------------------------------------
d2dr_human   KNGHAKDHPKIAKIFEIQTMPN------------------GKTRTSLKTMSRRKLS--
d1dr_human   ECSQPESSFKMSFK---------------------------------------------
```

```
acm2_human   TKQPAKKKPPPSREKKVTRTILAILLAFIITWAPYNVMVLINTFCA----------PC-I
b1ar_human   -----------REQKALKTLGIIMGVFTLCWLPFFLANVVKAFH-REL----------V
d4dr_human   ------------ERKAMRVLPVVVGAFLLCWTPFFVVHITQALCPA----------CSV
a2aa_human   GQN---------REKRFTFVLAVVIGVFVVCWFPFFFTYTLTAV---G---------CSV
a2ab_human   -QA---------REKRFTFVLAVVMGVFVLCWFPFFFIYSLYGICREA---------CQV
acm5_human   TK---RKRVVLVKERKAAQTLSAILLAFIITWTPYNIMVLVSTFCD---------KC-V
b2ar_human   -----------KEHKALKTLGIIMGTFTLCWLPFFIVNIVHVIQ-DNL----------I
d5dr_human   -----------KETKVLKTLSVIMGVFVCCWLPFFILNCMVPFCSGHPEGPPAGFPC-V
a2ac_human   --T--------REKRFTFVLAVVIGVFVLCWFPFFFSYSLGAICPKH---------CKV
b3ar_human   -----------REHRALCTLGLIMGTFTLCWLPFFLANVLRALGGPSL----------V
d2dr_human   -QQ---------KEKKATQMLAIVLGVFIICWLPFFITHILNIHC-D----------CNI
d1dr_human   -----------RETKVLKTLSVIMGVFVCCWLPFFILNCILPFCGS---GETQPF-C-I
                          * .    . ...*    * *.                       .
                                        TMS VI
acm2_human   PNTVWTIGYWLCYINSTINPACYALCNATFKKTFKHLL-MCHYKNI--------------
b1ar_human   PDRLFVFFNWLGYANSAFNPIIYC-RSPDFRKAFQGLLC-CARRAARRRHATHGDRPRAS
d4dr_human   PPRLVSAVTWLGYVNSALNPVIYTVFNAEFRNVFRKAL------RACC------------
a2aa_human   PRTLFKPFFWFGYCNSSLNPVIYTIFNHDFRRAFKKILCRGDRKRIV-------------
a2ab_human   PGPLFKFFFWIGYCNSSLNPVIYTVFNQDFRPSFKHILFRRRRRRGFRQ-----------
acm5_human   PVTLWHLGYWLCYVNSTVNPICYALCNRTFRKTFKMLL-LCRWKKKKVE-----------
b2ar_human   RKEVYILLNWIGYVNSGFNPLIYC-RSPDFRIAFQELLC--LRRSSLKAYG--------N
d5dr_human   SETTFDVFVWFGWANSSLNPVIYA-FNADFQKVFAQLLG-CSHFCSRT--PVETVNISNE
a2ac_human   PHGLFQFFFWIGYCNSSLNPVIYTIFNQDFRRAFRRILCRP-WTQTAW------------
b3ar_human   PGPAFLALNWLGYANSAFNPLIYC-RSPDFRSAFRRLLCRCGRRLPPEP-----------
d2dr_human   PPVLYSAFTWLGYVNSAVNPIIYTTFNIEFRKAFLKIL------H--C------------
d1dr_human   DSNTFDVFVWFGWANSSLNPIIYA-FNADFRKAFSTLLG-CYRLCPATNNAIETVSINNN
                *.  **. **   *    . .*.  *    *
                                        TMS VII
acm2_human   ------------------------------------------------------------
b1ar_human   GCLARPGPPPSPG-AASDDDDDDVVGATPPA--------------RLLEPWAGCNGGAA
d4dr_human   ------------------------------------------------------------
a2aa_human   ------------------------------------------------------------
a2ab_human   ------------------------------------------------------------
acm5_human   --------------------------------------------EKLY-----------
b2ar_human   GYSSNGNTGEQSGYHVEQEKENKLLCEDLPG--------------T--EDFVGHQGTVP
d5dr_human   ---LISYNQDIVFHKEIAAAYIHMMPNAVTPGNREVDNDEEEGPFDRMFQIYQTSPDGDP
a2ac_human   ------------------------------------------------------------
b3ar_human   ----------------------CAAARP--------------ALFPS-----GVPA
d2dr_human   ------------------------------------------------------------
d1dr_human   GAAMFSSHHEPRGSISKECNLVYLIPHAV--GSSEDLKKEEAAGIARPLE--KLSPALSV


