MOLECULAR CLONING OF A DOPAMINE RECEPTOR GENE FROM THE IXODID TICK *Amblyomma americanum* (L.) SALIVARY GLAND

By

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Kaohsiung, Taiwan

1986

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
May, 1995

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TICK *Amblyomma americanum* (L.)

SALIVARY GLAND

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ACKNOWLEDGMENTS

I would like at this time to express my cordial appreciation to my advisor Dr. Richard Essenberg for his excellent supervision, hearty assistance, and professional instruction. My sincere appreciation also comes to my other committee members Dr. John Sauer, Dr. Janis McSwain, Dr. Ulrich Melcher, and Dr. Eldon Nelson for their guidance, assistance, kindness, encouragement and friendship. I also want to thank Dr. John Sauer, Dr. Richard Essenberg, and Dr. Janis McSwain for giving me this research training opportunity and their generous financial assistantship.

Furthermore, I would like to express my sincere gratitude to Dr. John Cushman and Dr. Melanie Palmer for their suggestions and help. My thanks also go to Dr. Roushan Samad, Dr. Holly Schaeffer, Mr. Jim Tucker, Miss Marsha Pidherney, Mrs. Maryam Rafie, and Rocio Cruz-Ortega for their help and friendship and to Mrs. Janet Roger and Mrs. SueAnn Hudiburg for their accurate techniques for my oligo-primers and sequences.

In addition, I wish to give my special appreciation to my wife, Pei-Chen, for her spiritual support, love, and understanding throughout this whole process. And my best thanks go to my parents for their financial and moral support.

At last, I give my gratitude to the faculty of the department of Biochemistry and Molecular Biology for their support during my whole study here.

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LIST OF ABBREVIATIONS

AP	adaptor primer
AMP	adenosine monophosphate
ATP	adenosine triphosphate
Cys	cysteine
DNA	deoxyribonucleic acid
D_N receptor ($_{N=1,2,3,4,5,1A,1B,\ 2A,2B,}$or $_{2C}$)	dopamine receptor subtype $_N$
GDP	guanosine diphosphate
G-protein	guanine nucleotide binding protein
GSP	gene specific primer
GTP	guanosine triphosphate
PCR	polymerase chain reaction
5' RACE	rapid amplification of 5' cDNA end
3' RACE	rapid amplification of 3' cDNA end
RNA	ribonucleic acid
RT-PCR	reverse transcription and PCR
Thr	threonine
TMn (n=1 to 7)	transmembrane domain n
UAP	universal amplification primer
UDG	uracil DNA glycosylase

V-ATPase	vacuolar ATPase

CHAPTER I

INTRODUCTION

Ticks are economically important parasites of domestic animals due to their tenacious feeding behavior and their ability to harbor and transmit disease-causing pathogen and induce toxicosis or paralysis (Strickland et al., 1976). Tick paralysis and toxicosis as well as disease transmission in livestock and humans are related to the injection of saliva into the host by the feeding tick (Gregson, 1969).

Research on the physiology of fluid secretion by the salivary glands of ixodid ticks suggested that control of salivation may be related to the influence of nerves which release dopamine at the neuroeffector junction in the salivary glands (Sauer et al., 1979). Dopamine is a potent stimulant of *in vitro* and *in vivo* fluid secretion by salivary glands of ixodid ticks (Kaufman, 1977; McSwain et al., 1992).

A D_1 dopamine receptor linked to activation of adenylate cyclase has been identified in the salivary glands of ticks (Schmidt et al., 1981) and recent studies (Shipley et al., 1994) suggest that an additional dopamine receptor is present in the salivary glands.

Dopamine receptors are similar to other G-protein coupled receptors which make up a large family of integral membrane proteins that are involved in the specific perception of extracellular messengers (e.g., many neurotransmitters and hormones) or environmental signals (e.g., light or olfactory odor). Several structural features are shared between dopamine receptors and the other members of this important family of signal transducing proteins (Probst et al., 1992).

Dopamine receptors, like other G-protein coupled receptors, are composed of a single polypeptide with seven transmembrane domains (TM1 to TM7) joined by alternatingly

extracellular and intracellular loops. They begin with an extracellular amino terminal domain and end with a cytoplasmic carboxy-terminal tail. The transmembrane regions group together in the membrane to form "a cup" into which the ligand (i.e., agonist or antagonist) fits to activate the receptor that's coupled to a G-protein (Hall et al., 1994).

Dopamine receptors in ticks and vertebrates share some similar features in response to pharmaceutical agents . However, quantitative and qualitative differences may exist in their molecular structure and these differences will be assessed by cloning and sequencing dopamine receptors genes in tick.

CHAPTER II

LITERATURE REVIEW

Ticks are major arthropod parasites and vectors of disease-causing pathogens of humans and domestic animals (Kaufman, 1989). They exist almost everywhere in the world (Sonenshine, 1991). There are two stages of feeding in female ixodid ticks: the slow feeding phase and the rapid feeding phase (Sauer et al., 1979/1984).

The paired salivary glands of female ixodid ticks are their principal organs of osmoregulation. During feeding, female ixodid hard ticks ingest a large quantity of host blood and eliminate excess water and ions to concentrate the meal. Gregson (1967) proposed that the excess water and ions are returned to the host by the paired salivary glands. This process was later verified by Tatchell (1967) and Kaufman & Philips (1973a). Kaufman & Philips (1973b) also proposed that fluid elimination via the salivary glands is controlled by nerves. This hypothesis is supported by the ability of low concentrations of catecholamines to stimulate fluid secretion by glands *in vitro* (Kaufman & Philips, 1973b; Kaufman, 1976) and the finding of dopamine and norepinephine in salivary glands and the synganglion of *Boophilus microplus* (Megaw & Robertson, 1974). Furthermore, the intracellular level of cyclic AMP increased when glands were stimulated with 10^{-5} M dopamine (Sauer et al., 1979). An increase in cyclic AMP following gland stimulation with dopamine suggests the presence of a dopamine receptor coupled to adenylate cyclase. Studies on the physiology of fluid secretion by the salivary glands of ixodid ticks suggested that control of salivation may be through release of dopamine by nerves in the salivary glands (Sauer et al., 1979). Several workers had shown that injection of catecholamines into the hemocoel of ticks stimulates fluid secretion *in vivo*

(Hsu et al., 1975 & Kaufman, 1978).

Kaufman (1977) tested a series of derivatives of phenylethylamine and reported that dopamine was the most potent stimulant of fluid secretion *in vitro* by glands of ixodid ticks, followed by norepinephrine and epinephrine which were equipotent. Dopamine at low concentrations stimulated salivary fluid secretion *in vivo* by glands of several species of hardbodied ticks (Kaufman, 1976/1977), including *Amblyomma americanum* (Sauer et al., 1979), suggesting a similar means of control throughout the family Ixodidae.

Although the complete dopamine receptor genes have not been identified in the tick, they have been extensively characterized in humans and mice. Dopamine receptors belong to the G-protein coupled receptor superfamily, which also includes the adrenergic receptor, serotonin receptor, muscarinic receptor, SK (substance K) receptor, etc. (Libert et al., 1989). These receptors consist of a single protein with seven transmembrane domains (TM1 to TM7) joined by alternating extracellular and intracellular loops. They have an extracellular amino terminus and a cytoplasmic carboxy terminus and the transmembrane regions group together to form a "cup" into which the ligand fits to activate the receptor. The ligand may be a neurotransmitter, a peptide, or a hormone. Agonist binding to the receptor is thought to induce a conformational change within the transmembrane domains that is transmitted to the cytoplasmic face where interactions with a specific guanine-nucleotide binding-protein complex (G-protein) is known to occur (Hall et al., 1994).

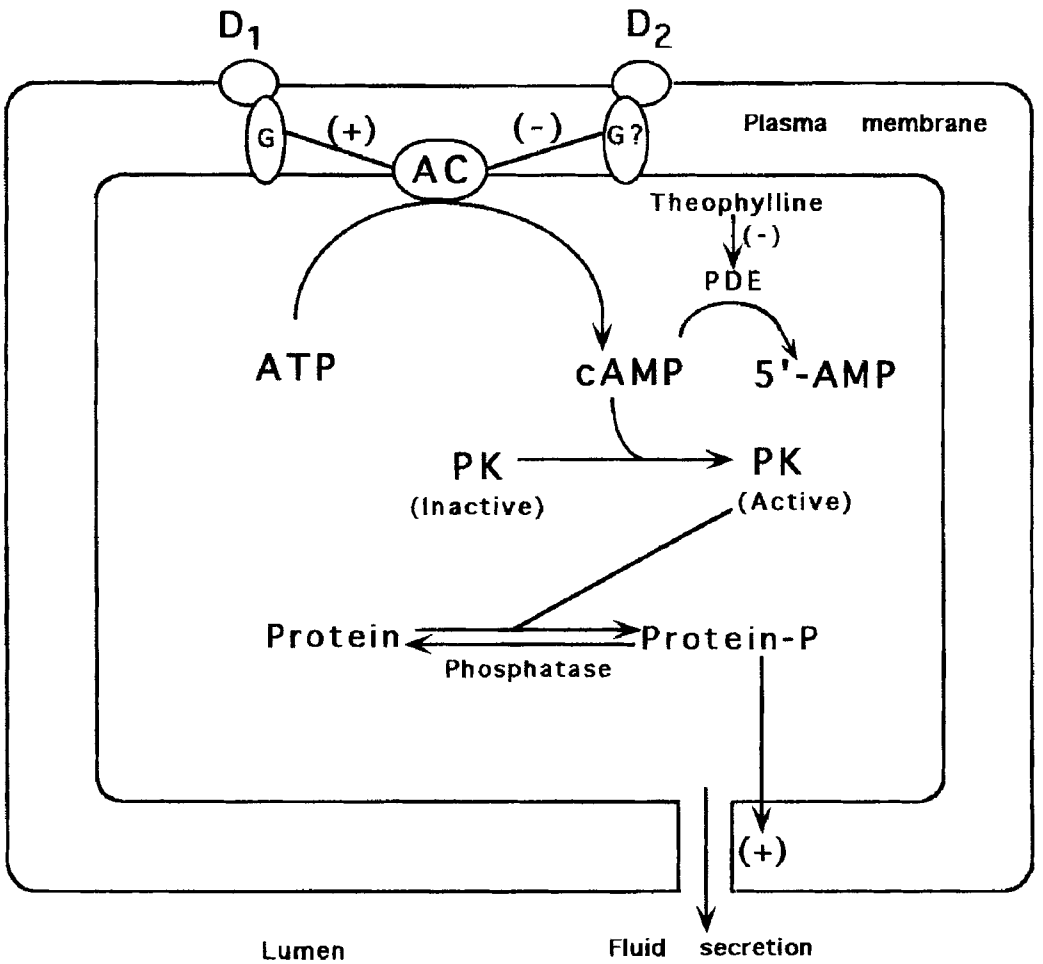
The inactive form of a G-protein binds GDP at rest. The G-protein complex consists of three subunits designated as: alpha (α), beta (β), and gamma (γ). Interaction of the G-protein complex with the activated receptor results in the replacement of GDP by GTP and activates the G-protein complex. Then, the GTP associated α subunit of the G-protein dissociates from $\beta\gamma$ subunits. The activated and dissociated α subunit interacts with one of the effector systems which include a variety of effectors like: phosphodiesterase, phospholipase C, adenylate cyclase (Fig. 1), phospholipase A_2, and

some ion channels. For some receptors, association with the βγ complex alone may facilitate phosphorylation by a kinase leading to immediate desensitization of the receptor. When the βγ complex dissociates from the receptor, it is then free to reassociate with the α subunit or in some cases to interact with other effectors. So, what kind of effector system these receptors can be coupled to and whether these interactions are stimulatory or inhibitory are determined by the G-protein involved. When the ligand dissociates from the receptor and the phosphate is removed from the receptor, the process is ready to begin again (Branden et al., 1991 & Hall et al., 1994).

The overall membrane topology and the interactions with the effector system have been suggested as applicable to all of the G-protein linked receptors that have been cloned. However, only for rhodopsin and the β_2-adrenoceptor is there biochemical evidence supporting this model (Sibley and Monsma, 1992).

In tick salivary gland, it's speculated (Shipley et al., 1994) that there are 2 dopamine receptors-D_1 and D_2 receptors; D_1 receptor is believed to couple a stimulatory G-protein and activate adenylate cyclase and D_2 receptor is thought to couple an inhibitory G-protein and inactivate adenylate cyclase. Activated adenylate cyclase can catalyze the conversion of ATP to cAMP (cyclic AMP) that can activate protein kinase. This enzyme can phosphorylate specific cellular proteins at serine and threonine residues. It is speculated that a conformational change following protein phosphorylation energizes the V-ATPase in the membrane or this change induces ion channels to pump the fluid out of the cell; the whole putative mechanism explains why the tick salivary gland can secrete fluid efficiently and the salivary gland cells won't swell to break due to the feeding of large amounts of the host blood (Fig. 1). The work done by Shipley et al. (1994) on salivary gland secretion in *Dermacentor variabilis* corroborates much of the previous work in other tick species. Dopamine at 10^{-5} M increased cAMP levels in the salivary glands of *Amblyomma americanum* (Sauer et al., 1979; McSwain et al., 1992). Wong and Kaufman (1981) reported enhanced secretion in isolated glands of *Amblyomma hebraeum* at a lower

Figure 1. Dopamine-induced signal transduction via G-protein coupled receptor and adenylate cyclase system to protein kinase effector system (from Shipley et al., 1994).


D1
D2
G
(+)
AC
(-)
G?
Plasma membrane
Theophylline
(-)
PDE
ATP
cAMP
5'-AMP
PK
(Inactive)
PK
(Active)
Protein
Protein-P
Phosphatase
(+)
Lumen
Fluid secretion

concentration (10^{-6} M). The most interesting effect seen in Shipley's work (1994) was the marked decrease in both cAMP and fluid secretion at the high dopamine concentration of 10^{-3} M. This result suggests the presence of a dopamine D_2-like receptor in the salivary glands with a lower binding affinity for dopamine than the D_1 receptor. This may explain in part why D_2 receptor antagonists like spiperone are able to potentiate the effect of dopamine in stimulating secretion (Wong and Kaufman, 1981).

