MOLECULAR CLONING OF DOPAMINE D2 RECEPTOR AND PROTEIN KINASE GENES FROM THE SALIVARY GLANDS OF *Amblyomma americanum* (L.)

By

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Bachelor of Science

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Shanghai, China

1989

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
December, 1996

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AND PROTEIN KINASE GENES FROM
THE SALIVARY GLANDS OF
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ACKNOWLEDGMENTS

I wish to express my sincere appreciation to my major advisor, Dr. Richard Essenberg for his supervision, inspiration and friendship. My sincere appreciation extends to my other committee members Dr. John Sauer and Dr. Franklin Leach, whose guidance, assistance, encouragement, and friendship are invaluable. I would like to thank Dr. John Sauer and Dr. Richard Essenberg for providing me this research opportunity and their generous financial support.

More over, I wish to express my gratitude to those who provided suggestions and assistance for this study: Dr. Janis McSwain, Dr. Melanie Palmer, Mr. Jim Tucker, Ms. Xiaoping Guo, Ms. Ying Qian, Mr. Kuichun Zhu and Mr. Jiayou Liu.

I would also like to give my special appreciation to my husband, Xuejie Qian, for his constant support, love and understanding throughout this whole process. Thanks also go to my parents for their support and encouragement.

Finally, I would like to thank the Department of Biochemistry and Molecular Biology for supporting during these two years of study.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
cAMP	cyclic AMP
cAPK	cAMP-dependent protein kinase
CNS	central nervous system
CPL	cytoplasmic loop
cDNA	complementary DNA
dCTP	deoxycytidine triphosphate
DNA	deoxyribonucleic acid
GDP	guanosine diphosphate
GTP	guanosine triphosphate
LB	luria broth
mRNA	messenger RNA
nm	nanometre
O.D.	optical density
PAGE	polyacrylamide gelelectrophoresis
PCR	polymerase chain reation
pfu	laque forming unit
RACE	rapid amplification of cDNA end
RNA	ribonucleic acid
RT-PCR	reverse transcription and PCR
TdT	terminal deoxynucleotidyl transferase
Tm	melting temperature
TM	transmembrane
X-gal	5-Bromo-4-Chloro-3-Indolyl-b-D-Galactoside

CHAPTER I

INTRODUCTION

Ticks are recognized worldwide as major vectors of arboviruses, rickettsiae, spirochaetes and parasitic protozoa of man and domestic animals (Kaufman, 1989). Ixodid ticks which are the family of the greatest economic and epidemiological importance are relatively long-term feeders on host animals. *Amblyomma americanum* (L.), commonly known as the lone star tick owing to the silvery spot on its dorsal surface, has the distinction of being the first tick described in the United States (Bequaret, 1946). In the south-eastern United States, climatic factors, vegetation and an abundance of the host animals favor growth in populations of *A. americanum* (L.) (Hair and Howell, 1970). *A. americanum* (L.) attacks a wide spectrum of host animals including domestic cattle, *Bos taurus* (L.). Beef producers are confronted with recurring and high levels of tick infestation on their cattle in Oklahoma, Arkansas, and Missouri (Barnard, 1986).

Because of the ticks' importance as a pest of man and his possessions in some regions of the United States, extensive work has been done on the ecology, behavior, and control (Hair and Bowman, 1986).

The important impact of ticks on man and animals results from its tenacious feeding behavior and the resulting inflammation. During feeding, female ticks ingest a large quantity of host blood and eliminate excess water and ions back to the host via its salivary glands (Gregson, 1967). Male ticks imbibe very little blood and their mechanism of fluid balance is not studied.

Secretions of the salivary glands are not only essential for tick feeding, but also are the major route via which pathogenic organisms and toxins access the vertebrate host

(Kaufman, 1989). At the same time, the glands contribute most of the antigens that stimulate the immunological defense of the host. In addition, they also play a vital role in ionic and osmotic regulation and carrying out many other functions (for review, see Sauer et al., 1995).

The salivary glands are morphologically complex and consist of three types of multicellular acini in female ticks (Fawcett et al., 1986). The glands are controlled by nerves (Kaufman and Phillips, 1973a). Dopamine appears to be the neurotransmitter at the neuro-effector junction. Dopamine activates an adenylate cyclase which causes the formation of cAMP. The protein kinases are activated by cAMP to phosphorylate proteins that are of importance to secretion. Examinations of the effects of known dopamine receptor agonists and antagonists on tick salivary glands indicate the presence of a dopamine D1 receptor in tick salivary glands. However, high concentrations of dopamine decreases in both cAMP and fluid secretion, together with the D2 antagonist increases cAMP on the study of *Dermacentor variabilis* suggest that dopamine control may be more complex than can be explained by a single dopamine receptor in tick salivary glands.

One purpose of the project was to study whether a dopamine D2 receptor is present in tick salivary glands. This could help explain many of the complex pharmacological results if additional dopamine receptors which couple to inhibition of adenylate cyclase or other inhibitory signal transduction pathways are present in the salivary glands. Another purpose was to clone, sequence and characterize protein kinases controlling phosphorylation/dephosphorylation events which regulate secretion fluid. The principal method for tick control has been limited to the treatment of hosts and their environment with chemical acaricides. Overall, this project would be informative in controlling ticks at molecular level.

CHAPTER II

LITERATURE REVIEW

Dopamine Receptors

Ticks are economically and epidemiologically important parasites of domestic animals and humans, and they may harbor and transmit pathogenic organisms, induce toxicosis and cause tick paralysis (Strickland et al., 1976). There are two major families of ticks: Ixodidae and Argasidae, and one minor one: Nuttalielidae. The feeding process of ixodid female ticks can be divided into two phases: the slow phase which occupies the first seven or more days in the adult, and the rapid phase which lasts only 12-24 hours (Kaufman, 1989). During feeding, female ixodid ticks ingest a large quantity of host blood and secrete excess water and ions back into the host's circulation through salivary glands (Gregson, 1967), thus concentrating the nutrient components of the meal and regulating haemolymph volume and ionic concentration. Salivary secretion is the major route via which pathogenic organisms and toxins access the vertebrate host. Therefore, the salivary glands are the focus of most medical and veterinary problems associated with ticks (Kaufman, 1989).