acm2_human   -----------------------GATR-- Acetylcholine Muscarinic receptor(M2)
b1ar_human   A---------DSDSSLDEPCRPGFASESKV β1-adrenergic receptor
d4dr_human   ----------------------------- dopamine receptor (D4)
a2aa_human   ----------------------------- α2-adrenergic receptor
a2ab_human   ----------------------------- α1-adrenergic receptor
acm5_human   -----WQ----------------GNSKLP Acetylcholine Muscarinic receptor (M5)
b2ar_human   S---------DNIDSQGRNCS---TNDSLL β2-adrenergic receptor
d5dr_human   VAESVWELDCEGEISLDKITPFTPNGFH-- dopamine receptor (D5)
a2ac_human   ----------------------------- α2-adrenergic receptor (type C2)
b3ar_human   A---------RSSPAQPRLCQR-LDG---- β3-adrenergic receptor
d2dr_human   ----------------------------- dopamine receptor (D2)
d1dr_human   I------LDYDTDVSLEKIQPITQNGQHPT dopamine receptor (D1)
```

The names used correspond to the names in the swiss protein data bank

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Appendix 2: Clustal alignment for the receptors for three pituitary hormones. Stars indicate matches across all sequences and dots are conservative substitutions. Name code is at the end of the alignment.

```
tshr_human    MR----PADLLQLVLLLD--LPRDLGGMGCSSPPCECHQEEDFRVTCKDIQRIPSLPPST
lshr_human    MKQRFSPLQLLKLLLLLLQAPLPRALRRL-CPEP-CNCVPDGALR--------APAPRPS
fshr_rat      M-----ALLLVSLLAFLGT------GSGCHHWLCHCSNRVFLCQDSKVTEIPTDLPRNA
              *      .  *. *. .*         *    *.*     .      . *. .


tshr_human    QTLKLIETHLRTIPSHAFSNLPNISRIYVSIDVTLQQLESHSFYNLSKVTHIEIRNTRNL
lshr_human    TRLSLAYLPVKVIPSQSFRGLNEVIKIEISQIDSLERIEANAFDNLLNLSEILIQNTKNL
fshr_rat      IELRFVLTKLRVIPKGSFAGFGDLEKIEISQNDVLEVIEADVFSNLPKLHEIRIEKANNL
                *..   ...**. .* .. .. .* .*    *. .*.. * **  .. .* * ...**


tshr_human    TYIDPDALKELPLLKFLGIFNTGLKMFPDLTKVYSTDIFFILEITDNPYMTSIPVNAFQG
lshr_human    RYIEPGAFINLPRLKYLSICNTGIRKFPDVTKVFSSESNFILEICDNLHITTIPGNAFQG
fshr_rat      LYINPEAFQNLPSLRYLLISNTGIKHLPAVHKIQSLQKVL-LDIQDNINIHIVARNSFMG
              **.*.*. .** *..* * ***.. .*.. *. * .  . *.* **  .  .. *.* *


tshr_human    LCNETLTLKLYNNGFTSVQGYAFNGTKLDAVYLNKNKYLTVIDKDAFGGVYSGPSLLDVS
lshr_human    MNNESVTLKLYGNGFEEVQSHAFNGTTLTSLELKENVHLEKMHNGAFRGA-TGPKTLDIS
fshr_rat      LSFESVILWLSKNGIEEIHNCAFNGTQLDELNLSDNNNLEELPNDVFQGA-SGPVILDIS
              . *...* *  **...... ***** *... *..* *.   ...* *. .** **.*


tshr_human    QTSVTALPSKGLEHLKELIARNTWTLKKLPLSLSFLHLTRADLSYPSHCCAFKNQKKIRG
lshr_human    STKLQALPSYGLESIQRLIATSSYSLKKLPSKQTFVNLLRATLHYPSHCCAFRN------
fshr_rat      RTKVHSLPNHGLENLKKLRARSTYRLKKLPNLDKFVTLMEASLTYPSHCCAFANLKRQIS
              *.. .**. *** .. * *  ... *****   .*. *  *.* ******* *


tshr_human    ILESLMCNESSMQSLRQRKSVNALNSPLHQEYEENLGDSIVGYKEKSKFQDTHNNAHYYV
lshr_human    --------------LPTK---------ELNFSHSISEN--------------------
fshr_rat      ELHPI-CNKSIL-----RQDIDDMT---------QIGDQRVSLID--------DEPSY--
                          .                    ...