Proposals for multiple types of dopamine receptors have been made, but only two subtypes, D_1 and D_2 receptors, had been definitively shown to exist by pharmacological & biochemical studies. Now, five pharmacologically distinct dopamine receptors have been identified through molecular cloning techniques and it seems the dopamine receptor family is more diverse than previously imagined (Sibley et al., 1992).

D_2 Receptor Subfamily

D_2 Receptor (D_{2A} Receptor)

The D_2 receptor was the first dopamine receptor to be cloned and has been studied the most. Bunzow et al. (1988) identified a full-length cDNA clone from a rat brain library using a β-adrenoceptor probe. The human homolog of the rat D_2 receptor was subsequently cloned, and its predicted protein sequence is 96% identical to that of the rat receptor (Dal Toso et al., 1989 & Stormann et al., 1990). There are several other structural features of the D_2 receptor that are worth notice. First, its N-terminal domain, lacking an apparent signal sequence, contains consensus sequences for three potential N-linked glycosylation sites, which means the D_2 receptor may be a glycoprotein. Secondly, the predicted size of the C-terminal region is rather small and it possesses a conserved Cys residue that may serve as a site for palmitoylation. Thirdly, between TM regions 5 and 6 there is a large cytoplasmic loop containing one potential site for phosphorylation by a cAMP-dependent protein kinase. A large third cytoplasmic loop and short C-terminal

domain are the characteristic features of most, but not all, receptors that inhibit adenylyl cyclase activity (Dohlman et al., 1991).

The regional and cellular localization of the D_2 receptor mRNA has been examined using Northern blot analysis and *in situ* hybridization histochemistry (Mansour et al., 1990 & Weiner et al., 1990). The areas of highest expression in the brain correspond to major dopaminergic projection areas. In the striatum, about 50% of the medium-sized cells express receptor mRNA (Weiner et al., 1990 & Gerfen et al., 1990); large diameter cells, mostly cholinergic interneurons, also express it (Le Moine et al., 1990).

The D_2 dopamine receptor has two protein isoforms that differ in length by 29 amino acids and are derived from the same gene by alternative RNA splicing. The location of this splice variation occurs within the third cytoplasmic loop of the receptor protein, approximately 30 residues from the fifth TM domain (Sibley et al., 1992).

The intracellular loops of the G-protein coupled receptors do not appear to be involved in ligand binding, and mutagenesis studies suggest the third cytoplasmic loop is important to G-protein coupling and effector regulation (Dohlman et al., 1991 & Strader et al., 1989). However, both of the D_2 receptor isoforms have the ability to inhibit adenylyl cyclase (Weiner et al., 1989 & Rinaudo et al., 1990), activate K^+ channel (Einhorn et al., 1990), potentiate arachidonic acid release (Kanterman et al., 1991) and undergo agonist-induced desensitization with equal efficiency (Rinaudo et al., 1990).

<u>D_3 Receptor (D_{2B} Receptor)</u>

The second receptor within the D_2 subfamily to be identified and cloned was the "D_3" receptor (Sokoloff et al., 1990). The predicted amino acid sequence as well as the proposed membrane topology of the rat D_3 receptor is very similar to that of the D_2 receptor. Overall, the rat D_3 receptor is 52% identical with the D_2 receptor; however, this identity increases to about 75% if only the TM regions are considered. As with the D_2 receptor, the D_3 receptor contains consensus sequences for N-linked glycosylation; two of

which are in the N-terminal region, but one is in the first extracellular loop (Sibley et al., 1992). The human D_3 receptor has 46 fewer amino acids in the third cytoplasmic loop than the rat D_3 receptor; nevertheless, human D_3 receptor is 97% identical with the rat protein within the TM regions (Giros et al., 1990). Radioligand binding experiments in transfected CHO cells indicate that the D_3 receptor's pharmacology is similar to, yet distinct from, that of the D_2 receptor. D_3 receptor activation in the CHO cells was reported to have no effect on adenylyl cyclase activity. These observations suggest the lack of appropriate D_3 receptor-G protein coupling in the CHO cells (Sokoloff et al., 1990).

D_4 Receptor (D_{2C} Receptor)

The D_4 receptor represents the latest receptor in the D_2 subfamily to be identified and cloned (Van Tol et al., 1991). The proposed membrane topology of the partially cloned D_4 receptor gene is similar to that seen with the D_2 and D_3 receptor but, as with human D_3 receptor, the D_4 receptor has a slightly smaller third cytoplasmic loop. The D_4 receptor contains one potential site for N-linked glycosylation in the N-terminus and one consensus cAMP-dependent phosphorylation site in the third cytoplasmic loop. As with the D_2 and D_3 receptors, the C-terminal tail has a conserved Cys residue (Sibley et al., 1992). Generally speaking, the D_4 receptor displayed similar or lower affinities for dopamine receptor antagonists and agonists compared with the D_2 receptor. However, the atypical antipsychotic clozapine, and its congener clorotepine, showed a tenfold higher affinity constant for the D_4 receptor. The fact that the affinity constant of clozapine is similar to the clozapine level in the patient's plasma water during antipsychotic therapy suggest that clozapine might exert its antipsychotic activity primarily by blocking the D_4 receptor (Van Tol et al., 1991).

D_2, D_3, and D_4 have similar pharmacological profiles and they exhibit picomolar to nanomolar affinity for the antagonist spiperone; hence, they belong to the same subfamily,

and sometimes, D_2, D_3, and D_4 are designated D_{2A}, D_{2B}, and D_{2C}, respectively (Sibley et al., 1992).

D_1 Receptor Subfamily

D_1 Receptor (D_{1A} Receptor)

The D_1 receptor is linked to the activation of adenylyl cyclase activity in either rat or human tissues. In contrast to the D_2 receptor subfamily, the D_1 receptor has a small third cytoplasmic loop and a long C-terminal domain. This seems to be a characteristic of receptors that are coupled to Gs (stimulatory G-protein) and activate adenylyl cyclase, such as the β-adrenoceptor. The D_1 receptor may also be a glycoprotein because it has two potential sites for N-linked glycosylation. Besides, there is one consensus site for cAMP-dependent phosphorylation in the third cytoplasmic loop and there is a conserved Cys in the carboxyl tail. The C-terminal domain also contains numerous Ser and Thr residues that may serve as additional sites of regulatory phosphorylation (Sibley et al., 1992). The characteristic of the D_1 receptor's pharmacological profile is that it has a saturable and high affinity binding of [^{3}H] SCH23390 or [^{125}I] SCH23982 (Monsma et al., 1990).

D_5 Receptor (D_{1B} Receptor)

The D_5 receptor is a second member of the D_1 receptor subfamily (Sunahara et al., 1991). Overall, the level of identity is about 50% between the D_1 and D_5 receptors, but this identity increases to about 80% within the membrane-spanning regions. The speculated glycosylation site, phosphorylation site , and a conserved Cys residue in the C-terminal domain are similar to those of the D_1 receptor (Sibley et al., 1992).

The results of the pharmacological analysis of D_1 and D_5 receptors are very similar except that dopamine is about ten-fold more potent at the D_5 receptor than at the D_1

receptor (Sunahara et al., 1991). This suggests that the D_5 receptor may be important in maintaining dopaminergic tone and arousal. Just like the D_2 subfamily, the D_1 and D_5 receptors belong to the same subfamily because they have similar pharmacological profile and exhibit nanomolar affinity for the prototypic antagonist ligand SCH23390 while the D_2 subfamily should exhibit picomolar to nanomolar affinity for the antagonist spiperone. Thus, D_1 and D_5 receptors can be designated D_{1A} and D_{1B} receptors, respectively (Sibley et al., 1992 & Tiberi et al., 1991& Monsma et al., 1991).

Approaches for Cloning Dopamine Receptors

There are two standard approaches in molecular biology which can be used for cloning new members of a gene family or for cloning the homologous gene from a different species once the first cDNA is available. These two methods are: reduced stringency library screening and the polymerase chain reaction (PCR) using degenerate or gene-specific primers. The work of Onai et al., 1989 & Arakawa et al., 1990 illustrates successful application of the first strategy. The newer method using PCR allows more rapid cloning and has some successful cases in the recent literature (Libert et al., 1989; Buck and Axel, 1991; Murtagh et al., 1993).

Reduced Stringency Library Screening

A genomic or cDNA clone, even a partial clone, or an oligonucleotide from the gene's protein coding sequence should be available to use for screening a library. If an oligonucleotide probe is used, it's necessary to design the probe(s) from the sequence areas which are most highly conseved across species, if known, or across receptor subtypes. Choosing the probe is the most important step for this approach. Introns and 5' and 3' untranslated regions of cDNA clones should be avoided (Hall et al., 1994).

Screening a cDNA library prepared from tissues or developmental stages, that are known to express the receptor of interest, should be effective; otherwise, a genomic library may be used because it includes segments of all the genes with almost equal frequency. The intron regions in the genomic clones can generally be recognized by codon usage (Gribskov et al., 1984) or third position codon bias analysis (Bibb et al., 1984). A preponderance of rarely used codons in a stretch of genomic DNA is a good indicator of a noncoding region. Reduced stringency hybridization conditions involve high salt and low temperatures during hybridization and washes (Hall et al., 1994).

The Polymerase Chain Reaction (PCR)

The reason of using PCR to clone genes is that degenerate primers can be used and the region to be amplified can be selected so that it will produce definitive regions of the genes of interest. Two different regions, TM3 to TM6 (Li et al., 1992) or TM6 to TM7 (Monnier et al., 1992) have been used successfully to amplify insect G-protein coupled receptors based on sequence information available from the mammalian literature. To account for cross species differences often requires the use of degenerate primers and reduced stringency of the annealing conditions by changing the annealing temperature or magnesium concentrations in the PCR reaction to allow for sequence mismatch (Hall et al., 1994).

The degenerate primers designed from the consensus amino acid sequences of TM3 and TM6 for a number of known mammalian biogenic amine receptors have been successfully used to clone many receptor genes from mammals and insects. For example, the degenerate primers used by Libert et al. (1989) to isolate four new members of the G-protein coupled receptor family from human thyroid tissues, and those primers designed by Broeck et al. (1993) to get a putative Locust G-protein coupled receptor, are all from the third and sixth transmembrane conserved segments of available receptors and these two primer pairs are very similar to each other in the strategy to design and the area used

(both primer pairs were designed from highly conserved regions in transmembrane domains TM3 and TM6 of the related receptors). New members of this heptahelical receptor class from *Drosophila* have also been cloned by Hall et al. (1994) using primers from highly conserved regions in transmembrane domains TM3 and TM6. Li et al. (1992) have also used a primer pair from these regions to clone a neuropeptide Y receptor from Drosophila. These primers amplify a region that includes the third cytoplasmic loop between TM5 and TM6. This loop varies in size among the different classes of heptahelical receptors giving different sized PCR products with the smallest generally being the peptide receptors and the largest being the muscarinic acetylcholine receptors. Nonetheless, the third cytoplasmic loop is not a very good diagnostic sequence because it varies across species and across different receptor types. However, the existence of the hydrophobic TM4 and TM5 domains between this primer pair (i.e. TM3 and TM6) is an easily recognizable diagnostic feature (Hall et al., 1994).

In addition, a clone of the tachykinin (NKD) receptor (Monnier et al., 1992) of *Drosophila* was obtained using degenerate receptor-specific primers from regions TM6 and TM7. The product was easier to amplify and sequence because these primers span a shorter sequence than the TM3/TM6 primer pair.

It seems the PCR approach is a very good resource to speed up the cloning process if some related DNA sequences are available, but one problem with both the reduced stringency library screening or the PCR methods is that many of the products isolated will be unrelated DNA sequences. Hence, it's necessary to confirm that the clones derived from these two approaches are the products of interest. In the case of PCR products, nested primers can be used to determine if there is an expected match lying within the amplified region. Alternatively, a cDNA probe from a related receptor clone of interest can be used to identify the most probable clone by Southern blotting & hybridization (Hall et al., 1994).

CHAPTER III

MATERIALS AND METHODS

Preparation of Genomic DNA from Tick Eggs

Tick eggs were used to prepare tick genomic DNA. Tick eggs were collected and kept at -70° C until used and no more than two grams of tick eggs were used for each preparation. The tick eggs were ground to a fine powder in a mortar in the presence of liquid nitrogen. The ground sample was then transferred to a 50 ml capped tube with 40 ml of 1xNIB (Nuclei Isolation Buffer, pH 7.4; 10 mM Tris, 60 mM NaCl, 10 mM EDTA, 0.5% Triton-X 100, 0.15 mM spermidine, 0.15 mM spermine) added. The diluted sample was then transferred to a 55 ml glass homogenizer and was homogenized by making several passes at moderately high speed. The sample was then transferred back to a 50 ml capped tube and centrifuged at 2500 xg for 15 sec to precipitate the hard and insoluble cuticle. The supernatant was transferred to a new 50 ml high speed polypropylene tube and the pellet was discarded. This process was repeated twice to remove the cuticle. The supernatant was centrifuged at 7.5K xg (SS34 rotor) for 7 minutes, and the pellet was resuspended in 50 ml of 1x NIB and recentrifuged at 7.5K xg for 7 minutes. This time, the pellet was resuspended in 14 ml of 1x NIB, and 4 mls of 10% sarcosyl (Sigma) was mixed with it with a glass rod. After 10 min incubation on ice, 21.26 gm of CsCl were added and the total volume was brought to 26.75 ml with 1xNIB. 270 ul of 10 mg/ml Ethidium Bromide was added after the CsCl was dissolved by slow stirring. The sample was centrifuged at 45K xg for 18 hrs. Then, the genomic DNA band was removed using

a 5 ml syringe with an 18 gauge needle using UV light to identify the band. The EtBr was removed by extracting several times with water-saturated butanol. CsCl was removed by dialysis against 1 liter of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) at 4 °C overnight. DNA was precipitated overnight at -20 °C with 1/10 volume of 3M sodium acetate (pH 4.6) and 2 volumes of room temperature ethanol. DNA was pelleted by centrifugation at 15,000 rpm for 30 min, then washed with 70% ethanol and air dried. The purified genomic DNA was then dissolved in TE buffer and its concentration and purity were determined by spectrophotometer (Model: SHIMADZU, UV160U) at 260 nm (for nucleic acid) and at 280 nm (for protein impurity). Aliquots of the genomic DNA were electrophoresesed on a 0.3% agarose gel at 4 °C in order to check the DNA's integrity.

