Characterization of the 5'-flanking region of the rat 5-hydroxytryptamine$_{1A}$ receptor gene

by

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Upstream DNA sequences of the
rat 5-HT_{1A} receptor gene

Abstract

To identify the genetic mechanisms underlying the cell specific expression displayed by the rat 5-hydroxytryptamine$_{1A}$ (5-HT$_{1A}$) receptor, over 3.2-Kb of 5'-flanking region were sequenced and characterized. A unique transcription initiation site was detected at -967 bp 5' to the translation initiation codon (+1 bp) and was specific to hippocampal tissues which are known to strongly express 5-HT$_{1A}$ receptors. By sequence analysis, a TATA consensus sequence was identified 58 bp upstream to the transcription initiation site and several other *cis*-acting elements were also observed. To assess potential transcriptional activity, deletion fragments of the 3.2-Kb 5-HT$_{1A}$ receptor gene 5' region were linked to a luciferase reporter gene and transiently expressed in mammalian cells (P19 and L tk) which do not endogenously express the 5-HT$_{1A}$ receptor gene. Results indicated a strong promoter located between nucleotides -1187 and -117, followed by an upstream silencer region (-1390/-1188), the repressor activity of which is attenuated by the presence of additional upstream sequences. I suggest that repression of transcription may be a major mechanism restricting the expression of the 5-HT$_{1A}$ receptor gene to the appropriate tissues.

Résumé

Le récepteur de la sérotonine de type 1A (5-HT_{1A}) est, dans le cerveau, exclusivement exprimé par l' hippocampe, le septum et les noyaux du Raphé. En vue de pouvoir etudier les méchanismes génétiques responsables pour cette spécificité d'expression, 3 2-Kb furent séquencés et charactérisés L'initiation de la transcription a ete détectée a un endroit bien précis qui est situé 967 nucléotides plus haut que le site d'initiation de la translation (désigné +1). Ce site de la transcription est spécifique pour les tissues de l' hippocampe et aucun autre site n' a été observé L' analyse de la séquence révèle plusieurs séquences générales repanduent sur l' ensemble de la région 5' du gène Pour déterminer si les séquences 5' du récepteur de la sérotonine de type 1A transmettent des activités quelconques sur la transcription, plusieurs fragments de differente longueur provenant de la séquence 5' du gène 5-HT_{1A}, liés à un gène reporteur (la luciferase), furent exprimés dans des cellules mammifères qui ne contiennent pas la machinerie necessaire pour produire des récepteurs 5-HT_{1A} Les résultats demontrent que les séquences 5' du gène codant le récepteur 5-HT_{1A} sont capable d' activer et d' entreprendre la transcription par eux-mêmes. Il semble que le méchanism par lequel le récepteur 5-HT_{1A} est exprimé, serait doter d' une dérépression selective aux tissues qui l'exprime.

To Ramani and my mother

"...you can't get something for nothing
you can't get freedom for free
you won't get wise with the sleep still in your eyes
no matter what your dreams might be "

Neil Peart

Contribution to Original Knowledge

This thesis provides novel information concerning the regulation of the rat 5-HT_{1A} receptor gene expression. Here, it is reported that sequences upstream of the rat 5-HT_{1A} receptor gene are capable of transcriptional activity and modulate the 5-HT_{1A} receptor gene expression. This finding is of important significance since the expression of the 5-HT_{1A} receptor has been shown to be highly specific in the mammalian central nervous system.

This thesis also provides valuable information concerning a transformed neuronal cell line which endogenously expresses 5-HT_{1A} receptors. Neuron cell lines endogenously expressing 5-HT_{1A} receptors have not been reported before. This cell line represent an ideal system to study the natural environment in which 5-HT_{1A} receptors are present and exert their functions.

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List of Abbreviations

5-HT, 5-hydroxytryptamine, serotonin
5,7,-DHT, 5, 7,-dihydroxytryptamine
8-OH-DPAT, 8-hydroxy-2-(Di-*n*-propylamino)tetralin
AD, antidepressant
ADP, adenosine di-phosphate
bp, base pair
cAMP, 3', 5', cyclic adenosine mono-phosphate
cDNA, complementary DNA
CNS, central nervous system
G protein, guanine nucleotide binding protein
GTP, guanine tri-phosphate
HTH, helix-turn-helix
Kb, kilo base
K_D, affinity constant
KDa, kilo Dalton
LSD, lysergic acid diethylamide
mACh, muscarinic acetylcholine receptor
PGE_2, prostaglandin E2
PT or PTx, pertussis toxin
RT-PCR, reverse transcription polymerase chain reaction
VIP, vasoactive intestinal peptide

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Note

This thesis is divided into two chapters that are based on the following manuscripts and which will be referred to by their Roman numerals

Chapter I

Charest, A , and Albert, P R 1992 Promoter and Silencer Activities of the Rat 5-Hydroxytryptamine$_{1A}$ Receptor Gene 5'-Region

submitted for publication to *Journal of Biological Chemistry*

Chapter II.

Charest, A., Wainer, B H , and Albert, P R 1992 Cloning of the Murine 5-Hydroxytryptamine$_{1A}$ Receptor cDNA and Functional Characterization in a Septal Cell Line

submitted for publication to *Molecular Pharmacology*

Introduction

Historical background

The serotoninergic field of research originated during the early 1950's with the isolation, identification and chemical synthesis of 5-hydroxytryptamine (5-HT; *serotonin*), a vasoconstrictor and smooth muscle contracting factor found in serum (Rapport et al , 1947), cells of the gastrointestinal tract (Erspamer, 1963), and brain extracts (Twarog and Page, 1953) The later observation that the blood brain barrier is not permeable to serotonin suggested that certain cells of the CNS were responsible for the synthesis of the serotoninergic content of the brain (Cooper et al , 1978) An entire decade passed before Dahlstrom and Fuxe unequivocally demonstrated the neuronal localization of serotonin (Frazer et al., 1990). During the late 60's, new developments in mapping techniques such as immuno- and histochemistry, made possible the detailed anatomy and topology of serotoninergic cells and their projections (Cooper et al , 1978) In the late 1970's, Peroutka and Snyder (1979) marked the history of central serotonin research by validating earlier observations (Gaddum and Picarelli, 1957) that serotonin's actions are mediated by multiple receptors In what is now a classic publication, they differentiated two distinct subpopulations of receptors using radioligand binding techniques The 5-HT_1 subpopulation displayed nanomolar affinities for [^{3}H] 5-HT and the 5-HT_2 subpopulation bore low affinity for [^{3}H] 5-HT but high affinity for [^{3}H] LSD This discovery initiated extensive research in the identification of multiple central receptor subtypes throughout the 1980's With the recent advent of molecular biology, the serotonergic research community witnessed the cloning of the first serotonin receptors in 1987 (Lubbert et al , 1987, Kobilka et al., 1987a) Since then, a plethora of receptors have been isolated, characterized and their distribution within the CNS obtained

Central 5-HT$_{1A}$ receptors: physiology

The serotonergic system is a remarkably sophisticated and intricate network. Virtually all regions of the brain are targeted by afferent fibers which are invariably terminals of neurons emerging from the raphé formation (Tork and Hornung, 1990). The density of serotonergic input is not uniform throughout the brain. Regions such as the cerebral cortex, the limbic structures, the basal ganglia and many regions of the brainstem are characteristically innervated by a higher degree of serotoninergic fibers (Tork , 1990). Therefore, the serotonergic system uses many different receptor classes to convey unique messages. These subpopulations of receptors are set apart by a distinct pharmacology, anatomical location and more recently, by distinct molecular structures and functions (Peroutka et al , 1990, Palacios et al., 1990, Weinshank et al , 1992, Hen, 1992, Aghajanian et al , 1990).

At least eighteen different serotonin receptors, each falling into five different subpopulations have been cloned and characterized from species such as dog, drosophila, rat, mouse and human (see table I). Of all the serotonin receptors, the 5-HT$_{1A}$ subtype has been the most studied for many reasons, one of them being the availability of a selective radioligand ([^{3}H]8-hydroxy-2-(di-*n* propyl-amino)tetralin or [^{3}H]8-OH-DPAT). This radioligand allowed specific labelling of 5-HT$_{1A}$ binding sites in the mammalian brain. Using receptor autoradiography, several groups were able to discretely map 5-HT$_{1A}$ binding sites in the rat to structures such as the hippocampal formation (mainly the pyramidal cells of the CA1 region and the dentate gyrus) the septum, the cerebral cortex (primarily the frontal and entorhinal cortex), certain amygdaloid nuclei, and to the raphé nuclei (mainly the dorsal raphé region) (Palacios et al , 1987, El Mestikawy et al , 1990, Biegon et al , 1982, Hamon et al , 1987, Pompeiano et al , 1992, Miquel and Hamon, 1992). However, the limited resolution of light microscopy receptor autoradiography did not permit the precise cellular localization of neurons bearing 5-HT$_{1A}$ binding sites. Such obstructions were counteracted by appending lesion experiments to receptor

Table I. Classification of serotonin receptors.

Type	Subtype	Species	Molecular status	References
5-HT_1	5-HT_{1A}	Human	Cloned, genomic	Kobilka et al., 1987a; Fargin et al., 1988; Parks et al., 1991
		Rat	Cloned, genomic	Albert et al., 1990
		Mouse	Cloned, cDNA	Charest et al., 1992
	5-HT_{1B}	Mouse	Cloned, genomic	Maroteaux et al., 1992
		Rat	Cloned, cDNA	Voigt et al., 1991
	$5\text{-HT}_{1D\alpha}$	Human	Cloned, genomic	Hamblin and Metcalf, 1991
		Dog	Cloned, cDNA	Libert et al., 1989
	$5\text{-HT}_{1D\beta}$	Human	Cloned, genomic	Demchyshyn et al., 1992
	5-HT_{1E}	Human	Cloned, genom'	McAllister et al., 1992
			Cloned, cDNA	Zgombick et al., 1992
5-HT_2	$5\text{-HT}_{1C/2A}$	Rat	Cloned, cDNA	Julius et al., 1988
		Mouse	Cloned, cDNA	Lübbert et al., 1987
	5-HT_{2B}	Human	Cloned, genomic	Chen et al., 1992
		Rat	Cloned, cDNA	Pritchett et al., 1988
		Rat	Cloned, cDNA	Julius et al., 1990
5-HT_3		Mouse	Cloned, cDNA	Maricq et al., 1991
5-HT_4		----	Pharmacologically defined only	Bockaert et al., 1992
5-HT_{dro}	5-HT_{dro1}	Drosophila	Cloned, genomic and cDNA	Witz et al., 1990
	5-HT_{dro2a}	Drosophila	Cloned, genomic	Saudou et al., 1992
	5-HT_{dro2b}	Drosophila	Cloned, genomic	Saudou et al., 1992
5-HT_{fund}	stomach fundus receptor	Mouse	Cloned, genomic	Foguet et al., 1992

autoradiography. Following specific destruction of serotonergic neurons by the neurotoxin 5,7-DHT, there was no alteration in 5-HT_{1A} binding sites in the forebrain areas, suggesting that these sites are of postsynaptic character Conversely, the high density of 5-HT_{1A} receptors occupying the raphé formation was completely eradicated, indicating an autoreceptor function for these presynaptic sites (Palacios et al., 1990, Vergé et al , 1985; Palacios et al , 1987) This latter observation was further ascertained by immunohistochemistry (Sotelo et al , 1990, El Mestikawy et al., 1990) using antibodies against 5-HT_{1A} receptors. These studies demonstrate that the 5-HT_{1A} receptors are localized to the cell body and dendrites of the serotonergic raphé neurons. In contrast, radioautography studies invariably demonstrate that the presynaptic serotonergic nerve terminals arising from the raphé nuclei and projecting to the postsynaptic forebrains areas are devoid of 5-HT_{1A} receptors but enriched in 5-HT_{1B} receptors (Vergé et al , 1985, Engel et al , 1986). Thus the 5-HT_{1A} receptor has a somatodendritic localization in both the pre- and postsynaptic neurons.

Dysfunctions of serotonin neurons have been implicated in a number of neuropsychiatric diseases such as major depression and anxiety disorders (Peroutka, 1988; Meltzer and Lowry, 1987; Blier et al , 1990a, Blier et al , 1987, Charney and Delgado, 1992) It has long been proposed that functional deficiency of 5-HT is responsible for these psychopathologies (Coppen, 1967, Lapin and Oxenkrüg, 1969). However, direct evidence of abnormal function of serotonin neurons as a direct cause of major depression and anxiety is lacking Interestingly, brain regions displaying the highest 5-HT_{1A} receptor density are components of limbic structures and pathways involved in the modulation of mood and behaviour (Palacios et al., 1990; Hamon et al , 1987, Blier et al , 1987) Therefore, the observation that several assorted 5-HT_{1A} agonists found to exert anxiolytic and antidepressant (AD) effects, both in appropriate animal models and humans (Sjoerdsma and Palfreyman, 1990, Charney et al , 1990, Kennett et al , 1987, Dourish, 1987; Gonzalez-Heydrich and Peroutka, 1990, Price et al , 1990) is

consistent with a role for this receptor in these disorders. Using electrophysiological studies, various classes of AD and anxiolytic drugs were shown to enhance serotonergic synaptic transmission (see table II), through either the modulation of agonist sensitivity and/or receptor density of 5-HT_{1A} sites, or via yet unknown mechanisms Despite relative evidences for the involvement of 5-HT in the clinical manifestation of AD responses, the events leading to depression may involve several abnormalities such as long term structural, chemical and molecular changes (Holden,1991).

Central 5-HT_{1A} receptors: molecular biology

The human 5-HT_{1A} was the first serotonin receptor to be successfully isolated The cloning strategy relied on low stringency hybridization of a size-fractionated human genomic library using a ^{32}P radiolabeled full length human β_2-adrenergic receptor gene as a means of detection (Kobilka et al , 1987a). Following this work, Albert et al used identical approaches to isolate the rat 5-HT_{1A} receptor gene using the coding region of the hamster β_2-adrenergic receptor gene as a probe (Albert et al , 1990) More recently, the cDNA coding for the mouse 5-HT_{1A} receptor was isolated from a brain library (Charest et al., 1992).

The deduced amino acid sequence of the three 5-HT_{1A} receptors (as well as all serotonin receptors) clearly indicates that they belong to the extended family of G protein-coupled receptors (Kobilka et al , 1987a, Albert et al., 1990; Hartig, 1989) which consist of a single polypeptide chain containing seven putative transmembrane α-helices (Julius, 1991, Dohlman et al , 1987; Lefkowitz et al., 1988) On the basis of structural homology with rhodopsin, mACh and the β_2-adrenergic receptors, it is likely that all 5-HT receptors bear an extracellular amino terminus, a cytoplasmic carboxy terminus, three extracellular and three intracellular loops (Kubo et al , 1986, Kobilka et al , 1987b; Yarden et al., 1986; Dixon et al., 1986, Nathans and Hogness, 1984)

Table II. Potential mechanisms of action of some psychotropic drugs.

(references. 1- de Montigny et al., 1990; 2- Blier et al., 1990a; 3- Welner et al ,1989, 4- Blier et al , 1987; 5- de Montigny et al., 1988; Blier et al , 1988)

Antidepressants	**synaptic nature**	**Observations and effects**	**Causes**
5-HT reuptake blockers[1,2,5]	**pre-**	firing activity same as in untreated due to a decreased sensitivity of somatodendritic autoreceptors	desensitized auto-receptors and/or decrease number of 5-HT_{1A} binding sites
		decrease sensitivity of terminal autoreceptors therefore increase amount of 5-HT to be released per impulse in the synaptic cleft	decrease function of terminal auto-receptors by unknown mechanisms[6]
	post-	sensitivity of postsynaptic sites are unaltered	N A.
Tricyclic antidepressants[2,4,5]	**pre-**	firing activity and responsiveness of somatodendritic receptors are unmodified	N.A
	post-	sensitized receptors in postsynaptic structures leading to an enhanced 5-HT signal transfer	increase number of 5-HT_{1A} binding sites in post-synaptic structures
Monamine Oxidase Inhibitors[2,4,5]	**pre-**	sensitivity of somatodendritic receptors decreased by threefold, hence firing rate of 5-HT neurons remains the same as untreated controls	unknown cause
	post-	5-HT neurotransmission greatly increased when 5-HT neurons discharge at their normal frequency	releasable pool of 5-HT enhanced by MAOIs
Anxiolytics/ Antidepressants			
5-HT_{1A} receptor agonists[2,4]	**pre-**	desensitization of 5-HT_{1A} binding sites resulting in a normal firing rate in the presence of agonists	decrease number of 5-HT_{1A} binding sites[3]
	post-	sensitivity of postsynaptic receptors is unaltered	N.A.

The predicted molecular weight of each of the three 5-HT_{1A} receptors is approximately 46 KDa (Kobilka et al , 1987a; Albert et al., 1990; Charest et al., in preparation) However, the actual size of both the human and rat receptors are much larger than the predicted sizes. Two different groups have indicated the size of the human and rat receptors as being 63 KDa by using immunoprecipitation and photoaffinity labeling techniques (Emerit et al., 1987; Ransom et al., 1986, Raymond et al , 1989) Such dichotomy in the predicted and actual molecular weight could arise from N-linked glycosylation at putative sites located on the extracellular amino terminus portion of the receptor and several possible phosphorylation sites situated within the third cytoplasmic loop, as observed in other G protein-coupled receptors

The degree of amino acid sequence homology between the eighteen different serotonin receptors varies considerably. However, receptors within the same subpopulation (e g the 5-HT_1 subclass) demonstrate a higher overall degree of homology This homology is highest within sequences that comprise the transmembrane domains (Hartig, 1989) For example, sequence comparison between the rat 5-HT_{1A} and 5-HT_{1B} reveals an overall homology of 45% and a transmembrane similarity of 60% (Voigt et al , 1991, Albert et al., 1990). On the other hand, inter-species variation in the protein sequence is extremely small. For example, the homology between the human, rat and murine 5-HT_{1A} receptors is as follows rat-human (89%, 97 8%), rat-mouse (94%, 98.9%) and human-mouse (86%, 97 3%) (the first percentage represents the overall homology and the second corresponds to homologies within the transmembrane region) (Charest et al , in preparation) Such exclusive sequence conservation between species suggests preserved physiological roles of these receptors

Functional expression of the three cloned 5-HT_{1A} receptor genes in mammalian cells has allowed for extensive study of their pharmacological profiles (Fargin et al , 1988, Albert et al , 1990; Charest et al., 1992). Membranes prepared

from transfected cells were examined for their ability to mimic the specific binding affinities of several previously established agonists for 5-HT_{1A} binding sites in the brain The resulting pharmacology was in agreement with what was obtained from brain membranes (Gozlan et al., 1983, Hall et al , 1985, Sills et al , 1984, Schlegel et al , 1986).

To understand the molecular interactions by which extracellular signals are transduced across the cell membrane so as to modify key intracellular effector mechanisms, a dissection of the structure-function relationship of the receptor itself is required Although much of the existing knowledge of this type of receptor system has originated from studies conducted on the adrenergic receptors (α's and β's), the acquired insights have often been successfully extrapolated to other receptor systems exhibiting close structural resemblance In fact, the current knowledge of the structure-function relationships of the 5 HT receptors relies entirely on the following paradigms Studies involving chimeric receptors, site directed mutagenesis and photoaffinity labelling demonstrated that the extracellular hydrophilic domains are not involved in determining ligand binding properties Instead, this function is mediated by the membrane spanning domains (Kobilka et al., 1987c, Frielle et al , 1988, Dohlman et al , 1988, Kobilka et al , 1988, Strader et al., 1987a, Strader et al , 1987b, Frazer, 1989, Strader et al , 1989) However, in light of the results from these different studies, it seems that the sites for ligand binding vary from receptor to receptor and from ligand to ligand Using similar approaches, the receptor domains involved in G protein coupling have also been studied. Although certain variations between receptors exist, the information indicates the importance of sequences of the third cytoplasmic loop and carboxyl-terminal tail located adjacent to transmembrane domains as being important in mediating receptor-G protein interactions (figure I) (Caron and Lefkowitz, 1991) The mammalian cell membrane contains a multitude of receptors regulating many different GTP-binding proteins as well as a common but diverse group of effector proteins such as ion channels and carriers, phospholipases, adenylyl cyclases,

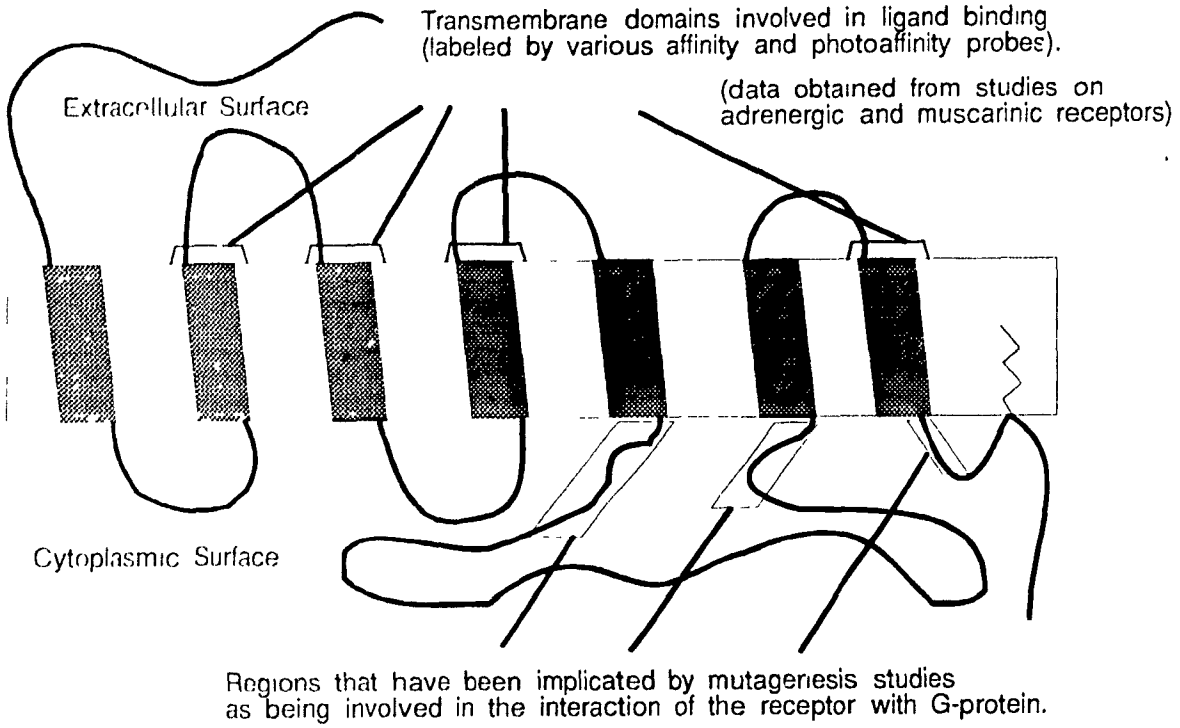


Figure I Schematic representation of the various regions of a receptor involved in ligand binding and G protein coupling

and phosphodiesterases (Ross, 1991). Furthermore, the regulation of each signal-transducing pathway is not independent of the others, resulting in complex extracellular-mediated cytoplasmic events.