tshr_human    FFEEQEDEIIGFGQELKNPQEETLQAFDSHYDYTICGDSEDMVCTPKSDEFNPCEDIMGY
lshr_human    -FSKQCESTV---------RKSELSGWD--YEYGFCLPKTPR-CAPEPDAFNPCEDIMGY
fshr_rat      ----------GKGSDMMY----------NEFDYDLCNEVVDVTCSPKPDAFNPCEDIMGX
                                          ..*..*      *.*..*.*********


tshr_human    KFLRIVVWFVSLLALLGNVFVLLILLTSHYKLNVPRFLMCNLAFADFCMGMYLLLIASVD
lshr_human    DFLRVLIWLINILAIMGNMTVLFVLLTSRYKLTVPRFLMCNLSFADFCMGLYLLLIASVD
fshr_rat      NILRVLIWFISILAITGNTTVLVVLTTSQYKLTVPRFLMCNLAFADLCIGIYLLLIASVD
              ..**...*....**. ** .. ** .* **.***.*********.***.*.*.********
                        TMS I                            TMS II
tshr_human    LYTHSEYYNHAIDWQTGPGCNTAGFFTVFASELSVYTLTVITLERWYAITFAMRLDRKIR
lshr_human    SQTKGQYYNHAIDWQTGSGCSTAGFFTVLASELSVYTLTVITLERWHTITYAIHLDQKLR
fshr_rat      IHTKSQYHNYAIDWQTGAGCDAAGFFTVFASELSVYTLTAITLERWHTITHAMQLECKVQ
              *...*.*.*.*******.**..*.******.********.******..** *..*. *..
                                    TMS III
tshr_human    LRHACAIMVGGWVCCFLLALLPLVGISSYAKVSICLPMDTETPLALAYIVFVLTLNIVAF
lshr_human    LRHAILIMLGGWLFSSLIAMLPLVGVSNYMKVSICFPMDVETTLSQVYILTILILNVVAF
fshr_rat      LRHAASVMVLGWTFAFAAALFPIFGISSYMKVSICLPMDIDSPLSQLYVMALLVLNVLAF
              ****  .*. **      *..*.  *.*.* ***** .***....*.   *... .*.**..**
                        TMS IV                          TMS V
tshr_human    VIVCCCHVKIYITVRNPQYNPGDKDTKIAKRMAVLIFTDFICMAPISFYALSAILNKPLI
lshr_human    LIICACYIKIYFAVRNPELMATNKDTKIAKKMAILIFTDFTCMAPISFFAISAAFKVPLI
fshr_rat      VVICGCYTHIYLTVRNPTIVSSSSDTKIAKRMATLIFTDFLCMAPISFFAISASLKVPLI
              ...* *...**..**** ....******.**.****** ******.*.** .. ***
                                    TMS VI
```

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```
tshr_human    TVSNSKILLVLFYPLNSCANPFLYAIFTKAFQRDVFILLSKFGICKRQAQA-YRGQRVPP
lshr_human    TVTNSKVLLVLFYPINSCANPFLYAIFTKTFQRDFFLLLSKFGCCKRRADPLYRRKDFSA
fshr_rat      TVSKAKILLVLFYPINSCANPFLYAIFTKNFRRDFFILLSKFGCYEMQAQ-IYRTE----
              **...*.*******.**************.*.**.*.****** ....*. ** .
```

TMS VII

```
tshr_human    KNSTDIQVQKVTHDMRQGLHNMEDVYELIENSHLTPKKQGQISEEYMQTVL
lshr_human    YTSNCKNGFTGSNKPSQSTLKLSTLH--CQGTALLDKTR------YTEC--
fshr_rat      -TSSATHNFHARKSHCSSAPRVTNSYVLV---PLNHSSQN-----------
              .*. .     .. . ....      *   ...
```


The names used are those of the swiss protein data bank.
tshr= thyrotropin receptor
lshr= lutropin-choriogonadotropic hormone receptor
fshr= follicle stimulating hormone receptor