Research on the physiology of fluid secretion by the salivary glands of ixodid ticks suggests that salivation is under nervous control, and the transmitter substance is a catecholamine (Sauer et al., 1979). Several other groups working on the salivary glands of ticks have provided evidence which supports this result. In early 1973, Kaufman and Phillips developed an *in vitro* preparation of the salivary gland which enables one to critically evaluate the effects of drugs on salivary secretion. They found that low

concentrations of catecholamines can stimulate fluid secretion (Kaufman and Phillips, 1973b; Kaufman, 1976). *In vivo*, injection of catecholamines into a tick's hemocoel stimulates secretion of saliva by partially fed females (Hsu and Sauer, 1975). The hypothesis is further supported by the anatomical evidence of nerves and synaptic-like areas in close association with salivary gland cells (Coons and Roshdy, 1973; Obenchain and Oliver, 1976; Megaw, 1977), and the finding of dopamine and norepinephrine in salivary glands and the synganglion of *Boophilus microplus* (Megaw and Robertson, 1974). Dopamine is the most potent stimulant of *in vitro* fluid secretion by glands of ixodid ticks (Kaufman, 1977). Salivary fluid secretion can be stimulated by low concentrations of dopamine in several species of hard ticks (Kaufman, 1976; Kaufman, 1977), including *A. americanum* (Sauer et al., 1979). It seems this is a common mechanism in the family Ixodidae.

Dopamine is an important neurotransmitter not only in ticks, but also is used widely in humans and other vertebrates. Dopamine is a neurotransmitter in both central and peripheral nervous systems. In the CNS, dopamine is involved in a variety of functions, including locomotor control, sexual function, cardiovascular homeostasis, endocrine regulation and cognition. The etiology of several diseases, including schizophrenia (Carlsson and Svensson, 1990; Davis et al., 1991), Parkinson's disease (Albanese, 1990; Robertson, 1992) and endocrine disturbances, have been linked to defective dopamine neurotransmission. In the periphery, dopamine has been ascribed multiple roles, including regulation of various aspects of cardiovascular function (Camsonne, 1987; Lokhandwala and Barrett, 1982; Lokhandwala et al., 1985).

Dopamine itself cannot cross the cell membrane. The effects of dopamine are mediated through two receptor subtypes, D1 and D2. The classification of receptor subtypes is based on different functional and physiological properties of these receptors. D1 receptors activate the enzyme adenylate cyclase and increase intracellular levels of cAMP. D2 receptors exert an inhibitory influence on adenylate cyclase (Kebabian and

Calne, 1979). D2 receptors may also be linked to additional second messenger systems including inhibition of phosphatidylinositol turnover, activation of potassium channels and inhibition of calcium influx (Vallar and Meldolesi, 1989). Besides the mode of receptor-effector coupling, dopamine D1 and D2 receptors can be distinguished by their abilities to bind and respond to selective dopaminergic agonists and antagonists. Dopamine D1 receptors bind with a high affinity to benzazepine-type compounds (such as SCH-23390 and SKF-38393), and with a poor affinity to classic neuroleptics (such as butyrophenone and substituted benzamide class). D2 receptors, on the other hand, exhibit a high affinity for numerous classes of drugs (including ergoline and napthoxazine derivatives), and substituted benzamide (sulpiride, raclopride and YM-09152) and butyrophenone (spiperone) class (Hyman et al., 1992).

Another conspicuous feature separating the D1 receptors from the D2 receptors is the inclusion of introns in the gene coding regions of the D2 receptors, but not in the D1 receptors. Introns are thought to allow the generation of receptor variants by alternative splicing (O'Dowd et al., 1989).

With the maturing of DNA technology, five different dopamine receptors have now been cloned and identified. These receptors are categorized as either D1-like (D1, D5) or D2-like (D2, D3, D4) using the aforementioned criteria and structural criteria. There are distinct similarities and dissimilarities between the structural and functional features of cloned dopamine receptors.

Genes encoding members of the dopamine receptor family are part of the gene superfamily of G-protein coupled receptors, which include many neurotransmitters, peptide hormones and other receptors. Some common structural motifs are shared by these structurally related transmembrane receptors. These receptors consist of a single protein with seven transmembrane domains (TM1 to TM7) joined by alternating extracellular and intracellular loops. The amino-terminal domain is outside and the carboxy-terminal tail is in cytoplasm side. The transmembrane regions can come together

to form a "cup" into which the ligand binding probably takes place. When the agonist binds to the "cup" formed by TM regions, the conformational change within these TM domains will be transmitted to the cytoplasmic face where G-protein binds. At rest, the G-protein which consists of three tigntly associated subunits, designated alpha (α), beta (β), and gamma (γ) binds GDP and is in an inactive form. Following agonist binding and conformational change, the α-subunit will exchange the bound GDP for GTP and disassociate from the $\beta\gamma$-subunits. The activated α-subunit will act on its effector. The $\beta\gamma$ complex will dissociate from the receptor and then be free to reassociate with the α subunit or in some cases to interact with other effectors. When the ligand dissociates from the receptor and the phosphate is removed from GTP, the process is ready to begin again (Hall et al., 1994).

Within the seven TM regions, there is a significant amount of amino acid sequence identity or homology between different classes of neurotransmitter receptors. The N-terminal stretch carries 1-6 putative N-linked glycosylation sites at asparagine residues. In some receptors, glycosylation sites may also exist in extracellular loops. The C-terminal stretch is about seven times longer for the dopamine D1-like receptors than for the dopamine D2-like receptors. This segment is rich in serine and threonine and may provide a substrate for phosphorylation. A highly conserved cysteine residue in the C-terminal of both D1-like and D2-like receptors is thought to undergo another type of post-translational modification, namely acylation, with palmitic acid. The variably sized cytoplasmic loop 3 (CPL3), coupled with the length of C-tail, is another structural characteristic of D1-like and D2-like receptors. The long CPL3 and short C-tail seem to be a common feature of receptors that inhibit adenylate cyclase by coupling through Gi-proteins. The CPL3 is the serine and threonine rich area which makes the receptor susceptible to protein kinase catalyzed phosphorylation and dephosphorylation (Jackson et al., 1994).

Little is known about the tertiary structure of G-protein coupled receptors. However, electron diffraction studies show that the TM regions are in a α-helical formation in the bacteriorhodopsin system, a protein with a topography similar to that of dopamine receptors (Henderson et al., 1990). More recently a mutagenesis approach has been utilized to show the alignment of TM regions (Suryanarayama et al., 1992). The results in α2-adrenergic (with a large CPL3 and a short carboxyl tail) and β2-adrenergic (with a short CPL3 and a large carboxyl tail) studies indicate that amino acids in the seventh TM region lie next to the first TM region. Disulfide bonds may exist to join two of the cytoplasmic loops, since many of the receptors in this family have cysteine residues in the cytoplasmic loops. The effect of the disulfide bonds will constrain TM3 in close proximity to TM5 (Strader and Dixon, 1992).

In spite of the common features of these cloned dopamine receptors, distinct characteristics may exist in the five different receptors.