In radioligand binding studies of the central 5-HT_{1A} receptors, modulation of agonist binding affinities by divalent cations and guanine nucleotides suggested that the receptors were associated with G proteins (Norman et al., 1985; Schlegel and Peroutka, 1986) which were later shown to belong to the pertussis toxin sensitive ($G_{i,o}$) family (Zgombick et al , 1989) Pertussis toxin ADP ribosylates the α subunit of G proteins thereby inactivating them. Direct confirmation of these earlier findings were made by several groups. They reported a phamacologically relevant negative influence of 5-HT_{1A} agonists on forskolin- or VIP-stimulated

adenylyl cyclase activity in primary cultures of mouse hippocampal and cortical neurons (Dumuis et al , 1988) and in hippocampal membranes prepared from rat and guinea pig (De Vivo and Maayani, 1986, Weiss et al , 1986, Adrien et al , 1989; Oksenberg and Peroutka, 1989, Cornfield et al , 1991) The recent heterologous expression of the rat and human 5-HT_{1A} in mammalian cells (Fargin et al., 1989; Albert et al., 1990) which also showed a dose-dependent negative coupling to adenylyl cyclase via pertussis toxin-sensitive $G_{i/o}$ proteins obviates the contradicting description of the stimulatory effect of 5-HT_{1A} receptors on adenylyl cyclase in rat hippocampal membranes suggested by Markstein and colleagues (Markstein et al., 1986)

In addition to the interaction with adenylyl cyclase, central 5-HT_{1A} receptors have been reported to control both K^+ and Ca^{2+} channels Electrophysiological studies performed on serotonergic neurons of the dorsal raphe obtained from brain slice preparations showed that the 5-HT_{1A} somatodendritic autoreceptor mediated hyperpolarization and decreased input resistance are caused by opening of K^+ channels (Aghajanian and Lakoski, 1984, Williams et al , 1988, Sprouse and Aghajanian, 1987; Van der Maelen et al , 1986, Trulson and Frederickson, 1987) In addition, the reduced responsiveness of the dorsal raphé serotonergic neurons to 5-HT following local or intracerebroventricular injection of pertussis toxin, demonstrates the G protein coupling nature ($G_{i/o}$) of the somatodendritic 5-HT_{1A} autoreceptors to K^+ channels (Blier et al , 1990b, Williams et al , 1988, Innis and Aghajanian, 1987; Innis et al., 1988) This G protein-mediated K^+ channel opening is not due to G protein-mediated effects on adenylyl cyclase (Innis et al , 1988) Furthermore, intracellular injection of a nonhydrolysable analog of GTP (GTP-γS) which constitutively activates G proteins, hyperpolarizes serotonin neurons and prevents additional actions of 5-HT (Innis et al , 1988), suggesting a direct coupling of the G protein to K^+ channels

Similar to the presynaptic autoreceptors, postsynaptic 5-HT_{1A} receptors mediate a hyperpolarization effect and a decrease in the input resistance of dorsal hippocampus pyramidal neurons resulting from an opening of K^+ channels (Colino

and Halliwell, 1987, Andrade and Nicoll, 1987). In addition, evidence suggests that the 5-HT_{1A} receptor-mediated effects of K^+ channels are the result of a pertussis toxin sensitive ($G_{i/o}$) G protein transducer as seen in doral raphé neurons (Andrade et al , 1986) A mechanism by which PT-sensitive G protein-coupled receptors act on potassium channels has recently been put forth by Armstrong and White (Armstrong and White, 1992).

The results concerning the possible involvment of 5-HT_{1A} receptors with Ca^{2+} channels remain inconsistent. The discrepancy arises from radioligand studies which demonstrated that 5-HT_{1A} receptors are not present on the presynaptic nerve terminals of dorsal raphé neurons (Vergé et al , 1985, Engel et al , 1986). Contradicting these results are the electrophysiological observations of Penington and Kelly where 8-OH-DPAT, (at pharmacologically relevant concentrations) elicited a reduction of calcium current presumably through raphé terminal 5-HT_{1A} autoreceptors (Penington and Kelly, 1990). More studies are required to unequivocally determine whether the 5-HT_{1A} receptor is involved in the modulation of calcium currents

Thus, activating both pre- and postsynaptic 5-HT_{1A} receptors results in inhibition of adenylyl cyclase and in a decrease in neuronal firing rate ensuing from enhanced K^+ conductance.

In addition to its neuron-specific expression in the CNS, the 5-HT_{1A} receptor is also under developmental regulation in the rat brain In particular, the density of 5-HT_{1A} binding sites is relatively abundant in the cerebellum of newborns and decreases with age, to non-detectable levels in adult and senescent animals (Hamon et al , 1990) This diminution of sites in the cerebellum is correlated with a progressive increase in receptor density in forebrain areas, notably the dentate gyrus of the hippocampus and the cerebral cortex (Hamon et al., 1990) The early postnatal development occuring in the cerebellum, in addition to the observations stated above, raises the question regarding the possible role of the 5-HT_{1A} receptors during CNS maturation (Hamon et al., 1990).

Eukaryotic transcription: General mechanisms

The process of transcribing DNA into RNA can be divided into three stages initiation, elongation and termination Initiation of transcription of eukaryotic genes is influenced by a numbers of regulatory elements termed enhancers, silencers and promoters (Johnson and McKnight, 1989) These elements are composed of specific DNA sequence motifs which are substrates for sequence-specific DNA-binding proteins, better known as transcription factors or *trans*-activating factors

Over the past decade, a tremendous amount of research has been dedicated to transcriptional complexes and their regulation This surge of interest in transcriptional mechanisms has arisen from two different perspectives First, transcriptional control represents a fundamental role in a broad variety of biological transitions resulting in sustained phenotypic changes in the cell Thus, a detailed knowledge of transcription provides a powerful handle towards understanding the molecular gears driving this transition machinery Second, the wide variety of distinct transcription factors comprising the transcriptional apparatus, their intricate interactions with each other and the regulation of gene expression by the resulting amalgams engender a myriad of mechanistic issues which are intellectually challenging.

The promoter region of a gene is defined as being the minimal DNA sequence capable of promoting and initiating transcription (Zubay, 1988) The promoter is most often located upstream (or 5') to the transcription initiation site Typically 100 bp in length, the promoter is required for accurate and efficient initiation of transcription (Maniatis et al , 1987) Molecular genetic analyses of many different promoters reveal a common pattern of organization Most typical promoters include an AT-rich region (designated as a TATA box) located 30-40 bp upstream of the initiator nucleotide The function of this region is to ensure that

transcription is accurately initiatied (Maniatis et al., 1987). The assembly of a preinitiation complex at the TATA element involves an ordered interaction of a string of general initiation factors (TFIIA, B. D. E. and F), in addition to RNA polymerase II. Assembly of the complex begins with the stable binding of TFIID to the TATA box which may be facilitated by TFIIA (Roeder, 1991). Following this, TFIIB and E assemble at the complex and are viewed as bridges between DNA-bound TFIID and the RNA pol II/TFIIF complex. The resulting core promoter protein complex is over 1000 KDa. This clearly indicates the existence of a complicated machinery with tremendous potential for a variety of functions such as basic initiation/elongation/termination, in addition to the modulation of regulatory interactions with other general and/or gene-specific effectors (Sawadogo and Sentenac, 1990, Roeder, 1991). Apart from their regular role of recruiting additional factors to the promoter, very little is known about other functions carried by these transcription initiator (Roeder, 1991).

Promoters are also endowed with sequences displaying spatial and compositional homologies referred to as UPEs (Upstream Promoter Elements). UPEs generally increase the rate of transcription, and mutagenesis studies suggest that the strength of certain promoters is determined by the number and type of UPEs present within them (Maniatis et al., 1987). A number of UPEs have been identified, and some of them (for example, the pentanucleotide CCAAT box, or the GC-rich SP-I box) are found within many different promoters. CCAAT boxes are usually located between 60 and 80 bp upstream of the initiation site (McKnight and Tjian, 1986). Several experimental paradigms have irrefutably demonstrated the importance of CCAAT boxes to promoter function (for reviews see Maniatis et al., 1987; La Thangue and Rigby, 1988). Although the detailed mechanisms through which CCAAT-binding proteins are still unknown, a number of recent studies have demonstrated that a heterogeneous population of DNA-binding proteins recognize and bind to CCAAT elements with high affinities (Chodosh et al., 1988a; Chodosh et al., 1988b; Hoolt van Huijsduijnen et al., 1990). Furthermore, these CCAAT-binding proteins are heteromeric in their composition, thus conferring them with a

greater variety of regulatory options. In addition, the interchange of subunits among such proteins would provide an additional level of combinatorial complexity to gene regulation.

In addition to promoter elements, a larger number of enhancer and repressor elements have been implicated in signal dependent regulation of transcription such as responses to hormones, growth factors and heat shock These *cis*-acting elements have no intrinsic promoter activity per se, but can activate or repress respectively, the transcriptional acitivty of the promoter in an orientation-independent manner. These elements can be located hundreds, or even thousands of base pairs away from initiation of transcription and still exert significant influence on transcriptional activity (Ptashne, 1988) The importance of a single *cis*-element can vary greatly in response to physiological inputs in different cell types presumably due to variation in the abundance or activity of the DNA-binding proteins which recognize and bind to the element (Mitchell and Tjian 1989). Moreover, the spatial organization of different combinations of binding sites for transcription factors on a gene can regulate the transcriptional activity in a manner which can be signal-dependent or cell-specific Although little is known about the actual structural details underlying such an interplay between the different sets of factors, it is believed that the combination of *cis*-elements arranged in a unique configuration is what confers each gene with an individualized spatial and temporal transcriptional program (Mitchell and Tjian, 1989). During the past few years, many new methods have allowed the detection, cloning and characterization of a myriad of transcription factors (Lamb and McKnight 1991) Structure-function relation studies have demonstrated that transcription factors are usually composed of separate DNA-binding and transcriptional activation domains (Ptashne, 1988; Mitchell and Tjian, 1989)

DNA-binding proteins are categorized according to the many different domains with which they bind DNA To this day, there are twelve identified DNA-binding motifs (He and Rosenfeld, 1991) The discovery of three specific DNA-binding motifs lead to the identification and characterization of many other DNA-

binding domains (Pabo and Sauer, 1992; Johnson and McKnight, 1989). The first DNA recognition motif to be discovered was the helix-turn-helix (HTH) motif. Examples of transcription factors harboring this motif are the lamda phage Cro protein, the E. Coli CAP protein, and the Lac repressor. In eukaryotes, the HTH motif is not a separable, stable domain and unlike the other motifs, the HTH domain cannot function by itself but rather, requires other components from a larger DNA-binding structures. For example, such larger DNA-binding structures are seen in a structural component referred to as the homeodomain. The homeodomain is a stable, folded structure of 60 amino acids in length, capable of binding DNA by itself, and was first recognized in proteins regulating the development of *Drosophila* Although homeodomains are capable of binding DNA on their own, it appears that the precise DNA-binding specificity is modulated by additional regions of the protein. For instance, many homeodomain proteins contain extra sequence motifs which flank the homeodomain and are conserved within specific subfamilies It was observed that both the N- and C-terminus of the homeodomain are located near the DNA. Therefore, neighboring residues from either ends could easily contact the flanking regions of the DNA and thereby modulate the specificity of binding. For example, the POU proteins contain a POU-specific domain of 65-75 residues located at the N-terminal side of a homeodomain (Ruvkun and Finney, 1991; He et al., 1989; Treacy et al., 1991; Pabo and Sauer, 1991). This POU-specific domain, first observed in the Pit-1, Oct-2 (both of mammalian origin) and Unc-86 (*C. elegans*) transcription factors, contacts DNA with a set of bases adjacent to those involved in homeodomain binding (Rosenfeld, 1991; Ruvkun and Finney, 1991).

Zinc finger proteins were first identified in the *Xenopus* transcription factor TFIIIA and represent another type of DNA-binding structure. There are two types of zinc fingers found in the DNA-binding motifs of transcription factors participating in RNA pol II mediated trancription. TFIIIA-like zinc fingers consist of ~30 residues with two cysteine and two histidine residues stabilizing the domain by tetrahedrally coordinating a Zn^{2+} ion. A region of ~12 amino acids characterized by conserved

hydrophobic and basic residues, separates the two cysteine and histidine pairs The mammalian SP-I transcription factor, which binds to a GC-rich region (also refered to as the SP-I box) contains three tandem zinc fingers in its DNA-binding domain The second class of zinc fingers are mainly found in the DNA-binding domain of steroid receptors. They structurally differ from their TFIIIA-like conterparts by carrying two cysteine pairs instead of one cystein and one histidine pair. Both types of zinc fingers require Zn^{2+} for their DNA binding activity and exhibit a variety of DNA sequence specificities Although the highly conserved residues of the zinc fingers confer a scaffold for the binding domain, the determinants of binding specificity are likely to lie elsewhere For example, the non-conserved residues at the base of the finger region of several steroid receptors are intimately involved in various binding specificities of these proteins (Mader et al , 1989)

The third eukaryotic DNA-binding motif, is the leucine zipper This motif plays an important role in differentiation and development (Landschulz et al., 1988). The leucine zipper motif was first identified as a conserved sequence pattern in several eukaryotic transcription factors (Landshulz et al , 1988) and subsequently, in several other eukaryotic *trans*-acting factors The DNA-binding domain of the leucine zipper proteins consists of 60 to 80 amino acid residues and is divided into two subdomains: a leucine zipper region which is required for dimerization and an adjacent basic region which is necessary for DNA binding Analysis of leucine zipper sequences reveals that they are characterized by a heptade repeat of leucine over a region of 30-40 residues McKnight and colleagues have biochemically shown that leucine zipper proteins dimerize by forming two parallel α helices in a coiled-coil arrangement which are stabilized by hydrophobic interactions between the two closely positioned α helical leucine repeat regions (Pabo and Sauer, 1992; Mitchell and Tjian, 1989) Leucine zipper proteins can form homo- and heterodimers as a result of their bipartite nature Although many different transcription factors exhibit similar dimerization domains, the heterodimerization process is not promiscuous Certain factors will have higher

affinities for different members and lower affinities for others The rules governing the specificity of heterodimerization are still poorly understood (Lamb and McKnight, 1991) Members of the leucine zipper family include transcription factors such as v-FOS, c-JUN, C/EBP, FRA-I and c-MYC (Johnson and McKnight, 1989).

The dual nature of transcription factors is demonstrated by the presence of both DNA binding domains and transcriptional activation domains. The latter are made up of 30-100 amino acid residues and are separate from the previously mentioned DNA-binding motifs.

Transcription factors frequently contain more than one activation domain whose function is confered by several unrelated structural motifs (Mitchell and Tjian, 1989) The first eukaryotic activation domains to be defined were those of the yeast GAL 4 and GCN 4 transcription factors Ptashne and his colleagues, using domain-swap experiments, defined the activating regions of GAL 4 and GCN 4 as stretches of amino acids bearing significant net negative charge which have the ability to form amphipathic α-helical structures (Ptashne, 1988; Mitchell and Tjian, 1989). Fitting this model is a group of mammalian hormone-responsive activators (human estrogen, rat and human glucocorticoid receptors), each consisting of at least one transcriptional activation domain of the short amphipathic α helix type (Ptashne, 1988) It has been proposed that acidic activation domains can stimulate transcription initiation by interacting with a general component of the initiation complex such as TFIID (Ptashne and Gann, 1990).

A second characterization of activation domains came from deletion analyses of the SP-I transcription factor, which revealed four discrete regions carrying transcriptional ativation functions. The two most potent activation domains were composed of ~25% glutamine residues and few charged amino acids (Mitchel and Tjian, 1989) Gln-rich regions are also seen in extablished and putative transcription factors from the *Drosophila* Antennapedia, Ultrabithorax and Zeste proteins, in yeast HAP I, HAP 2 and GAL II as well as in mammalian OCT-I, OCT-2, JUN, AP-2 and SRF proteins (Mitchell and Tjian, 1989). The important structural feature of a third potential activator domain consist of a region rich in proline

residues. Domain-swap experiments demonstrated that this proline-rich region was involved in activating transcription of certain genes. This domain was discovered in CTF/NF I and was subsequently noted in AP-2, JUN, OCT-2 and SRF (Mitchell and Tjian, 1989)

The idea that all activation domains mediate their enhancement activity by directly interacting with the same element of the initiation complex (such as TFIID) seems unlikely It is therefore assumed that a strict mechanistic categorization of actions of the many different transcription factors is impossible Each transcription factor (albeit common structural features) is likely to regulate transcription in a unique fashion (Ptashne and Gann, 1990; Mitchell and Tjian,1989)

As a general phenomenon, transcription factors whose activities can be regulated by cellular events recognize and bind to specific DNA sequences in a spatial and orientation independent fashion However, by yet unidentified mechanisms, they are known to enhance or repress transcription

Initially, crude knowledge of the structure of DNA often led to an erroneous representation of it as being a naked string of sequences floating freely in the nucleus An important point that is omitted from these simplistic representations is that transcription occurs in an environment in which DNA is intricately packaged into a structure called chromatin (for a review see Morse, 1992) This three-dimentional aspect of the DNA structure is likely to play a major role in determining the complex interactions between transcription factors

In chromatin, nucleosome-free regions, known as *nuclease hypersensitive sites* are believed to be the protein-free openings through which transcription factors have acess to their corresponding *cis*-acting DNA sequences (Weisbrod, 1982; Gross and Garrard, 1988) These windows (generally from 50 to 400 bp in length) are functionally defined by their enhanced sensitivity to nuclease cleavage or chemical modification (Elgin, 1988)

In virtually every case which has been thoroughly studied, hypersensitive sites are associated with sequences involved in replication, recombination, chromosome segregation and, transcription (Gross and Garrard, 1988) *Cis*-acting

elements are often found with many inducable, tissue-specific, and developmentally regulated hypersensitive sites (Gross and Garrard, 1988). In fact, from an extensive study of all reported cases of hypersensitivity, it is possible to generalize that if a *cis*-acting element is involved in any chromosomal process, it will be associated with a hypersensitive site. However, hypersensitivity is necessary but obviously not sufficient for the underlying DNA sequences to exert their function (Gross and Garrard, 1988). To date, the precise structure of any given hypersensitive site is not known, even at the level of DNA conformation and protein composition Thus, future knowledge of the *cis*-acting sequences and *trans*-acting factors involved in the generation and/or maintenance of hypersensitive sites will allow a better understanding of the role of the latter during transcription.

Gene expression arises from a complex, specific and dynamic machinery whereby a multitude of factors and DNA sequences which interact with each other and with an active chromatine scaffold, result in the ultimate process known as transcription

Gene expression in mammalian brain

The mammalian central nervous system is generally regarded as the most complex of all organ systems It is composed of a heterogeneous population of cell types of which neurons are the most extensively studied for many reasons. It is estimated that the CNS consists of as many as 10^{12} neurons each displaying biochemical and biophysical heterogeneity (McKay, 1989). Neurons differs from one another in their signalling capabilities, neuronal and hormonal responsiveness and in the distinct genes they express It is the diversity of neuronal characteristics and actions that define the world as we know it.

The complexity of the mammalian central nervous system arises from the multitude of genes dedicated to endow the brain with its unique function and anatomy Roughly 30 000 distinct genes are expressed in the mammalian brain, of which 65% are brain-specific (Sutcliffe, 1987; Takahashi, 1992). This differential

expression of genes in distinct temporal and spatial patterns is undoubtedly associated with the presence of various brain specific transcription factors (Struhl, 1991; He and Rosenfeld, 1991). Owing to the scarce number of *in vitro* systems available, very little is known regarding gene expression in the mammalian brain compared to other organ systems Nevertheless, the mechanisms governing the expression of a few brain-specific genes have been recently characterized and analysed. In each example mentioned below, sequences upstream to the transcription initiation site were fused to a reporter gene whose measured activity is directly proportional to the transcriptional strength of the sequences driving its expression in transiently transfected cultured cells (Nordeen, 1983, Brasier et al , 1989). Depending on the environment in which the 5' sequences are present, modulation of the transcription of the reporter gene will occur

For example, analysis of three mouse neurofilament 5' deletion mutants shows that each gene has a non discriminatory promoter which is readily expressed in neuronal and non-neuronal cells. The activities of the promoters of the three genes, are reduced by yet uncharacterized negative elements localized further upsteam (Shneidman et al., 1992) The human D_{1A} dopamine receptor gene 5'-flanking region was evaluated in a similar manner The promoter of this gene showed high activity in cells carrying the nuclear machinery necessary for the expression of the gene, but displayed low activity in cells which do not express the receptor. In addition, as in the previous case, sequences upstream of the promoter exert enhancer and repressor functions (Minowa et al , 1992) Comparable results were noted from upstream regions of two other neuron-specific genes, the rat GAP-43 and the brain type II sodium channel genes (Nedivi et al , 1992, Maue et al., 1990). In both cases, upstream sequences negatively modulated a minimal core promoter in non-neuronal cells and were inactive in neuronal cells Most recently, the neural-specific expression of the sodium channel type II has been shown to be mediated by a 28 bp silencer element located in the region exhibiting this cell-specific repression (Kraner et al , 1992; Mori et al , 1992)

The following examples dissect minimal elements using techniques such as

site-directed mutagenesis, band shift assays and DNAase I footprinting assays. The tissue-specific transcription of the rat tyrosine hydroxylase gene requires a well characterized cis-acting element (AP-I motif) synergising with an E-box containing element. It was shown that these two elements are both necessary and sufficient to confer cell specificity, and that a unique DNA-protein complex is formed at these two sites. The authors claim that the proteins that form this unique complex are likely to direct the tissue-specific expression of tyrosine hydroxylase (Yoon and Chikaraishi, 1992).

Although relatively simple *in vitro* experiments allow initial characterization of certain DNA elements, transgenic animal models are undoubtedly the ultimate system used to validate the tissue-specific functions of these elements. For instance, the 5'-flanking region of the neuron-specific rat SCG10 gene, a growth-associated protein expressed early in the development of the nervous system, exhibits superimposable actions both *in vitro* and *in vivo* (Wuenschell et al., 1990; Mori et al , 1990). Studies of SCG10 regulation in transfected cell lines and in transgenic mice revealed a minimal core promoter, which is active in a non tissue-specific manner, and is differentially repressed by the presence of additional upstream sequences only in cells which do not express endogeneous SCG10. Most recently, a 21 bp cell type-specific silencer element has been localized within the upstream region which selectively represses SCG10 expression in non-neuronal cells and tissues Surprisingly the SCG10 silencer element exhibits striking homology to the sodium channel type II negative element described aboved (Mori et al., 1992). In both cases, (SCG10 and Na^{2+} channel type II) a similar protein complex was shown to specifically bind the negative elements.

Gene expression relies on complex communications between the physiological state of a cell and its nuclear content and arrangement Although a tremendous amount of information concerning the regulation of gene expression has been revealed over the past decade, more knowledge is needed in order to fully understand and therefore rectify defects ensuing form abnormal gene expression.

Objective of study

The interests in studying the 5-HT_{1A} receptor gene are numerous. First the 5-HT_{1A} receptor is a key element in the regulation of the overall serotoninergic tone. Therefore, understanding the mechanisms underlying the regulation of 5-HT_{1A} receptor expression might provide further insight as to how the serotonergic structure is involved in brain functions Second, since the expression of the receptor appears to be developmentally regulated, the characterization of the elements necessary for this temporal display will provide new important insights into the research of mammalian brain development. And lastly, the identification of neuron-specific *cis*-acting elements could potentially serve as vectors in future gene therapy treatments

This study is divided into two parts. The first part of this research was to localize the initiation of transcription of the rat 5-HT_{1A} receptor gene and to assess its 5'-flanking sequences for transcriptional activity Given the high degree of specificity in the spatial expression of this gene, characterization of the factors (*cis*- and *trans*-acting) necessary for such display is of primordial importance in order to understand and hence modify the underlying mechanisms of regulation

Since most of the strategies involved in the study of the regulation of gene expression rely heavily on *in vitro* systems, the goal of the second part of this study was to identify a cell line carrying all the necessary epigenetic material to support endogeneous expression of the 5-HT_{1A} receptor gene In addition, a functional and molecular characterization of the expressed receptor was undertaken.