Southern Blot

I used mouse genomic DNA (gift from Dr. Melanie Palmer, Dept. of Entomology, Oklahoma State University) as control DNA. Two cDNA probes (rat DNA coding the D_1 receptor cloned into the HindIII and SacI sites of pGEM Blue: described in Zhou et al., 1990; and full length rat cDNA coding the D_2 dopamine receptor cloned into EcoRI site of pGEM Blue: described in Bunzow et al., 1988) were used to hybridize to tick and mouse genomic DNA. The D_1 receptor clone encodes the rat D_1 dopamine receptor gene and the D_2 receptor clone (with a D_2 cDNA recombinant plasmid) encodes a rat D_2 dopamine receptor gene; the probes were gifts from Dr. Oliver Civelli (Vollum Institute, Portland, Oregon).

Enzyme Digestion and Electrophoresis

Tick genomic DNA and mouse genomic DNA were digested separately by EcoRI, SmaI, and HindIII (from Gibco BRL). In each digestion, 10 ug of genomic DNA were used and 1/10 of total volume of enzyme buffer and 2.5 ul of one of the enzymes (25

units) were added. The digestions were performed at 37 °C for 3-4 hours. After that, an appropriate amount of dye was added and the samples were loaded on a 0.7% agarose gel and electrophoresesed in a GibcoBRL Horizon 11-14 EP kit at 20 volts overnight at 4 °C with the 1 kb DNA ladder on a separate lane to be the standard and the D_1 and D_2 insert DNA(s) on separate lanes to be the positive controls.

Capillary Transfer of DNA to Nylon or Nitrocellulose Membrane

After electrophoresis, the DNA in the agarose gel was depurinated by 0.25N HCl, denatured by 1.0M NaCl/0.5M NaOH, and neutralized by 1M NH_4CH_3COO following the standard protocol (Maniatis et al., 1987, or the protocol about transfer and immobilization of nucleic acids to S & S solid supports from Schleicher & Schuell company). The DNA was then transferred to nylon membrane (Magna NT nylon transfer membrane from MSI, Micro Separation Inc.) by capillary transfer overnight, using 20x SSPE (2.8M NaCl, 0.2M NaH_2PO_4, 0.02M EDTA, pH 7.4) as transfer buffer. DNA was then fixed on the membrane by UV-crosslinking (UV Stratalinker 1800, setting: 0.12 Joules/cm^2, 45 sec). Alternatively, the DNA in the gel could be bidirectionally (one membrane above the gel and another membrane beneath the gel) transferred to the nitrocellulose membranes for about 3 to 4 hours; but after that, the membranes were baked at 80 °C in vacuum for about 30 minutes to fix the DNA.

Preparation and Labeling of the Probe

The DNA fragment to be used as the probe was removed from the plasmid by restriction digestion and separated by agarose gel electrophoresis, the insert was then cut from the gel and purified with the QIAquick GEL Extraction Kit. Initially, I used a photobiotin labeled probe and the instruction manual of Photogene™ Nucleic Acid Detection System from BRL (Life Technologies, Inc.) to perform my experiment. But, it seemed the photobiotin-labeled probes were not sensitive and the blots could not easily be

exposed twice. As a result of these inconveniences, I chose to use a radioisotope (^{32}P) labeled probe because it's more sensitive, and the membrane can be washed again easily to obtain a definitive autoradiography film if I found the image intensity on the X-ray film was too strong.

cDNA probes were labeled by the random priming method (Maniatis et al., 1987). The cDNA (50 ng in a total volume of 7.1 ul) was heated at 95 °C for 5 minutes in a 1.5 ml eppendorf tube and then immediately cooled on ice for 5 minutes. The tube with denatured DNA in it was then centrifuged briefly and combined with the following reagents: 11.4 ul LS (HEPES/DTM/OL in a ratio of 25/25/7, HEPES: 1M, pH 6.6; DTM: 100 uM dATP, 100 uM dGTP, and 100 uM dTTP in 250 mM Tris pH 8.0, 250 mM $MgCl_2$, 50 mM β-mercaptoethanol; OL: 1mM Tris pH 7.5, 1mM EDTA pH 8.0, 90 OD units/ml of oligonucleotide hexamers), 1 ul of 10 mg/ml BSA (bovine serum albumin), 5 ul α-^{32}P-dCTP (3,000 Ci/mmol, NEN), 0.5 ul Klenow (2.5 units, BRL). The mixture was incubated at room temperature for at least 3 hours. The unicorporated nucleotides were separated by Sephadex G-50 or Biogel P-60 spin column chromatography (Maniatis et al., 1989a).

If the probe used was an oligonucleotide, it was end-labeled with the enzyme T4 polynucleotide kinase (Maniatis et al., 1989b). The oligonucleotide (1 ul of ~ 100 ng/ul) was combined with 27 ul of distilled H_2O, 5 ul of 10x T4 kinase buffer, 15 ul of γ-^{32}P-dATP (0.01 mCi/ul), and 2 ul of 4U/ul T4 polynucleotide kinase. The mixture was incubated at 37 °C for 45 minutes and the enzyme was denatured at 68-70 °C for 10 minutes. The reaction sample was then diluted with another 50 ul STE buffer (0.1 M NaCl; 10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0) to make 100 ul of total volume and the whole solution was subject to spin column chromatography to remove uni-corporated nucleotides.

Prehybridization and Hybridization with cDNA Probes

The prehybridization and hybridization buffers were: 5x SSPE (diluted from 20x SSPE stock), 5x Denhardt's (diluted from 50x stock), 0.5% SDS, and 50 ug/ml denatured salmon sperm DNA. Prehybridization was carried out at 42 °C for 2 to 3 hours.

The hybridization solution contained the radioisotope-labeled probe and the same buffer as the prehybridization buffer. Before applying the column purified probe to the hybridization buffer and membrane, the probe was denatured again by boiling for 10 minutes, then immediately cooled on ice for 5 minutes. Hybridization was completed at 42 °C for about 17 hours.

Prehybridization and Hybridization with Oligonucleotide Probes

The prehybridization and hybridization buffers were: 6x SSPE (diluted from 20x SSPE), 1x Denhardt's (diluted from 50x stock), 0.5% SDS, 100 ug/ml denatured salmon sperm DNA, 1mM EDTA (pH 8), and 0.01 M sodium phosphate (pH 6.8). Other procedures were the same as using cDNA probes except that the membrane was hybridized for 3 or 4 hours only using the oligonucleotide probe and hybridization temperatures were carefully calculated using the Tm of each oligonucleotide probe..

Washing the Membrane Probed by cDNA

After hybridization, the membrane was washed at progressively higher stringency to decrease the background. First, the probed membrane was washed twice in 5x SSPE and 0.1% SDS at 42 °C for 30 minutes, then washed twice at 52 °C or twice at 62 °C if the background was high. After every wash, radioactivity on the membrane was assessed with a Geiger-Muller counter; if the signal indicated some spots on the membrane, the membrane was exposed to X-ray film.

Washing the Membrane Probed by Oligonucleotide

Due to the weak annealing force between the oligonucleotide probe and the DNA on the membrane, washing was started at very low stringency (e.g. 6x SSPE, 0.1% SDS at room temperature for 30 minutes twice), then the washing stringency was increased (if the radioactive signals from the membrane background were still very strong) with the membrane monitored by a Geiger counter to determine when to expose it to X-ray film (the washing was stopped at the time when some specific spots on the membrane were detected with strong signals).

Polymerase Chain Reaction (PCR)

PCR of Tick Genomic DNA by Using Degenerate Primers Designed from Available G-protein Coupled Receptors

The oligonucleotides for the degenerate primers were synthesized by the Recombinant DNA/Protein Resource Facility at Oklahoma State University. The primer pair (Libert et al., 1989) consisted of:

5' CTGTG(CT)G(CT)(CG)AT(CT)GCI I T(GT)GA(CT)(CA)G(CG)TAC 3" (Designed from TM3; either deoxynucleotide in the parentheses has 50% probability to appeaar in that specific position of this "degenerate" oligo and this "degenerate" oligo has 2^8 =256 different kinds of DNA sequences) and 5' A(TG)G(AT)AG(AT)AGGGCAGCCAGCAGA I(GC)(GA)(TC)GAA 3' (Designed from TM6; same as above, this "degenerate" oligoprimer has 2^6 =64 different kinds of DNA sequences)

For the PCR reaction, a master mix was prepared by adding ingredients in the following order: sterile H_2O, 10x PCR buffer, 10x $MgCl_2$ buffer, dNTPs, and primer sets; it was necessary that all tubes and reagents be kept on ice at all times before placing into the thermocycler machine. Two units of Taq polymerase from BRL was applied to the reaction mixture (final volume: 50 ul). The templates were from tick genomic DNA and

from 3 day or 6 day-feeding salivary gland cDNA libraries (prepared in Dr. Sauer's lab by Jim Tucker, Dept. of Entomology, OSU). Mouse genomic DNA and human blood genomic DNA were used as controls for this experiment. In addition, a negative control (no DNA) was always included as the last tube in the reactions. The thermocycler used for PCR was either from MJ Research Inc. (PTC-100™ Programmable Thermal Controller) or from Perkin Elmer Cetus (DNA Thermal Cycler). After 5 minutes' heating at 94 °C, the thermal cycling consisted of 30 cycles with a denaturation step of 1 min 30 sec at 93 °C, and an annealing step of 2 min at 55 °C, and an extension time of 4 min at 72 °C. The annealing step was varied from 45 °C up to 65 °C depending on the Tm (melting temperature) of the primer pair used and the mismatches between the primers and the templates.

RT-PCR, 5' RACE, and 3' RACE

Total RNA or messenger RNA (having poly-A tail) isolated from tick salivary glands were used in these experiments. cDNA was synthesized using reverse transcriptase. The sample was then treated with RNAse H to degrade the RNA and leave cDNA that would be used as the template for the PCR with different primer pairs (Fig. 2 & Fig. 3). The procedures followed those outlined in the manuals provided by GIBCO BRL. The schemes for these RACE experiments are shown in Fig. 2 and Fig. 3.

Screening

Screening of the Uni-ZAP™ XR cDNA Library

About one million clones from a Uni-ZAP™ XR tick salivary gland 6-day feeding cDNA library were screened. XL1-Blue bacteria were grown in LB-broth (1% NaCl, 1% Bacto-Tryptone, 0.5% Yeast Extract) containing 4% maltose at 37 °C overnight. The cells were centrifuged at 5Kxg for 5 minutes, and resuspended in sterile 10 mM $MgSO_4$.

Figure 2. Overview of the 5' RACE Procedure (cited from GIBCO BRL's instruction manual; cat. no. 18374-025).