Appendix 3: Alignment of the neuropeptide receptors. Stars indicate identity and dots conservative substitutions. Name code is at the end of the alignment.

```
ratskr        MGTRAIVS--DANILSGLESNATGVTAFSMPGWQLAL-----WATAYLALVLVAVTGNAT
mussubkrec    MGAHASVT--DTNILSGLESNATGVTAFSMPGWQLAL-----WATAYLALVLVAVTGNAT
guipigsprec   MDN-VLPV--DSDLFPNISTNTSEPNQFVQPAWQIVL-----WAAAYTVIVVTSVVGNVV
humsubpra     MDN-VLPV--DSDLSPNISTNTSEPNQFVQPAWQIVL-----WAAAYTVIVVTSVVGNVV
humneypepy    MNSTLFSQVENHSVHSNFSEKNAQLLAFENDDCHLPLAMIFTLALAYGAVIILGVSGNLA
gpisprec      MDN-VLPV--DSDLFPNISTNTSEPNQFVQPAWQIVL-----WAAAYTVIVVTSVVGNVV
humsprlong    MDN-VLPV--DSDLSPNISTNTSEPNQFVQPAWQIVL-----WAAAYTVIVVTSVVGNVV
bosskr        MGACVVMT--DINISSGLDSNATGITAFSMPGWQLAL-----WTAAYLALVLVAVMGNAT
              *..        . ..  .........    .*   . ... *      . **  ..... .* ** .
                                                                    TMS I
ratskr        VIWIILAHERMRTVTNYFIINLALADLCMAAFNATFNFIYASHNIWYFGRAFCYFQNLFP
mussubkrec    VIWIILAHERMRTVTNYFIINLALADLCMAAFNATFNFIYASHNIWYFGSTFCYFQNLFP
guipigsprec   VMWIILAHKRMRTVTNYFLVNLAFAEASMAAFNTVVNFTYAVHNEWYYGLFYCKFHNFFP
humsubpra     VMWIILAHKRMRTVTNYFLVNLAFAEASMAAFNTVVNFTYAVHNEWYYGLFYCKFHNFFP
humneypepy    LIIIILKQKEMRNVTNILIVNLSFSDLLVAIMCLPFTFVYTLMDHWVFGEAMCKLNPFVQ
gpisprec      VMWIILAHKRMRTVTNYFLVNLAFAEASMAAFNTVVNFTYAVHNEWYYGLFYCKFHNFFP
humsprlong    VMWIILAHKRMRTVTNYFLVNLAFAEASMAAFNTVVNFTYAVHNEWYYGLFYCKFHNFFP
bosskr        VIWIILAHQRMRTVTNYFIVNLALADLCMAAFNAAFNFVYASHNIWYFGRAFCYFQNLFP
              .. ***  ..  **.*** ...**....  .* .    .*.*.   . * .*    *  .. . .
                                          TMS II
ratskr        ITAMFVSIYSMTAIAADRYMAIVHPFQPRLSAPSTKAIIAGIWLVALALASPQCFYSTIT
mussubkrec    VTAMFVSIYSMTAIAADRYMAIVHPFQPRLSAPSTKAVIAVIWLVALALASPQCFYSTIT
guipigsprec   IAAVFASIYSMTAVAFDRYMAIIHPLQPRLSATATKVVICVIWVLALLLAFPQGYYSTTE
humsubpra     IAAVFASIYSMTAVAFDRYMAIIHPLQPRLSATATKVVICVIWVLALLLAFPQGYYSTTE
humneypepy    CVSITVSIFSLVLIAVERHQLIINPRGWRPNNRHAYVGIAVIWVLAVASSLPFLIYQVMT
gpisprec      IAAVFASIYSMTAVAFDRYMAIIHPLQPRLSATATKVVICVIWVLALLLAFPQGYYSTTE
humsprlong    IAAVFASIYSMTAVAFDRYMAIIHPLQPRLSATATKVVICVIWVLALLLAFPQGYYSTTE
bosskr        ITAMFVSIYSMTAIAADRYMAIVHPFQPRLSAPGTRAVIAGIWLVALALAFPQCFYSTIT
              ... .**.*.. .* .*.  *..*   *  ..   . .* **...*.  . *    *  . .
                   TMS III                          TMS IV
ratskr        VDE---------GATKCVVAWPNDNGGKMLLLYHLVVFVLIYFLPLLVMFGAYSVIGLT
mussubkrec    VDQ---------GATKCVVAWPNDNGGKMLLLYHLVVFVLIYFLPLVVMFAAYSVIGLT
guipigsprec   TMP---------GRVVCMIEWPSHPDKIYEKVYHICVTVLIYFLPLLVIGYAYTVVGIT
humsubpra     TMP---------SRVVCMIEWPEHPNKIYEKVYHICVTVLIYFLPLLVIGYAYTVVGIT
humneypepy    DEPFQNVTLDAYKDKYVCFDQFPSDSHRL---SYTTLLLVLQYFGPLCFIFICYFKIYIR
gpisprec      TMP---------GRVVCMIEWPSHPDKIYEKVYHICVTVLIYFLPLLVIGYAYTVVGIT
humsprlong    TMP---------SRVVCMIEWPEHPNKIYEKVYHICVTVLIYFLPLLVIGYAYTVVGIT
bosskr        TDE---------GATKCVVAWPEDSGGKMLLLYHLIVIALIYFLPLVVMFVAYSVIGLT
              .    *   ..*..       *   . .* ** **   .   *   . .
                                      TMS V
```