Results and Discussion

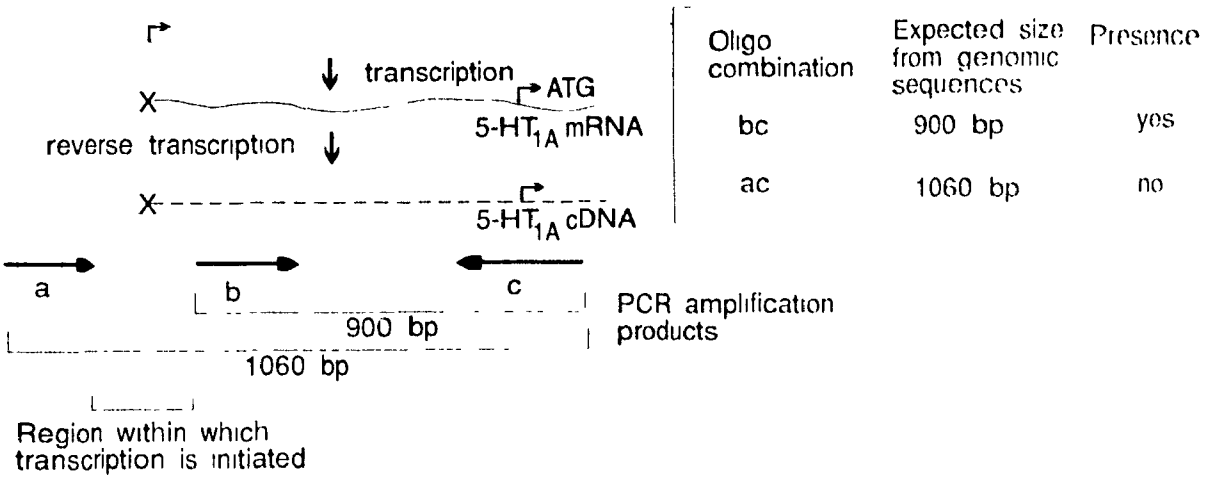
Isolation and determination of the 5'-flanking region sequences

The cloning of the rat 5-HT_{1A} receptor gene has been previously described (Albert et al., 1990) The strategy behind the construction of vectors from the 5-HT_{1A} receptor gene 5'-flanking region used for sequencing is detailed under "Materials and Methods" of chapter I. The resulting sequence is depicted in figure 1-chapter I

Initiation of transcription

In order to define the region within which transcription initiation occurs, an RT-PCR strategy was employed. 0.3 ug of total RNA from both hippocampus and cortex was reverse transcribed following the supplier's specifications (Gibco-BRL). Subsequent PCR amplification using an antisense oligonucleotide, the sequence of which encompasses the translation initiation codon (ATG), combined with sense oligonucleotides located in a 5' sequential manner, revealed the region carrying the transcription start site (see figure 2) Using this PCR walking strategy, it was observed that a small stretch of DNA situated between nucleotide -1038 and -886 (relative to the translation initiation codon, the A of ATG as being designated +1) carried the transcription start site(s). To delineate the discrete site(s) at which transcription initiates, RNase protection and primer extension techniques were used as described under "Materials and Methods" of chapter I.

In a first primer extension experiment, a ^{32}P radiolabeled antisense oligonucleotide, whose sequence is complementary to genomic sequences falling between nucleotides -808 and -783 (referred to as oligo #55) was hybridized to 10 µg of total RNA from hippocampus and cortex and subsequently reverse



Oligo combination	Expected size from genomic sequences	Presence
bc	900 bp	yes
ac	1060 bp	no

Figure 2 Schematic representation of a PCR walking strategy Oligonucleotides were derived from genomic sequences The strategy relies on the presence of absence of corresponding mRNA sequences for the sense oligonucleotides

transcribed (see figure 2A-chapter I) The resulting single specific fragment of 174 bp (as determined by comigration with M13 sequencing markers), which was unique to hippocampus, suggests that the initiation site is localized at -957 bp from the translation initiation codon (see figure 2B-chapter I)

In order to substantiate the latter result, another primer extension experiment was performed, whereby an oligonucleotide situated further upstream from oligo #55 was used Oligo #53 (see figure 2C-chapter I) was extended by 11 bp only in hippocampal RNA and not from cortex or liver (which served as a control) RNA samples, suggesting that the transcription start site was located at -967 bp from the first ATG

To investigate the discrepancy emerging from the primer extension experiments, an RNase protection assay was performed Using α^{32}P-UTP body labeled RNA molecules whose sequence are complementary to the 5-HT_{1A} receptor gene (probe A, described under "Materials and Methods" of chapter I) (see figure 3A-chapter I) and total RNA from hippocampus, cortex and liver, a fragment of 98 bp was found to be protected (see figure 3B-chapter I) The experiment, when performed several times using different RNA samples exhibited

the same results These RNAse protection experiments correlated precisely with the primer extension experiment utilizing oligo #53, indicating that the transcription start site of the rat 5-HT_{1A} receptor gene is unique and is situated at a nucleotide 967 bp upstream of the translation initiation (ATG) codon The sequence surrounding this transcription initiation site exhibits the canonical TATA and CCAAT consensus sequences which are located 58 and 167 bp upsteam of the deduced initiation site respectively (see figure 1-chapter I).

A conflicting observation arose from the above results The inability of oligo #55 to detect the -967 bp initiation site can be ascribed to unexplainable differences in migration patterns during the separation acheived by electrophoresis RNA degradation, as well as poor enzymatic activity of the reverse transcriptase as being potential sources of defects are ruled out since the same RNA sample was used in the RNase protection experiments In addition, the result of an exhausted enzyme would be represented as a ladder of bands which was not observed at any time

The transcription initiation site is unique and specific

The transcription start site, localized at -967 bp from the translation initiation codon, is unique Primer extension experiments utilizing antisense oligonucleotides located downstream of oligo #55, failed to revealed the presence of any additional initiation sites Furthermore, by RNase protection analysis, there were no indications of other sites being present upstream of the -967 nucleotide site, as the full length probe was not protected

In order to verify if the -967 nucleotide site is specific to tissues capable of endogeneous expression of the receptor, a fragment of DNA comprising this initiation site was transfected into two cell lines incapable of tissue-specific expression (the murine P19 and Ltk$^-$ cells) An RNase protection assay using probe A (see figure 3A-chapter I), performed on RNA extracted from the transfected cells, failed to detect the hippocampus-specific transcription initiation

site (see figure 6A-chapter I) As a control, a northern blot analysis was carried out to verify the presence of messages originating from the transfected fragment (see figure 6B-chapter I)

Intronless nature of the rat 5-HT_{1A} 5' untranslated region

A characteristic feature of most serotonin receptors is the absence of introns within their coding regions (ref. to references of Table I) Since the coding sequence of the rat 5-HT_{1A} receptor gene had already been demonstrated to be devoided of introns, it was decided to verify whether the large 967 bp long 5' untranslated fragment contains introns, as their presence has been shown to increase both the stability of mRNAs and their transport rate out of the nucleus (Zubay, 1988) Again, an RT-PCR scheme was applied to this task As shown in figure 3, amplification using an antisense oligonucleotide overlapping the translation initiation codon, combined with a sense oligonucleotide located close to the transcription initiation start site was performed on cDNAs derived from hippocampal RNA and genomic DNA This resulted in fragments of similar sizes (figure 4-chapter I), a fact that is consistent with an absence of introns The presence of smaller fragments from the cDNA sample would indicate the presence of intron(s). The functions of this large 5' untranslated region are yet to be discovered One could certainly speculate that this 5' untranslated region may have a significant function in RNA stability or even in translational control

Promoter activity of the 5'-flanking region

The usage of reporter genes in promoter activity studies is two fold First,

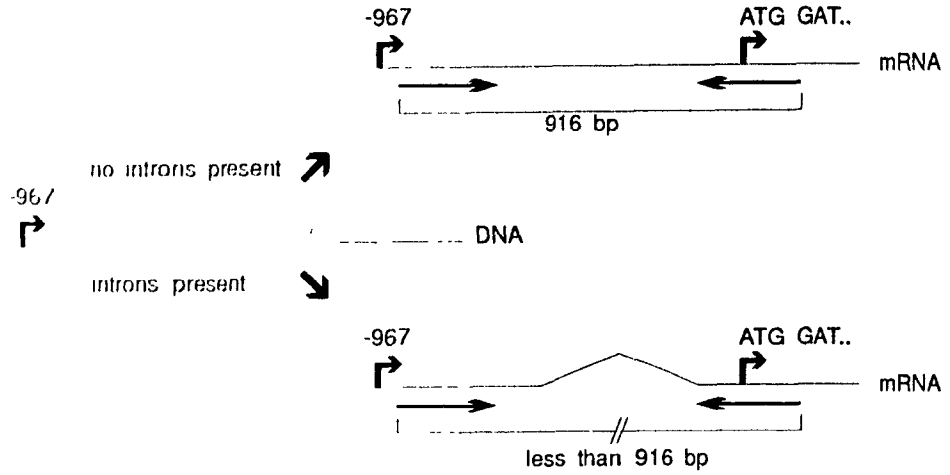


Figure 3 Schematic representation of the strategy used for the elucidation of the intronless nature of the rat 5-HT_{1A} receptor 5' untranslated region

they are of non-mammalian origin and therefore any activity emanating from cells tranfected with reporter genes has to arize from this exogeneous gene. Second, they are easy to assay, where the enzymatic activity of a transfected cell extract, in presence of excess substrate for the reporter enzyme, is directly proportional to the amount of enzyme present which is, by definition, directly related to the strength of the DNA sequences driving its expression.

In the present case, 5' as well as 3' sequential deletion fragments of the rat 5-HT_{1A} receptor gene 5'-flanking region driving the expression of a firefly luciferase gene were assayed for transcriptional activities in mammalian cells (described under "Materials and Methods" of chapter I). Murine embryonal carcinomal P19 and Ltk transformed fibroblast cell lines, both of which do not endogeneously express the 5-HT_{1A} receptor gene, were utilized throughout this study. P19 and Ltk^- cells were chosen on the account of two main reasons: they are both very good recipients of exogeneous genetic material and their growth rate is relatively rapid, requirements dictated by tranfection protocols.

A 2.6-Kb genomic fragment (PIBI) spanning the rat 5-HT_{1A} receptor gene 5'-region from positions -2719 to -117 directs expression of the luciferase gene (see figure 5A-chapter I) both in P19 and Ltk^- cells. This demonstrates, when compared

to the control plasmid pKS⁺-L, that the 2.6-Kb fragment contains transcriptional activity. When sequences from position -2719 to -1516 are removed, the transcriptional activity of the resulting construct (SIBI-L, see figure 5A-chapter I) declines dramatically both in P19 and Ltk This decrease in strength of transcriptional activation is not alleviated by further removal of sequences located between -1515 and -1390, as the activity displayed by H2BI-L is still weak (see figure 5A-chapter I) However, removal of sequences from -1390 to -1187 reveals a fragment (TIBI-L) carrying the strongest transcriptional activity (see figure 5A-chapter I) in both cell systems.

From these tissue culture paradigms, only qualitative analyses can be extracted It is clear, albeit substantial quantitative differences between the two cell lines, that repression of transcription is displayed by sequences situated between the Sma I and Taq I (-1516 to -1187) restriction sites Such repression is alleviated when additional upstream sequences are present as they show transcriptional activity on their own (see figure 5B- chapter I)

Therefore, in an environment lacking the special machinery which characterizes the tissue specificity of 5-HT_{1A} receptor gene expression, the large 5' untranslated region displays a prevailing intrinsic promoter activity This marked activity is strongly weakened by a fragment of 337 base pairs (Sma I-Taq I) which appears to act as a repressor. The repressor activity is partly alleviated when additional sequences are present immediately upstream to it In order to assess the promoter activities of these additional sequences, 3' deletion constructs, all of which are lacking the 5' untranslated region displaying the strongest activity (TIBI fragment) were transiently expressed in P19 (see figure 5B-chapter I) When compared to the pKS⁺-L control, which accounts for cryptic promoter sequences intrisinc to the plasmid backbone, each construct displayed higher relative transcriptional activity This denotes the capacity of the various constructs depicted in figure 5B-chapter I to exercise promoter activity even in the absence of the earlier defined TIBI promoter This property readily explains their potent repressive features on the Sma I-Taq I repressor element Figure 5B-chapter I also indicates

that the Sma I-Taq I silencer fragment is dependent on the presence of the TIBI strong promoter to exert its repressing activity, as its 3' sequential deletion does not dramatically affect the resulting constructs' activities.

Analysis of the 5'-upstream region sequences

The definitions of both the transcription initiation site and the regions required for transcription activity, enable one to characterize the sequences involved in the regulation of the 5-HT_{1A} receptor gene expression. A putative TATA consensus sequence (as defined in Warkman and Roeder, 1987) is present 58 bp upstream of the -967 bp start site (-1025 TATAA -1021). Furthermore, a CCAAT box as well as an additional TATAA consensus sequence are located 167 and 183 bp upsteam of this initiation site respectively These ubiquitous promoter elements may be involved in the transcriptional regulation of the gene

The presence of other TATA and CCAAT consensus elements scattered throughout the sequence upstream of the Sma I restriction site is what may confer these fragments with the observed transcriptional activities in P19 and Ltk$^-$ cells (see figures 1-chapter I and 5B-chapter I).

A computer-aided search for the occurence of consensus *cis*-acting elements known to be recognized by characterized transcription factors revealed a pleiad of sequences (see table 1-chapter I and figure 1-chapter I). Although none have been shown to elicit any control on the transcription of the 5-HT_{1A} receptor gene, the intrinsic activity which define them may be relevant to the 5-HT_{1A} receptor gene Over twenty five different transcription factor binding sites are present within the 3.2 Kb of 5'-flanking sequence. Some of them reflect a ubiquitous pattern of expression (AP-I (2), SP-I (2), and CREB (1)). AP-I consensus sequence binding sites are recognized by proteins of the AP-I family. In mammals, these proteins are encoded by multiple genes including c-*jun*, *jun*B, *jun*D, *fos* and *fra*-I. Homo- and heterodimerization of AP-I proteins play an important role in transcriptional regulation by creating a variety of transcription

factors with different functional properties. Therefore, transcriptional regulation of genes containing AP-I sites may be very complex (Mitchell and Tjian, 1989) SP-I *cis*-acting elements are recognized by the SP-I transcription factor. It has been observed that SP-I binding sites, present in TATAA-less promoters, are capable of modulating initiation of transcription (Ptashne and Gann, 1990, Anderson and Freytag, 1991). However, their function within promoters exhibiting TATAA and CCAAT consensus sequences is less well understood The presence of a CREB (cAMP response element) binding site within the 5'-flanking region of the 5-HT_{1A} receptor gene indicates that the expression of this gene could be regulated by cAMP levels (the mechanisms by which cAMP indirectly regulates gene expression are reviewed in Habener, 1990). Since cAMP levels are potently modulated by 5-HT_{1A} receptor activities, one could imagine the indirect intogenous regulation of expression of the 5-HT_{1A} receptor gene In fact, several other G protein coupled receptors have been shown to indirectly modulate their own expression (Collin et al., 1992; Hadcock and Malbon, 1991)

Other cis-acting elements present in the 5'-flanking region of the 5-HT_{1A} receptor gene are characterized as being brain-specific or even most abundant in brain tissues. Three GATA-3 (Orkin, 1990; Yamamoto et al , 1990, Ho et al , 1991, Ko et al., 1991), one AP-2 (Williams and Tjian, 1991, Mitchell et al , 1991, Courtois et al., 1990; Winning et al , 1991), one EGR-I (Lemaire et al , 1990, Cao et al , 1990; Christy and Nathans, 1989), and three CCAGG consensus sequences are found at different positions within the 5'-flanking region of the 5-HT_{1A} receptor gene (see table I-chapter I) CCAGG is a functional orphan pentanucleotide consensus sequence which has been observed in several neuron-specific gene promoters (Maue et al., 1990). In *Drosophila,* this element is part of a larger 14 bp fragment present in the dopa decarboxylase gene which has been shown to be necessary but not sufficient for gene expression in the central nervous system (Scholnick et al., 1986; Johnson et al , 1989) The function of the CCAGG element present in different mammalian promoters as well as in the 5'-flanking region of the rat 5-HT_{1A} receptor gene is not known. It has yet to be determinated whether the presence

of these *cis*-acting elements exerts any influences on the actual modulation and cellular discrimination of the 5-HT_{1A} receptor gene expression.

In addition to many consensus sequences, the rat 5-HT_{1A} receptor gene 5'-flanking region exhibits other important structural features. The first, is a stretch of 151 bp (from nucleotides -1841 to -1690) which shares 80% homology with a fragment of similar size from the murine tumor necrosis factor β (TNFβ) 5'-flanking region (Gray et al , 1987) A possible functional role has not been attributed to this trait yet, but one can be postulated from a correlation between the mature T cell nature of TNFβ expression (Fashena et al, 1990; English et al, 1991) and the initial report demonstrating the expression of the 5-HT_{1A} receptor gene in lymphoid tissues (Kobilka et al , 1987a)

The second feature is a stretch of 26 (GT) dinucleotides spanning nucleotides -1289 to -1238 embeded within the Sma I-Taq I fragment whose negative transcriptional activity has been shown in P19 and Ltk$^-$ cells . Although the poly (GT) has not been directly proven to exert the repression aspects of the fragment, a causative nature is not eliminated since it has recently been demonstrated that certain type of transcription factors (particularly of the EGR type) (Bickmore et al , 1992) can bind poly (GT) stretches with high affinity. Therefore, one could easily conceive the existence of repressor factors acting through this poly (GT) tract In fact, a possible role for poly (GT) in transcription has been previously reported (Braaten et al., 1988)

The rat 5-HT_{1A} receptor gene 5'-flanking region exhibits many necessary structures involved in basal and modulatory transcription. Moreover, in cells which do not express the essential machinery conferring the gene with its tissue-specific expression, the 5'-flanking region displays transcriptional activity which seems to be modulated by repressing upstream sequences, thereby suggesting a mechanism of tissue-specific expression analoguous to the reported selective derepression systems (Mori et al., 1990, Wuenschell et al., 1990; Kraven et al., 1992; Mori et al , 1992)

In order to determine the exact nature of the above postulated mechanism

of action concerning the expression of the 5-HT_{1A} receptor gene, similar experiments need to be conducted in a system capable of sustaining endogeneous expression of the 5-HT_{1A} receptor gene. Until recently, the existence of a transformed cell line endogeneously expressing the 5 HT_{1A} receptor has never been reported. The characterization of such a cell line is communicated in the second chapter of this thesis. Unfortunately, as will be discussed below, the cell line is not suitable for 5-HT_{1A} receptor gene expression studies

A neuroblastoma X septum hybrid cell line expressing the 5-HT_{1A} receptor

Several murine hybrid cell lines, derived from the fusion of primary cultures of pre- and postnatal hippocampal and septal neurons with a neuroblatoma cell line ($N18TG_2$), kindly provided by Dr. Bruce H Wainer (U of Chicago), were screened by RT-PCR for the presence of mRNA coding for the 5-HT_{1A} receptor An intrinsic feature of these cell lines is the potential inducibility of certain phenotypic neuronal markers upon treatment of the cells with certain drugs (e g Retinoic acid (RA) or dibutiryl cAMP). Of the different hybrid clones screened, only one (SN48) showed an increased level of 5-HT_{1A} receptor gene expression in the differentiated state (RA-treated) over the non differentiated one The SN48 line is derived from a 21 day postnatal C57BL/6 mouse primary culture of septal neurons fused with the $N18TG_2$ neuroblastoma cell line (Lee et al , 1990)

Correlation of neuronal phenotype and 5-HT_{1A} receptor expression

In the non-differentiated state, the SN48 cells do not exhibit any morphological features of neurons (see figure 1A-chapter II) Forty-eight hour treatment with 10 μM RA resulted in a substantial neuronal morphologic differentiation (see figure 1B-chapter II). Immunofluorescence of SN48 cells directed against neurofilament proteins, which serve as neuron-specific markers, disclosed unequivocally a phenotype of neuronal nature (Lee et al , 1990)

Concomitant with this morphological inducibility, is the increase in the level of mRNA coding for the 5-HT_{1A} receptor. Northern blot analysis of RNA harvested from differentiated and non-differentiated SN48 displays a considerable increase in the level of 5-HT_{1A} receptor mRNA molecules upon differentiation of these cells (see figure 2-chapter II) The mechanisms underlying this phenomenon are unknown and likely to involve complex systems. The size of the 5-HT_{1A} receptor mRNA present in the differentiated SN-48 cells, which is 10.9 Kb in length, is larger than species observed for the rat and human receptors. Northern blot analysis of rat brain tissues detects three message sizes (3.9, 3.6, and 3.3 Kb) encoding the 5-HT_{1A} receptor and analysis of fetal human peripheral tissues demonstrates a single receptor mRNA species of ~6.0 Kb which is weakly expressed in lymphoid tissues (Albert et al., 1990; Kobilka et al., 1987a) These differences in size between the three species is most likely the result of variation in the length of 5'- and 3'-untranslated regions.

Functional characterization of the 5-HT_{1A} receptors

The concomitant increase in the number of 5-HT_{1A} receptor mRNA molecules with the acquisition of neuronal features from differentiated SN48, correlates with the presence of functional 5-HT_{1A} receptor proteins. As reviewed in the introduction, the 5-HT_{1A} receptor negatively couples to an adenylyl cyclase enzyme thereby reducing the formation of its product (cAMP) upon receptor activation

Using the assay described under "Materials and Methods" of chapter II, the effects of the 5-HT_{1A} receptors on adenylyl cyclase as measured by cAMP levels, were studied in both non-differentiated and differentiated SN48 cells. Figure 3A-chapter II clearly demonstrates the presence of functional 5-HT_{1A} receptors in the differentiated state but not in the undifferentiated one, as the inhibition of either PGE_2- or VIP-stimulated cAMP levels by a pharmacologically relevant concentration of 8-OH-DPAT (a selective 5-HT_{1A} ligand) is only observed in the

differentiated SN48 cells.

This inhibition of elevated cAMP levels is mediated via a pertussis toxin (PT) sensitive G protein since 16 hours pretreatment of the differentiated cells with 250 ng/ml of PT abolished the 5-HT_{1A}-mediated inhibition of stimulated cAMP levels (see figure 3A-chapter II). This pertussis toxin sensitivity is specific to 5-HT_{1A} receptor containing cells as PT did not exert any effect on undifferentiated cells

To strengthen the observation of the existence of functional 5-HT_{1A} receptors in differentiated SN48 cells, a dose-response experiment was conducted Figure 3B-chapter II unequivocally shows the dependence of 5-HT_{1A} receptor mediated actions on agonist (8-OH-DPAT) concentrations with an estimated EC_{50} of 10-30 nM, consistent with previously reported values (Dumuis et al, 1988)

Cloning of the mouse 5-HT_{1A} receptor cDNA

In order to verify the existence of 5-HT_{1A} receptors in the differentiated population of SN48, a fragment of the 5-HT_{1A} receptor mRNA was cloned by RT-PCR from total RNA isolated from differentiated SN48 cells The sequences of the oligonucleotides used in the RT-PCR strategy, were derived from the highly conserved transmembrane domain regions of the receptor Sequence analysis of the cloned fragment showed striking similarity with corresponding regions of the rat and human 5-HT_{1A} receptor genes, and furthermore, showed identical homology to an analogously isolated fragment from mouse hippocampus total RNA. Using this fragment as a probe, a mouse brain λgt11 cDNA library (kindly provided by DR. Jean-Pierre Julien, McGill U.) was screened under high stringency and one 2.4-Kb clone was isolated.

Analysis of the cDNA clone revealed a corresponding region showing identical (100%) homology with the sequences derived from the mouse brain and differentiated SN48, indicating that the 2 4-Kb cDNA clone does indeed code for a 5-HT_{1A} receptor. Overall sequence homology with the rat and human 5-HT_{1A} receptor gene is as follows: mouse-rat, 93% and 94% and mouse-human, 80%

and 86%, where the first and second percentages indicate homologies at the nucleotide and amino acid levels respectively. The deduced amino acid sequence and structural topology are represented in figure 5B-chapter II.