The D2 receptor is the first member of dopamine receptor family to be cloned, and consequently the most studied to date. Bunzow and co-workers (1988) identified a full-length cDNA from a rat brain library using β-adrenoceptor gene as a probe. The cDNA encoded a protein of 415 amino acids that exhibits appropriate radioligand binding activities with the pharmacological characteristics that are expected for D2 receptors. Topographic analysis shows that the cDNA encoded protein spans the membrane seven times with a large CPL3 and a very short carboxyl tail which is the characteristic of most receptors that inhibit adenylate cyclase activity (Dohlman et al., 1991). The distribution of the D2 receptor was found by Northern blot analysis and *in situ* hybridization (Bunzow et al., 1988; Weiner et al., 1989). In general, D2 receptor mRNA is present in various tissues and brain regions. The areas of highest expression in the brain correspond to major dopaminergic projection areas. Cellular localization of the D2 receptor mRNA has also been investigated in the medium-sized (Gerfen et al., 1990; Weiner et al., 1990) and large-diameter striatum cells (Le Moine et al.. 1990).

The unique feature of D2 receptor is that it exists as two isoforms, which are generated through alternative splicing of mRNA. The two forms of the receptor are identical except in length of the large CPL3 with the D2L receptor having 29 amino acids more than the D2S receptor. Since studies with other catecholamine receptors by mutagenesis have suggested that the CPL3 is important for G protein coupling and effector regulation (Dohlman et al., 1991; Strader et al., 1989), this could imply functional and regulational differences of the two receptor species. The differences between the two isoforms have been described in both their biological activities (Dal Toso et al., 1989) and tissue distributions (Neve et al., 1991), but not yet in their pharmacological profiles (Giros et al., 1989). Some conflicting results on the subregions and the illnesses involving of D2 receptor (for example, alcoholism) have come from different groups. The characterization of the D2 receptor still requires further investigation.

A cDNA clone encoding the dopamine D3 receptor was obtained from a rat library using a probe derived from the D2 receptor sequence. This gene has a similar structure to the D2 receptor but is distinguished from the D2 receptor by the binding patterns of several dopamine receptor agonists and antagonists. Regional analysis of D3 receptor mRNA in the brain shows that it is much less abundant and more narrowly distributed than that of D2 receptor (Sokoloff et al., 1990). Interestingly, the rat D3 receptor gene exists in two non-functional RNA splice variants. It is possible that both of these D3 receptor splice variants undergo incorrect protein folding and insertion into the plasma membrane (Giros et al., 1991). In addition, the D3 receptor is insensitive to G-protein and has no effect on adenylate cyclase activity (Sokoloff et al., 1990). These might be caused by the lack of appropriate D3 receptor-G protein coupling.

The D4 receptor represents the latest receptor to be identified and cloned in the D2 subfamily. This was accomplished through screening a human neuroblastoma cell cDNA library using the rat D2 receptor cDNA as a probe (Van Tol et al., 1991). It has a slightly smaller CPL3 than other D2 receptors. The D4 receptor mRNA in the brain is

expressed at a lower level than that of the D2 receptor by Northern blot analysis (Van tol et al., 1991). Six additional allelic variant forms of D4 receptor which were discovered in the human population (Van Tol et al., 1992; Shaikh et al., 1993) interest researchers. These polymorphic forms of the D4 receptor provide new approaches in the study of preclinical and clinical aspects of dopamine receptor function.

In 1990, four laboratory groups simultaneously reported the cloning of genes encoding a D1 receptor in either human (Zhou et al., 1990; Dearry et al., 1990; Sunahara et al., 1990) or rat (Monsma et al., 1990; Zhou et al., 1990). This receptor differs considerably from the D2-like dopamine receptors. Structural distinctions include a short CPL3 and a long C-terminus, and the lack of introns in the coding region of the dopamine D1 receptor gene. The D1 receptor is coupled to Gs and activates adenylate cyclase. D1 receptor mRNA is not as widely distributed in brain as D2 receptor mRNA as analyzed by Northern blot analysis and *in situ* hybridization (Gerfen et al., 1990; Monsma et al., 1990).

A second member of the D1 receptor subfamily, termed the D5 receptor, has recently been isolated and cloned by screening a human genomic library. The probe is a fragment of the human D1 receptor DNA (Sunahara et al., 1991). The membrane topography of the seven TM regions of the D5 receptor is similar to that of the D1 receptor. Apart from the brain, dopamine D5 receptor mRNA has also been detected in rat kidney (Grandy et al., 1992). In general, the level of expression of the D5 receptor is much lower than that of the D1 receptor. There are at least two more human D5-like receptor genes with high sequence homology to the D5 receptor (Nguyen et al., 1991; Grandy et al., 1991). This is the first example of such aberrations in the family of G-protein coupled receptors. The functional significance of these pseudogenes is not clear.

On the basis of structure, function, distribution and pharmacology, the dopamine D5 receptor shares extensive similarities with the dopamine D1 receptor, while dopamine D3 and D4 receptors more closely conform to the features of the dopamine D2 receptor.

Usually people designate these two groups as dopamine D1-like and D2-like receptors, respectively.

Although the identification and classification of dopamine receptors in the invertebrates is inconclusive based on existing pharmacological and biochemical data, dopamine receptors are believed to exist in the invertebrate nervous system (Sugamori et al., 1994). Most actions of catecholamines are thought to result from changes in the intracellular level of cAMP through adenylate cyclase in a variety of tissues (Sutherland and Robison, 1966; Robison et al., 1968). In tick salivary glands, adenylate cyclase is stimulated by several derivatives of phenylethylamine while dopamine has the highest potency. The enzyme exhibits much higher affinity with phenothiazine drugs (thioridazine, chlorpromazine, and fluphenazine) which are thought to be dopamine D1 receptor antagonists than that of the butyrophenone drug (haloperidol) which is an antagonist of dopamine D2 receptor. This indicates that adenylate cyclase may be associated with a D1 type dopamine receptor (Schmidt et al., 1981). Interestingly, the intracellular level of cAMP increases when glands are stimulated with 10^{-5} M dopamine (Sauer et al., 1979). However, there is a marked decrease in both cAMP and fluid secretion at dopamine concentration of 10^{-3} M in *Dermacentor variabilis* which is another species of ixodid family (Shipley et al., unpublished). This result may suggest that dopamine control is complex and there may exist more than one type of receptor in ixodid salivary glands. The complete dopamine D2 receptor genes have not been identified in ticks, or in other invertebrates. The study of dopamine D1 and D2 receptors will help understand the disease-transmitting behavior of ticks.

Cyclic AMP-dependent Protein Kinase

Cyclic AMP-dependent protein kinase (cAPK) is another important regulatory enzyme that has effects on tick fluid secretion. This enzyme can phosphorylate specific cellular proteins at serine and threonine residues.