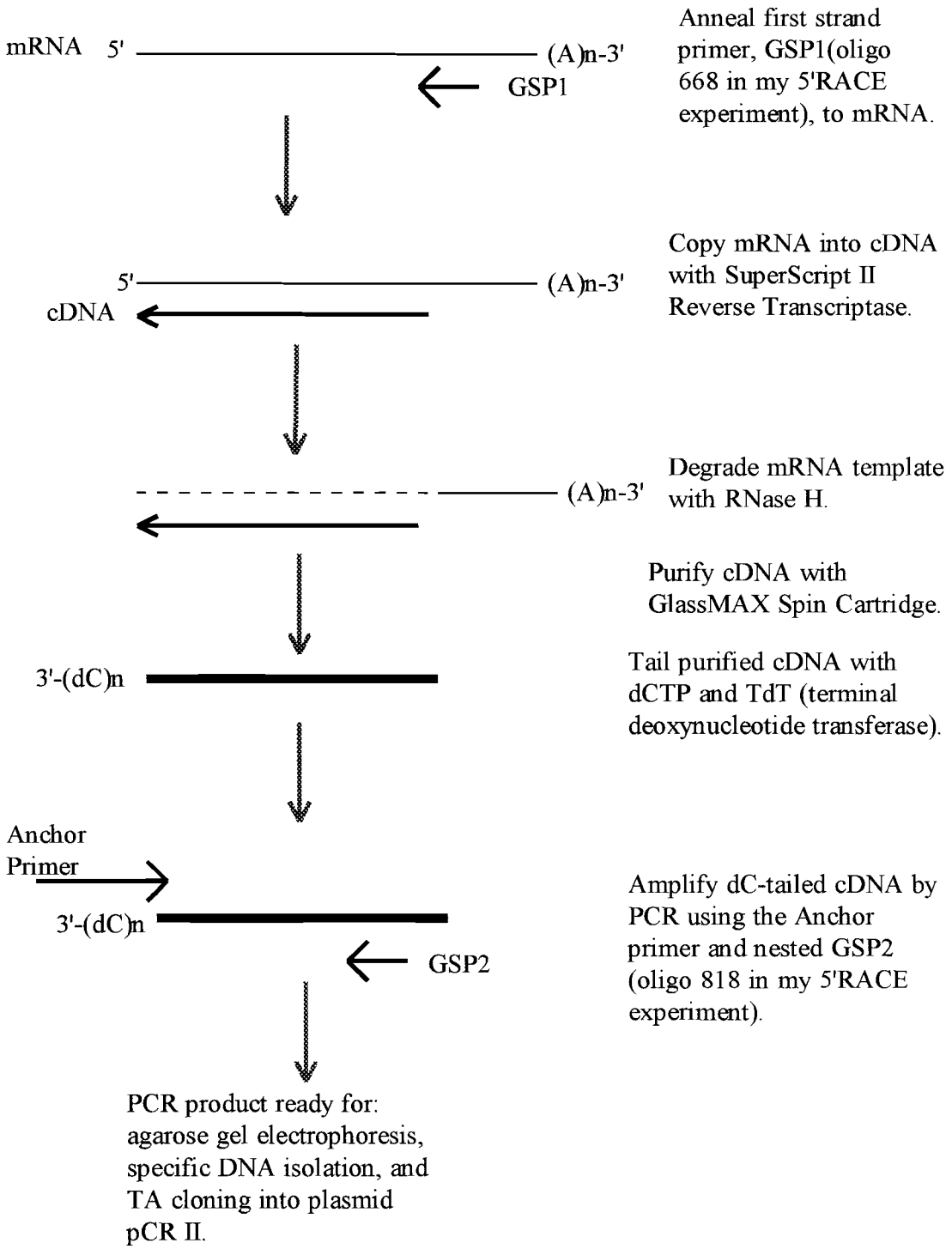
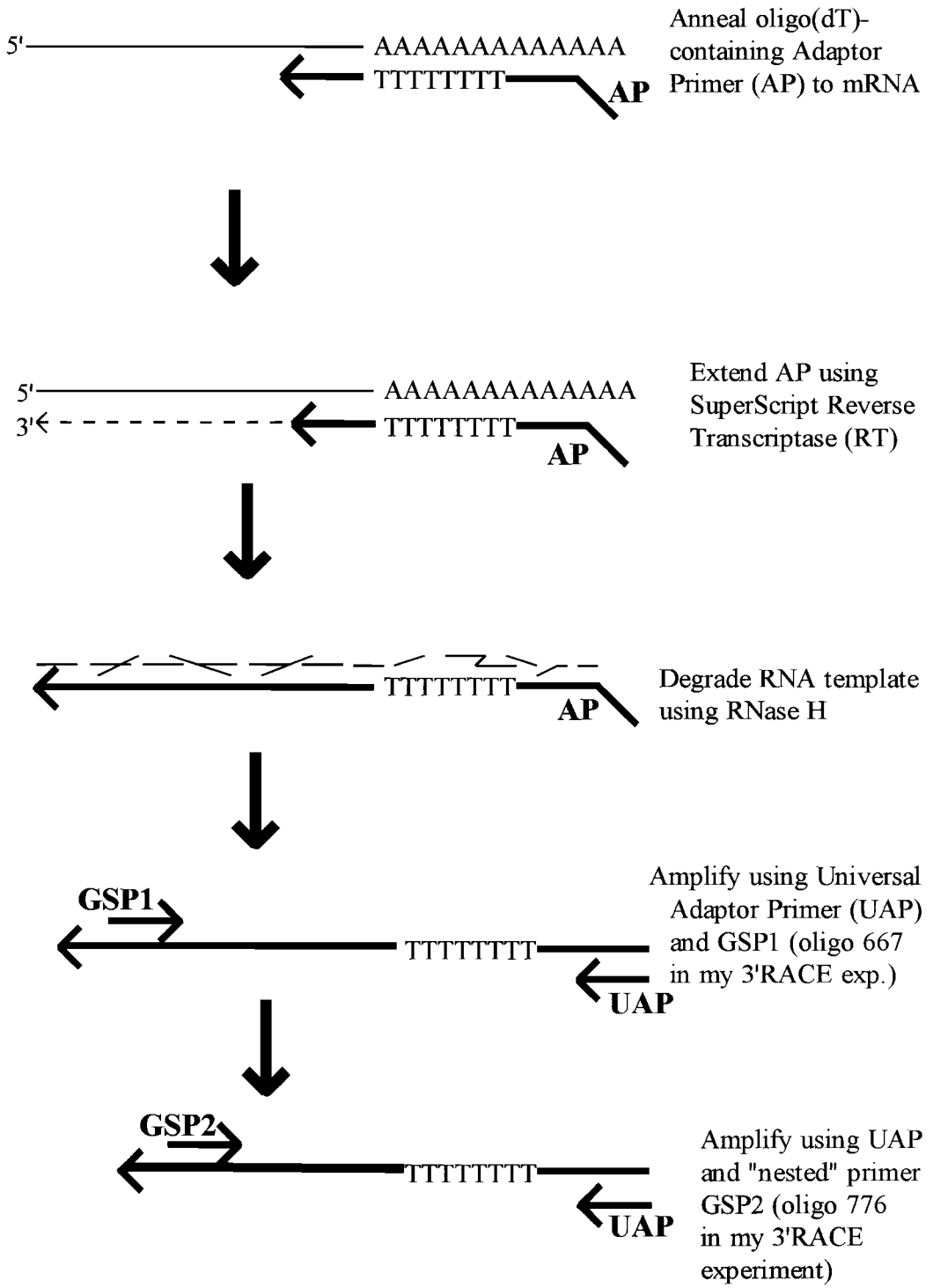

mRNA 5'
(A)n-3'
GSP1
Anneal first strand primer, GSP1(oligo 668 in my 5'RACE experiment), to mRNA.
5'
(A)n-3'
cDNA
Copy mRNA into cDNA with SuperScript II Reverse Transcriptase.
(A)n-3'
Degrade mRNA template with RNase H.
Purify cDNA with GlassMAX Spin Cartridge.
3'-(dC)n
Tail purified cDNA with dCTP and TdT (terminal deoxynucleotide transferase).
Anchor Primer
3'-(dC)n
GSP2
Amplify dC-tailed cDNA by PCR using the Anchor primer and nested GSP2 (oligo 818 in my 5'RACE experiment).
PCR product ready for: agarose gel electrophoresis, specific DNA isolation, and TA cloning into plasmid pCR II.

Figure 3. Summary of the 3' RACE System procedure (cited from GIBCO BRL's instruction manual; cat. no. 18373-019).


5'
AAAAAAAAAAAAA
TTTTTTTT
AP
Anneal oligo(dT)-containing Adaptor Primer (AP) to mRNA
5'
3'
AAAAAAAAAAAAA
TTTTTTTT
AP
Extend AP using SuperScript Reverse Transcriptase (RT)
TTTTTTTT
AP
Degrade RNA template using RNase H
GSP1
TTTTTTTT
UAP
Amplify using Universal Adaptor Primer (UAP) and GSP1 (oligo 667 in my 3'RACE exp.)
GSP2
TTTTTTTT
UAP
Amplify using UAP and "nested" primer GSP2 (oligo 776 in my 3'RACE experiment)

Three-tenth ml of cells were mixed with an appropriate amount of phage solution to give 10,000-50,000 plaques/150 mm plate. The mixture was incubated at 37 °C for 15 minutes with constant shaking, then plated in 7 ml top agar (0.7% agarose in LB-broth). Plates were incubated at 37 °C for 11 hours to let phages grow, then cooled at 4° C for 3 hours. Plaques were then lifted onto nitrocellulose filters (MagnaGraph, Micron Separations Inc., Westboro, MA). Two duplicate membranes were used for each plate. Three pieces of Whatmann 3MM paper were soaked respectively with denaturation buffer (0.5M NaOH, 1.5 M NaCl), neutralization buffer (0.5M Tris pH 8.0, 1.5M NaCl), and 2x SSC (diluted from 20x stock, 2.8M NaCl, 0.3M sodium citrate pH 7.0). Membranes were placed on the denaturation paper for 5 minutes, the neutralization paper for 5 minutes, then the 2x SSC paper for 2 minutes with the DNA side up. Membranes were allowed to dry 10 minutes, then DNA was fixed on the membrane by UV-crosslinking (UV Stratalinker 1800).

The processed membranes were prehybridized for 2 hours in 35% formamide, 5x SSPE, 5x Denhardt's, 0.5% SDS, 100 ug/ml salmon sperm DNA at the amount of 4 ml/150 mm filter. The prehybridization solution was replaced by fresh hybridization buffer and the radioisotope labeled probes were added to make the concentration to 0.5-0.75x10^6 cpm/ml. Hybridization was carried out at 37 °C overnight.

The membranes were washed first at 42 °C, then at 52 °C, and finally at 62 °C in 5x SSPE and 0.1% SDS, each for 30 minutes. Membranes were autoradiographed for one or two days depending on the signal intensity.

The clones which showed positive spots on both of the duplicate filters were removed from the plates with a sterile Pasteur pipette, placed in 1 ml SM buffer (0.1M NaCl, 0.017M $MgSO_4$, 0.05M Tris-HCl, 0.01% gelatin pH 7.5) with 20 ul chloroform, and incubated 2 hours at room temperature to release the phage. The phages from positive clones were replated and screened a second or a third time to purify. The final positive clones were removed from the plates and resuspended as before to use for subcloning.

In Vivo Excision of pBluescript from Uni-ZAP^{TM} XR (Subcloning)

I followed the protocol outlined by Stratagene for this step. After the phagemid was rescued, 200 ul and 20 ul of phagemid were mixed with 200 ul XL1-Blue at $O.D._{600}$=1, and 100 ul of each mixture was plated on LB plate containing 100 ug/ml ampicillin separately. The colonies grown on the plate after incubating at 37 °C overnight contained pBluescript double stranded phagemid with the cloned DNA insert, which was used for plasmid DNA preparation.

Cloning and Sequencing

TA Cloning

The TA Cloning^{TM} kit (from Invitrogen Company) takes advantage of the nontemplate-dependent activity of thermostable polymerase used in PCR that adds a single deoxyadenosine to the 3' ends of duplex molecules. These 3' A-overhangs are used to insert the PCR product into a pCR^{TM} II Vector which contains single 3' T-overhangs at its insertion sites.

The compositions of the ligation reaction were: 5 ul of sterile water, 1 ul of 10x ligation buffer, 2 ul of resuspended pCR^{TM} II vector (25 ng/ul), 1 ul of PCR product, and 1 ul of T_4 DNA ligase. The amount (X ng) of PCR product of "Y" base pairs to be ligated to the pCR^{TM} II vector with a 1:1 molar ratio was calculated according to the equation below:

X ng PCR product/Y bp PCR product=50 ng pCR^{TM} II vector/size in bp of the pCR^{TM} II vector (3932 bp)

The ligation reaction was incubated at 14-15 °C for a minimum of 4 hours.

TA Cloning Transformation

One ul of each TA Cloning ligation reaction (containing ligated recombinant plasmid DNA) was combined with 50 ul ONE SHOT™ competent cells. After adding 450 ul of prewarmed SOC medium to each vial, the cells were incubated at 37 °C for exactly 1 hour with shaking at 225 rpm. 25 ul and 100 ul from each transformation vial was then plated out on the LB agar plates containing antibiotic ampicillin (50 ug/ml) and X-gal (25 ul of 40 mg/ml X-gal stock was spread on the plates 1 hour before spreading the cells). After incubating at 37 °C overnight, the white colonies were lifted and grown in 3 ml or 150 ml of LB-ampicillin medium (ampicillin conc. : 50 ug/ml) at 37 °C overnight.

Plasmid MidiPreparation

The cells from 150 ml LB-ampicillin culture were harvested by centrifugation at 4000 g for 30 minutes, and plasmid DNA was purified by QIAGEN kit (QIAGEN Inc., Studio City, CA). The protocol supplied with the kit was followed. After the purified DNA was redissolved in TE buffer, its concentration was measured with a SHIMADZU spectrophotometer (model: UV160U).

Wizard™ Miniprep DNA Purification System from Promega

Two ml cells from 3 ml cultures (the remaining 1 ml was saved for frozen cell stocks) were treated with cell resuspension solution, cell lysis solution, then the neutralization solution and centrifuged to precipitate the cell debris. The plasmid in the supernatant was then purified by using the Wizard Minipreps DNA purification resin and the Wizard minicolumn.

Sequencing

Originally, sequencing reactions were done manually according to the protocol supplied with the Sequenase Version 2.0 kit (from USB). Later, the DNA was sequenced

by the Recombinant DNA/Protein Resource Facility (Core Facility) at OSU on a 373A DNA Sequencer from Applied Biosystems. The primers used for the clones in the pCR™ II vector were SP6 and T7 primers; those used for the clones from Uni-ZAP™ XR Subcloning were T3 and T7 primers.

Analysis of the DNA Sequences and Designed Oligonucleotides

The finished DNA sequences were analyzed with the MacVector™ program version 4.1.4 on the Core's Macintosh IIsi computer. The DNA was analyzed to find open reading frames (ORF) and its restriction enzyme maps. The analyzed DNA was also translated in all 6 protein reading frames and the molecular weight, pI, amino acid composition, antigenic structure, hydrophilic domains, and hydrophobicity plots of these six proteins were determined. Hydropathy index (profile) is a particularly important tool to predict the transmembrane domains in the G-protein coupled receptors.

The DNA or protein sequences were also sent to NCBI by E-mail to be aligned with DNA or protein databases by the Blast E-mail server; or the sequences were analyzed or aligned by using the GCG program sets in the OU VAX system, especially by using the Fasta and Pileup subprogram.

The oligonucleotides designed for use as internal probes for Southern blot analysis or as primers for PCR or primer walking sequencing were chosen from the conserved areas of the available protein sequences by counting the numbers of codon choices of the amino acids (e.g., met and trp have 1 codon choice; phe, tyr, and his have 2 codon choices; ile has 3 codon choices; val, pro, thr, ala, and gly have 4 codon choices; leu, ser, and arg have 6 codon choices) to select the areas containing the least gene coding peptides (Maniatis et al., 1989c). The oligonucleotides designed in this way will be more specific to the gene of concern. They were analyzed by using the Oligo 4.0 program to obtain their Tm and GC content and to determine if there was any dimer formation or hairpin loop formation in the

3' end of the designed oligonucleotide. Predicted oligonucleotides were synthesized by the OSU Core facility.

Northern Blot

Probe Preparation

A 152 bp DNA fragment I obtained from an RT-PCR experiment was amplified and radiolabeled by PCR radioactive labeling system from GIBCO BRL (Life Technology Inc.) with primer pair TM4/TM5 (see fig. 10 & fig. 14).

The recombinant plasmid DNA containing the 152 bp DNA insert was also transcribed with T_7 RNA polymerase to obtain the RNA fragment covering the 152 bp insert by using Ambion company's MAXIscript *in vitro* Transcription kit. The vector was linearized using a Hind III restriction site in the polylinker near the 5' end of the coding strand of this 152 bp DNA.

Preparation of Formaldehyde Gel

One gram of agarose was boiled in 75 ml DEPC treated H_2O for approximately 3 minutes in the microwave. Five ml of 20x RNA running buffer (83.72 g of MOPS, 13.6 g of $NaCH_3COO.3H_2O$, and 40 ml of pH 8.0-0.5M EDTA to make 1 liter solution) was mixed and the solution was cooled to 60 °C. Twenty ml of formaldehyde was then added and mixed by swirling and the gel was poured.

Electrophoresis and Blotting

There were 5 total RNA samples from different feeding stages (unfed, 20-40 mg, 50-100 mg, 100-200 mg, and over 250 mg) and 2 messenger RNA samples (from 50-100 mg and 100-200 mg feeding stages). These RNA samples and an RNA standard were electrophoresesed in the same gel.