When expressed in mouse Ltk$^-$ fibroblast cells, the cloned murine 5-HT_{1A} receptor cDNA exhibited binding features characteristic of 5-HT_{1A} receptors. For instance, saturation binding experiments using [^{3}H]8-OH-DPAT as a ligand, revealed a calculated K_D of 1 68 nM $\pm$ 0.36 which is well within the range of values observed for the rat and human receptors (Albert et al., 1990; Fargin et al., 1988). It can therefore be concluded that the functional receptors present on the differentiated SN48 cells, are of 5-HT_{1A} type as defined by their sequence and pharmacology Several structure-function postulates can be extracted from the comparison of the amino acid sequence between the mouse, rat and human 5-HT_{1A} receptor The most variable regions of the receptor are the third cytoplasmic loop and the extracellular N-terminal domains (see Table III). However, the regions within the third cytoplasmic loop which are involved in G protein coupling are fully conserved between the three species (see figure 1 and 5B-chapter II). This structural feature reinforces the prediction that all three receptors couple to the same family of G proteins ($G_{i/o}$) On the other hand, the most conserved regions of the receptor are found within the transmembrane domains. Their highly identical homology correlates with the comparable pharmacology displayed by the three 5-HT_{1A} receptors since the ligand binding sites reside within these transmembrane domains Further indepth characterization of the receptors will ultimately reveal functional differences between the three receptors. Only then will the structural differences be accounted for Knowing the specific structural components of the receptor necessary to elicit certain functional aspects will allow the design of drugs endowed with more specific and discrete mode of actions.

Table III. Structural homology between the rat, mouse and human 5-HT$_{1A}$ receptors. The values are represented as percentage of homology.

Species	Overall		Transmembrane domains	N-terminus	Third cytoplasmic loop
	Amino acid	Nucleotide	Amino acid	Amino acid	Amino acid
Mouse-rat	94	93	98.9	77.8	89.1
Mouse-human	86	80	97.3	66.7	72.7
Rat-human	89	82	97.8	72.2	75.8

Conclusion

The expression of the rat 5-HT_{1A} receptor by specific neurons is most likely a result of the unique nature of the 5'-flanking sequences of the gene that encodes the receptor In the light of initial results, I proposed a mechanism responsible for the high degree of neural specificity of the rat 5-HT_{1A} receptor gene expression. To ascertain the molecular components behind such mechanism, a system in which endogeneous expression of the 5-HT_{1A} receptor gene takes place is inevitably required. This work was initiated by the discovery and characterization of a hybrid cell line which embodied 5-HT_{1A} receptor expression upon induced morphological differentiation Unfortunately, this SN48 cell line can not be employed in such a task for several reasons. First, the morphological and most likely biochemical heterogenous nature of the cell population resulting from the differentiation procedure would be detrimental to any interpretation of results originating from transcription studies. And second, the density at which the cells are maintained to obtain optimal differentiation, is not compatible with the requirements dictated by protocols of exogeneous expression of genetic material.

However detrimental to transcription studies the SN-48 cell line may be, it nevertheless remains an excellent system to study the natural environment in which 5-HT_{1A} receptors act Moreover, due to their inducible differentiation properties, the SN-48 cells represent an excellent model system to study neuronal differentiation leading to the development of sensitivity to serotoninergic input

The results obtained from my studies can be summarized as follows:

1- the 5'-flanking region of the rat 5-HT_{1A} receptor gene carries promoter activity

2- transcription is initiated at a unique and tissue-specific site

3-the regulation of expression seems to follow a selective derepression mechanism.

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CHAPTER I

Promoter and Silencer Activities of the Rat 5-Hydroxytryptamine$_{1A}$ Receptor Gene 5'-Flanking Region

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ABSTRACT

The serotonin$_{1A}$ (5-HT$_{1A}$) receptor displays an exquisite cell specificity of expression, being localized to discrete regions of the brain, such as the hippocampus, septum, and raphé nuclei. To identify genetic mechanisms directing receptor expression, over 3 3 kb of 5' flanking region of the rat 5-HT$_{1A}$ receptor gene was sequenced and characterized A unique site of transcriptional initiation was localized at -967 base pairs (bp) 5' to the start of translation (+1 bp) by RNAase protection, primer extension, and RT-PCR with progressively 5' primers, and was specific for hippocampal RNA These analyses indicated an absence of introns in this region of the gene and no evidence of upstream start sites was found By sequence analysis, a TATAA box was identified (-1025 bp) close to the start site, as well as other TATAA boxes, CCAAT boxes, SP-1 and AP-2 sites, several neural-specific consensus sequences, and a 120-bp region with high homology to the tumor necrosis factor-ß promoter. To assess promoter activity, deletion constructs of the 3 3-kb 5-HT$_{1A}$ fragment were linked to firefly luciferase and transfected into cells which do not endogenously express the 5-HT$_{1A}$ receptor. Results concurred in two cell lines (P19 and Ltk-), indicating the presence of a strong promoter located between nucleotides -1187 and -127, followed by an upstream silencer region (between -1521/-1188), which suppressed the activity of the promoter to below basal activity. Further promoter activity was detected upstream (-2719/-1522) from the silencer Regulation of the 5-HT$_{1A}$ receptor via a repressor element in non-expressing tissues is analogous to the arrangement in promoters from other, more widely-distributed neuron-specific genes. We suggest that repression of transcription may be a major mechanism in restricting the expression of diverse neuron-specific genes to the appropriate cell types.

INTRODUCTION

The regulation of eukaryotic transcription results from an interplay between diverse DNA binding proteins which is dependent upon the relative loccalization of cis-acting regulatory DNA sequences to which these proteins bind (Johnson and McKnight, 1989; Mitchell and Tjian, 1989) While certain promoter elements, such as TATA, GC, and CCAAT boxes, are common to many protein-encoding genes, specific enhancer and promoter elements confer cell-specificity and signal dependent transcriptional regulation in the presence of appropriate activator or repressor proteins (Maniatis et al , 1987, Ptashne, 1988) With the recent cloning of a variety of brain-specific Pit-1-related POU-domain proteins (Rosenfeld, 1991, He et al., 1989; Ruvkun and Finney, 1991), the identification of target genes for these DNA binding proteins awaits the characterization of multiple brain-specific gene promoters, such as that of the 5-HT_{1A} receptor gene

Several genes which are expressed in the brain have been studied already, and they fall into two broad classes pan-neuronal genes such as neurofilament, GAP-43, SCG-10, peripherin, and sodium channel type II genes (Shneidman et al , 1992; Mori et al., 1992; Kraner et al., 1992, Nevidi et al , 1992, Wuenschell et al , 1990, Mori et al., 1990; Maue et al , 1990), which are expressed in all neurons, but only in neurons, and cell type-specific genes, such as tyrosine hydroxylase or dopamine D1 receptor genes (Yoon and Chikaraishi, 1992, Minowa et al , 1992), which are expressed in subsets of neurons as well as in other tissues Certain general similarities among the structures of the promoters of these two classes of genes are emerging. the pan-neuronal genes possess neuron-specific enhancer elements as well as ubiquitous promoters, and neuron-specificity may involve repression of expression in non-neuronal tissues, the type-specific genes have promoter and enhancer elements which direct their expression in particular cell

types. We wished to examine the control of a gene which was both neuron-specific and cell type-specific

The expression of the 5-HT_{1A} receptor gene is brain-specific, and expression in peripheral tissues of the adult is undetectable by Northern blot analysis However, unlike pan-neuronal genes, the neuronal distribution of 5-HT_{1A} receptor is localized to specific neuronal subtypes (Albert et al., 1990; Pompeiano et al., 1992), especially neurons in the raphé, hippocampus, and septum. The receptor plays a key role in tonic and dynamic control of the serotoninergic neuronal activity (Frazer et al , 1990). The raphé nuclei, which form the major serotonergic input of the brain, are under the inhibitory regulation of 5-HT_{1A} autoreceptors The 5-HT_{1A} receptor is also expressed in several target tissues to which neurons of the raphé nuclei project, particularly in hippocampal, septal and cortical regions. Thus, activation of the 5-HT_{1A} receptor has a widespread influence on brain functions which are regulated by serotonergic neurons of the raphe nuclei. In particular, agonists of this receptor such as buspirone or gepirone are effective in the treatment of major depression and generalized anxiety disorders (Blier et al., 1990; Charney et al , 1990, Blier and de Montigny, 1987), which may result from abnormal serotonergic regulation, perhaps due to aberrant expression of 5-HT_{1A} receptors

In order to understand the regulation of the 5-HT_{1A} receptor gene, we sought to identify and characterize specific DNA regulatory elements in the 5'-flanking region of the gene. Chimeric constructs of 5-HT_{1A} receptor gene 5' flanking sequences with a firefly luciferase cDNA as a reporter gene (deWet et al., 1987) were transiently expressed in cultured mammalian cells and assayed for promoter activity. We found that in cells which do not express the receptor, a strong promoter is repressed by an adjacent 5'-region of the gene which may represent the tissue-specific region The presence of this repressor element suggests that suppression of transcription may play a role in regulating the expression of the 5-HT_{1A} receptor or other brain-specific genes

MATERIALS AND METHODS

Construction of Luciferase Plasmids and Sequencing- A 5 57 Kb Pst I fragment of genomic λ-EMBL3 clone D (the rat 5-HT_{1A} receptor (Albert et al , 1990)) was subcloned into pGEM-blue (Promega Biotech) and named 63P The 3.2 Kb Bam HI fragment of 63P was further subcloned into pBluescript KS_+ (Stratagene, LaJolla, CA) in both orientations (clones #1 and #2) From clone #1, P1B1, S1B1, and H2B1 were all constructed by digestion with the appropriate restriction enzymes followed by internal ligation The 1 06 kb Taq I/Bam HI fragment of clone #1 was ligated into Cla I/Bam HI-digested pBluescript KS_+ to generate T1B1. The firefly luciferase cDNA (deWet et al , 1987) was isolated from pGEM-LUC (a gift from Dr. M Szyf, McGill University) using Bam HI/Sac I endonucleases and ligated into Bam HI/Sac I-cut P1B1, S1B1, H2B1, T1B1 and pBluescript KS+ to generate P1B1-L, S1B1-L, H2B1-L, T1B1-L, and $pBKS_+$-L, respectively. P1T1 originated from a 2 6 Kb Kpn I/Bam HI fragment of P1B1 which was purified and then redigested with Taq I and ligated into Pst I/Cla I-cut pBluescript KS+. P1T1 was then digested with Hinc II/Eco RV or Sma I/EcoRV to give rise to P1H2 or P1S1, respectively The 1 8 Kb Bam HI/Sac I luciferase cDNA was inserted in P1T1, P1H2, and P1S1 as described above to give rise to P1T1-L, P1H2-L, and P1S1-L. Clone #1 was digested with Hind III and internally ligated with T4 DNA ligase to give H3B1 which was treated similarly as P1B1 to yield H3T1, with subsequent Sma I digestion and internal ligation to give H3S1 The luciferase cDNA was the inserted as outlined above giving rise to H3S1 L DNA sequence from both coding and non-coding strands were analysed by obtaining single stranded phagemid DNA from clones #1 and #2 and subclones and sequenced by the Sanger dideoxy chain termination method using a Sequenase sequencing kit (US Biochemicals)

Primer Extension- A 19-mer oligonucleotide (5'-GGCTATAGGGATGCGG GAT-3') (oligo #53) located 938 bp upstream of the first ATG of the rat 5-HT_{1A} receptor and a 25-mer oligonucleotide (5'-CCCGCCTCCTACCTAGCA

AACTAGA-3') (oligo #54) located 784 bp upstream of the initiating ATG codon were 5' end-labeled with [g-32P] ATP (Amersham) by T4 polynucleotide kinase (New England Biolabs) and purified by reverse phase chromatography with Sep-Pak C18 cartridges as described by the manufacturer (Waters-Millipore, Milford, MA) Total RNA was isolated from tissues of 200-300 g male Sprague-Dawley rats by the guanidium isothiocyanate protocol (Sambrook et al., 1989) The reverse transcription reaction was as follows: 10 µg of total RNA prepared from various sources was denatured in 10 mM MeHgOH for 10 min. at room temperature The MeHgOH was then neutralized with the addition of 116 mM of ß-Mercaptoethanol (Sigma, St. Louis, MI) and incubated at room temperature for 5 min The denatured RNA was hybridized with 0.1 µg of labeled oligonucleotide (5 min, room temperature) and incubated for 2h at 42°C in 50 mM TrisHCl (pH 7.5) 75 mM KCl, 3 mM MgCl2, 2 mM dNTP (Pharmacia), 2.5 mM dithiothreitol, and 200 units of Superscript reverse transcriptase (BRL, Gaithersburg, MD.) in a 40 µl reaction volume. The resulting extension products were subjected to electrophoresis on a 6% polyacrylamide/urea gel (Sambrook et al , 1989), with the sequence of the M13 phage as a size index.

RNase Protection- Probe A was synthesized by PCR using oligo #54 (antisense) and the sequencing reverse primer (5'-AACAGCTATGACCATG-3') of pBluescript (sense), with T1B1 as template. The resulting 409 bp fragment was then inserted into the Eco RV site of pBluescript KS+ (plasmid A). The labelling of the cRNA probe was as follows: 1 µg of Xho I-digested (linearized) plasmid A DNA was transcribed in the presence of 10 mM dithiothreitol, 0.5 mM of each ATP, CTP, and GTP (Pharmacia), T7 transciption buffer, 50 µCi of 400-800 Ci/mmol [a-32P] UTP (Amersham) and 10 units of T7 RNA polymerase at 35°C for 1 hour The resulting transcripts were then treated with 2 units of RNase-free DNase I at 37°C for 15 min , purified on a mini-sequencing gel, and eluted overnight at 37°C in 0.5 M ammonium acetate, 1 mM EDTA, and 0 2% SDS. The cRNA probe (0.5 µCi) was coprecipitated with 10 µg of total RNA and resuspended in 50% formamide, 0.4 M NaCl, 0 04 M PIPES (pH 7.5), and 1 mM EDTA (pH 6.0), heat-denatured at 85°C for 5 min, and incubated overnight at 50°C. The RNase

digestion conditions were performed as described (Krieg and Melton, 1987). The resulting protected fragments were analyzed on a DNA sequencing gel with M13 phage DNA sequence as the size marker.

Cell Culture and Transient Transfections- Murine embryonal carcinoma P19 cells obtained from Dr. M. Featherstone (McGill University) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum at 37°C in 5% CO_2 Murine Ltk- fibroblasts were grown in alpha-modified Eagle's medium with 10% (v/v) heat-inactivated fetal calf serum also at 37°C in 5% CO_2. Prior (4-6 h) to transfections, cells were plated at a density of 10^4 cells/cm^2 on 3.5-cm dishes Plasmid DNA (20 µg/ml) and internal control ß actin promoter-Lac Z plasmid (5 µg/ml) (kindly provided by Dr G Almazan, McGill University) were co-precipitated with calcium phosphate (Sambrook et al , 1989). After 14-16 hours of incubation the plates were rinsed with PBS, fresh medium was added and the cells were incubated for another 24 hours before assaying for luciferase activity. All plasmids used for transfection were purified by CsCl-equilibrium gradient centrifugation (Sambrook et al , 1989)

Luciferase Assays- Cells were washed once with cold PBS and lysed on the plates by addition of 200 µl of 1% Triton X-100, 25mM glycylglycine (pH 7 8), 15 mM MgSO4 and 4 mM EGTA solution. The supernatants were collected and fractions assayed for luciferase and ß-galactosidase activity as described before (Brasier et al., 1989; Sambrook et al., 1989) Values represent the integrated area under the light output curve divided by units of activity of the corresponding ß-galactosidase fractions The data are reported relative to the positive control which is pRSV-L (deWet et al., 1987) and are arbitrarily labeled as relative light units. Data are presented as the mean ± SE of triplicate samples and are representative of at least 3 different experiments for each cell line

RNA Isolation and Analysis- Total cellular RNA from P1B1-L transfected cells was extracted by the fast cytosolic RNA preparation method and Northern blot analysis conducted as described elsewhere (Sambrook et al , 1989)

RESULTS

Sequence of the 5'-Flanking Region of the 5-HT_{1A} Receptor Gene- The 3.2 Kb Pst I/Bam HI genomic fragment of the rat 5-HT_{1A} receptor gene was transferred in both orientations into pBluescript KS+ as described under "Materials and Methods" The resulting sense and antisense clones (#1 and #2 respectively) were rendered single-stranded and both strands were sequenced. The resulting sequences are depicted in figure 1. We suspect that the elements necessary for the proper transcription initiation are present within this 3.2 Kb genomic fragment.

Mapping the Transcription Initiation Site-In order to determine the 5' end of the rat 5-HT_{1A} transcript we first mapped the region of initiation in hippocampus to a distinct area (between -1038 and -886) by RT-PCR using the translation initiation site primer (antisense) and progressively further 5' sense primers (data not shown) We then used labeled oligonucleotide #55, in a primer extension experiment and found that the initiation site was approximately -957 bp upstream to the translation initiation codon as shown in figure 2B This transcript was specific to the hippocampus, since the cortex contains no such transcript This suggests that the primer extension product is derived from the 5-HT_{1A} receptor RNA since the receptor RNA is highly enriched in hippocampus, present at low levels in cortex (Albert et al , 1990) In order to more accurately localize the start site, we repeated the primer extension experiment with an oligonucleotide which hybridizes closer to the putative start site. Oligo #53 which overlaps the initiation site shown with oligo #55 was extended by only 11 nucleotides, again specific to hippocampus (Fig 2C), suggesting that the start site is located at -967. To corroborate this putative initiation position, we used RNase protection assays. In figure 3B, a unique protected fragment of 98 nucleotides from Probe A (described under "Materials and Methods") was identified in hippocampal RNA, but absent in RNA from both cortex and liver. This protected fragment corresponds to a start site located at -967 bp, and substantiated the hippocampus-specific transcription

start site found by primer extension analysis with oligo #53 We conclude that transcriptional initiation of the 5-HT_{1A} receptor RNA is at -967 bp upstream from the translational initiation codon. This site is located 58 bp downstream from a consensus TATAA box which is preceded by a CCAAT box sequence (Fig 1).

Finally, we determined whether the large (967 bp) 5' untranslated region of the 5-HT_{1A} receptor RNA transcript contains introns. Using primers which span the 5' untranslated region, RT-PCR analysis of hippocampal RNA was performed (Fig 4). RT-PCR of hippocampal RNA yielded a unique 916 bp fragment, with a molecular weight identical to the fragment generated by PCR of the 5-HT_{1A} receptor genomic plasmid construct (Fig 4) This fragment was not obtained from RNA which was not reverse transcribed, indicating that the fragment was derived from 5-HT_{1A} receptor RNA present in the sample, and not due to amplification of minute genomic DNA contaminants Thus, the entire genomic sequence in this region is transcribed to RNA, and we conclude that the 5'-untranslated fragment of the 5-HT_{1A} receptor lacks introns.

Promoter Activities of the 5' Region of the Rat 5-HT_{1A} Gene-To assess promoter activity, the luciferase structural gene was ligated downstream from a series of 5'-deleted fragments of the 5-HT_{1A} receptor containing upstream sequences, the transcription start site and a large portion of the 5'-untranslated sequence (Fig 5A) These constructs were transiently transfected into 5-HT_{1A} receptor-negative P19 embryonal carcinoma or Ltk- fibroblast cells, and the cells assayed 48h later for luciferase activity While it would have been preferable to select a cell line which does express 5HT_{1A} receptors, no such cells have been identified The activity of these constructs was compared to the activity of the control plasmid, pKS+-L, which had significant promoter activity (12% of RSV-L) in the P19 cells The construct containing 2 6 kb of flanking region (P1B1) displayed promoter activity in the Ltk- cells, but negligible activity in the P19 cells when compared to control (PKS+-L). Truncation of 1 2 kb of 5'-flanking sequence located between the Pst I and Sma I sites giving rise to S1B1-L resulted in a

tremendous loss of luciferase activity in both cell lines. This suggested the presence of enhancer activity in the upstream region, which was lost upon deletion. In the P19 cells, the activity was one third of control levels, suggesting an active repression of basal promoter activity in these cells. Further deletion of 132 bp (from the Sma I site to the Hinc II site) in H2B1 slightly attenuated the inhibition observed in S1B1, however repression of activity remained in the P19 cells. Removal of a further 203 bp segment to generate T1B1 resulted in the strongest transcriptional activity observed in the four constructs, clearly indicating the presence of a promoter in this region. Since the transcription start site is located 200 bp downstream from the beginning of T1B1, we propose that the promoter is located in this 200 bp region which contains a CCAAT box followed by a TATA box. The data indicate that a repressor region is located between 200-300 bp upstream, and further upstream there may be additional promoter or enhancer elements

To examine the gene in greater detail, a series of 3' deletion constructs were made and tested in transient transfection experiments (Figure 5B). Remarkably, truncation of the Taq I/Bam HI region (P1T1-L) resulted in the unmasking of significant promoter activity (as measured by luciferase enzyme activity), which was absent in the P1B1 fragment in P19 cells (Figure 5A). Further truncation of the putative silencer regions between Sma I and Taq I (P1H2-L and P1S1-L) resulted in a 1.5-fold enhancement of the cryptic upstream promoter, while deletions further 5' (H3T1-L and H3S1-L) led to significantly decreased luciferase activity These data suggest that promoter activity is present upstream of the Sma I site, and that the Sma I/Taq I segment is the sole mediator of the silencing of the 5-HT_{1A} receptor gene in P19 cells. The promoter activity of the P1T1 fragment indicates that elements present downstream, possibly in the 5'-untranslated region of the gene, are required for silencing of the gene.

P19 and Ltk- do not Use The Same Transcription Initiation Site as

Hippocampal Cells- To determine whether the T1B1 minimal promoter fragment contained elements dictating use of the hippocampal transcription start site, RNA isolated from P19 and Ltk- cells transiently transfected with T1B1-L was subjected to RNase protection assay using probe A. As shown in figure 6A, the 98-nucleotide protected fragment found in hippocampal RNA was not present in transfected P19 or Ltk- cells. The construct was effectively transcribed in the transfected cells as evidenced by the presence of luciferase RNA (Figure 5B), indicating that the lack of signal in RNase protection analysis was not due to a lack of RNA, but probably to different or diffuse sites of transcriptional initiation. This suggests that site of initiation of transcription is tissue-specific and may depend upon the presence of additional DNA elements or specific transcription factors found in tissues which endogenously express the gene.

Putative cis-Acting Elements of the 5-HT_{1A} Receptor in Promoter/Repressor Regions- Computer-aided analysis of the 5'-flanking sequence (Figure 1) was used to identify putative *cis*-acting elements present in the 5-HT_{1A} promoter as shown in Table 1. Of particular interest are the presence of three consensus neural-specific enhancer elements, one immediately upstream from the transcription start site, and one downstream in the 5'-untranslated region adjacent to an AP-1 sequence. Just downstream from the AP-1 site is a sequence bearing striking homology to the recently-identified repressor element (Mori et al., 1992). The juxtaposition of these elements suggests complex regulator interactions may occur in this portion of the 5'-untranslated region. The silencer region (Sma I/Taq I) contains only a salient (GT)26 repeat, whereas further upstream regions contain a number of putative enhancers. Further upstream, consensus sequences for a number of *cis*-acting elements were identified. Curiously, a search of the EMBL-Genebank identified a 150-nucleotide sequence (from nucleotides -1841 to -1690) with 80% identity to sequence found in the 5'-flanking region of the mouse tumor necrosis factor ß gene (Gray et al., 1987). Additional constructs and transfections are necessary to determine the functional significance of these

sequences in the regulation of the 5-HT_{1A} receptor gene.