The cAPK is one member of the protein kinase family. The reception of an extracellular signal and then the translating and amplifying of that signal inside the cell involves protein phosphorylation. The phosphate group is most often donated from the γ-phosphate of ATP. Based on the acceptor hydroxyl group, protein kinases can be divided into two classes: those proteins that transfer phosphate to Ser or Thr and those proteins that transfer phosphate to Tyr (Krebs and Beavo, 1979). A third class of protein kinase which can phosphorylate both Ser/Thr and Tyr has been reported recently (Lindberg et al., 1992).

Protein kinases are a diverse family. This diversity is obvious in peptide recognition, mechanism of activation, and in structure in terms of size, subunit structure, and subcellular localization. At the same time, protein kinases are evolutionarily related (Barker and Dayhoff, 1982) and may have evolved divergently from a common origin (Hanks et al., 1988). About 260 residues in the catalytic core, including the residues for catalysis and for MgATP binding, are conserved in all of the eukaryotic protein kinases. It seems that all these protein kinases have a common mechanism for catalysis.

The cAPK can serve as a framework for the entire protein kinase family, since it is the simplest and best-understood biochemically. In an inactive form, the cAPK holoenzyme is a tetramer consisting of two catalytic and two regulatory subunits. There are at least three major functional sites in the catalytic subunit: the MgATP binding site, the peptide or protein substrate binding site, and the catalytic site where the phosphotransfer reaction occurs. Two cAMP binding sites, a dimer interaction site, and in some cases an autophosphorylation site are located in the regulatory subunits. In addition, interaction sites between the regulatory and catalytic subunits may exist in the holoenzyme. Following the elevation of the cAMP level due to the binding of agonists, two molecules of this second messenger cAMP bind to each regulatory subunit. The tetramer complex will dissociate and release free and active monomeric catalytic subunits (Beebe and Corbin, 1986, Taylor et al., 1990) which are free to phosphorylate substrate

proteins. The free catalytic subunit can also translocate to the nucleus where it regulates transcription factors (Fantozzi et al., 1992).

There are at least three types of cAPK based on the forms of the catalytic subunit associated with the regulatory subunit: type I, which is the predominant form in bovine skeletal muscle; type II, which is the major form in bovine heart; and a neural type II, which is found in bovine brain (Lohmann and Walter, 1984; Weldon et al., 1985). The catalytic subunits of three types protein kinases have similar physical and enzymatic properties (Flockhart and Corbin, 1982; Zoller et al., 1979).

The cAMP is most widely studied in the protein kinase family. The first protein kinase to be sequenced was bovine type II cAPK (Shoji et al., 1983). Some essential residues have been identified in the protein by affinity labeling and group specific labeling. For example, Lys72 was shown to be essential for MgATP binding (Zoller et al., 1981), and is invariant in all protein kinases. Mutagenesis of this lysine leads to the loss of kinase activity (Kamps et al., 1984).

Recently cAPK became the first protein kinase with a known crystal structure. The solved structure is the mouse recombinant catalytic subunit (Slice and Taylor, 1989). Overall, it is a slightly ellipsoid protein with two lobes. Between the two lobes there is a cleft where catalysis occurs. The smaller amino terminal N-lobe is important for protein-protein interactions. It is dominated by a five stranded antiparallel β-sheet and a long α-helix. There is an additional helix between the third β-sheet and the helix in cAPK compared to other PKs. The conserved glycine-rich sequence in the first two β-sheets contributes to protein kinase function in many ways. The large lobe is associated with peptide binding and catalysis. This lobe consists of seven α-helices and four short β-strands, with a central bundle of four antiparallel α-helices. The sixth and ninth β-strands come to form the bottom of the cleft. The catalytic loop and the metal binding loop between β strands are primarily important for the active site of PKs.

The partial sequence of the catalytic subunit of cAPK in tick salivary glands has been identified. Initially a 667 base pair fragment was obtained using reverse transcriptase polymerase chain reaction (RT-PCR). For the missing 5' end, rapid amplification of cDNA ends was employed successfully to clone 125 more base pairs. The sequence has 77% homology with the *Drosophila* and mammalian clones at the DNA level. This gene is expressed in all stages of tick feeding (McSwain et al., unpublished). However, the 3' end and 42 base pairs of 5' end is still missing. Completing the sequence of catalytic subunit of cAPK can help characterize the action of enzyme thoroughly.

CHAPTER III

MATERIALS AND METHODS

Materials

The rapid total RNA isolation kit and the mini-oligo(dT) cellulose spin column kit were purchased from 5 Prime ---> 3 Prime, Inc. All gene specific primers were synthesized by the Oklahoma State University Core Facility. The reverse transcriptase polymerase chain reaction kit was from GibcoBRL. The TA Cloning kit was obtained from Invitrogen. The Gel Extraction kit was from QIAGEN. The Wizard Minipreps DNA purification system was from Promega. The 3' RACE and 5' RACE kit for rapid amplification of cDNA ends were bought from GibcoBRL. The GeneTrapper™ cDNA positive selection system was from GibcoBRL. All enzymes (Taq DNA polymerase, T4 polynucleotide kinase, T4 RNA ligase, EcoRI, BamHI and HindIII) were purchased from GibcoBRL, except Klenow DNA polymerase from Promega. MagnaGraph hybridization transfer membranes were from Micron Separations Inc. and NEN Research Products. The tick embryo library and mouse genomic DNA were provided by Dr. Melanie Palmer (Department of Entomology, Oklahoma State University). The bovine genomic DNA was provided by Joel Yelich (Department of Animal Science, Oklahoma State University). Poly-prep chromatography columns were bought from Bio-Rad. SCH23390 was from Research Biochemicals International. Spiperone was from Life Science Products. α-^{32}P-ATP, γ-^{32}P-ATP and α-^{32}P-dCTP were obtained from NEN Research Products. All other chemicals and reagents were from Sigma, or Fisher Scientific.

Methods

RNA Extraction

Total RNAs from five feeding stages of the tick were prepared by Jim Tucker using the rapid total RNA isolation kit (small scale). Poly(A)$^+$ mRNA was isolated by using a mini-oligo(dT) cellulose spin column. The procedure was performed according to the manufacturer's recommendation.

Primer Design

Degenerate primers were designed based on sequences of animal dopamine D2 receptors. A total of five animal dopamine receptor nucleotide sequences (mouse, rat, cow, human, and *Xenopus laevis*) were retrieved from the GenBank database using the MacVector software (IBI-Kodak). Their nucleotide and deduced amino acid sequences were aligned using the Clustal software via a OU GCG client.

From these alignments, several conserved regions were identified and examined further for primer design using the Oligo 4.0 primer analysis software. Effort was made to most closely meet the following criteria for good primer pairs: a minimized degeneracy and a high Tm in order to increase the specificity of the PCR reaction; perfectly matched 3' end of primers to the target to ensure elongation; no stable dimers and hairpins, particularly at the 3' end; a good internal stability (high GC content at the 5' end and low GC content at the 3' end) and closely matched Tm of two set primers.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was employed to amplify an internal fragment of dopamine D2 receptor cDNA. The procedure was performed according to the GibcoBRL instruction manual and was basically as follows.