After electrophoresis (20 Volt was applied to the Horizon 11-14 electrophoresis kit from GibcoBRL and the samples were electrophoresesed overnight), the RNA was transferred to the GeneScreen nylon membrane (NEN Research Products) and fixed according to the GeneScreen protocol. The hybridization and membrane washing conditions also followed the GeneScreen manufacturer's protocol.

CHAPTER IV

RESULTS AND DISCUSSION

Based on pharmacological studies, dopamine receptors in ticks and vertebrates are similar in their functions. However, quantitative and qualitative differences may exist in their molecular structure and these differences can be assessed by cloning and sequencing dopamine receptors in tick and comparing to the published sequences of vertebrate receptors.

Tick Genomic DNA Southern Blot

We obtained two available complementary DNA (cDNA) clones for both D_1(Zhou et al., 1990) and D_2 (Bunzow et al., 1988) receptors from Dr. Oliver Civelli, Vollum Institute, Portland. Genomic Southern blotting with these two probes was performed to determine if tick has genes for these two receptors in its genome.

Recombinant plasmid DNAs of both D1 and D2 clone were digested with the appropriate enzymes and separated by agarose gel electrophoresis with Ethidium Bromide staining (Fig. 4). A 2 Kb insert of the D_1 clone and a 2.4 Kb insert of the D_2 clone were cut from the agarose gel and purified (QIAquick Gel Extraction Kit). Tick and mouse genomic DNAs were digested with EcoRI, SmaI, and HindIII separately. The resulting fragments were separated according to size by 0.7% agarose gel electrophoresis (Fig. 5). The DNA on the gel was treated according to the standard protocol as described in the *Molecular Cloning Lab Manual* and transferred from the gel to a solid support (nylon

Figure 4. Confirmation of the D_1 and D_2 insert in the plasmid. The 2 lanes on the left side of the ladder contained the rat D_1 receptor insert (about 2.0 Kb long) and the 2 lanes on another side (right) contained the rat D_2 receptor insert (about 2.4 Kb long). The vectors of both clones all belong to pGEM Blue vector (about 2.8 Kb long).

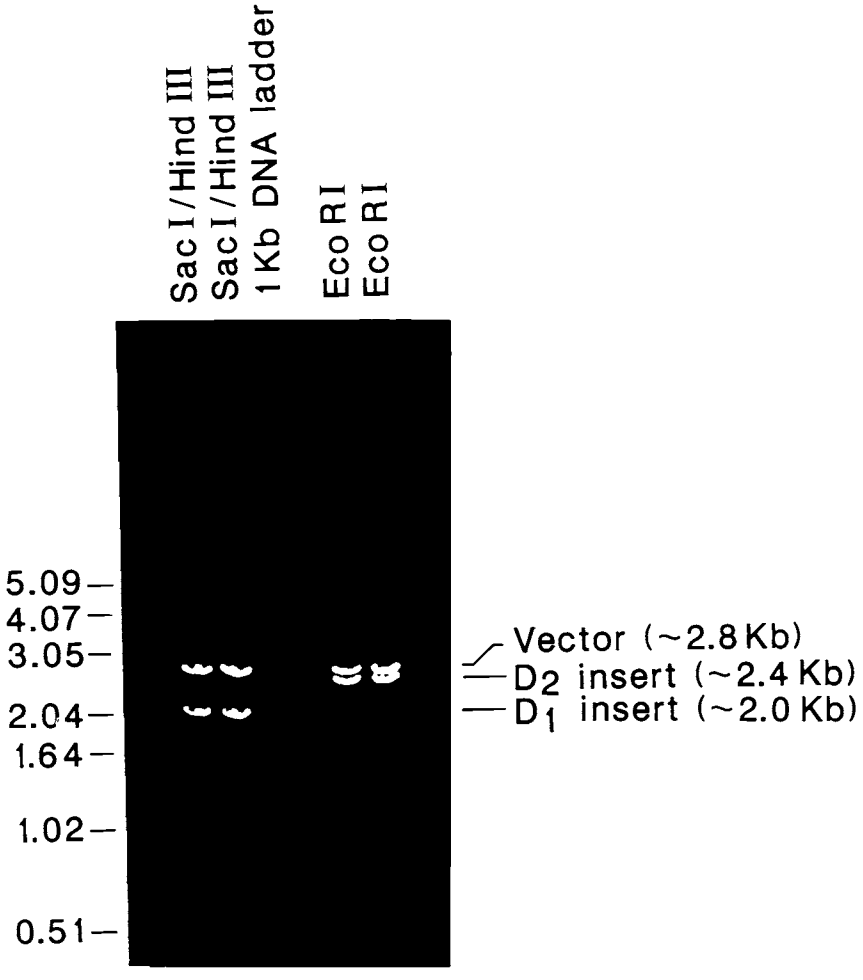
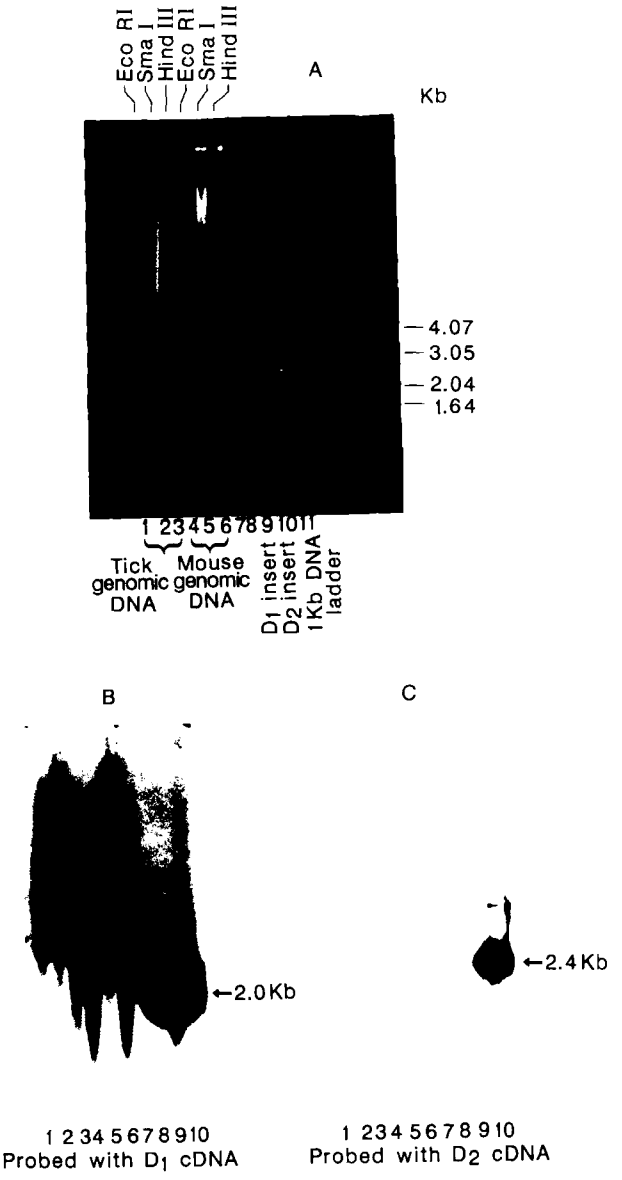

Sac I/Hind III
Sac I/Hind III
1 Kb DNA ladder
Eco RI
Eco RI
5.09
4.07
3.05
2.04
1.64
1.02
0.51
Vector (~2.8 Kb)
D2 insert (~2.4 Kb)
D1 insert (~2.0 Kb)

Figure 5. Tick genomic DNA Southern blot. A. tick and mouse genomic DNA were digested with EcoRI, SmaI, and HindIII respectively and separately. They were electrophoresesed on 0.7% agarose gel with the positive control D_1, and D_2 insert. B. autoradiographic film with D_1 cDNA insert (after 1 day exposure). C. autoradiographic film with D_2 cDNA probe (after 1 hour exposure).


Eco RI
Sma I
Hind III
Eco RI
Sma I
Hind III
A
Kb
4.07
3.05
2.04
1.64
1 2 3 4 5 6 7 8 9 10 11
Tick genomic DNA
Mouse genomic DNA
D1 insert
D2 insert
1Kb DNA ladder
B
C
2.0Kb
2.4Kb
1 2 3 4 5 6 7 8 9 10
Probed with D1 cDNA
1 2 3 4 5 6 7 8 9 10
Probed with D2 cDNA

membrane). Two membranes (from two gels) were prepared in this way; one was hybridized to the D_1 probe, another was hybridized to the D_2 probe. The final autoradiographs are shown in fig. 5. The membrane probed with rat D_1 cDNA was exposed to X-ray film for about 1 day and some nonspecific bindings were seen on the tick and mouse genomic DNA. The membrane probed with rat D_2 cDNA was exposed to X-ray film for 1 hour only and the strongest binding signal was seen on the probe itself.

The probes did not hybridize specifically to the tick genomic DNA or control genomic DNA from mouse, but only hybridized strongly to the probes themselves. The possible reasons why the D_1 and D_2 probes failed to hybridize specifically to tick or mouse genomic DNA are: 1. Maybe the clones were contaminated in the process of transformation. 2. There were some degradations both in the mouse and tick genomic DNA. 3. There were some interferences derived from the introns of the genomic DNA because the probes used were cDNAs and had exons only.

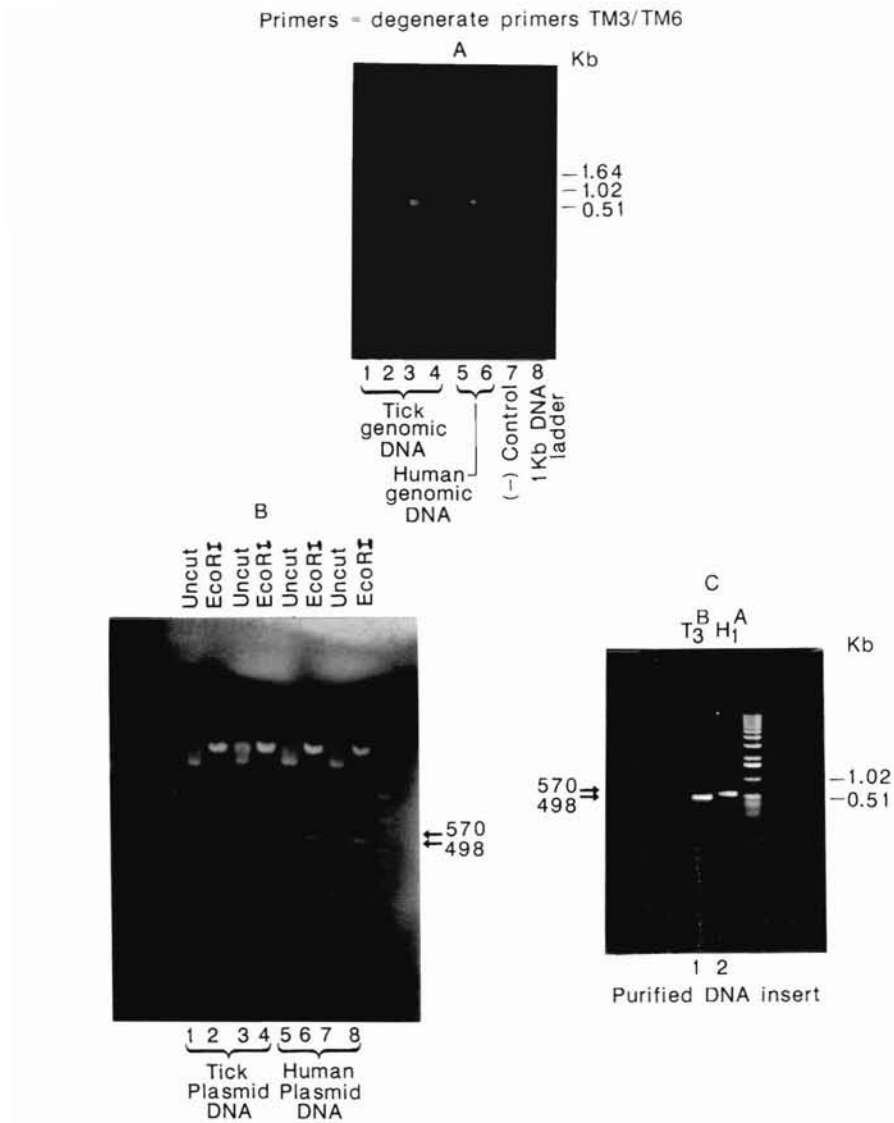
Genomic DNA PCR

An approach based on PCR with degenerate primers had been devised to clone new members of the G-protein coupled receptor family (Libert et al., 1989). So this approach was pursued to isolate the G-protein coupled receptor from tick genomic DNA using degenerate primers for PCR.

Several distinct bands were obtained from the mouse and human genomic DNA (controls) but very faint bands were present when the tick genomic DNA was used as template. I cut the specific bands out of the gel from the tick or human PCR products. I obtained 4 clones from the tick DNA and 2 clones from the human DNA (Fig. 6).

The TA cloning system was used to ligate these fragments to the pCR™ II vector. It seemed the fragment no. 3 (on lane 3 in fig. 6-A) identified from tick DNA PCR products (T_{3B}) and first fragment (on lane 5 in fig. 6-A) from human DNA PCR products

Figure 6. Genomic DNA PCR. A. genomic DNA PCR of tick and human samples with degenerate primers; T_{3B} clone was from DNA fragment on lane 3 and H_{1A} clone was from DNA fragment on lane 5. B. confirmation of the tick and human genomic DNA PCR products (T_{3B} and H_{1A}) after TA cloning and Wizard Miniprep. C. purified DNA inserts of T_{3B} and H_{1A} fragments.