DISCUSSION

The cloning and functional characterization of the rat 5-HT_{1A} receptor has led to the development of valuable tools for understanding the distribution and regulation of this gene in the central nervous system. In particular, receptor-derived oligonucleotides, riboprobes, and antibodies have elucidated the cell-specific expression of this receptor in the central nervous system The next challenge involves understanding the factors which determine this distribution, and how they are regulated in health and disease states, and by therapeutic interventions Toward this aim, we report the sequence and preliminary functional characterization of the rat 5-HT_{1A} receptor gene

We have identified a unique and cell-specific transcriptional start site, followed by 1 kb of intronless 5'-untranslated sequence. Like the β_2-adrenergic receptor RNA transcripts, the $5HT_{1A}$ receptor gene lacks introns, both in its translated portion and in the 5'-untranslated region (Kobilka et al., 1987). The functional significance of the intronless nature of these receptors is unclear Evidence from RNAase protection and primer extension experiments indicated a single transcription start site, which does not explain the multiple (3 3, 3.6, and 3.9 kb) species of RNA observed by Northern analysis Because the gene is intronless over 3.2 kb (ie., 1 kb 5'-untranslated, and 2 2 kb translated and 3'-untranslated sequence) we suggest that the different sizes may arise from alternate use of poly-adenylation sites further 3' from the portions we have sequenced Finally, we found that the start site was specific to tissues which normally express the gene, since transfected reporter constructs did not have the same transcriptional start site as hippocampus

Studies with the luciferase constructs elucidated a pattern of expression controlled by a proximal promoter, an upstream repressor, followed by enhancer and promoter activity located further upstream. Within 200 bp upstream of the

transcriptional start site, a minimal promoter containing CCAAT and TATAA boxes, directed expression of the gene The nearest TATAA was -58 from the transcription start site, further than the standard -25 to -30 found in most genes but similar to the localization in other neural-specific genes (eg , SCG-10 -70 TO -65, GAP-43, -43). Upstream from the TATAA box was a CCAGG box, a sequence found in several neuron-specific genes, which alone is insufficient to confer neuronal specificity. Our results show that the minimal promoter is not cell-specific, despite the presence of this element, since the T1B1 construct was active in both receptor negative cell lines Furthermore, the CCAGG box present in the 5'-untranslated region did not confer cell specificity

The 330-bp segment upstream to the proximal promoter (the Sma I/Taq I region) strongly repressed the expression of the reporter constructs This element repressed expression below even the basal expression of the luciferase plasmid construct, suggesting that it actively silences the gene, rather than abrogating the activity of the proximal promoter In certain neuron specific genes, such as SCG-10 and type II sodium channel genes, it is clear that a similarly localized repressor determines neural-specific expression The repressor is active in cells which do not express the gene, but in neurons the repressor is inactive allowing neuronal expression mediated by the promoter elements Since our luciferase studies have been done in non-expressing cells, we propose that the repressor region we have identified may confer neuron-specific expression to this gene This is difficult to test, however, due to a lack of cell lines which express endogenous 5-HT_{1A} receptors Preliminary results in N18TG2 neuroblastoma cells which do not express 5-HT_{1A} receptors indicate that the repressor is active in these cells, suggesting that the repressor is not only neuron-specfic, but discriminates among different subtypes of neurons.

Recently, a neural-specific repressor element has been identified in the SCG-10, sodium channel type II, and synapsin I genes which mediates tissue specific silencing of gene expression (Mori et al , 1992) Tissues which do not express these genes are enriched in proteins which bind specifically to this

consensus sequence and repress gene expression Neuronal tissues which express these genes lack the repressor proteins, and the gene can be expressed in these tissues As shown in figure 7, we have identified a sequence in the 5-HT_{1A} gene which contains a core sequence identical to the core consensus sequence of this silencer element, and thus may participate in determining the cell-specific expression of the 5-HT_{1A} receptor gene. However this element was found in the 5'-untranslated region, and not in the putative repressor region

The only element found within the 330 bp Sma I/Taq I sequence was a poly-GT repeat, which has been found in other genes (eg , the GAP-43 gene). The function of the GT repeat is unclear, however it has a structure compatible with the formation of Z-DNA structures It is possible that the presence of Z-DNA could open the chromatin structure to expose cryptic promoter or repressor elements, such as those described above This may explain why this element is required for repression, yet is unable to repress in the absence of the 5'-untranslated region (Figure 5B) Thus, we hypothesize that the consensus silencer located in the 5'-untranslated region is primarily responsible for the repression of the gene, but is normally inaccessible to repressor proteins in the absence of the poly-GT repeat (eg , in the T1B1 construct), perhaps because of competition from adjacent CCAGG and AP-1 enhancer elements Further dissection of this complex array of DNA elements will be required to determine the precise role of each element in the transcriptional control of the 5-HT_{1A} receptor gene

Further upstream from the repressor element, we identified a distal promoter capable of inducing luciferase expression. The sequence indicates the presence of CCAAT and TATAA boxes, and several enhancer elements including a weak CCAGG box Immediately following a consensus TATAA element at -1848 a 150 bp sequence with 80% homology to nucleotides -893 to -1045 of the mouse tumor necrosis factor ß (TNF-ß) gene was identified The role of this segment in transcriptional control of the TNF-ß gene is unknown, but suggests a possible correspondence between 5-HT_{1A} receptor and TNF-ß expression in some tissues

It is unclear whether at some time in development 5-HT_{1A} receptors are expressed in the T cells which express TNF-ß In human fetal tissue the receptor has been detected in spleen and thymus, tissues rich in T cells Conversely, little is known about the expression of TNF-ß in the central nervous system

From these studies, we have identified a pattern of promoter and silencer activities which is remarkably similar to the general pattern found for a subclass of neuron-specific genes such as SCG-10 and sodium channel type II genes Namely, a ubiquitous proximal promoter is followed by an upstream silencer region which shuts gene expression off in inappropriate host cells We propose that the silencer elements we have identified are active only in cells which do not express the 5-HT_{1A} receptor, and that ubiquitous repressor proteins similar to those described, restrict the expression of this gene to the appropriate neuronal subpopulations Further studies in transgenic animals must be attempted to test how faithful this simple model of the 5-HT_{1A} receptor gene is to the highly specific tissue distribution found in vivo

Figures Legends

Figure 1. Nucleotide sequences of 5'-flanking region of 5-HT_{1A} receptor gene The sequence of the 3.3 Kb Pst I-Bam HI genomic fragment from the 5' region of the rat 5-HT_{1A} receptor gene was determined by the Sanger dideoxy termination chain reaction as described under "Materials and Methods" The transcription initiation site is indicated by an arrowhead TATAA and CCAAT consensus sequences are circled. Consensus sequences for other putative *cis*-acting elements are indicated as boxed residues The neuron-specific CCAGG consensus elements are boxed by a dashed line and indicated by a star The poly GT repeat is underlined and sequences homologous to the TNF-ß promoter are in brackets

Figure 2 Delimiating the transcription start site of the rat 5-HT_{1A} receptor gene using primer extension methodology A, schematic representation of the oligonucleotides used to map the initiation start site. The cartoon is drawn to scale. B. primer extension experiment using oligonucleotide #55 A fragment of 174 nucleotides was seen in hippocampus tissue only On the left are size markers generated by M13 sequences (not shown). C, primer extension experiment using oligonucleotide #53 Oligo #53 was elongated by 11 nucleotides only in hippocampal tissue Shown on the left is the 5-HT_{1A} receptor gene sequences. Size markers were generated by an M13 sequence (not shown).

Figure 3 RNase protection analysis of the 5'-region of the rat 5-HT_{1A} receptor gene. A, schematic representation of the 5'-region of the receptor gene The full length Probe A is depicted below the map as well as the predicted protected fragment of 98 nucleotides The figure is drawn to scale B, 10 ug of total RNA form hippocampus, cortex and liver were subjected to RNase protection analysis as described under "Materials and Methods" A 98 nucleotides long fragment was protected only in hippocampal tissue (arrowhead).

Figure 4 Intronless nature of the 5' untranslated region of the rat 5-HT_{1A} receptor gene 300 ng of total RNA from hippocampal tissue were reverse transcribed as described under "Materials and Methods". The resulting cDNA was then amplified with Oligonucleotides #51 (-886 to -867) and #34 (+30 to +1) in a polymerase chain reaction (Hippocampus + lane) The resulting PCR products were ran on a 1 3% agarose gel, transferred to filters, and probed with ^{32}P-labelled T1B1 fragment As a control (Hippocampus C lane), RNA from hippocampus was omitted from the RT reaction prior to amplification. Lane "Clone #1 +" represents amplification of 300 ng of clone #1 (genomic construct). The size of the fragment is 916 bp as indicated by the arrowhead The ordinate indicates the migration of DNA molecular weight standards

Figure 5: Promoter activity of the 5'-region of the rat 5-HT_{1A} receptor gene The physical maps of the luciferase constructs are shown relative to the 5-HT_{1A} receptor gene 5'-region. Indicated are TATAA and CCAAT consensus sequence elements and the transcription start site The maps are drawn to scale A, P19 and Ltk- and B, P19 only, were transiently transfected with the indicated plasmids as described under "Materials and Methods". Transfection efficiency was normalized using the ß-actin promoter-Lac Z plasmid as an internal control, and the data were expressed relative to the activity of the RSV-L construct in each experiment Each value is presented as mean ± S E M and is representative of more than 3 different experiments.

Figure 6: The tissue-specific nature of the transcription initiation site of the rat 5-HT_{1A} receptor gene A, 10 ug of total RNA from hippocampus tissue and P19 and Ltk- cells transfected with the plasmid TIBI-L were sujected to RNase protection analysis using Probe A (described under "Materials and Methods") The expected 98 nucleotides fragment is only present in hippocampus B, northern blot analysis of transfected P19 and Ltk- with TIBI-L (+ lanes) versus non transfected cells (lanes) 25 ug (P19) and 15 ug (Ltk-) of total RNA from both transfected and non-transfected cells were analysed The size of the resulting band was determined by calculation from its co-migration with molecular weight standards

Figure 7 Sequence of a consensus neuron-specific element The sequence of a putative neuron-specific element present in the 5-HT_{1A} receptor 5' flanking region is shown Boxed nucleotides indicate identity between the rat 5-HT_{1A} receptor sequence and the neuron-specific negative element consensus sequence "X" denotes a lack of nucleotide

Table 1: Homologies between known and characterized regulatory consensus sequences and the rat 5-HT_{1A} receptor gene 5'-region sequences Binding

recognition sequences from previously characterized transcription factors (Faisst and Meyer, 1992) were searched in the 5' upsteam region of the 5-HT_{1A} receptor gene using the Quick Global Search for a Subsequence program form PC/Gene (Intelligenetics)

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Wuenschell, C W , Mori, N , and Anderson, D. J. 1990. Analysis of SCG10 gene expression in transgenic mice reveals that neural specificity is acheived through selective derepression *Neuron* **4**.595-602

Figure 1

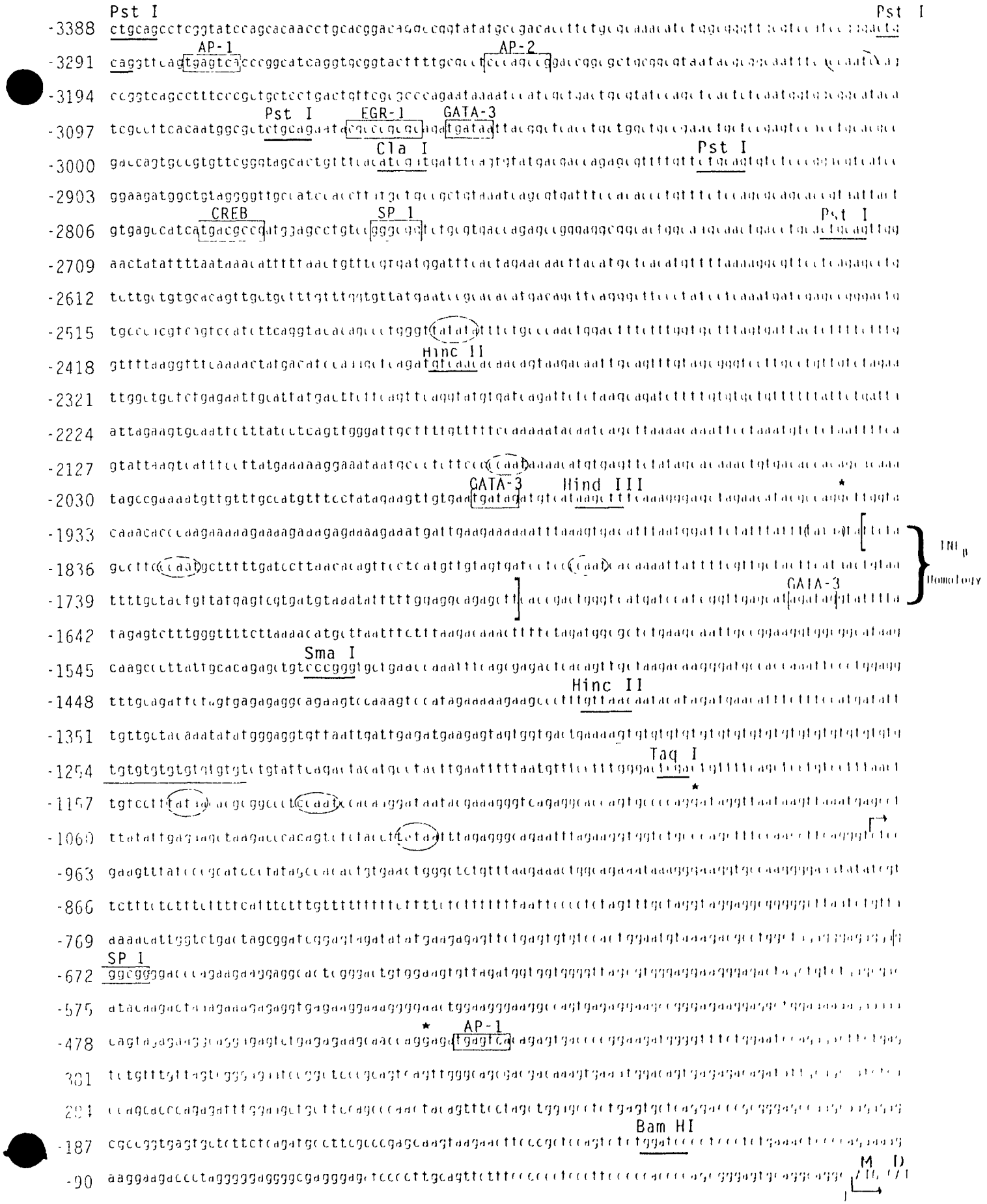
Pst I
Pst I
AP-1
AP-2
Pst I
EGR-1
GATA-3
Cla I
Pst I
CREB
SP 1
Pst I
Hinc II
GATA-3
Hind III
INR Homology
GATA-3
Sma I
Hinc II
Taq I
SP I
AP-1
Bam HI
M D

Figure 2A

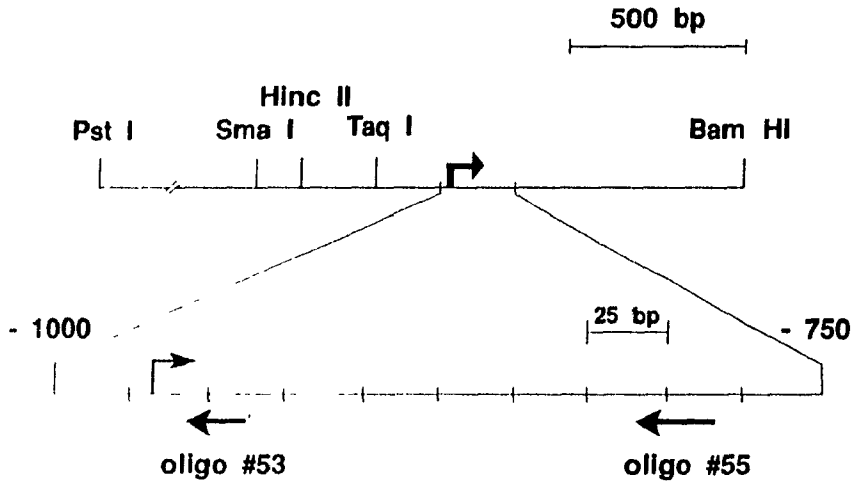
500 bp
Hinc II
Pst I
Sma I
Taq I
Bam HI
- 1000
25 bp
- 750
oligo #53
oligo #55

Figure 2B

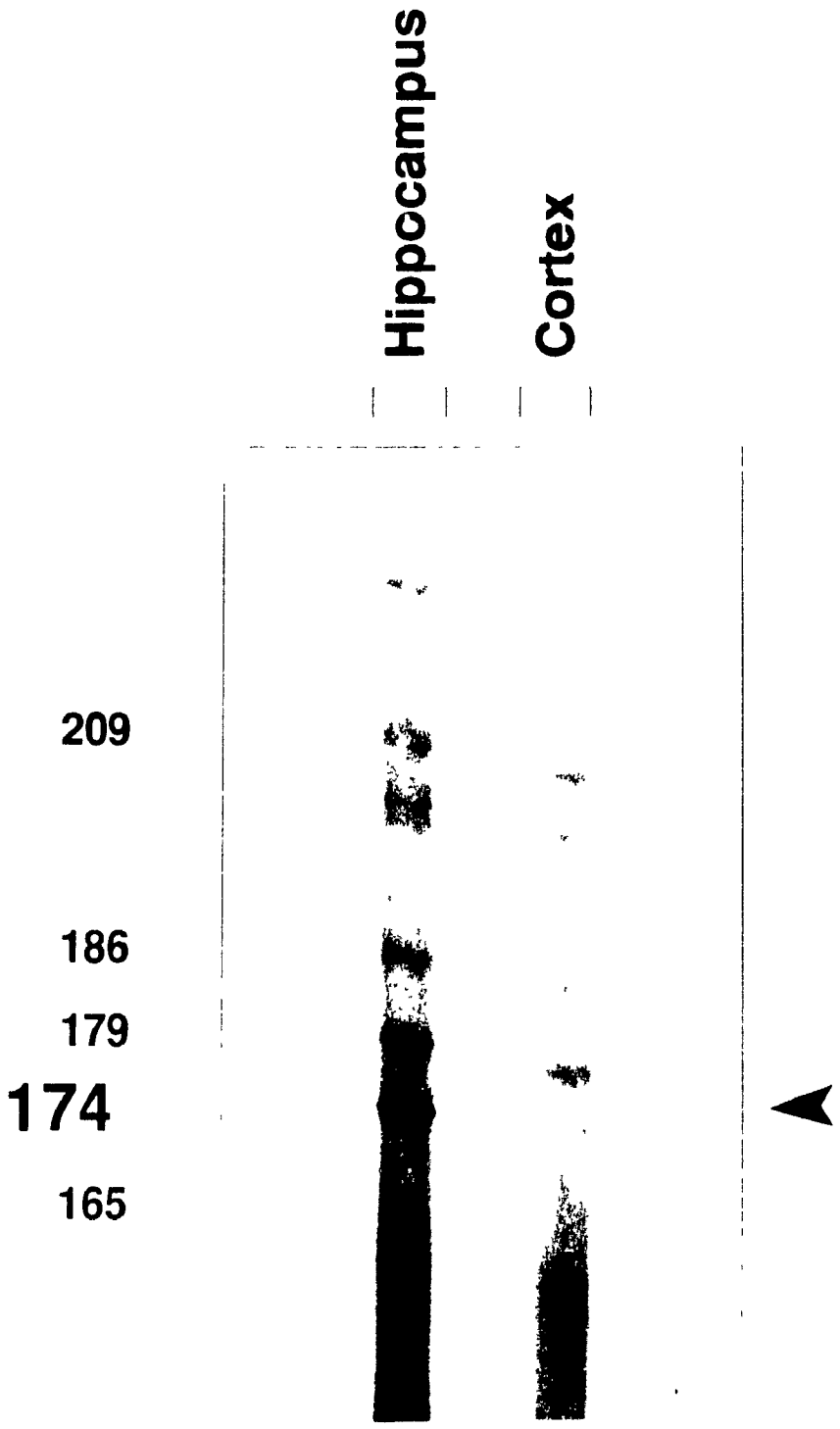

Figure 2C

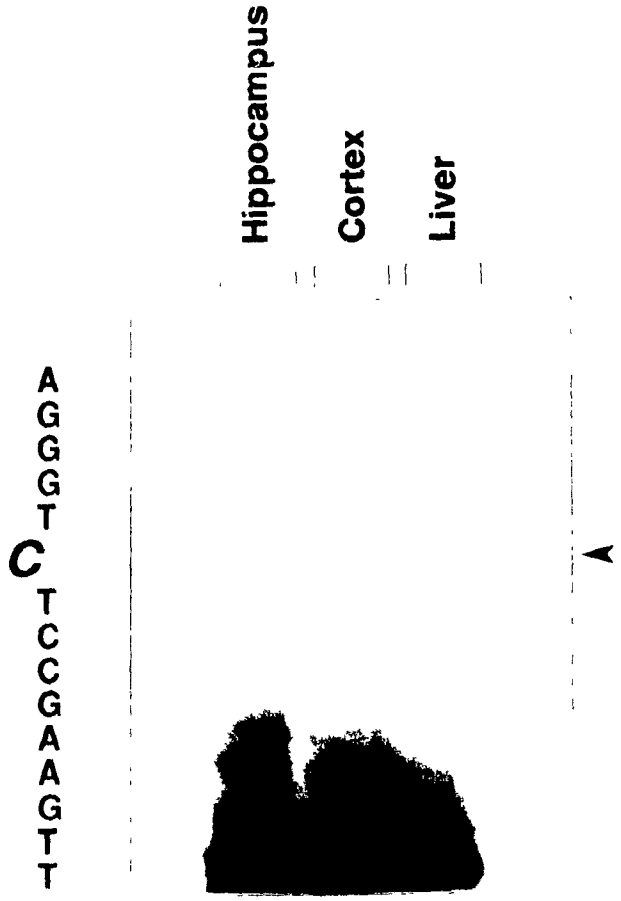

Figure 3A

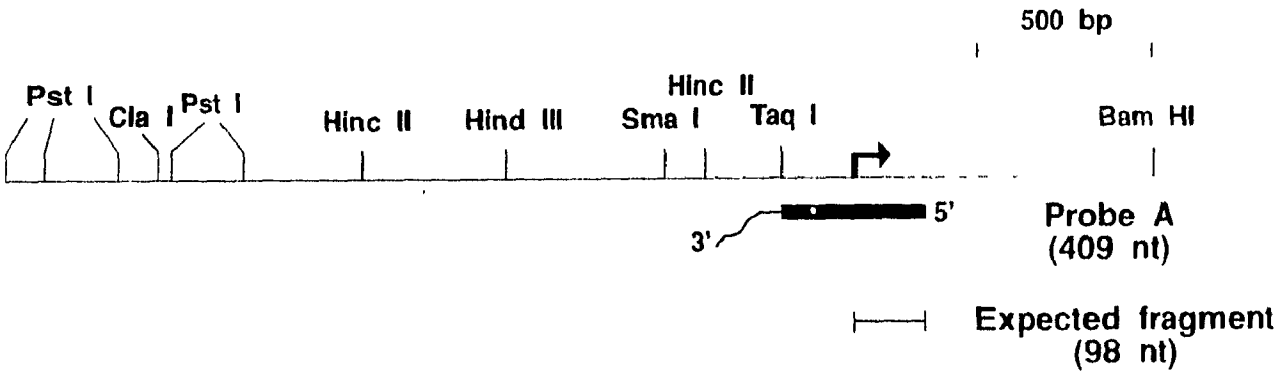
500 bp
Pst I
Cla I
Pst I
Hinc II
Hind III
Hinc II
Sma I
Taq I
Bam HI
3'
5'
Probe A
(409 nt)
Expected fragment
(98 nt)