First strand cDNA was synthesized from 1-5 μg total RNA or purified poly(A)$^+$ RNA of different feeding stages. The primer for cDNA synthesis was Oligo(dT), random hexamers or a designed primer. A negative control with no reverse transcriptase added in

cDNA synthesis was also included. An aliquot of the cDNA synthesis mixture was directly used as a template for PCR amplification. A standard PCR cocktail (50 µl or 100 µl final volume) was set up as recommended by the Taq polymerase manufacturer (GibcoBRL).

Two different PCR programs were tested in this experiment. One, designed for gene specific primers, is as follows: denaturation at 94 °C for 1 minute, annealing for 1 minute at varied temperatures, and extension at 72 °C for 2 minutes. This cycle was repeated 35 times followed by a 10 minute final extension at 72 °C. The annealing temperature was dependant on the Tm of the primer pair used and the possible mismatches between the primers and the templates. Another program was designed for degenerate primers. This is almost the same as above except the annealing step. The annealing temperature was 5 °C lower for the first three cycles than that of the following 25 cycles.

Following amplification, aliquots from each reaction were electrophoresed on an agarose gel to determine the size of the products.

Capillary Transfer of DNA to Nylon Membrane

The transfer process was performed according to the Salt Transfer Protocol of GeneScreen™. After the PCR products were separated on a 1.5% agarose gel, the DNA in the gel was depurinated in 0.25 N HCl for 10 minutes, denatured in 0.4 N NaOH containing 0.6 M NaCl for 30 minutes, and neutralized in 0.5 M Tris-HCl (pH 7.5) containing 1.5 M NaCl for another 30 minutes. The DNA was then transferred onto a nylon membrane by overnight capillary transfer, using 10 x SSC (1.5 M NaCl and 0.15 M sodium citrate, pH 7.0) as transfer buffer. After transfer, DNA was fixed on the membrane by UV-crosslinking (UV Stratalinker 1800, setting: 0.12 Joules/cm2, 45 sec).

Probe Preparation

Oligonucleotides were labeled using the polynucleotide kinase end labeling method (Maniatis et al., 1989). The 50 µl reaction contained 100 ng of oligonucleotide, 1 x T4

kinase buffer, 0.15 mCi γ-^{32}P-ATP and 8U of T4 polynucleotide kinase. The mixture was incubated at 37 °C for 45 minutes, then the enzyme was denatured at 68-70 °C for 10 minutes.

The several hundred base pair fragment was labeled by the random primer labeling method (Maniatis et al., 1989). The DNA sample (25-50 ng in a total volume of 7.1 µl) was heated at 95 °C for 5 minutes, then immediately cooled on ice for 5 minutes. After a brief spin, the denatured DNA was combined with 11.4 µl of LS (HEPES/DTM/OL in a ratio of 25/25/7, HEPES: 1 M, pH 6.6; DTM: 100 µM dATP, 100 µM dGTP, 100 µM dTTP in 250 mM Tris pH 8.0, 25 mM $MgCl_2$, 50 mM β-mercaptoethanol; OL: 1 mM Tris pH 7.5, 1 mM EDTA pH 8.0 at 90 $O.D_{600nm}$ of random oligonucleotide hexamers), 1 µl of 10 mg/ml bovine serum albumin (BSA), 5 µl of α-^{32}P-dCTP (3000 Ci/mmol), and 0.5 µl of Klenow fragment (2.5 units). The mixture was incubated at room temperature for three hours to overnight.

The unincorporated nucleotides were separated through a Sephadex G-50 column. Before use, the probes were heated at 95 °C for 10 minutes, then kept on ice.

Prehybridization, Hybridization and washing with Oligonucleotide Probes

Prehybridization and hybridization solutions were the same: 6 x SSPE (0.9 M NaCl, 60 mM NaH_2PO_4, pH 7.4 and 6 mM EDTA, pH 7.4), 0.01 M sodium phosphate (pH 6.8), 1 mM EDTA (pH 8.0), 0.5% SDS, 100 µg/ml denatured salmon sperm DNA, 1 x Denhardt's solution. Prehybridization was performed at 42 °C for at least 3 hours. Then hybridization was performed with labeled probe in fresh hybridization solution for another 3 hours at 42 °C.

The washing process started at very low stringency. The membrane was washed twice at room temperature for 30 minutes each using 6 x SSPE and 0.1% SDS. If necessary, the washing stringency could be increased. The membrane was then exposed to X-ray film at -70 °C.

Cloning, Sequencing and Data Analysis

To clone PCR products, the TA Cloning Kit (Invitrogen) was used and the kit-provided protocol was followed. This kit takes advantage of the fact that Taq polymerase adds a single template-independent adenosine to the 3' ends of PCR products at very high frequency. Thus, these PCR products can be easily ligated into linearized vectors that contain a single thymidine overhang at both 3' ends.

The PCR products which were hybridized with probe were isolated from an agarose gel using QIAquick gel extraction kit. Then the appropriate amount of the DNA was mixed with 1 µl of 10 x ligation buffer, 2 µl of PCR™ II vector and 1 µl of T4 DNA ligase for ligation. The mixture was incubated at 14 °C from 4 hours to overnight.

One µl of each TA cloning ligation solution was added into 50 µl ONE SHOT™ competent cells and 2 µl of 0.5 M β-mercaptoethanol. After incubation on ice for 30 minutes and heat shock for 30 seconds at 42 °C, 450 µl of SOC medium was added and then incubated at 37 °C for 1 hour with shaking. Twenty five µl to 200 µl of transformation aliquot was plated on LB plates containing 1 mg ampicillin and 1 mg X-gal. The plates were incubated at 37 °C for overnight.

The white colonies which contained inserts were picked and grown in 3 ml LB liquid medium with 25 µg/ml ampicillin. The plasmid was isolated and purified from these cultures by using the Wizard Minipreps kit.

Since some sequence heterogeneity often occurs in PCR products, a DNA blot analysis was conducted to screen for positive clones. Each isolated plasmid was restricted with EcoRI and separated on an agarose gel, then blotted onto a nylon membrane for hybridization as described above.

Four positive clones were sent to the OSU core facility for automatic-sequencing (ABI). Sequence analyses was carried out using the on line Blast client and the MacVector software (IBI-Kodak).

Rapid Amplification of cDNA Ends (RACE)

3' RACE (GibcoBRL) was employed to amplify the 3' end of dopamine D2 receptor. Overall, the procedure from cDNA synthesis until sequence analysis was essentially the same as that in the section "RT-PCR" except for the following changes.