Primers = degenerate primers TM3/TM6
A
Kb
1.64
1.02
0.51
1 2 3 4 5 6 7 8
Tick genomic DNA
Human genomic DNA
(-) Control
1 Kb DNA ladder
B
Uncut
EcoRI
Uncut
EcoRI
Uncut
EcoRI
Uncut
EcoRI
570
498
1 2 3 4 5 6 7 8
Tick Plasmid DNA
Human Plasmid DNA
C
T3B H1A
Kb
570
498
1.02
0.51
1 2
Purified DNA insert

(H_{1A}) were in the right size range of 500 to 600 bp between the TM3 and TM6 primers, and these clones (T_{3B} and H_{1A}, see fig. 6) were partialy sequenced by manual method. The sequencing was completed by Core's DNA sequencer. The DNA sequence of H_{1A} clone (fig. 7) has stop codons and there are two explanations for this phenomenon: 1. The H_{1A} clone was sequenced only once from both directions (the sequencer can read accurately for about 150 bp from either direction), so there were some misreadings of the bases in sequencing process. 2. Because this clone was ampified from the genomic DNA, there would be some introns in the sequence and stop codons would happen in the middle of the sequence. However, the H_{1A} clone was very similar to human 5-HT1D-type serotonin receptor gene (97% identity in the DNA sequences). In contrast, the T_{3B} insert (fig. 8) had no high similarity to other receptor genes according to the nucleic acid similarity search based on Fasta program and the analyses based on the hydropathy plots from MacVector's protein analysis toolbox.

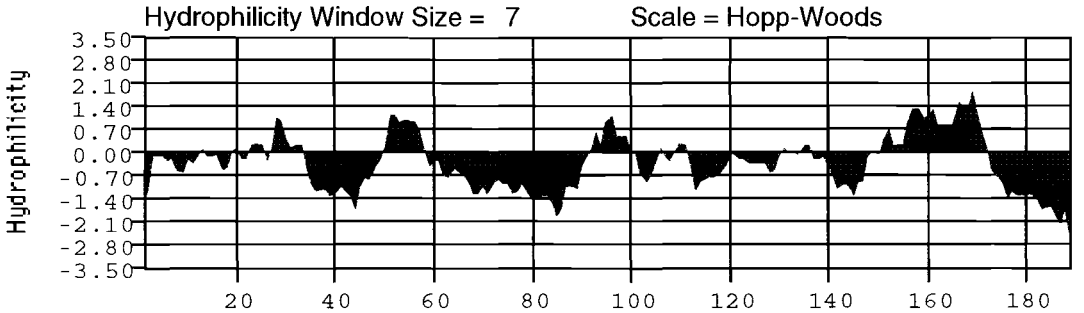
The results suggest that degenerate primers designed from the available mammalian receptor sequences might not be homologous enough for use in PCR with the tick genomic DNA which has a less close relationship with the human or mouse DNA sequences in the phylogenetic tree.

PCR Amplification of Tick cDNA from a 3-Day and 6-Day Tick Salivary Gland cDNA Library

The same degenerate primer pair was used to do the PCR with the tick cDNA library as template. There were promising bands in the PCR products (Fig.9). Those PCR products near 500 bp (500 to 600 bp is the predicted size range between the TM3 and TM6) were also cloned into the pCR™ II vector. Nevertheless, they did not prove to be a tick receptor gene after completing the sequencing. They appeared to be contaminants from some bacterial DNA or vector DNA of cDNA library because after these sequences

Figure 7. Cloned H_{1A} gene and hydropathy profile. A. hydropathy profile of H_{1A} peptide. B. H_{1A}'s DNA sequence and translated amino acids (below the DNA sequences) from 5' to 3' ends.

A.



B.

```
T GTG TGT CAT CGC GGT GGA TAG GTA CTG GGC AAT CAC AGA TGC CCT GGA
   V   C   H   R   G   G   *   V   L   G   N   H   R   C   P   G

ATA CAG TAA ACG CAG GAC GGC TGG CCA CGC GGC CAC CAT GAT CGC CAT
 I   Q   *   T   Q   D   G   W   P   R   G   H   H   D   R   H

TGT CTG GGC CAT CTC CAT CTG CAT CTC ATC CCC CCG CTC TTC TGG CGG
 C   L   G   H   L   H   L   H   L   I   P   P   L   F   W   R

CAG GCC AAG GCC CAG GAG GAG ATG TCG GAC TGT CTG GTG AAC ACC TCT
 Q   A   K   A   Q   E   E   M   S   D   C   L   V   N   T   S

CAG ATC TCC TAC ACC ATC TAC TCC ACC TGT GGG GGC TTC TAC ATT CCC
 Q   I   S   Y   T   I   Y   S   T   C   G   G   F   Y   I   P

TCG GTG TTG CTC ATC ATC CTA TAT GGC CGT ATC TAC CGG GGT GGC CGG
 S   V   L   L   I   I   L   Y   G   R   I   Y   R   G   G   R

AAC CGC ACC CTG AAT CCA ACC TCA CTC TAT GGG AAG CGC TTC ACC CCG
 N   R   T   L   N   P   T   S   L   Y   G   K   R   F   T   P

GCC CAC CTC ATC ACA GGC TCT GCC GGG TCC TCG CCC TGC TCG CTC AAC
 A   H   L   I   T   G   S   A   G   S   S   P   C   S   L   N

TCC AGC CTC CAT GAG GGG CAC TCG CAC TCG GCT GGC TCC CCT CTC TTT
 S   S   L   H   E   G   H   S   H   S   A   G   S   P   L   F

TTC AAC CAC GTG AAA ATC AAG CTT CCT GAC AGT GCC CTG GAA CGC AAG
 F   N   H   V   K   I   K   L   P   D   S   A   L   E   R   K

AGG ATT TCT GCT GCT CGA GAA AGG AAA GCC ACT AAA ATC CTG GGC ATC
 R   I   S   A   A   R   E   R   K   A   T   K   I   L   G   I

ATT CTG GGG GCC TTC ACC CTC TGC TGG CTG CCC TTC TTC CT
 I   L   G   A   F   T   L   C   W   L   P   F   F   L
```

Figure 8. T_{3B} sequence from 5' to 3' direction.

5'-----

CTGTGTGTGATTGCGGTGGATAGCTACAACCTCTTTATCGTCTCGAGAAGTGGTGGTGCCATT

CCCAGAGTACCAAGGCGGTGGAAGAGAGTTTCGTGCGGAACGTTATCATAAGCCTTTTCGAC

GTCCAGAAAGCAGCAGATGAGTCTCCTGCCTTCCTTCATGGCGATGTCTATACAGCTGGTGAG

TACGAAGAGGATGTCTTCTAGCTGTCTGCCCGGACGAAACCCATTCTGCAGCTTCGTTAGGAC

GTGCACTGATCCTACCCATCCGCTGATCCAGGCTTTCAAGACTTGCATGAAAACTCGGTACAC

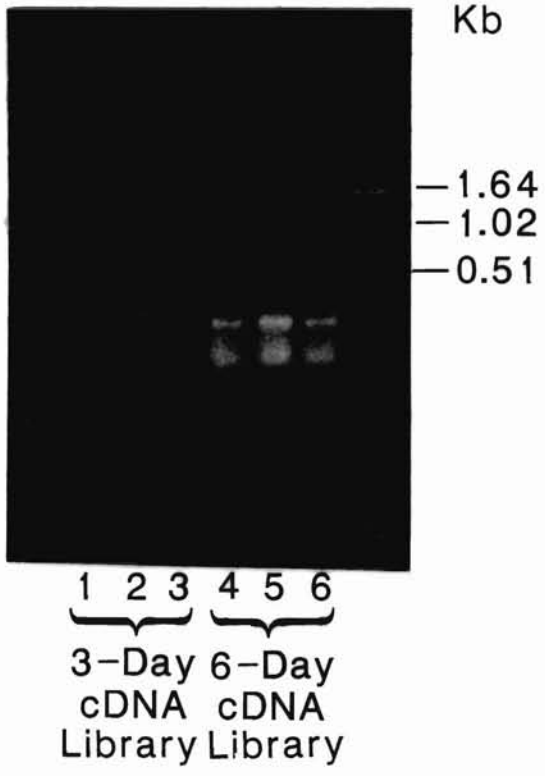
CACGCTGGTGACCGTGAGTGCCCAGTAGTCACCGATGTGGGCTGCATTGCCGCCGTGCTTCAA

CAGGAGCACCACCCTGCCATTGCGCCAGTCTTCGGGTATGTCTCCGCCGTTGGTAATGGTGGT

AAACAACTCGGCCAGCTGTTCTCTCGAGTTTCACCCTCTGCTGGCTGCCCTACTTCAT-----3'

Figure 9. PCR amplification of tick cDNA from a 3-DAY and 6-DAY tick salivary gland cDNA library.

Primer set = degenerate primers TM3/TM6



were aligned with DNA databases they were similar to some bacterial or phage sequences.

Screening

Up to this point, the only result obtained was the human 5-HT1D-type serotonin receptor gene fragment (from TM3 to TM6). So, I tried to use this H_{1A} gene (Fig. 7) to probe and screen the tick's Uni-ZAP XR cDNA library. Two phagemid clones were noted with the strongest hybridization signal. After the pBluescript phagemids were rescued (see Materials and Methods) and transformed to XL1-Blue cells I did the plasmid DNA preparation and tried to get their sequences. Unfortunately, they did not turn out to be receptor genes (data not shown) because their hydropathy profiles of translated proteins (6 frames) did not have the typical pattern like that of G-protein coupled receptor (i.e., 7 hydrophobic regions representing 7 transmembrane domains in receptor); besides, their DNA similarity searches in the database turned out to have nothing to do with the G-protein coupled receptors. Maybe I should lower down the hybridization and washing stringency and pick up more clones to sequence.

RT-PCR

I next used RT-PCR method because the cDNA derived from the direct reverse transcription of the RNA may have more complete information about the gene than the cDNA library, and it would not have interferences from introns that may be present in the genomic DNA. Tick total RNA was used as template and cDNA was synthesized using reverse transcriptase. Then the degenerate primer pair was used for the PCR reaction. The oligonucleotides, originally designed to be the internal probes from the TM4 (sense strand) and TM5 (anti-sense strand) conserved segments of the rat D_2 dopamine receptor gene (Fig. 10), were also used as primers to complete the secondary PCR of those PCR

Figure 10. Rat D_2 receptor gene (GeneBank accession no.: M36831; Document ID: 203905) and translated amino acid sequences; the areas of DNA sequences used for TM4 and TM5 primers are underlined.

```
1
ATG GAT CCA CTG AAC CTG TCC TGG TAC GAT GAC GAT CTG GAG AGG CAG AAC TGG AGC CGG
 M   D   P   L   N   L   S   W   Y   D   D   D   L   E   R   Q   N   W   S   R
61
CCC TTC AAT GGG TCA GAA GGG AAG GCA GAC AGG CCC CAC TAC AAC TAC TAT GCC ATG CTG
 P   F   N   G   S   E   G   K   A   D   R   P   H   Y   N   Y   Y   A   M   L
121
CTC ACC CTC CTC ATC TTT ATC ATC GTC TTT GGC AAT GTG CTG GTG TGC ATG GCT GTA TCC
 L   T   L   L   I   F   I   I   V   F   G   N   V   L   V   C   M   A   V   S
181
CGA GAG AAG GCT TTG CAG ACC ACC ACC AAC TAC TTG ATA GTC AGC CTT GCT GTG GCT GAT
 R   E   K   A   L   Q   T   T   T   N   Y   L   I   V   S   L   A   V   A   D
241
CTT CTG GTG GCC ACA CTG GTA ATG CCG TGG GTT GTC TAC CTG GAG GTG GTG GGT GAG TGG
 L   L   V   A   T   L   V   M   P   W   V   V   Y   L   E   V   V   G   E   W
301
AAA TTC AGC AGG ATT CAC TGT GAC ATC TTT GTC ACT CTG GAT GTC ATG ATG TGC ACA GCA
 K   F   S   R   I   H   C   D   I   F   V   T   L   D   V   M   M   C   T   A
361
AGC ATC CTG AAC CTG TGT GCC ATC AGC ATT GAC AGG TAC ACA GCT GTG GCA ATG CCC ATG
 S   I   L   N   L   C   A   I   S   I   D   R   Y   T   A   V   A   M   P   M
421
CTG TAT AAC ACA CGC TAC AGC TCC AAG CGC CGA GTT ACT GTC ATG ATT GCC ATT GTC TGG
 L   Y   N   T   R   Y   S   S   K   R   R   V   T   V   M   I   A   I   V   W
                                                     TM4 Primer
481
GTC CTG TCC TTC ACC ATC TCC TGC CCA CTG CTC TTC GGA CTC AAC AAT ACA GAC CAG AAT
 V   L   S   F   T   I   S   C   P   L   L   F   G   L   N   N   T   D   Q   N
541
GAG TGT ATC ATT GCC AAC CCT GCC TTT GTG GTC TAC TCC TCC ATT GTC TCA TTC TAC GTG
 E   C   I   I   A   N   P   A   F   V   V   Y   S   S   I   V   S   F   Y   V
601
CCC TTC ATC GTC ACT CTG CTG GTC TAT ATC AAA ATC TAC ATC GTC CTC CGG AAG CGC CGG
 P   F   I   V   T   L   L   V   Y   I   K   I   Y   I   V   L   R   K   R   R
TM5 Primer
661
AAG CGG GTC AAC ACC AAG CGC AGC AGT CGA GCT TTC AGA GCC AAC CTG AAG ACA CCA CTC
 K   R   V   N   T   K   R   S   S   R   A   F   R   A   N   L   K   T   P   L
721
AAG GAT GCT GCC CGC CGA GCT CAG GAG CTG GAA ATG GAG ATG CTG TCA AGC ACC AGC CCC
 K   D   A   A   R   R   A   Q   E   L   E   M   E   M   L   S   S   T   S   P
781
CCA GAG AGG ACC CGG TAT AGC CCC ATC CCT CCC AGT CAC CAC CAG CTC ACT CTC CCT GAT
 P   E   R   T   R   Y   S   P   I   P   P   S   H   H   Q   L   T   L   P   D
841
CCA TCC CAC CAC GGC CTA CAT AGC AAC CCT GAC AGT CCT GCC AAA CCA GAG AAG AAT GGG
 P   S   H   H   G   L   H   S   N   P   D   S   P   A   K   P   E   K   N   G
901
CAC GCC AAG ATT GTC AAT CCC AGG ATT GCC AAG TTC TTT GAG ATC CAG ACC ATG CCC AAT
 H   A   K   I   V   N   P   R   I   A   K   F   F   E   I   Q   T   M   P   N
961
GGC AAA ACC CGG ACC TCC CTT AAG ACG ATG AGC CGC AGA AAG CTC TCC CAG CAG AAG GAG
 G   K   T   R   T   S   L   K   T   M   S   R   R   K   L   S   Q   Q   K   E
1021
AAG AAA GCC ACT CAG ATG CTT GCC ATT GTT CTC GGT GTG TTC ATC ATC TGC TGG CTG CCC
 K   K   A   T   Q   M   L   A   I   V   L   G   V   F   I   I   C   W   L   P
1081
TTC TTC ATC ACG CAC ATC CTG AAT ATA CAC TGT GAT TGC AAC ATC CCA CCA GTC CTC TAC
 F   F   I   T   H   I   L   N   I   H   C   D   C   N   I   P   P   V   L   Y
1141
AGC GCC TTC ACA TGG CTG GGC TAT GTC AAC AGT GCC GTC AAC CCC ATC ATC TAC ACC ACC
 S   A   F   T   W   L   G   Y   V   N   S   A   V   N   P   I   I   Y   T   T
1201
TTC AAC ATC GAG TTC CGC AAG GCC TTC ATG AAG ATC TTG CAC TGC TGA
 F   N   I   E   F   R   K   A   F   M   K   I   L   H   C   *
```