Figure 3B

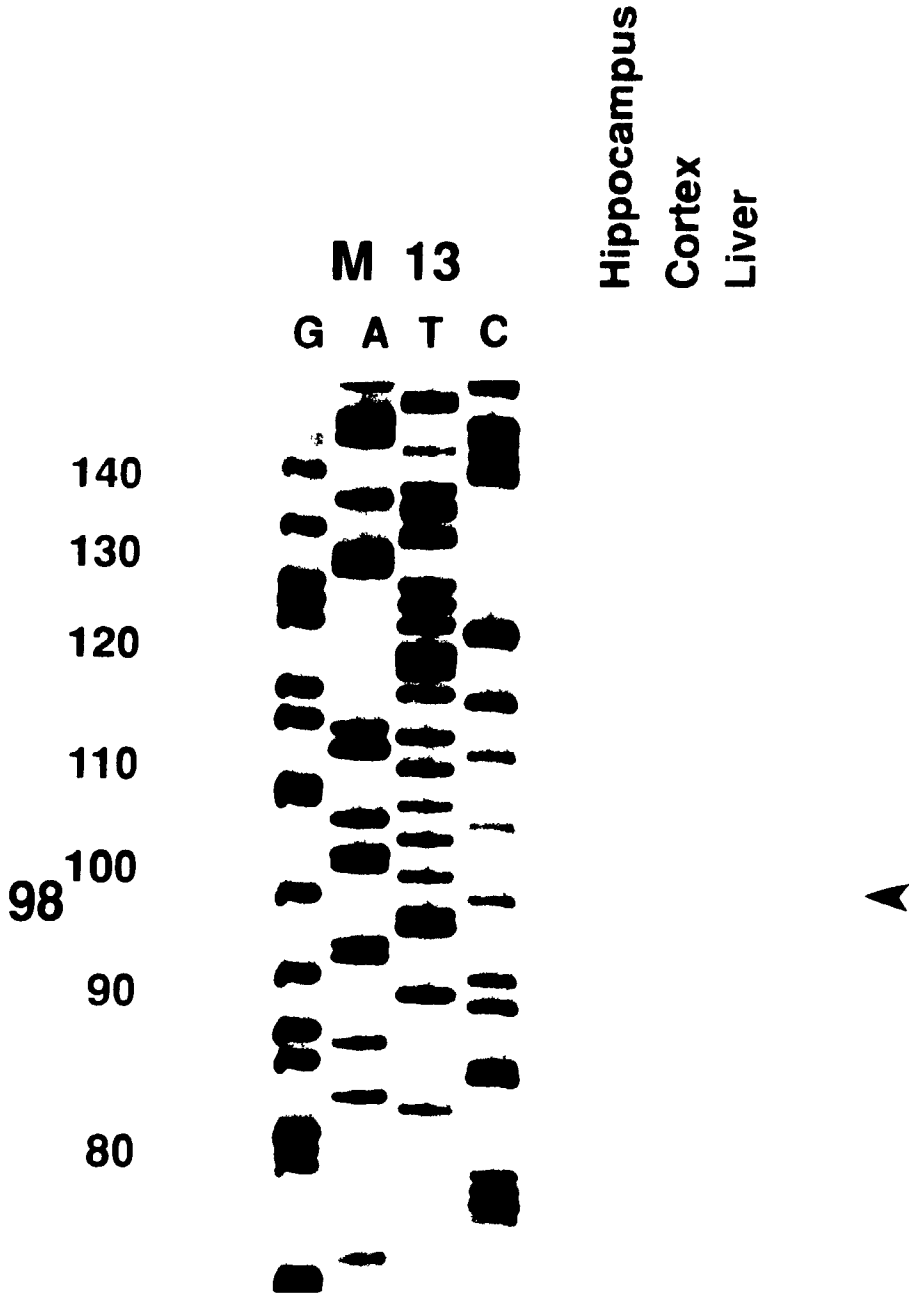
M 13
G A T C
Hippocampus
Cortex
Liver
140
130
120
110
100
98
90
80

Figure 4

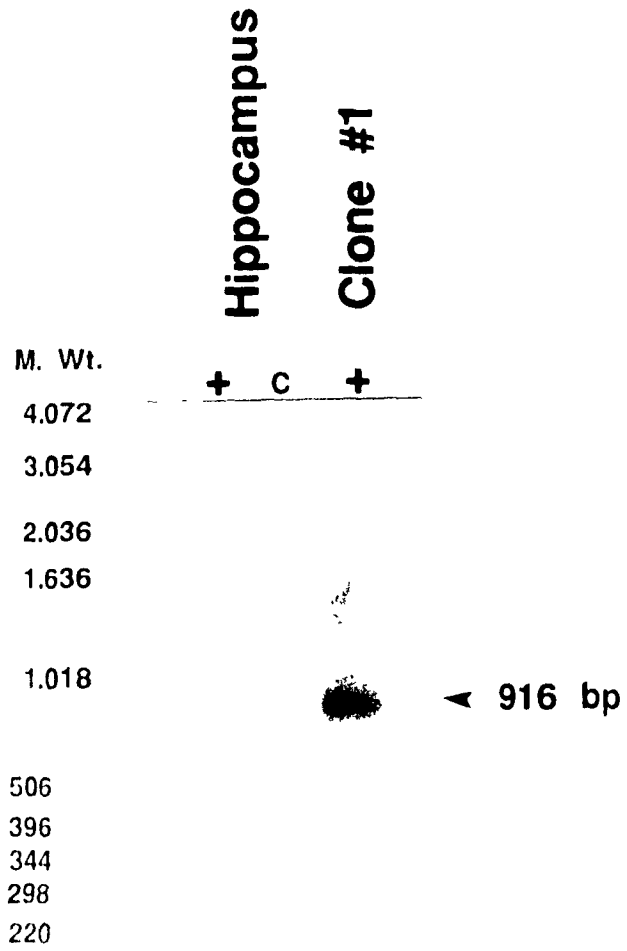
Hippocampus
Clone #1
M. Wt.
+
C
+
4.072
3.054
2.036
1.636
1.018
916 bp
506
396
344
298
220

Figure 5A

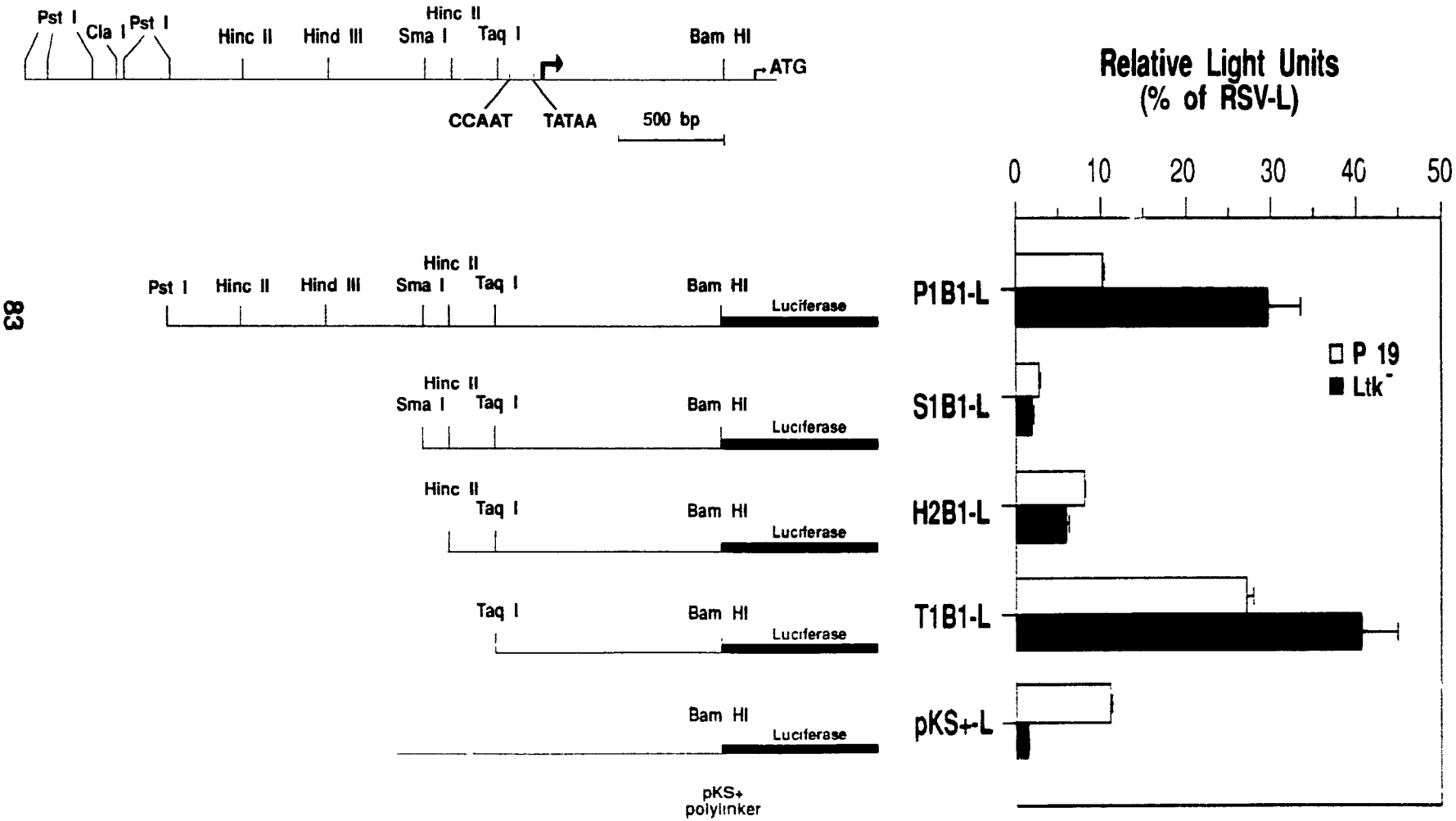
Pst I
Cla I
Pst I
Hinc II
Hind III
Hinc II
Sma I
Taq I
Bam HI
ATG
CCAAT
TATAA
500 bp
Luciferase
pKS+
polylinker
Relative Light Units
(% of RSV-L)
0
10
20
30
40
50
P1B1-L
S1B1-L
H2B1-L
T1B1-L
pKS+-L
P 19
Ltk-

Figure 5B

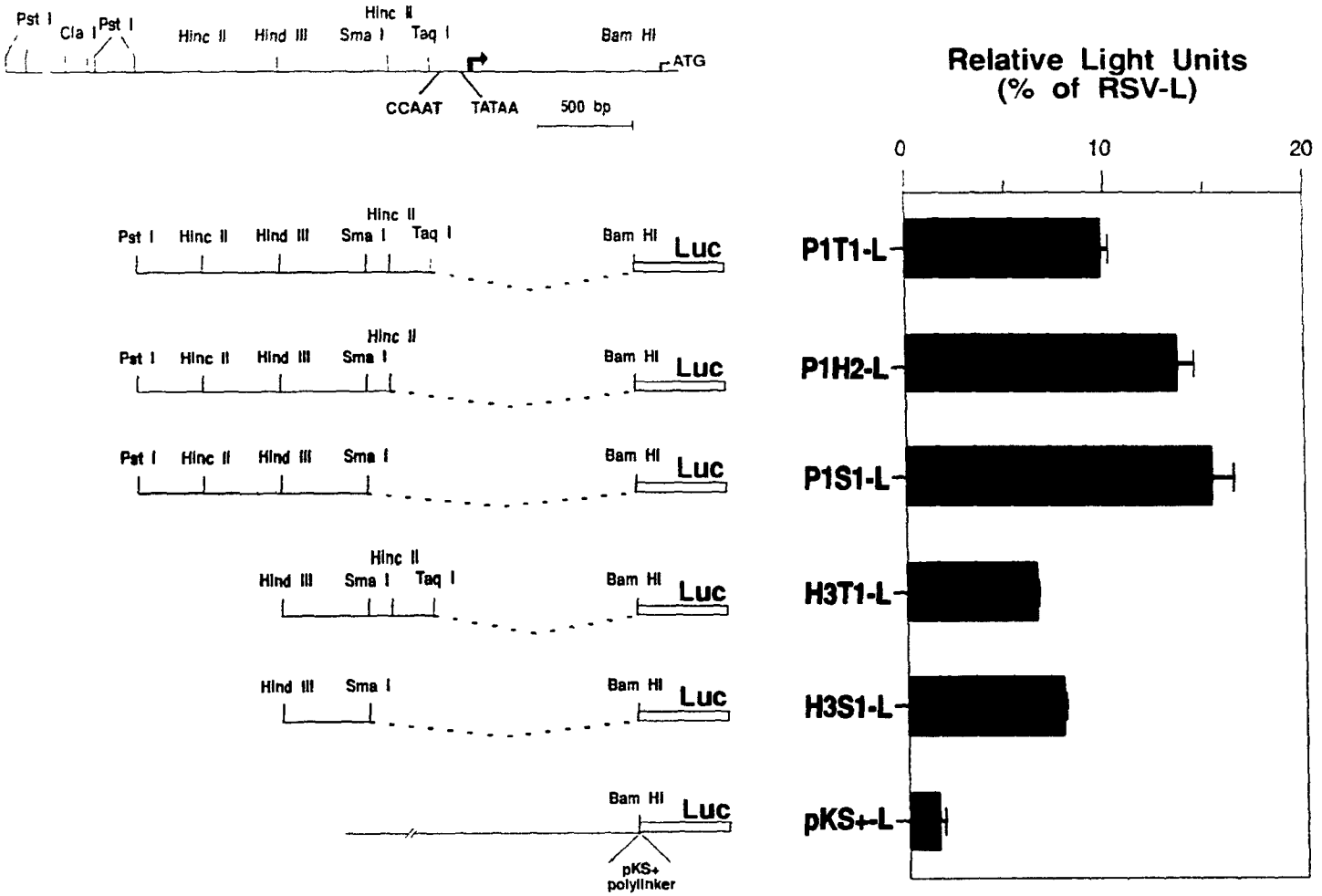
Pst I
Cla I
Pst I
Hinc II
Hind III
Hinc II
Sma I
Taq I
Bam HI
ATG
CCAAT
TATAA
500 bp
Hinc II
Pst I
Hinc II
Hind III
Sma I
Taq I
Bam HI
Luc
Hinc II
Pst I
Hinc II
Hind III
Sma I
Bam HI
Luc
Pst I
Hinc II
Hind III
Sma I
Bam HI
Luc
Hinc II
Hind III
Sma I
Taq I
Bam HI
Luc
Hind III
Sma I
Bam HI
Luc
Bam HI
Luc
pKS+
polylinker
Relative Light Units
(% of RSV-L)
0
10
20
P1T1-L
P1H2-L
P1S1-L
H3T1-L
H3S1-L
pKS+-L

Figure 6A

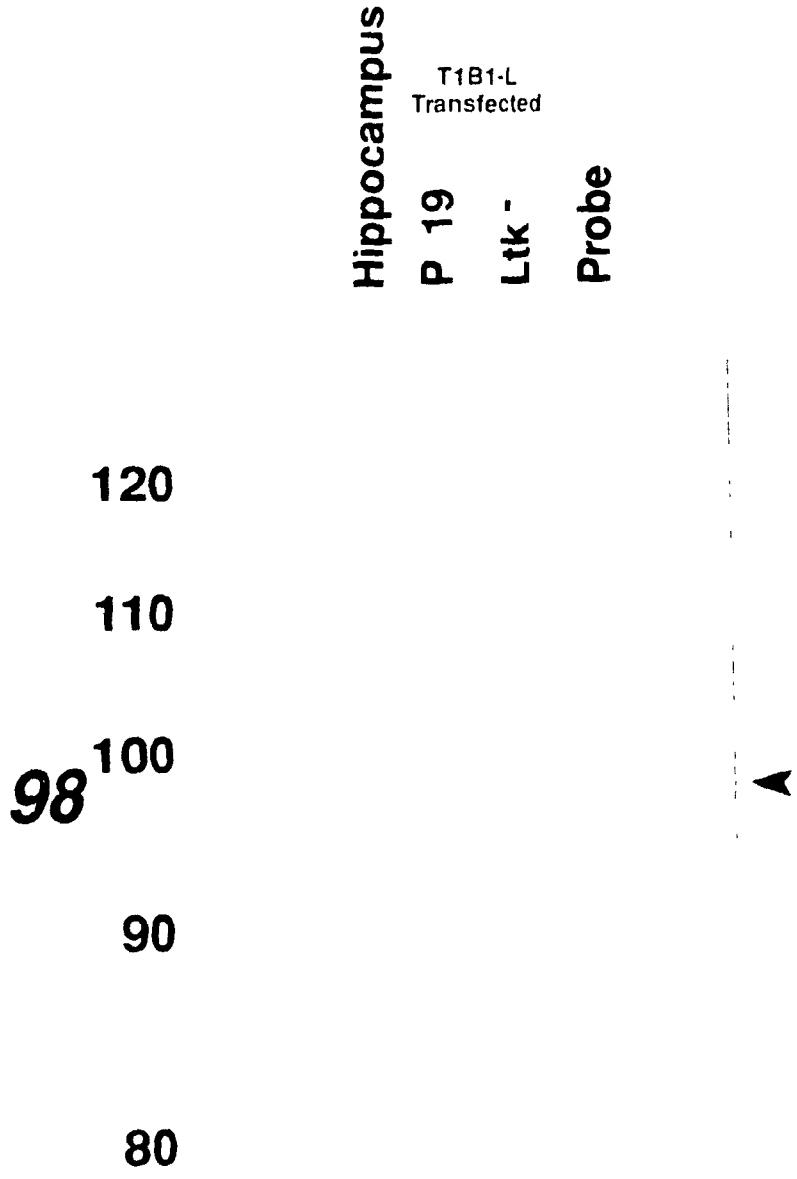
Hippocampus
T1B1-L
Transfected
P 19
Ltk -
Probe
120
110
100
98
90
80

Figure 6B

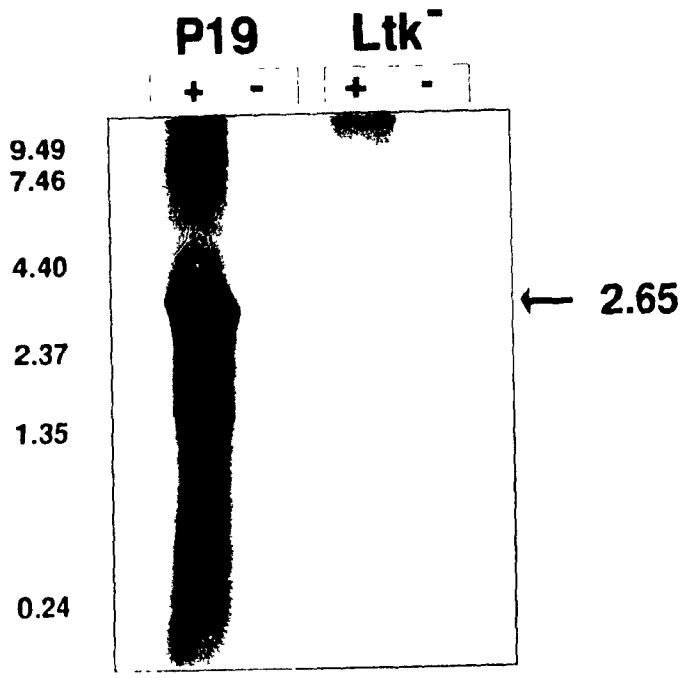

Figure 7

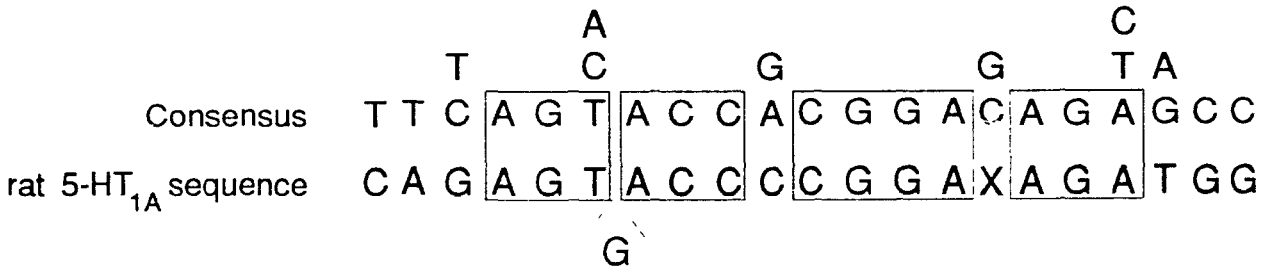
A
C
T
C
G
G
T A
Consensus
T T C A G T A C C A C G G A C A G A G C C
rat 5-HT1A sequence
C A G A G T A C C C C G G A X A G A T G G
G

Table I. Homologies between known and characterized regulatory consensus sequences and the rat 5-HT receptor gene 5'-region sequences.

AP-1	TGAG/CTC/AA
	-3282 TGAGTCA -3276
	-436 TGAGTCA -430
AP-2	CCCA/CNG/CG/CG/C
	-3245 CCCAGCCG -3238
CCAGG	CCAGG
	-1945 CCAGG -1941
	-1019 CCAGG -1086
	-444 CCAGG -440
CREB	TGACGC/TC/AG/A
	-2795 TGACGCCG -2788
EGR-1	CGCCCC/GCGC
	-3068 CGCCCGCGC -3060
GATA-3	T/AGATAG/A
	-3056 TGATAA -3051
	-1986 TGATAG -1981
	-1656 AGATAG -1651
SP-1	G/AGGCG/TG/A
	-2774 GGGCGG -2769
	-673 GGGCGG -668

CHAPTER II

Cloning of the Murine Serotonin$_{1A}$ Receptor cDNA and Functional Characterization in a Septal Cell Line.

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[¶]Funded by US PHS NS-25787

[§]Supported by the Medical Research Council of Canada, and recipient of Chercheur Boursier of the FRSQ, Quebec. To whom correspondence should be addressed.

[1]The abbreviations used are: 5-HT, 5-hydroxytryptamine (serotonin); 8-OH-DPAT, 8-OH-2-(di-*n*-propylamino) 1,2,3,4-tetrahydronaphthalene; bp, base pair(s); Kb, kilobase(s); RT-PCR, reverse transcription-polymerase chain reaction; cAMP,3', 5'-cyclic adenosine monophosphate; BSA, bovine serum albumin; RA, retinoic acid; PTx, pertussis toxin; VIP, vasoactive intestinal peptide; PGE_2, prostaglandin E_2; K_D, affinity constant.

Abstract

Using a reverse transcription-polymerase chain reaction (RT-PCR) approach, we have identified a mouse septum X neuroblastoma fusion cell line (named SN-48) which endogeneously express low amount of 5-hydroxytryptamine$_{1A}$ (5-HT$_{1A}$) receptor mRNA. These cells proliferate in a non-differentiated state, but differentiate into neurofilament-positive cells following 24-96 h treatment with 10 μM retinoic acid in low serum Northern blot analysis using a 5-HT$_{1A}$ receptor probe demonstrated a single 10 9 kilo base (Kb) mRNA species which was not detected in undifferentiated SN-48 cells, but clearly present in cells which were morphologically differentiated with retinoic acid Elevation in receptor message induced by differentiation correlates with the appearance of functional responses to 5-HT$_{1A}$ agonists. In undifferentiated SN-48 cells, 8-OH-2-(di-*n* propylamino) 1,2,3,4-tetrahydronaphthalene (8-OH-DPAT) had no effect on basal, VIP- or PGE$_2$-stimulated cAMP accumulation. However, in RA-differentiated cells, 8-OH-DPAT inhibited VIP- and PGE$_2$-stimulated levels of cAMP by > 50 % with an EC$_{50}$ of 10-20 nM. This inhibitory action of 8-OH-DPAT on cAMP accumulation was completely abolished by 16 h pretreatment of the cells with Pertussis Toxin (PTx) (250ng/ml). These observations are consistent with the known pharmacology of 5-HT$_{1A}$ receptors. In order to verify that the large 10 9 Kb species in SN-48 truly corresponded to the mouse 5-HT$_{1A}$ receptor mRNA, a RT-PCR-derived fragment of the 5-HT$_{1A}$ receptor mRNA obtained from the differentiated SN-48 cells, was used to probe a mouse brain cDNA library. A single 2 4 Kb cDNA clone having a predicted amino acid sequence with high homology to the human (86%) and rat (94%) 5-HT$_{1A}$ receptor genes was isolated. Radioligand binding studies on membranes derived from mouse Ltk$^-$ fibroblast cells stably-transfected with the 2 4 Kb cDNA in eukaryotic expression vector displayed a pharmacology specific to 5-HT$_{1A}$ receptors, indicating that the 2.4 Kb cDNA clone encodes the murine 5-HT$_{1A}$

receptor Since the sequences of the cDNA cloned matched the PCR fragments isolated from SN-48 cells, we conclude that upon differentiation, the SN-48 cell express RNA encoding functional 5-HT_{1A} receptors The SN-48 septal cells provide a cellular model system for investigating aspects of neuronal differentiation leading to the development of sensitivity to serotonergic input.