First, an oligo(dT)-adapter primer was used to prime the first strand cDNA synthesis. Then, a gene specific primer which was located in the internal fragment and a universal amplification primer that was complementary to the adapter region of all cDNA species were used for amplifying the target cDNA.

Second, a 20 bp oligonucleotide downstream of the first gene specific primer used in PCR or the full cloned internal fragment was used as a probe in DNA blot analysis to confirm the identity of the 3' PCR product.

In an attempt to obtain the missing 5' end of the dopamine D2 receptor, 5' RACE (GibcoBRL) was employed. The whole procedure was performed according to the instruction manual.

The first strand cDNA was synthesized using an antisense gene specific primer which was designed from the internal fragment. RNA templates were then digested away with RNase H. The cDNA was tailed with dCTP by terminal deoxynucleotidyl transferase (TdT) and served as the PCR template for an anchor primer (with a complementary poly(dG) tail) and a nested gene specific primer. The identity of the products was then confirmed as in 3' RACE except the oligonucleotide probe was located upstream of the gene specific primer used in PCR.

cRACE (circular or concatemeric first strand cDNA-mediated RACE) was also tried to obtain the missing 5' end. In principle, this method makes use of the RNA ligase activity in ligating single strand DNA, so that specific first strand cDNA primed by a gene specific primer is ligated into circular or concatemeric form. Either form of the cDNA can be amplified in PCR with two gene specific primers, instead of one in standard 5' RACE PCR.

Southern Blot of Tick Genomic DNA

Ten μg of tick genomic DNA was digested by EcoRI, BamHI, and HindIII, separately. Mouse and bovine genomic DNAs were used as positive controls. After digestion, an appropriate amount of dye was added. The samples were separated on a 0.7% agarose gel.

After electrophoresis, the DNA was transferred to nylon membrane, and hybridized with the internal fragment of the dopamine D2 receptor cDNA.

Northern Analysis of Dopamine D2 Receptor

The Northern blotting of dopamine D2 receptor was carried out by Jim Tucker. Ten μg of total RNA from two feeding stages (50-100 mg and 100-200 mg) of tick were denatured in formamide/formaldehyde buffer, fractionated through a 1.2% agarose, 7.4% formaldehyde gel, and transferred to nitrocellulose membrane.

Labeled single stranded RNA probe was synthesized with T7 RNA polymerase from the cloned RT-PCR product. Transcription reactions were performed according to the manufacturer's recommendation (Promega).

RNA/RNA hybridization was carried out at 55 °C overnight in a hybridization solution containing 50% formamide, 0.75 M NaCl, 0.15 M Tris (pH 8.0), 10 mM EDTA, 0.2 M sodium phosphate (pH 6.8), 1 x Denhardt's solution, 10% dextran sulfate, and 0.1% SDS. Approximately 1 μg of labeled RNA was used as the probe. The blot was washed in buffer containing 0.75 M NaCl, 0.15 M Tris, 10 mM EDTA, 25 mM Na_3PO_4, 0.1% NaPPi, and 0.1% SDS at 65 °C for 1 hour. Both low and high washing stringency were tried.

Amplification of Uni-ZAP™ XR cDNA Library

To make a large, stable quantity of a library stock with a high titer, it is usually desirable to amplify a library prepared in lambda vectors.

XL1-Blue was used as host strain. Aliquots of the Uni-ZAP™ XR library suspension containing approximate 50,000 recombinant bacteriophage were mixed with

600 µl of host cells (O.D.600 = 0.5) and incubated for 15 minutes at 37 °C to allow infection. Then the infected bacteria were mixed with 6-7 ml top agar and spread evenly onto a NZY 150 mm plate of bottom agar. The plates were incubated at 37 °C for 5-8 hours until the plaques just touched each other, then 10 ml of SM buffer was added. After an overnight incubation at 4 °C with gentle shaking, the bacteriophage suspension from each plate was recovered. The cell debris was removed by centrifugation for 5 minutes at 4000 X g.

In vivo excision of pBluescript from Uni-ZAP™ XR

The lambda vector for this library construction was designed to allow automatic excision and recircularization of any cloned insert from the lambda vector to form a phagemid containing the insert. An appropriate amount of λZAP phage stock, XL1-Blue and R408 helper phage were mixed at 37 °C for 15 minutes. Then 5 ml of 2 x YT media was added and incubated for 4-6 hours at 37 °C with shaking. After heating at 70 °C for 20 minutes, the cell debris were removed by centrifugation at 1000 X g for 5 minutes. The supernatant contained the pBluescript plasmids packaged in M13 phage particles.

GeneTrapper™ cDNA Positive Selection

An obvious advantage of the GeneTrapper™ cDNA positive selection system is rapid isolation of target cDNA clones from a cDNA library without prior cDNA library screening. In this method, an oligonucleotide which was biotinylated at the 3' end is allowed to hybridize with the complementary target ssDNA. The hybrids are captured on streptavidin-coated paramagnetic beads which are retrieved from the solution using a magnet. The captured ssDNA is then released from the biotinylated oligonucleotide and converted to dsDNA by using a nonbiotinylated specific primer. The whole procedure was performed according to the manufacturer's recommendation (GibcoBRL).

An oligonucleotide complementary to a segment of the target cDNA was designed following certain principles. The percentage of G + C in a 16-25 bp nucleotide was between 50% and 60% in order to decrease the background. Vector-like sequences were

avoided to get rid of the false-positive signal. For the best results, the oligonucleotide was located in amino acid-encoding region. The formation of dimers or hairpin structures at the 3' end which would eliminate the activity of TdT was also avoided. The oligonucleotide was biotinylated at the 3' end with biotin-14-dCTP using TdT. The probe was purified by polyacrylamide gelelectrophoresis (PAGE).

At the same time, the Uni-ZAP^{TM} XR cDNA library was amplified and excised to create the ssDNA. Twenty ng denatured probe was added to 5 μg denatured ssDNA to hybridize for 1 hour at 37 °C.

To capture the target DNA, the hybridization solution was mixed well with the magnetic beads, then incubated for 30 minutes at room temperature. The paramagnetic beads were captured with a magnet and washed three times with wash buffer. The beads were then incubated with elution buffer for 5 minutes at room temperature. The supernatant which contained the captured cDNA clone was purified by phenol/chloroform extraction and precipitated with ethanol.