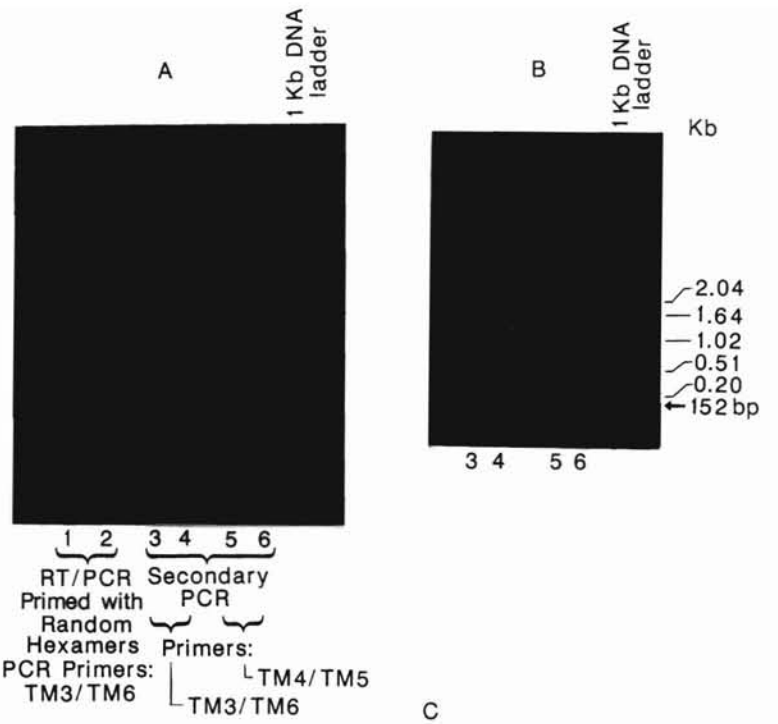
products derived from tick's cDNA with degenerate primers. In this experiment, 3 fragments from the secondary PCR with degenerate primers (TM3/TM6) and one very specific band (~152 bp) from the secondary PCR with oligonucleotide primers (TM4/TM5) were obtained (Fig. 11). After the Southern blot analysis by the H_{1A} probe (Fig. 11), it appeared that some of the fragments had high similarity with the H_{1A} probe, especially the small fragment (~152 bp) derived from the secondary PCR with internal primers (TM4/TM5). TA cloning (Fig. 12) and subsequent sequencing (Fig. 13 and Fig. 14) of this DNA showed that the small fragment (~ 152 bp) had 98% similarity to the rat D_2 receptor gene at the cloned and sequenced area (between TM4 and TM5).

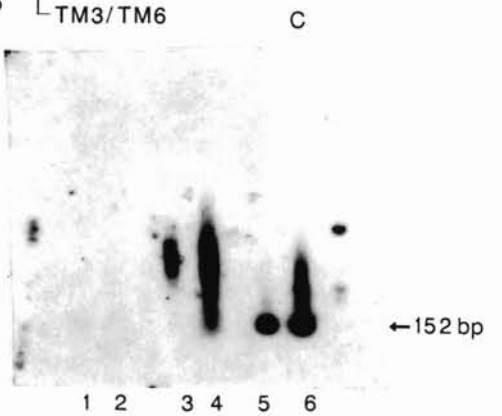
Extension of the Cloned Gene Fragment to Full Length

Next, I tried to extend the tick-derived D_2 receptor gene fragment (152 bp DNA) to complete its length and obtain the full-length gene. I tried to use the primer pair, which consisted of one primer from TM4 or TM5-a.s. and another from T3 or T7 primer of the vector, to do the PCR of the tick's cDNA library to see if I could get a longer piece of the tick-derived D_2 dopamine receptor gene. Several PCR fragments were obtained but none showed similarity to dopamine receptor gene (Fig. 15) after their sequences were determined and analyzed based on the blastn and MacVector programs (data not shown). In addition, there were no matches or any alignments between the sequences of the PCR fragments and the 152 bp DNA. Maybe the D_2 receptor gene fragments in tick cDNA library doesn't cover the area from TM4 to TM5. Finally, I tried to use the 5' RACE & 3' RACE (Fig. 2 & Fig. 3) to extend this 152 bp tick-derived D_2 receptor gene fragment.

I did not see any bands from the 1st run of my 5' RACE reaction, but I saw a specific band near 1 Kb after another 2 runs of PCR (Fig. 16). Unfortunately, this fragment was not the extended gene of the 152 bp DNA because this fragment had no match sequences with the 152 bp DNA except in the primer site (data not shown). I obtained a specific

Figure 11. Southern blot of RT-PCR products with H_{1A} probe. A. RT-PCR of tick's total RNA. lanes 1,2: random hexamer for reverse transcription, degenerate primers (TM3 and TM6) for PCR; lanes 3,4: PCR of lanes 1,2's products again with degenerate primers; lanes 5,6: PCR of lanes 1,2's products again with TM4/TM5 primer set. B. high resolution view of left picture's lanes 3,4, 5, and 6. C. autoradiographic film with H_{1A} probe.





PROBE: Human 5-HT1-D-type Serotonin Receptor Gene Fragment

Figure 12. Southern blot of the RT-PCR products with H_{1A} probe after TA cloning. A. confirmation of the secondary PCR products of RT-PCR after TA cloning and Wizard Miniprep by EcoRI digestion. B. Southern blot of the secondary PCR DNA of RT-PCR with H_{1A} probe.

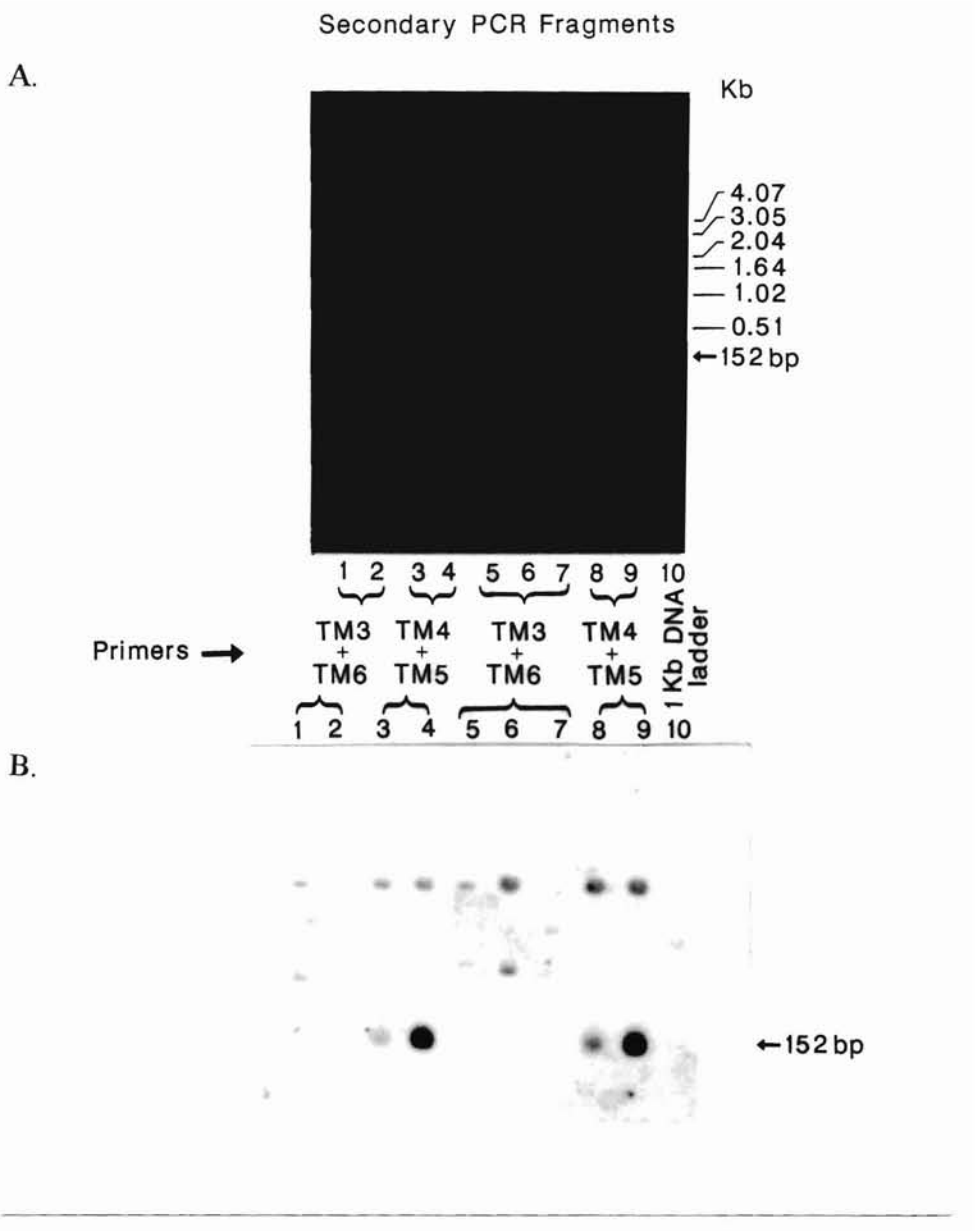
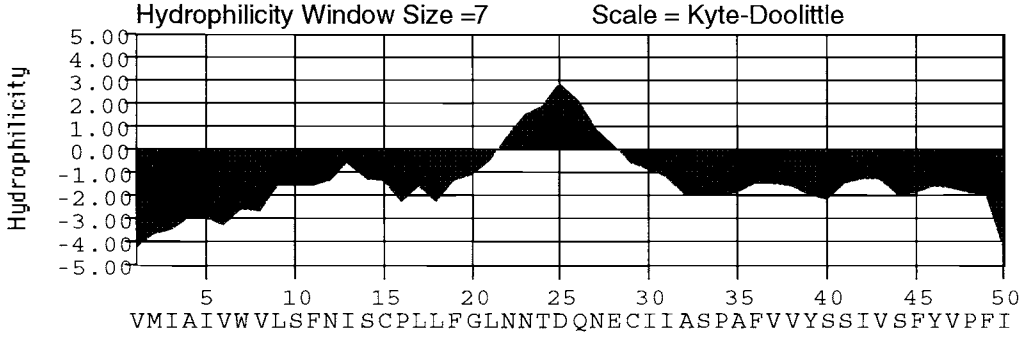

Secondary PCR Fragments
A.
Kb
4.07
3.05
2.04
1.64
1.02
0.51
←152 bp
1 2 3 4 5 6 7 8 9 10
Primers →
TM3 + TM6
TM4 + TM5
TM3 + TM6
TM4 + TM5
1 Kb DNA ladder
1 2 3 4 5 6 7 8 9 10
B.
←152 bp

Figure 13. Alignments of the tick-derived 152 bp DNA sequence with the rat D_2 (genebank accession No. X17458) and human retinal D_2 (genebank accession No. S69899) dopamine receptor sequences in the same area (using Pileup and Pretty subprograms in GCG program).

```
           1                                                   50
      Rat  GTCATGATTG CCATTGTCTG GGTCCTGTCC TTCACCATCT CCTGCCCACT
     Tick  GTCATGATTG CCATTGTCTG GGTCCTGTCC TTCAaCATCT CCTGCCCACT
    Human  GTCATGATct CCATcGTCTG GGTCCTGTCC TTCACCATCT CCTGCCCACT
Consensus  GTCATGATTG CCATTGTCTG GGTCCTGTCC TTCACCATCT CCTGCCCACT

           51                                                  100
      Rat  GCTCTTCGGA CTCAACAATA CAGACCAGAA TGAGTGTATC ATTGCCAaCC
     Tick  GCTCTTCGGA CTCAACAATA CAGACCAGAA TGAGTGTATC ATTGCCAGCC
    Human  cCTCTTCGGA CTCAAtAAcg CAGACCAGAA cGAGTGcATC ATTGCCAGCC
Consensus  GCTCTTCGGA CTCAACAATA CAGACCAGAA TGAGTGTATC ATTGCCAGCC

           101                                                 150
      Rat  CTGCCTTtGT GGTCTACTCC TCCATTGTCT CATTCTACGT GCCCTTCATC
     Tick  CTGCCTTCGT GGTCTACTCC TCCATTGTCT CATTCTACGT GCCCTTCATC
    Human  CTGCCTTCGT GGTCTACTCC TCCATTGTCT CATTCTACGT GCCCTTCATC
Consensus  CTGCCTTCGT GGTCTACTCC TCCATTGTCT CATTCTACGT GCCCTTCATC

           151
      Rat  GT
     Tick  GT
    Human  GT
Consensus  GT
```

Figure 14. Tick-derived 152 bp D_2 receptor gene fragment and hydropathy plot. A. The hydropathy profile of tick-derived D_2 receptor fragment's amino acids and the amino acid alignments with those of rat, human, and xenopus' D_2 receptors in this area. Unconserved amino acids are italicized and bolded. B. Tick-derived receptor gene fragment and translated amino acids between TM4 and TM5. The DNA sequences used as primers are bolded: TM4 and TM5 were used for RT-PCR; TM4, GSP872 and GSP776 were used for 3' RACE; TM5, GSP871 and GSP818 were used for 5' RACE.