Introduction

5-hydroxytryptamine (serotonin, 5-HT^1) is a neurotransmitter believed to play a role in various cognitive functions such as feeding, sleep, pain, depression, learning and anxiety (Blier et al., 1987; Curzon, 1992, Charney et al., 1990). This diversity of actions can be related to the fact that serotonergic neurons send afferents to almost every structure of the brain and especially to the limbic regions (Törk, 1990) Serotonin actions are mediated via activation of a growing family of serotonin receptors This receptor family has been divided into four classes designated 5-HT_1, 5-HT_2, 5-HT_3 (Julius, 1991) and 5-HT_4 (Bockaert et al , 1992) on the basis of pharmacological properties, intracellular signaling systems, structural homology and tissue distribution. The 5-HT_1 group is the most heterogeneous and is divided into at least four subtypes; 5-HT_{1A} (Fargin et al , 1988, Kobilka et al , 1987; Albert et al., 1990), 5-HT_{1B} (Maroteaux et al , 1992; Voigt et al , 1991), 5-HT_{1D} (Libert et al., 1989; Hamblin and Metcalf, 1991, Demchyshyn et al., 1992), and 5-HT_{1E} (McAllister et al , 1992; Zgombick et al., 1992) of which the 5-HT_{1A} subtype has been the most extensively studied for two reasons First, the availability of a highly selective radioligand (8-OH-DPAT) which allow an extensive biochemical, physiological and pharmacological characterization of the receptors (Hamon et al , 1987), second, the observation that certain 5-HT_{1A} agonists exert anxiolytic and antidepressant effects in clinical paradigms (Blier et al , 1987, Blier et al , 1990a).

5-HT_{1A} receptors are expressed in discrete neuronal cell type within the mammalian central nervous system In fact, radioligand binding studies correlated with in situ hybridisation (Pompeiano et al , 1992) indicates that the highest level of expression of the receptor is found in structures such as the dorsal raphe nucleus, septum, hippocampal formation (especially the pyramidal neurons of the CA1 region and the dentate gyrus), and the entorhinal cortex The 5-HT_{1A} receptor has been shown to couple negatively to adenylyl cyclase through pertussis toxin (PTx) sensitive G proteins (Zgombick et al , 1989, Albert et al , 1990) and positively to potassium channels through a different PTx sensitive $G_{i/o}$ protein (Blier et al., 1990b, Williams et al , 1988; Innis et al , 1988, Innis and Aghajanian, 1987), the latter process inducing membrane hyperpolarization, causing a decrease in neuronal firing rate.

To avoid problems of heterogeneity and complexity associated with most brain preparations, neuronal cell lines provide an abundant and homogeneous source of neurons These intrinsic features of neuronal cell lines facilitate the biochemical analysis of various neuron-specific proteins in their natural environment (Choi et al , 1992, Blusztajn et al , 1992) However, none of these cell lines have been reported to express functional 5-HT_{1A} receptors Recent progress has led to the construction of cell lines derived from different regions and developmental stages of the central nervous system (Lee et al , 1990a, b, Fredericksen et al., 1988; Morimoto and Koshland, 1990, Renfranz et al , 1991, Hales et al., 1992). We took advantage of hybrid cell lines derived from pre- and postembryonal hippocampus and septum, areas richest in 5-HT_{1A} receptors, to screen for cell lines which express the 5-HT_{1A} receptor

We report the characterization of a 5-HT_{1A} receptor from a hybrid cell line (SN-48) derived from 21-day postnatal mouse septal neurons fused with a murine neuroblastoma ($N18TG_2$). We also describe the cloning, molecular and pharmacological characterization of the mouse 5-HT_{1A} receptor cDNA

Materials and Methods

Cell culture- SN-48 (Lee et al., 1990b) fusion cells were maintained in Dubelco's modified Eagle's medium (D-MEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) (Gibco), 2 mM glutamine (Gibco) and antibiotics (Gibco) at 37° C in 5% CO_2. Differentiation was acheived by plating the cells at a density of 10^4 cells/cm^2 in D-MEM supplemented with 1% (v/v) heat-inactivated FCS, 10 µM retinoic acid (all *trans*-) (Sigma, St-Louis MI) and 2 mM glutamine. Ltk$^-$ mouse fibroblast cells were maintained in α-modified Eagle's medium (α-MEM) supplemented with 10% (v/v) heat-inactivated FCS and antibiotics at 37° C in 5% CO_2.

Cloning of the Mouse 5-HT$_{1A}$ receptor gene- The 918 bp fragment from the differentiated SN-48 5-HT$_{1A}$ mRNA obtained by RT-PCR was as follow: total RNA was isolated from 48 h differentiated SN-48 by a method previously described (Sambrook et al., 1989). 0.3 µg of this RNA was reverse transcribed with random primers (Pharmacia) by the Superscript reverse transcriptase following the supplier's specifications (BRL). An aliquot of the resulting cDNA was used as template during the subsequent PCR amplification with sense oligonucleotide (#45, 5' GTGCTGGTGCTGCCCATGG 3') and antisense oligonucleotide (#44, 5' GGGAGTTGGAGTAGCCTAGCC 3') derived from sequences of the 2nd and 7th predicted transmembrane domains of the rat and human 5-HT$_{1A}$ receptor genes The 918 bp product of the amplification was isolated, and cloned into the Eco RV site of pBluescript KS+ (Stratagene). This 918 bp fragment was then radiolabeled using a random primed labeling kit (Bhoeringer Manheim). An 18-day old mouse λgt11 brain cDNA library (generously provided by Dr. J.-P. Julien, McGill University, Montreal) was probed at high stringency with the radiolabeled 918 bp fragment as described elsewhere (Sambrook et al., 1989) and a single clone (clone A) containing a 2.4 Kb cDNA insert was isolated and subcloned into the Eco

RI site of pBluescript KS+. Both strands of the cDNA insert were sequenced by the Sanger dideoxytermination method using a T_7 polymerse-based DNA sequencing kit (Pharmacia).

Expression of Clone A in Ltk⁻ cells- The 2.4 Kb Eco RI fragment of clone A was subcloned into Eco RI-cut pcDNA mammalian expressing vector (Invitrogene) and 20 µg of it co-transfected by the calcium phosphate method (Sambrook et al., 1989) with 2 µg of pRSV-*neo* plasmid. Clones were selected in medium containing 500 µg/ml of G418 (Geneticin, Gibco) and screened for expression of the transfected 5-HT_{1A} receptor by RT-PCR. Positive clones were further screened for level of functional receptors by measurement of Ca^{2+} release from intracellular pools upon activation of the receptors as described previously (Liu and Albert, 1991). One clone (LM1A-5) was isolated for further characterization.

Radioligand binding studies- Membranes from the positive LM1A-5 clone were harvested and assayed for binding as described previously (Albert et al., 1990). The results were analysed by the LIGAND computer software (Biosoft, Cambridge, UK).

cAMP assay- Measurement of cAMP was performed as described elsewhere (Albert et al., 1990). In brief, SN-48 cells were differentiated in six-well 35-mm dishes; medium was changed 24h prior to experimentation. After removal of the medium, cells were incubated in 1 mL of serum-free medium containing 100 µM IBMX (Sigma, St-Louis, MI) with the various test compounds for 20 min at room temperature. The medium was discarded and the cells were lysed by addition of 1 ml of serum-free medium containing 0.05 % of Triton X-100 and 100 µM IBMX The media were collected, centrifuged (2000 g x 5 min) and the supernatant assayed for cAMP. cAMP was assayed by a specific radioimmunoassay (ICN) as described previously (Albert et al., 1990) with antibody used at 1:500 dilution After 16h of incubation at 4° C, 20 µL of 10% BSA and 1 mL of ice-cold 95% ethanol

were added sequentially to precipitate the antibody-antigen complex. Data is represented as mean ± S.E.M. for triplicate wells.

Northern blot analysis- Total RNA (extracted from differentiated and non-differentiated SN-48 cells, and from LM1A-5 cells), poly A^+ selection of mRNA species and northern blot analysis were performed as described before (Sambrook et al , 1989).

Results and Discussion

A neuroblastoma X septum hybrid cell line expressing the 5-HT_{1A} receptor

The characterization of neuronal hybrid cells derived from the fusion of primary cultures of pre- and postnatal hippocampal and septal neurons with a neuroblastoma cell line ($N18TG_2$) has been described (Lee et al., 1990a,b). An intrinsic feature of these cell lines is the potential inducibility of certain phenotypic neuronal markers (e. g. neurofilaments) upon treatment of the cells with differentitation-inducing agents (e.g. Retinoic acid (RA) or dibutiryl cAMP). Although the mechanisms underlying this phenomenon are unknown and likely to involve complex systems, inducibility of neuronal phenotype by chemical inducers is a well established property of various systems (Jones-Villeneuve et al., 1982; McBurney et al., 1988; Rudnicki and McBurney, 1987; Hammond et al., 1986). Several murine hybrid cell lines were screened by RT-PCR for the presence of mRNA coding for the 5-HT_{1A} receptor. Of the different hybrid clones screened, only one (SN-48) showed an increased level of 5-HT_{1A} receptor gene expression in the differentiated state (RA-treated) over the non differentiated one (data not shown). The SN-48 cell line is derived from a 21 day postnatal C57BL/6 mouse primary culture of septal neurons fused with the $N18TG_2$ neuroblastoma cell line (Lee et al., 1990b) and appears to be endowed with GABAergic properties (D. N. Hammond, personal communication). The SN-48 cell line was chosen for further

characterization of the mouse 5-HT_{1A} receptors.

Correlation of neuronal phenotype and 5-HT_{1A} receptor expression

In the non-differentiated state, the SN-48 cells are rapidly dividing and do not exhibit morphological features of neurons (see figure 1A) However, forty-eight hour treatment with 10 μM RA (see "Materials and Methods") resulted in neuronal differentiation, as assessed by the appearance of long, axon-like processes (see figure 1B). Since the concentration of RA used was not toxic to the cells, the differentiation observed is induced and not due to selection of a minor population of differentiated cells pre-existing within the undifferentiated population Concomitant with this morphological change, the appearance of mRNA encoding for the 5-HT_{1A} receptor was observed by northern blot analysis of RNA harvested from differentiated for 48h and from non-differentiated SN-48 cells (see figure 2) The temporal association of 5-HT_{1A} receptor expression with the development of the neuronal phenotype suggests that the increase in 5-HT_{1A} receptor message may be due to indirect actions of RA which result in a phenotypic differentiation of the cells into neurons capable of 5-HT_{1A} receptor expression, although direct action of RA on receptor expression cannot be ruled out. The size of the 5-HT_{1A} receptor mRNA present in the differentiated SN-48 cells, which is 10 9 Kb in length is larger than species observed for the rat and human receptors Northern blot analysis of rat brain tissues detects three message sizes (3 9, 3 6, and 3 3 Kb) encoding the 5-HT_{1A} receptor and analysis of fetal human peripheral tissues demonstrates a single receptor mRNA species of ~6.0 Kb which is weakly expressed in lymphoid tissues (Albert et al., 1990, Kobilka et al , 1987) These differences in size between the three species is most likely the result of variation in the length of 5'- and 3'-untranslated regions.

Functional characterization of the 5-HT_{1A} receptors

We determined whether the increase in 5-HT_{1A} receptor mRNA which precedes morphologic neuronal differentiation was associated with expression of function responses to 5-HT_{1A} receptor activation in SN-48 cells by measuring effects on intracellular cAMP accumulation (see "Materials and Methods"). In the non differentiated SN-48 cells, both VIP and PGE_2 markedly elevated basal cAMP levels, but 8-OH-DPAT (a selective 5-HT_{1A} agonist) (10 μM) had no effect on basal, VIP- or PGE_2-stimulated cAMP accumulation (figure 3A). This suggests that 5-HT_{1A} receptors are not expressed by undifferentiated SN-48 cells, consistent with the lack of 5-HT_{1A} receptor mRNA in northern blot analysis (see figure 2). In contrast, the undifferentiated cells apparently express PGE_2 and VIP receptors. However, in the differentiated state, 8-OH-DPAT significantly decreased both the VIP- and the PGE_2-stimulated levels of cAMP by 54% and 37% respectively (figure 3A). This inhibition is mediated through Pertussis toxin (PTx) sensitive G proteins ($G_{i/o}$) since 16 h pretreatment of the differentiated SN-48 cells with 250 ng/ml of PTx completely abolish the 8-OH-DPAT mediated inhibition of stimulated cAMP accumulation (figure 3A). PTx pretreatment did not, however, exert any effect in the undifferentiated cells (data not shown).

In order to strengthen the specificity of 8-OH-DPAT mediated actions, a dose-response experiment was conducted on differentiated SN-48 cells. Different concentrations of 8-OH-DPAT were tested for their potency to inhibit PGE_2-stimulated cAMP levels (figure 3B). An EC_{50} of 10-20 nM was estimated, consistent with a previously reported value of 10 nM from mouse hippocampal membrane preparations (Dumuis et al,.1988). At low concentrations (1 μM) 8-OH-DPAT appeared to enhance slightly PGE_2-induced cAMP accumulation, however this was not statistically significant.

These results correlate with the increase in 5-HT_{1A} receptor mRNA levels, and strongly suggest that the differentiated SN-48 cells express functional 5-HT_{1A} receptors which are coupled to PTx-sensitive G proteins to inhibit adenylyl cyclase. The GABAergic phenotype of these cells is consistent with the recent observation that 5-HT_{1A} receptors are located on certain GABAergic neurons of the rat septum

(Pompeiano et al., 1992), the neurons from which the SN-48 cells may be derived.

Cloning the mouse 5-HT_{1A} receptor cDNA

Because of the large size of the mouse 5-HT_{1A} receptor mRNA, we wished to determine the nature of the coding sequence in SN-48 and mouse brain A 918 bp fragment of the 5-HT_{1A} receptor mRNA from the differentiated SN-48 cell population was isolated by RT-PCR as described under "Materials and Methods". Sequence analysis of the cloned fragment showed striking similarity with corresponding regions of the rat and human 5-HT_{1A} receptor genes and identical homology to an analogously isolated fragment from mouse hippocampus total RNA (data not shown). Using this fragment as a probe, an 18-day old mouse brain λgt11 cDNA library was screened under high stringency and a single clone containing a 2.4-Kb cDNA insert (clone A) was isolated and sequenced (figure 4)

Analysis of the cDNA clone A revealed a corresponding region with identity (100%) to the sequences derived from the mouse brain and differentiated SN48 cells 918 bp RT-PCR fragment (data not shown). Overall sequence homology with the rat and human 5-HT_{1A} receptor gene (nucleotide/amino acid) is as follows. mouse-rat: 93%/ 94%; mouse-human: 80%/ 86%. The sequence of clone A contained a single open reading frame encoding a 421-amino acid residue protein of predicted molecular weight of 46 KDa. Computer-aided hydrophobicity analysis of the predicted amino acid composition of clone A indicates a seven transmembrane α helical topology (figure 5A and 5B) consistent with the structure predicted for G protein-coupled receptors (Caron and Lefkowitz, 1991) The strong sequence similarity of clone A with the rat and human 5-HT_{1A} receptors suggest that clone A encodes the mouse 5-HT_{1A} receptor. In order to substantiate this hypothesis, radioligand binding assays were performed.

Ligand binding assay

Clone A was subcloned in an mammalian expression vector and stably expressed in mouse Ltk fibroblast cells as described under "Materials and Methods" Membranes prepared from the cloned cells LM1A-5, exhibited binding features characteristic of 5-HT_{1A} receptors. One-site model Scatchard analysis of saturation binding experiments using [^{3}H]8-OH-DPAT as ligand, indicate a K_D of 1 68 nM ± 0 36 (see figure 6). This calculated K_D for a single affinity state of the receptor is well within the range of those seen in membrane preparations of mammalian cells tranfected with the human and rat 5-HT_{1A} receptor genes (Fargin et al., 1988; Albert et al., 1990). Moreover, ligand competition binding experiments demonstrate an order of potency of the following drugs; 8-OH-DPAT>5-HT>spiroxatrine>spiperone, as observed for the rat and human 5-HT_{1A} receptors.

Conclusion

Based on the pharmacological profile exhibited by clone A when expressed heterologously in mammalian cultured cells, as well as its strong homology with the human and rat 5-HT_{1A} receptors, we conclude that clone A encodes the mouse 5-HT_{1A} receptor cDNA. In addition, we have identified an inducible neuronal cell line which expresses endogeneously the murine 5-HT_{1A} receptor upon morphological differentiation This cell line represents an excellent model system to investigate the natural environment in which the 5-HT_{1A} receptor exerts its actions. Moreover, the SN-48 cells provide a powerful paradigm for investigating the aspects of neuronal differentiation leading to the development of sensitivity to serotoninergic input through 5-HT_{1A} receptors.

Acknowledgments

We are indebted to Dr. Jean-Pierre Julien who kindly provided us with a 18-

day old mouse brain λgt11 cDNA library.

Figures legends

Figure 1. Morphological representations of SN-48 cells. A, non-differentiated SN-48 cells and B, 48h RA-differentiated SN-48. The differentiation procedure is described under "Materials and Methods".

Figure 2. Northern blot analysis of the mouse 5-HT_{1A} receptor from SN-48 cells. 10 µg of poly A^+ selected RNA isolated from differentiated and non differentiated SN-48 cells were loaded in the designated lanes. The blot was probed with ^{32}P-labeled cloned 2.4 Kb mouse 5-HT_{1A} receptor cDNA (clone A- see below) M wt standard (Gibco/BRL) is indicated at left.

Figure 3. 5-HT_{1A} mediated inhibition of stimulated cAMP accumulation A, 8-OH-DPAT mediated effects on stimulated cAMP levels from non differentiated, differentiated and PTx treated/differentiated SN-48 cells. The data are representated as fold increase over basal B, Dose-response relationship between different concentrations of 8-OH-DPAT and their corresponding effect on PGE_2-stimulated cAMP levels All the data are representative of three different experiments and the error bars represent ± S E M of triplicate samples

Figure 4. Restriction map and sequence of the mouse 5-HT_{1A} receptor cDNA A, restriction endonuclease map of the 2.4 Kb cDNA clone A isolated from a mouse brain λgt11 library. The shaded box represent the position of the coding sequences. B, nucleotide sequence of the mouse 5-HT_{1A} receptor cDNA Underlined are the putative seven transmembrane domains and capital bold underlined italic nucleotides represent a putative polyadenylation signal sequence

Figure 5 Analysis of the deduced amino acid sequence of the mouse 5-HT_{1A} receptor cDNA A, computer generated hydropathy plot of the mouse β_2-adrenergic (Nakada et al , 1989) and 5-HT_{1A} receptors. The analysis was performed on a 21 residues window as described by Kyte and Doolittle (Kyte and Doolittle, 1982) using the Intelligentics PC/GENE AASCALE program. The *y* axis represents hydrophobic moments and the *x* axis represent the amino acid composition. Bars represent the transmembrane domains. B, schematic representation of the mouse 5-HT_{1A} receptor. The amino acid residues which differs from the rat and/or human receptor are indicated. Putative N-linked glycosylation sites are indicated by small arboreous drawings.

Figure 6 Specific binding of [^{3}H]8-OH-DPAT to membranes from LM1A-5 cells. Saturation analysis of specific binding of [^{3}H]8-OH-DPAT to membranes prepared fron LM1A-5 cells The *abscissa* represent the free concentration of [^{3}H]8-OH-DPAT corrected by substracting the amount bound specifically to membranes. The *ordinate* is the amount of bound [^{3}H]8-OH-DPAT *Inset*, transformation of the above data for Scatchard analysis. The data was best fit by a one-site model (plotted line) with a K_D of 1 68 $\pm$ 0.36 nM and a B_{max} of 0.19 $\pm$ 0.05 pmol/mg protein.

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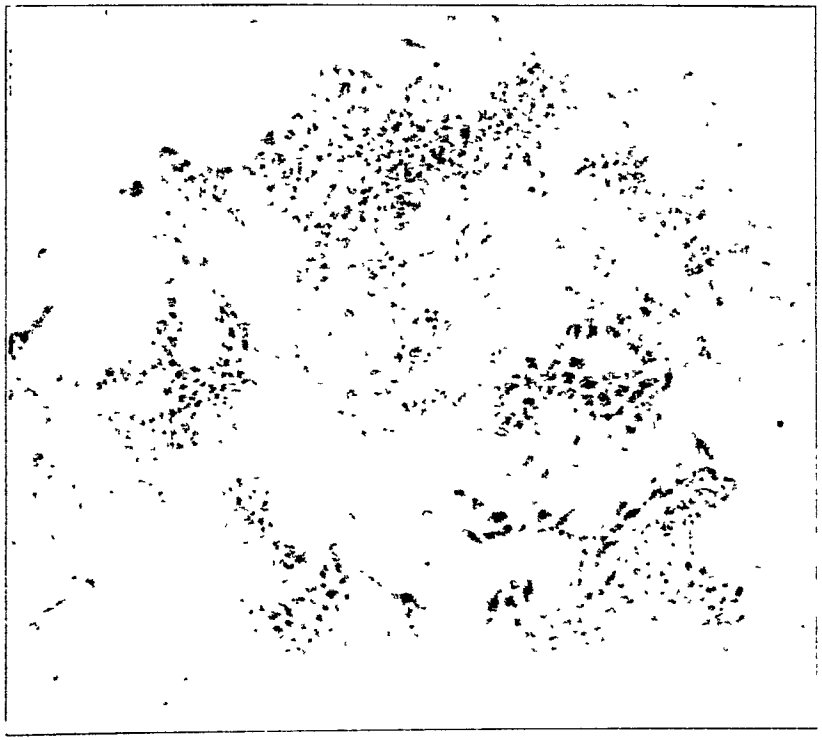
Figure 1

A



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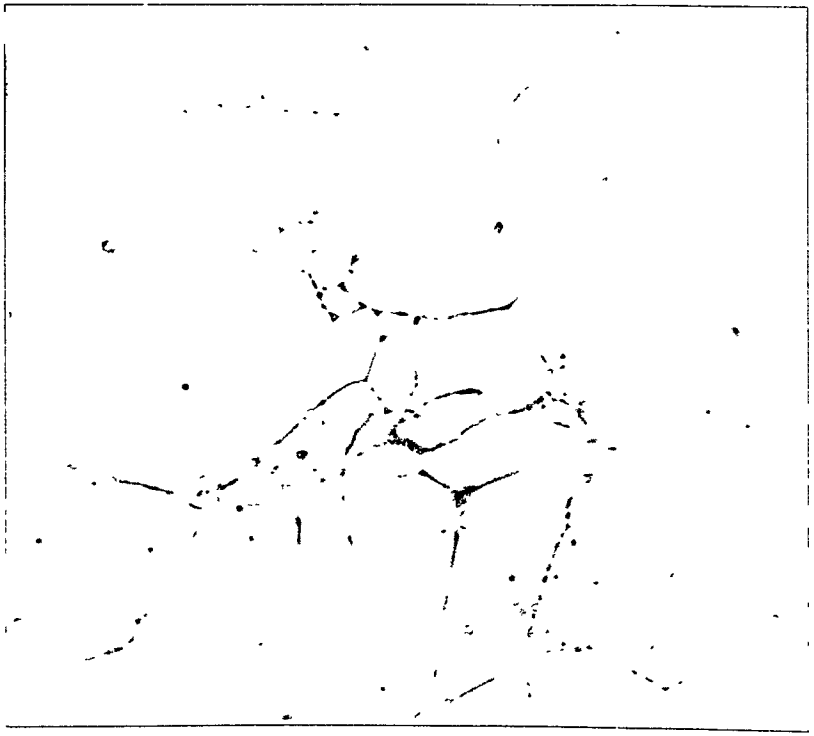