To convert the ssDNA to dsDNA, a repairing mix containing 1.5 μl of 10 x repair buffer, 0.5 μl of 10 mM dNTP, 0.5 μl of repair enzyme in a final volume of 15 μl for one sample was prewarmed at 70 °C. At the same time, the DNA primer mix containing 1.5 μl of 10 x repair buffer, 5 μl of captured DNA, 50 ng of nonbiotinylated oligonucleotide in a final volume of 15 μl was incubated at 95 °C for 1 minute followed by an incubation at 70 °C for another 1 minute. Fifteen μl of prewarmed repair mixture was added to the DNA primer mix, then incubated at 70 °C for 15 minutes to allow primer extension. The enriched DNA was extracted with phenol/chloroform and precipitated with ethanol again, then dissolved in 10 μl of TE buffer.

High Efficiency Electro-transformation of E. coli

Electroporation provides a method of *E. coli* transformation with much higher efficiency than available chemical methods. An appropriate volume of LB media was inoculated with 1/100 volume of a fresh overnight culture. The cells were grown at 37 °C

with vigorous shaking until O.D.(600nm) = 0.5 to 0.8. After chilling on ice for 15 to 30 minutes, the cells were centrifuged in a cold rotor at 4000 X g for 15 minutes. The pellets were washed twice and then resuspended to 1/50 volume using cold sterile water.

The cells, sterile cuvettes and white chamber slide were kept on ice as much as possible. The Gene Pulser apparatus was set to 25 μF, Pulse Controller to 200 W, Gene Pulser apparatus to 1.50 to 1.80 KV for the 0.1 cm cuvettes. The cell suspension was mixed with 2 μl of DNA and kept on ice for 1 minute before transferring into the cuvette. After the pulse, one ml of SOB was added to the cuvette immediately to quickly resuspend the cells, and then they were incubated at 37 °C for 1 hour with shaking. LB medium with ampicillin was used for plating.

Screening of the Uni-ZAP™ XR cDNA Library

About one million phages from a tick embryo cDNA library constructed in Uni-ZAP™ XR were plated out on NZY agar plates at a density of about 50,000 pfu per 150 mm plate. The plates were kept at 37 °C for about 8 hours until the plaques just touched. After the plates had been chilled at 4 °C for 2 hours, plaques from each plate were transferred to a pair of nylon membranes. Each membrane was then washed successively with 0.5 N NaOH containing 1.5 M NaCl; 0.5 M Tris-HCl (pH 8.0) containing 1.5 M NaCl; and 2 x SSC containing 0.2 M Tris-HCl (pH 7.5). The plaque DNA was then cross-linked to the membrane by UV cross-linking. Prehybridization and hybridization were carried out in the presence of 50% formamide, 2 x 1,4-piperazine-diethanesulfonic acid (PIPES) buffer, 0.5% (w/v) SDS and denatured, sonicated salmon sperm DNA (100 mg/ml). After a minimum of 2 hours prehybridization, the solution was replaced by fresh hybridization solution containing radioactive probe at 1.0×10^7 cpm/ml. The hybridization was carried out at 37 °C overnight. The membranes were washed with 0.1 x SSC containing 0.1% (w/v) SDS at 50-60 °C with shaking.

Positive plaques were picked out from the original plates. Secondary screening was done essentially the same way as the primary screening, except that plaque density

was reduced to below 500 pfu per 80 mm plate. If necessary, tertiary screening would be performed. Well resolved plaques were then isolated and stored individually.

Adenylate Cyclase Activity Assay

Adult female slow feeding *A. americanum* (L.) ticks were used. Salivary glands were removed from ticks as described by Needham and Sauer (1975). Cold 50 mM Tris-HCl (pH 7.4) was used as dissection buffer. Cold 50 mM Tris-HCl (pH 7.4) containing 3.84 mM $MgCl_2$, 0.77 mM MIX (3-isobutyl-1-methylxanthine), and 0.096 mM EDTA (ethylenediaminetetraacetic acid) was used as homogenization buffer. The excised glands were kept on ice until homogenization. Tissues were homogenized for 2 minutes in an all-glass homogenizer using a loose-fitting pestle. Tick membrane proteins were prepared as described by McSwain et al. (1987). The homogenates were centrifuged at 900 X g for 10 minutes. The supernatant was centrifuged again at 11,500 X g for another 10 minutes. The resulting pellet was then washed twice in homogenization buffer. The isolated membranes could be frozen at -70 °C. The protein concentration was determined by the method of Lowry et al. (1951), with BSA as the standard.

Adenylate cyclase activity was determined by measuring the conversion of α-^{32}P-ATP to cyclic ^{32}P-AMP. The reaction was performed according to the Schmidt et al. (1982). The standard mixture contained 5 mM $MgCl_2$, 0.77 mM IBMX (3-isobutyl-1-methylxanthine), 0.1 mM EDTA, 0.5 mM cAMP, 20 mM creatine phosphate, 50 U/ml creatine phosphokinase, 0.1 mM α-^{32}P-ATP, 1 mM dithiothreitol in 25 mM Tris-HCl buffer (pH 7.4) in a final volume of 40 µl. Assays were initiated by the addition of different concentrations of agonists or antagonists and 50 µg isolated membranes to standard mixture that had been pre-incubated at 30 °C for 3 minutes. After another 15 minutes incubation at 30 °C, the reaction was stopped by adding 100 µl of stopping solution (2% SDS, 40 mM ATP, 1.4 mM cAMP) followed by adding 100 µl of [^{3}H]cAMP recovery standard (0.25 µCi/ml in water) and 700 µl water. Separation of ^{32}P-cAMP from labeled ATP, ADP, AMP, and Pi employed a two column system (Farndale

et al., 1992). The first column was negatively-charged Dowex 50 which adsorbed cAMP by a non-specific interaction. The reaction solution was loaded and allowed to run to waste. The column was washed twice using 1 ml of water. Then 6 ml of washing water was added to run from the Dowex 50 column to the second neutral alumina column to waste. At this time, the cAMP was washed onto the alumina column to which it adsorbed together with any remaining ATP. After the alumina column was washed by 1 ml of 0.3 M imidazole buffer, 1.75 ml of same buffer was run through the column into 3 ml of scintillant for counting.

CHAPTER IV

RESULTS AND DISCUSSION

Primer Design

The primers were synthesized based on the criteria described in methods of primer design (see materials and methods). The sequences and orientation of such designed primers are shown in Fig. 1 and Fig. 2.

RT-PCR and Confirmation of PCR Products

Different primer sets and PCR conditions were tested. Total RNA or mRNA of five feeding stages (embryo, 20-40 mg, 50-100 mg, 100-200 mg, >250 mg) served as template. Many PCR products' bands were obtained after primary and secondary PCR. Only two fragments of secondary PCR products, which were amplified with degenerate primer 426 and 427 for primary PCR, the nested degenerate primer set 667 and 668 for secondary PCR, were able to hybridize with oligonucleotide probe 776. The 152 bp fragment which was close to the expected size (Fig. 3A and 3B) was cloned.