A.




```
Tick     VMIAIVWVLSFNISCPLLFGLNNTDQNECIIASPAFVVYSSIVSFYVPFI
Rat      VMIAIVWVLSFTISCPLLFGLNNTDQNECIIANPAFVVYSSIVSFYVPFI
Human    VMISIVWVLSFTISCPLLFGLNNADQNECIIANPAFVVYSSIVSFYVPFI
Xenopus VMISVVWVLSFAISCPLLFGLNNTGSKVCIIDNPAFVIYSSIVSFYVPFI
```

B.

```
                (TM4)
     5'-----------667----------->3'                ----------872---------->
5'-----GTC ATG ATT GCC ATT GTC TGG GTC CTG TCC TTC AAC ATC TCC TGC CCA
        V   M   I   A   I   V   W   V   L   S   F   N   I   S   C   P

                                 --------------776------------>
CTG CTC TTC GGA CTC AAC AAT ACA GAC CAG AAT GAG TGT ATC ATT GCC
 L   L   F   G   L   N   N   T   D   Q   N   E   C   I   I   A
                              <-------------818-------------

AGC CCT GCC TTC GTG GTC TAC TCC TCC ATT GTC TCA TTC TAC GTG CCC
 S   P   A   F   V   V   Y   S   S   I   V   S   F   Y   V   P
    <---------871----------                    3'<-----668------
                                                    (TM5)

TTC ATC GT-----3'
 F   I   V
<---------5'
```

Figure 15. Products of PCR using 3-day feeding tick salivary gland cDNA library to amplify 5' or 3' end of the D_2 152 bp gene. The primer pairs used are indicated above the figure.

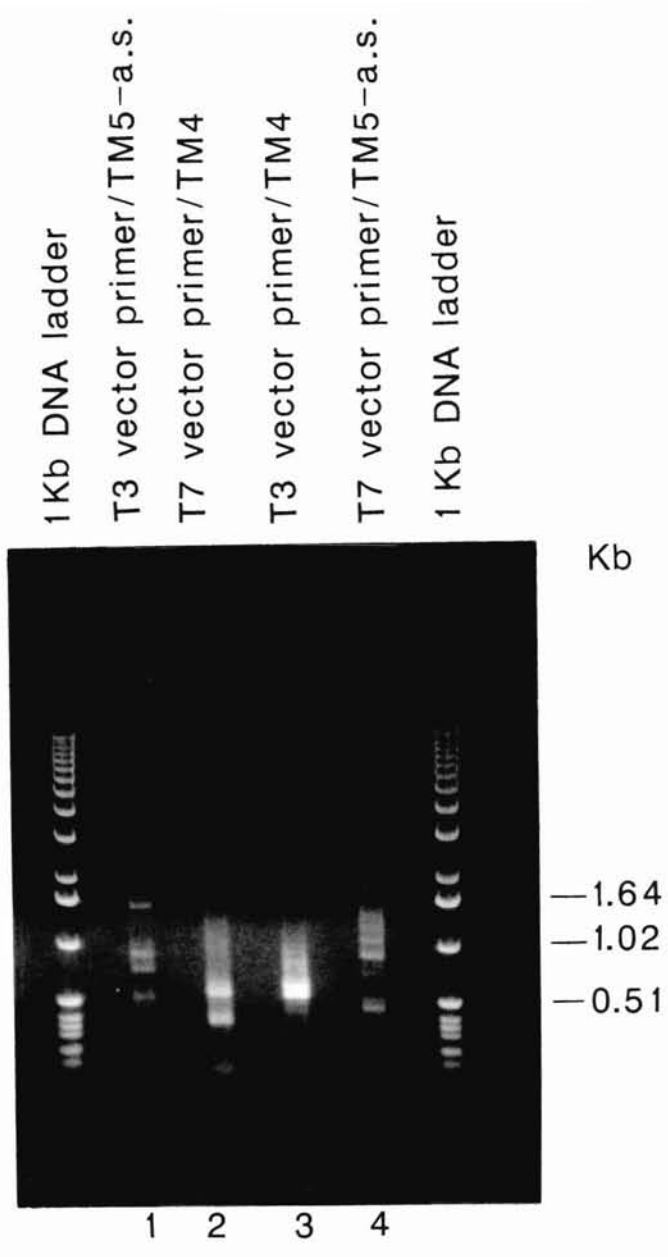
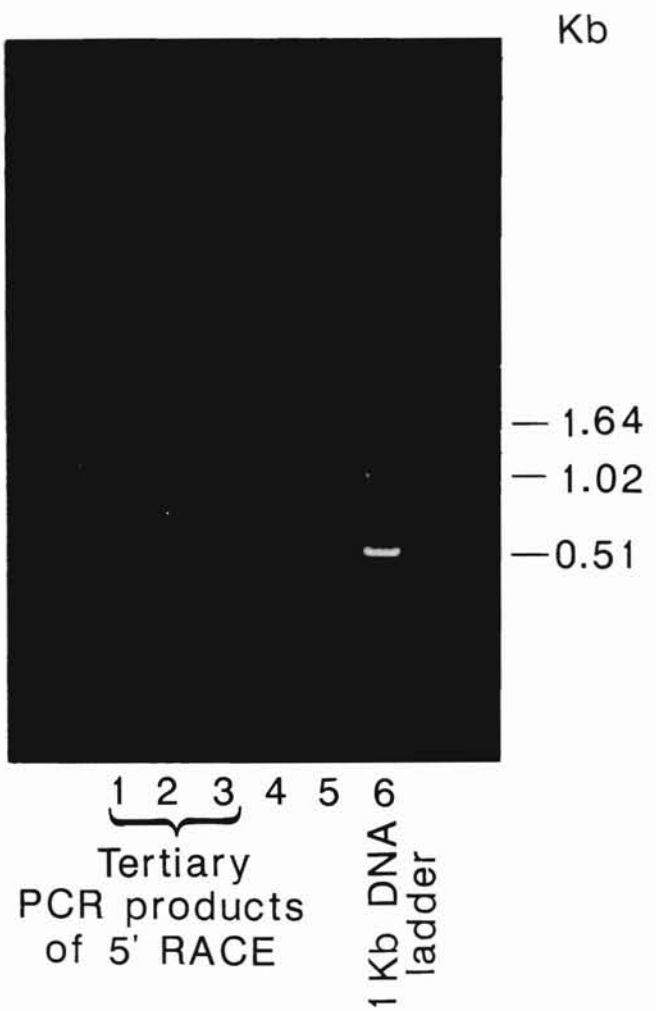

1Kb DNA ladder
T3 vector primer/TM5-a.s.
T7 vector primer/TM4
T3 vector primer/TM4
T7 vector primer/TM5-a.s.
1 Kb DNA ladder
Kb
1.64
1.02
0.51
1
2
3
4

Figure 16. Rapid amplification of 5' cDNA ends of tick-derived D_2 receptor gene fragment from total RNA. Primers used are indicated above the figure.

Primer for reverse transcription = 668
Primer set for PCR = 818/UAP

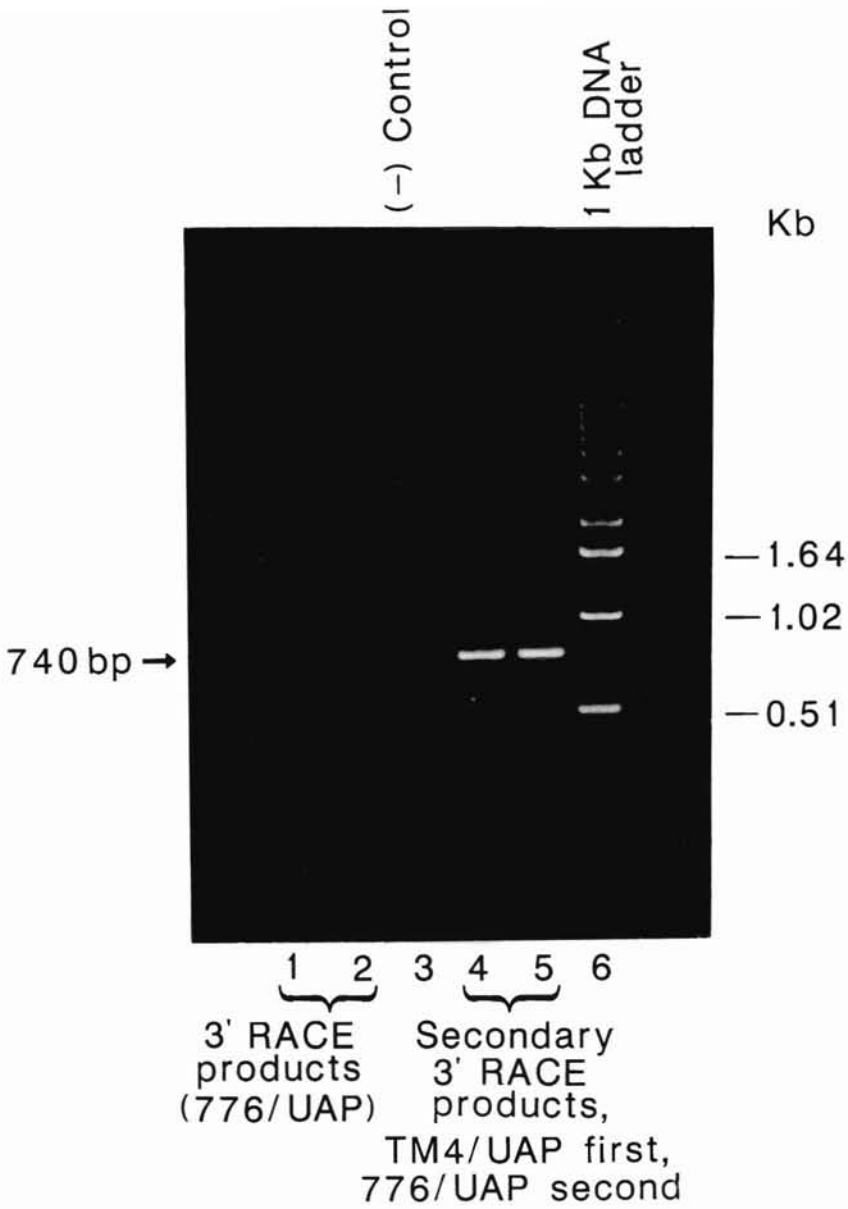


band near 740 bp after one run of 3' RACE reaction (Fig. 17). However, this was not the gene I wanted after cloning and sequencing were finished because there were no match sequences between this fragment and the 152 bp DNA except in the primer site (data not shown). Maybe I should change the conditions (e.g., used primer locations, PCR parameters) and redo the 5' and 3' RACE or try another approach to finish this tick-derived D_2 receptor whole sequence.

Northern Blot

In this point, we doubted if the 152 bp DNA sequence was the real gene in tick genome. The Northern blotting was performed trying to confirm it. Both RNA and DNA probes derived from this 152 bp DNA fragment (see Materials and Methods for probe preparation) were used to hybridize with different stages of tick RNA samples, but none of the autoradiograms showed promising bands (data not shown). It's speculated that either the tick genome doesn't express this gene or this receptor sequence is existent in very low copy in tick mRNA.

Figure 17. Products of 3' RACE of tick-derived D_2 receptor gene fragment with 776/UAP primer pair.


(−) Control
1 Kb DNA ladder
Kb
1.64
1.02
0.51
740 bp
1 2 3 4 5 6
3' RACE products (776/UAP)
Secondary 3' RACE products, TM4/UAP first, 776/UAP second

CHAPTER V

SUMMARY AND CONCLUSIONS

Ticks are economically important parasites of domestic animals due to their tenacious feeding behavior and their ability to harbor and transmit disease-causing organisms, including toxicosis and tick paralysis. They are obligatory blood feeding, facultative ectoparasites. Much evidence indicates that the fluid elimination via the tick's salivary glands in the process of feeding is controlled by nerves. A dopamine receptor plays a major role in controlling fluid secretion; dopamine or biogenic amine stimulates the dopamine receptor and the signal is transduced to the interior of the cell to trigger series of reaction to control the ion channel or other physiological functions like elimination of water from the blood or neutralization of some antibody from the host.

So, the cloning of the receptor gene of the dopamine or other neurotransmitters in the tick salivary glands becomes very important because the salivation processes may involve specific receptor sites that could provide novel targets for pharmaceutical control agents and provide ecologically more targeted means of biorational tick control.

My experiment of the Southern blotting of the tick genomic DNA by using the D_1 and D_2 probes failed due to the accuracy of the probes or the preparation of the genomic DNA. Furthermore, it seemed not easy to get the right clone by just using the degenerate primers to do the PCR; and sometimes, the scientific discovery happens in occasional situation just like that the primer pair I used to get the putative tick D_2 receptor gene fragment was originally designed for the use as internal oligo-probes to do the Southern blotting experiment.

The 152 bp D_2 dopamine receptor gene fragment, which may be the first reported finding of dopamine receptor in tick, has high similarity (98% identity) to the rat D_2 receptor gene and Gotzes et al. also found a *Drosophila* dopamine receptor with high similarity to human $D_{1/5}$ receptors (unpublished, see Entrez, Document ID: 479127). This means that the arthropod dopamine receptor may have similar function and structure to mammal's; anyway, the truth will be found out when the whole dopamine receptor sequence in tick is finished.

Because the 152 bp gene fragment I cloned from tick was difficult to extend to full length, could not significantly hybridize to the tick RNA, and it has only 3 nucleotides (Fig. 13) different from the rat D_2 receptor gene that was used in the tick genomic DNA Southern blot, this 152 bp gene is suspected to be a contaminant from the PCR reaction. Besides, from the view of the evolutionary process as shown by the phylogenetic tree, it seems not possible that the tick sequence has about 98% similarity to the rat D_2 receptor gene in that 152 bp sequence area (human D_2 receptor DNA sequence has only 91% similarity to rat D_2 receptor DNA sequence in this area) even if this area is so short and highly conserved in different species. So, I can only say, this 152 bp DNA fragment may not be in the tick genome at all or tick has a very low copy of this D_2 receptor gene.

The identification of five separate genes encoding pharmacologically distinct dopamine receptors in the bioorganism by other researchers indicates heterogeneity within this receptor system that was unexpected as of a few years ago. It is thus logical to say that even more dopamine receptor subtypes might be existent in the biological organism and the life is controlled by a very complex system that can be coordinated so well.

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