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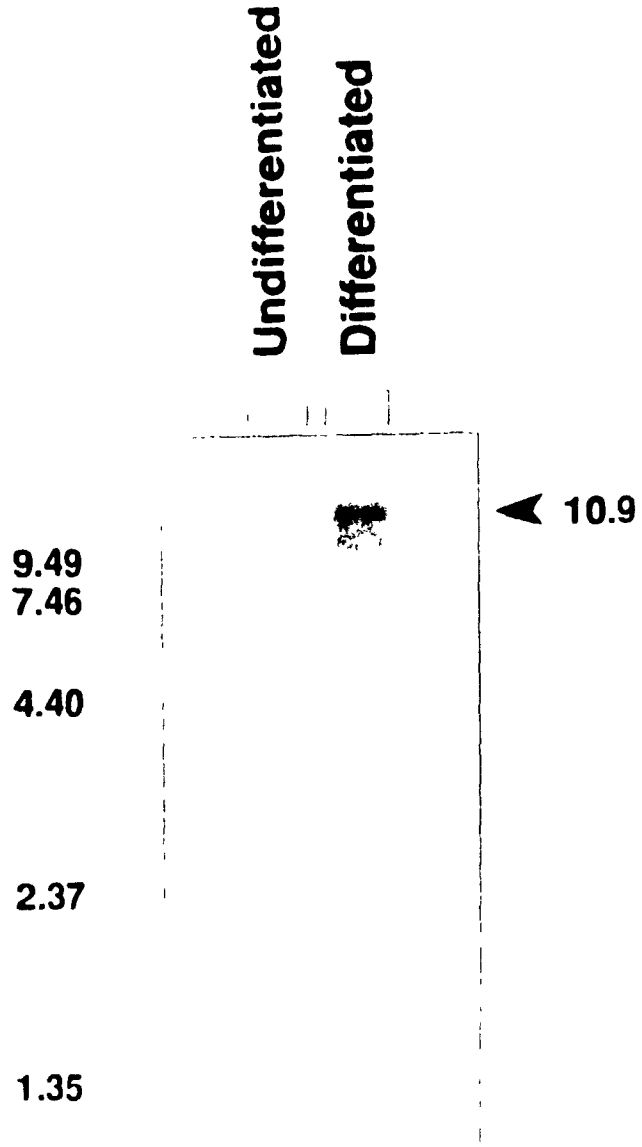

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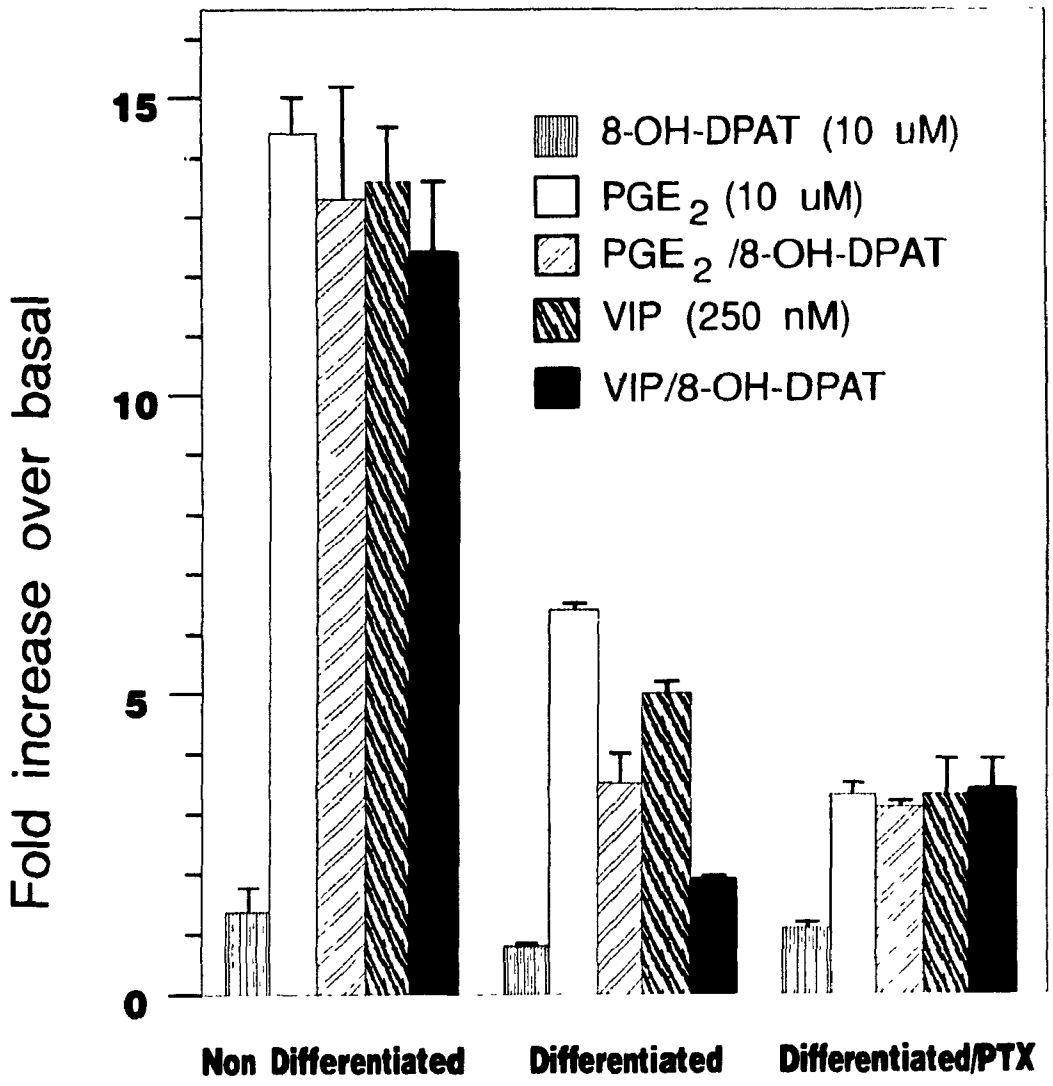
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10
5
0
8-OH-DPAT (10 uM)
PGE2 (10 uM)
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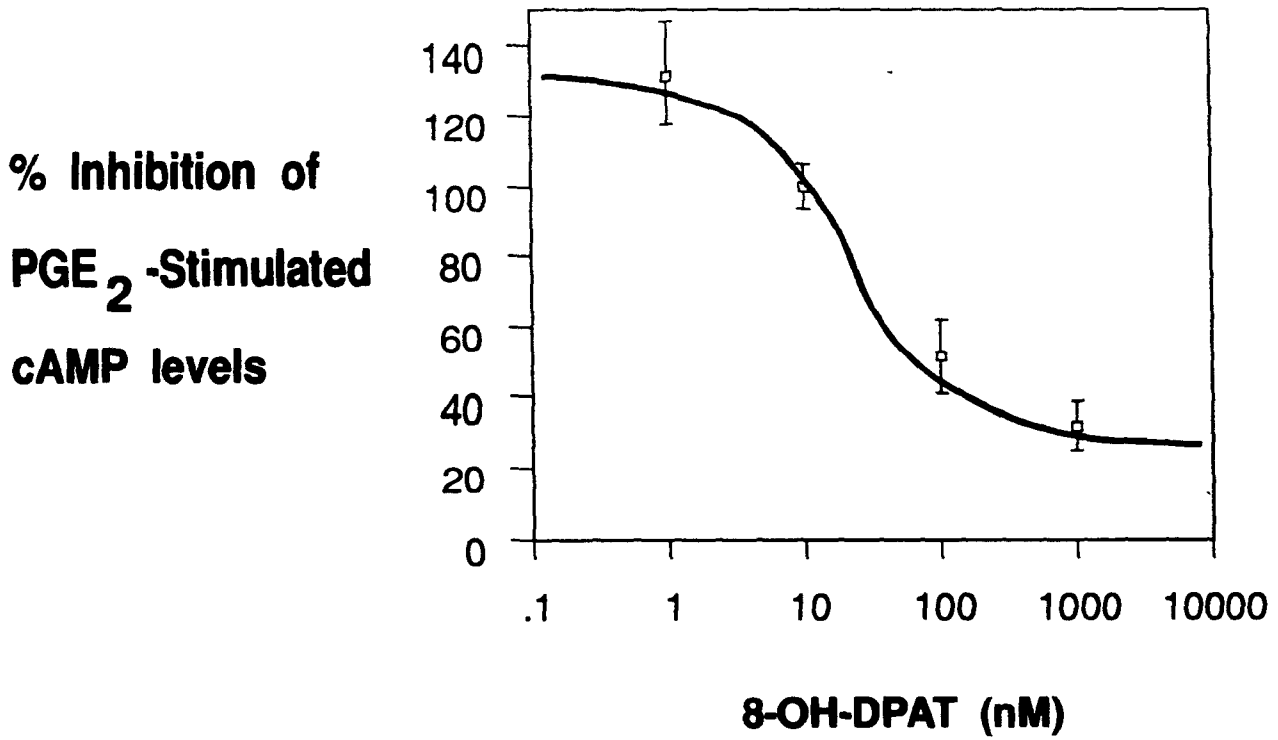
VIP (250 nM)
VIP/8-OH-DPAT
Non Differentiated
Differentiated
Differentiated/PTX

Figure 3B


% Inhibition of
PGE2-Stimulated
cAMP levels
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100
80
60
40
20
0
.1
1
10
100
1000
10000
8-OH-DPAT (nM)

Figure 4A

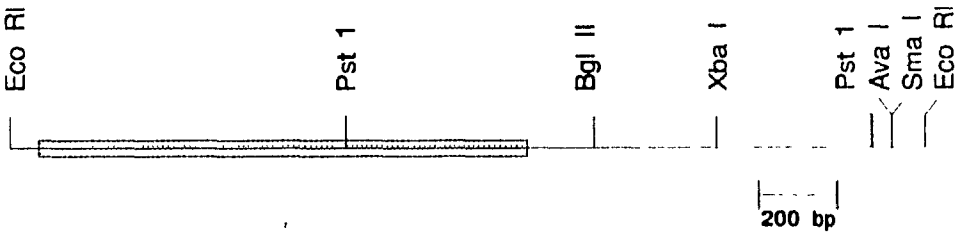
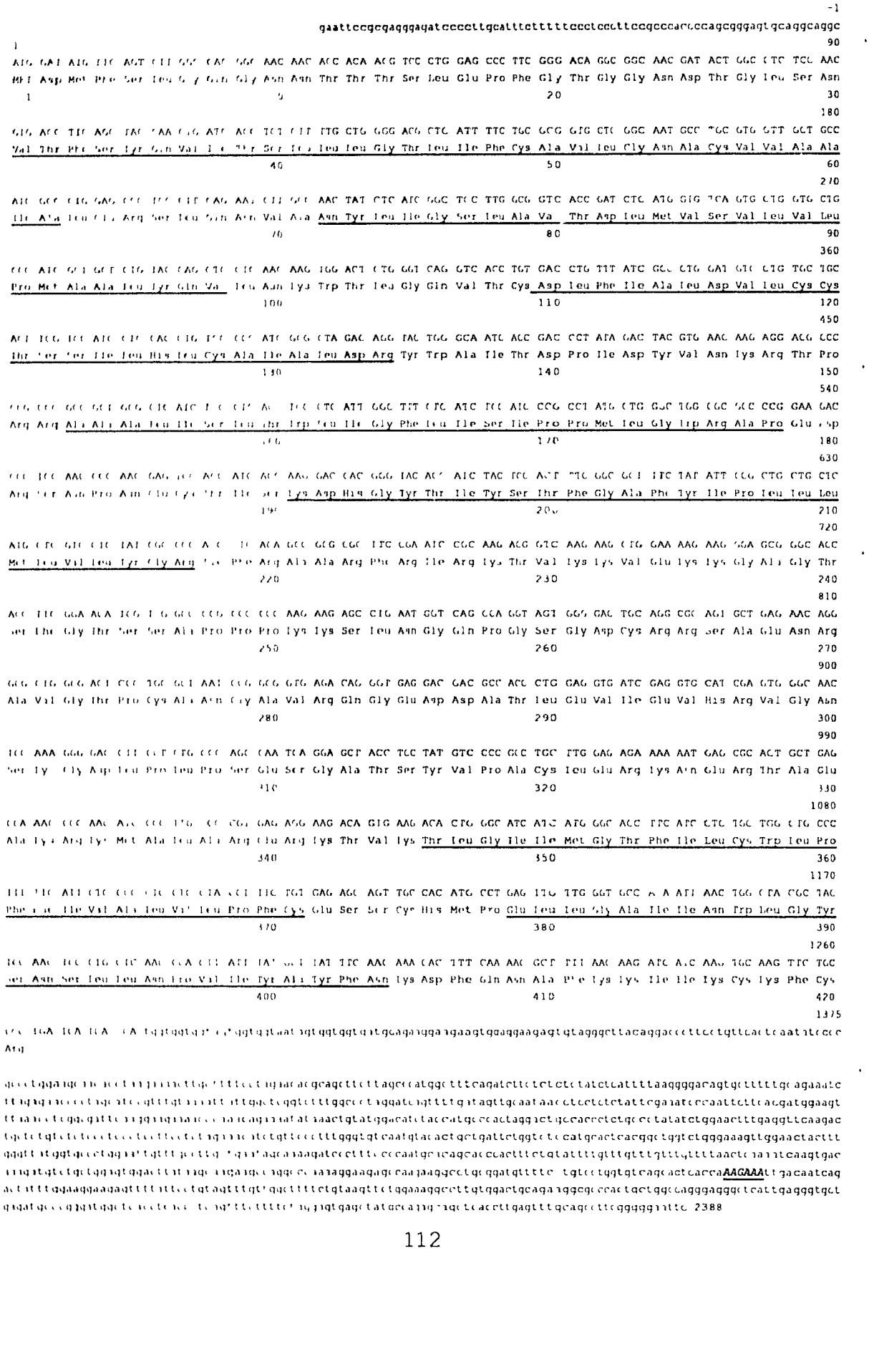


Figure 4B



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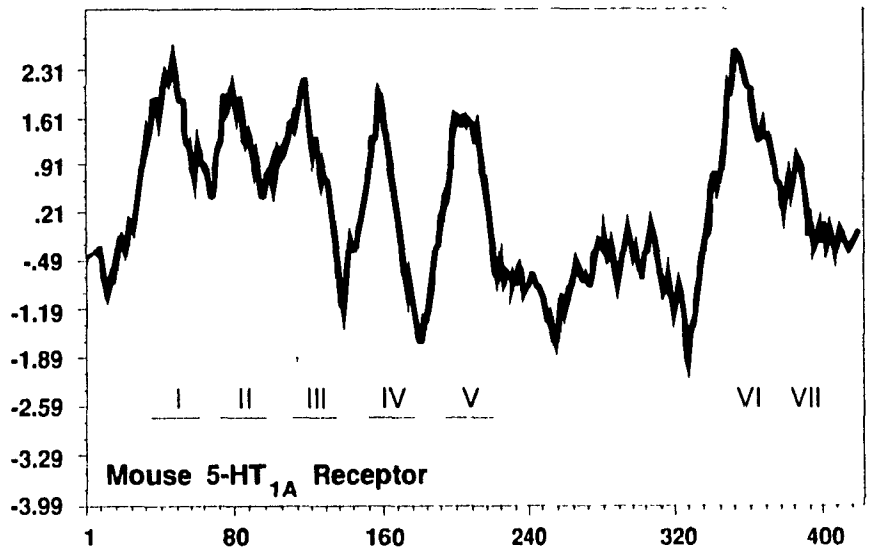
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I
II
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VII
Mouse 5-HT1A Receptor
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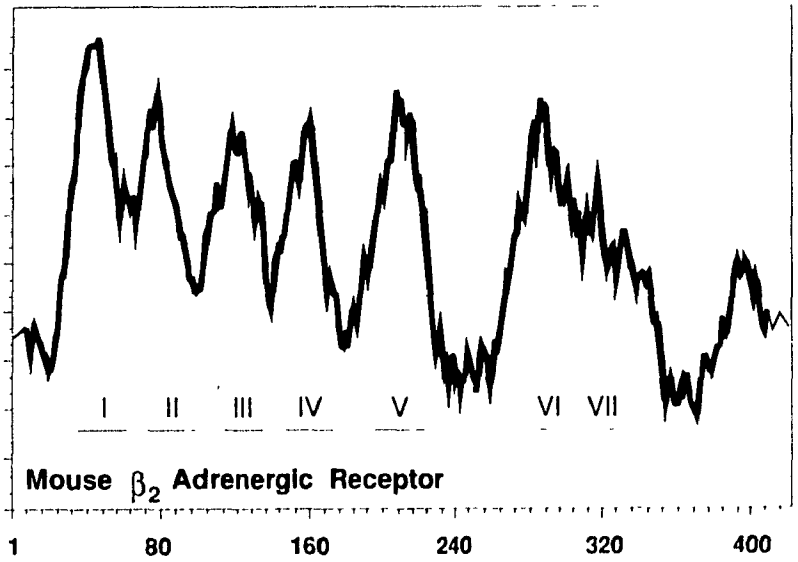
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Mouse β2 Adrenergic Receptor
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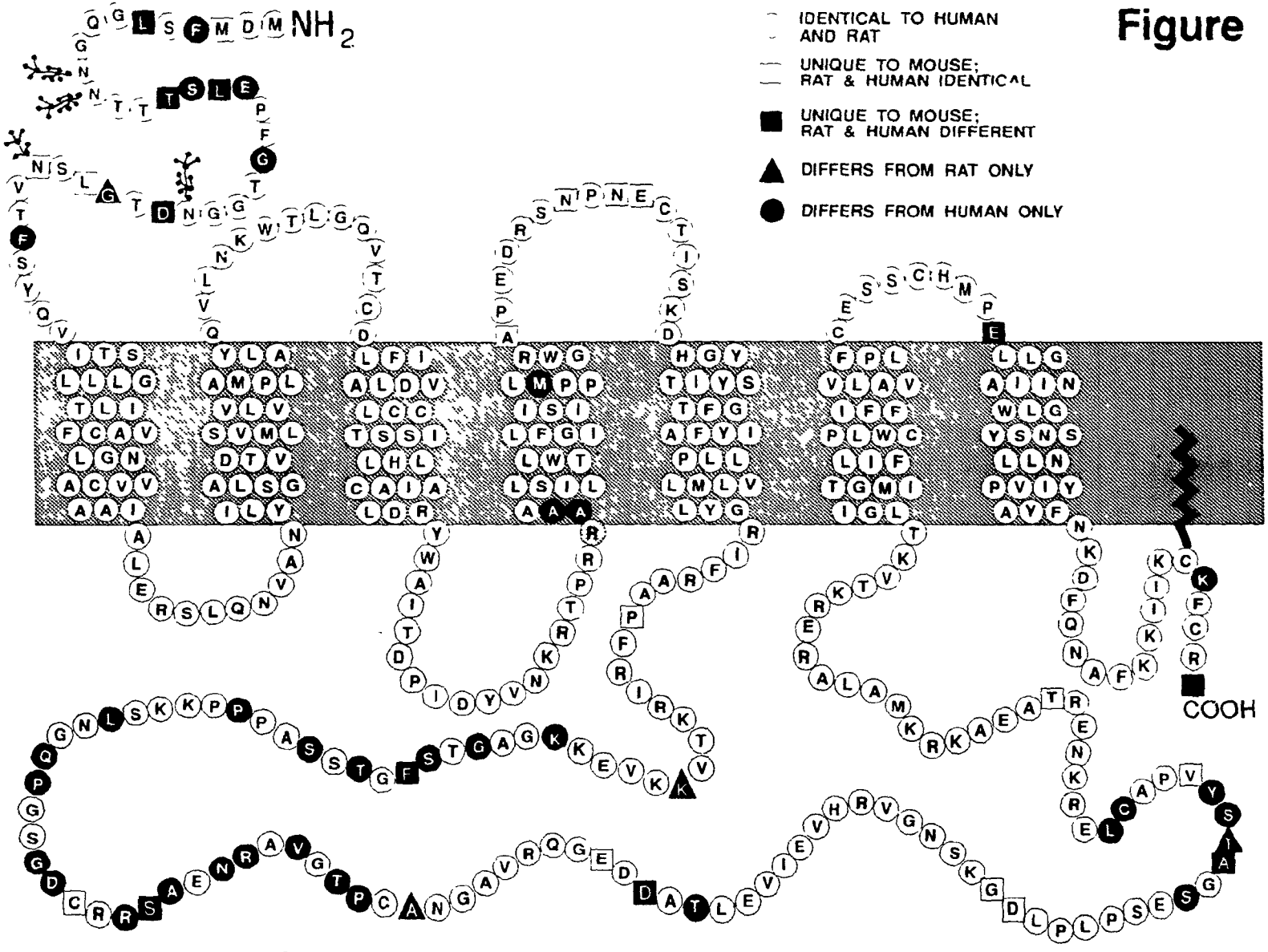
IDENTICAL TO HUMAN AND RAT
UNIQUE TO MOUSE; RAT & HUMAN IDENTICAL
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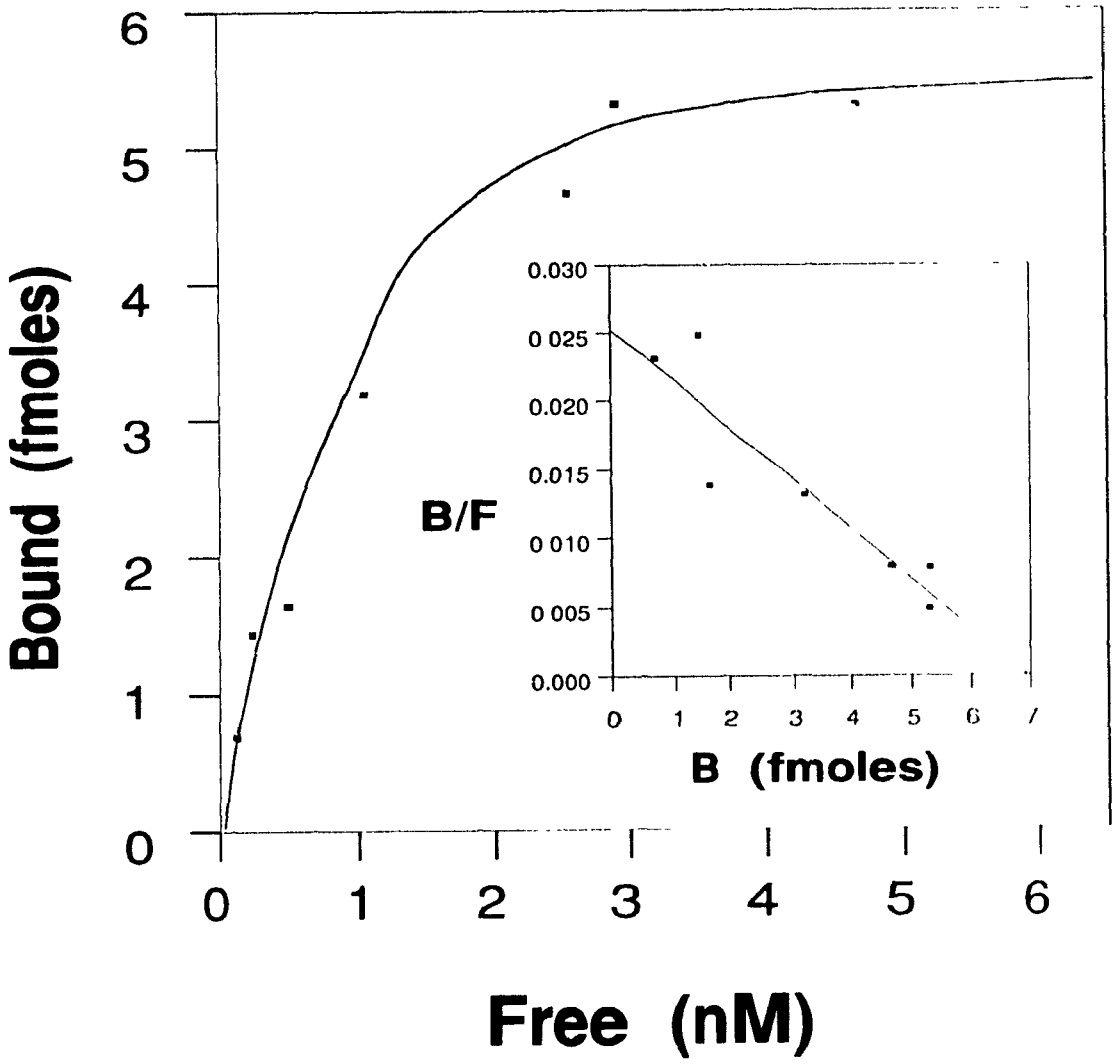
DIFFERS FROM RAT ONLY
DIFFERS FROM HUMAN ONLY
NH2
COOH

Figure 5B

Figure 6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B/F
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0.000