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```
ratskr          LWKRAVPRHQAHGANLRHLQAKKKFVKAMVLVVLTFAICWLPYHLYFILGTFQEDIYYHK
mussubkrec      LWKRAVPRHQAHGANLRHLQAKKKFVKAMVLVVVTFAICWLPYHLYFILGTFQEDIYYRK
guipigsprec     LWASEIPGDSSDRYH-EQVSAKRKVVKMMIVVVCTFAICWLPFHIFFLLPYINPDLYLKK
humsubpra       LWASEIPGDSSDRYH-EQVSAKRKVVKMMIVVVCTFAICWLPFHIFFLLPYINPDLYLKK
humneypepy      LKRRNNMMDKMRDNKYRSSETKRINI-MLLSIVVAFAVCWLPLTIFNTVFDWNHQIIATC
gpisprec        LWASEIPGDSSDRYH-EQVSAKRKVVKMMIVVVCTFAICWLPFHIFFLLPYINPDLYLKK
humsprlong      LWASEIPGDSSDRYH-EQVSAKRKVVKMMIVVVCTFAICWLPFHIFFLLPYINPDLYLKK
bosskr          LWRRSVPGHQAHGANLRHLQAKKKFVKTMVLVVVTFAICWLPYHLYFILGTFQEDIYCHK
                *    ..     .        .*.  .  .. .**.****  ..   .   . ..
                                                        TMS VI
ratskr          FIQQVYLALFWLAMSSTMYNPIIYCCLNHRFRSGFRLAFRCCPWVTPTEE-DRLELTHTP
mussubkrec      FIQQVYLALFWLAMSSTMYNPIIYCCLNHRFRSGFRLAFRCCPWGTPTEE-DRLELTHTP
guipigsprec     FIQQVYLAIMWLAMSSTMYNPIIYCCLNDRFRLGFKHAFRCCPFISAA-DYEGLEMKSTR
humsubpra       FIQQVYLAIMWLAMSSTMYNPIIYCCLNDRFRLGFKHAFRCCPFISAG-DYEGLEMKSTR
humneypepy      NHNLLFLLCHLTAMISTCVNPIFYGFLNKNFQRDLQFFFNFCDFRSRDDDYETIAMSTMH
gpisprec        FIQQVYLAIMWLAMSSTMYNPIIYCCLNDRFRLGFKHAFRCCPFISAA-DYEGLEMKSTR
humsprlong      FIQQVYLAIMWLAMSSTMYNPIIYCCLNDRFRLGFKHAFRCCPFISAG-DYEGLEMKSTR
bosskr          FIQQVYLALFWLAMSSTMYNPIIYCCLNHRFRSGFRLAFRCCPWVTPTEE-DKMELTYTP
                . ..*       ** **  ***.*  **..*.  ...   *. *  . . . . .... .
                                  TMS VII
ratskr          SLSRRVNRCHTKETLFMTGDM--THSEATNGQVGSPQDGEPAGPIC--------------
mussubkrec      SISRRVNRCHTKETLFMTGDM--THSEATNGQVGGPQDGEPAGPx---------------
guipigsprec     YF-------QTQGSVYKVSRLETTISTVVGAHEEDPEEGPKATPSSLDLTSNGSSRSNSK
humsubpra       YL-------QTQGSVYKVSRLETTISTVVGAHEEEPEDGPKATPSSLDLTSNCSSRSDSK
humneypepy      TDVSKTSLKQASPVAFK--------------KINNNDDNEK------------------
gpisprec        YF-------QTQGSVYKVSRLETTISTVVGAHEEDPEEGPKATPSSLDLTSNGSSRSNSK
humsprlong      YL-------QTQGSVYKVSRLETTISTVVGAHEEEPEDGPKATPSSLDLTSNCSSRSDSK
bosskr          SLSTRVNRCHTKEIFFMSGDV--APSEAVNGQAESPQAGVSTEP---------------
                     ..      ..             . .. ...

ratskr          ---------KAQA  rat substance K receptor
mussubkrec      -------------  mouse substance K receptor
guipigsprec     TVTESSSFYSNMLS guinea pig substance P receptor
humsubpra       TMTESFSFSSNVLS human substance P receptor protein mRNA
humneypepy      ------------Ix human neuropeptide Y receptor (type Y1)
gpisprec        TVTESSSFYSNMLS porcine substance P receptor
humsprlong      TMTESFSFSSNVLS human substance P receptor long form
bosskr          ------------- bovine substance K receptor
```

The names used are those from the gp data base

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