To further confirm the 152 bp PCR product, a total of thirteen purified plasmids which had correctly sized inserts were chosen for hybridization again after being released from the vector by EcoRI digestion (Fig. 4A and 4B). Four clones showing a positive hybridization signal were sequenced by the OSU core facility.

The four clones had the same sequence. These four sequences, together with the rat and the human dopamine D2 receptor DNA sequence, were aligned using the GCG

Figure 1. Primer sequences. The primers 426, 427, 667, 668 and 776 were designed from consensus DNAs of five animals (mouse, rat, cow, human, *Xenopus laevis*). The specific primers 1394, 1395 and 1396 were synthesized from the 152 bp fragment.

Number	Primer Sequence (5'--->3')
426	CTGTG(C/T)G(C/T)(C/G)AT(C/T)GCIIT(G/T)GA(C/T)(C/A)G(C/G)TAC
427	A(T/G)G(A/T)AG(A/T)AGGGCAGCCAGCAGAI(G/C)(G/A)(T/C)GAA
667	GTCATGATTGCCATTGTCT
668	ACGATGAAGGGCACGTAGAA
776	AACAATACAGACCAGAATGAGTG
1394	GGAGGAGTAGACCACAAAGG
1395	GTCCGAAGAGCAGTGGGCAG
1396	CATTGTCTGGGTCCTGTCCT

Figure 2. Primer orientation. Primer set 426 and 427, and nested primer set 667 and 668 were used for amplification of 152 bp fragment. Primer set 1394 and 1396 was designed for confirmation of the 152 bp fragment. Primers 776 and 1395 were used as internal probe. Sense primer 1396 was used in amplification of the firsrt strand cDNA and oligonucleotide 1395 was used as probe in 3'RACE. Antisense primer 1394 was used in synthesis first strand cDNA, nested antisense primer 1395 was used in amplification of the first strand cDNA, and oligonucleotide 1396 was used as probe in 5'RACE.

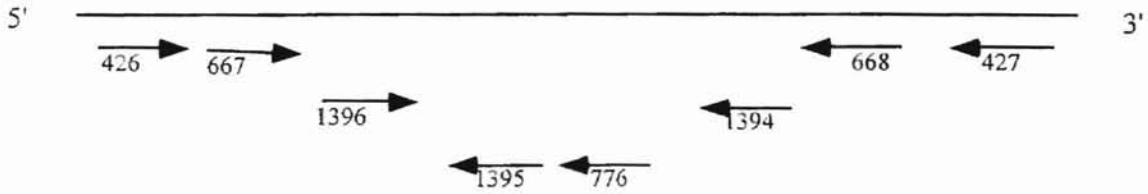
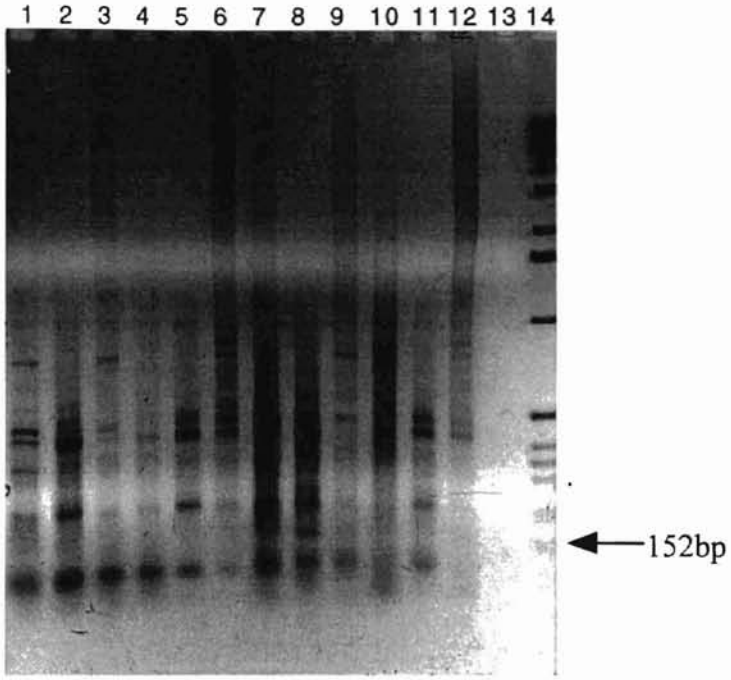

5'
3'
426
667
668
427
1396
1394
1395
776

Figure 3. Southern blot of RT-PCR products with probe 776. A. RT-PCR of tick's RNA. lanes 1-6: primary PCR with degenerate primers 426 and 427; lanes 1,3,6: Oligo(dT) for reverse transcription, lanes 2,4,5: random hexamer for reverse transcription; lanes 7-12: secondary PCR using primer set 667 and 668, and 1-6 PCR products as template, respectively; lane 13: negative control with no cDNA added; lane 14: 1 kb DNA ladder. B. autoradiographic film with oligonucleotide probe 776.

A



B

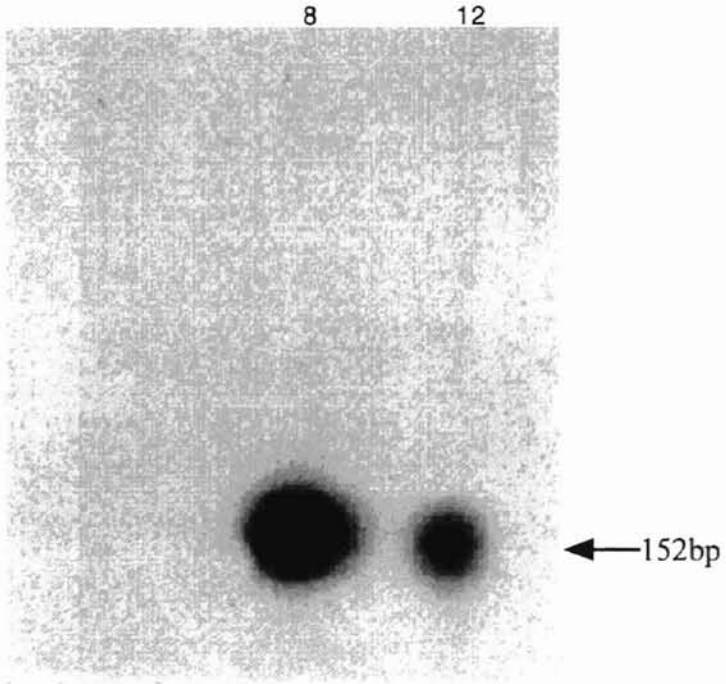


Figure 4. Southern blot of the RT-PCR products with oligonucleotide probe 776 after TA cloning. A. confirmation of the secondary PCR products after TA cloning and Wizard Miniprep by EcoRI digestion. B. Southern blot of the secondary PCR DNA with oligonucleotide probe 776.

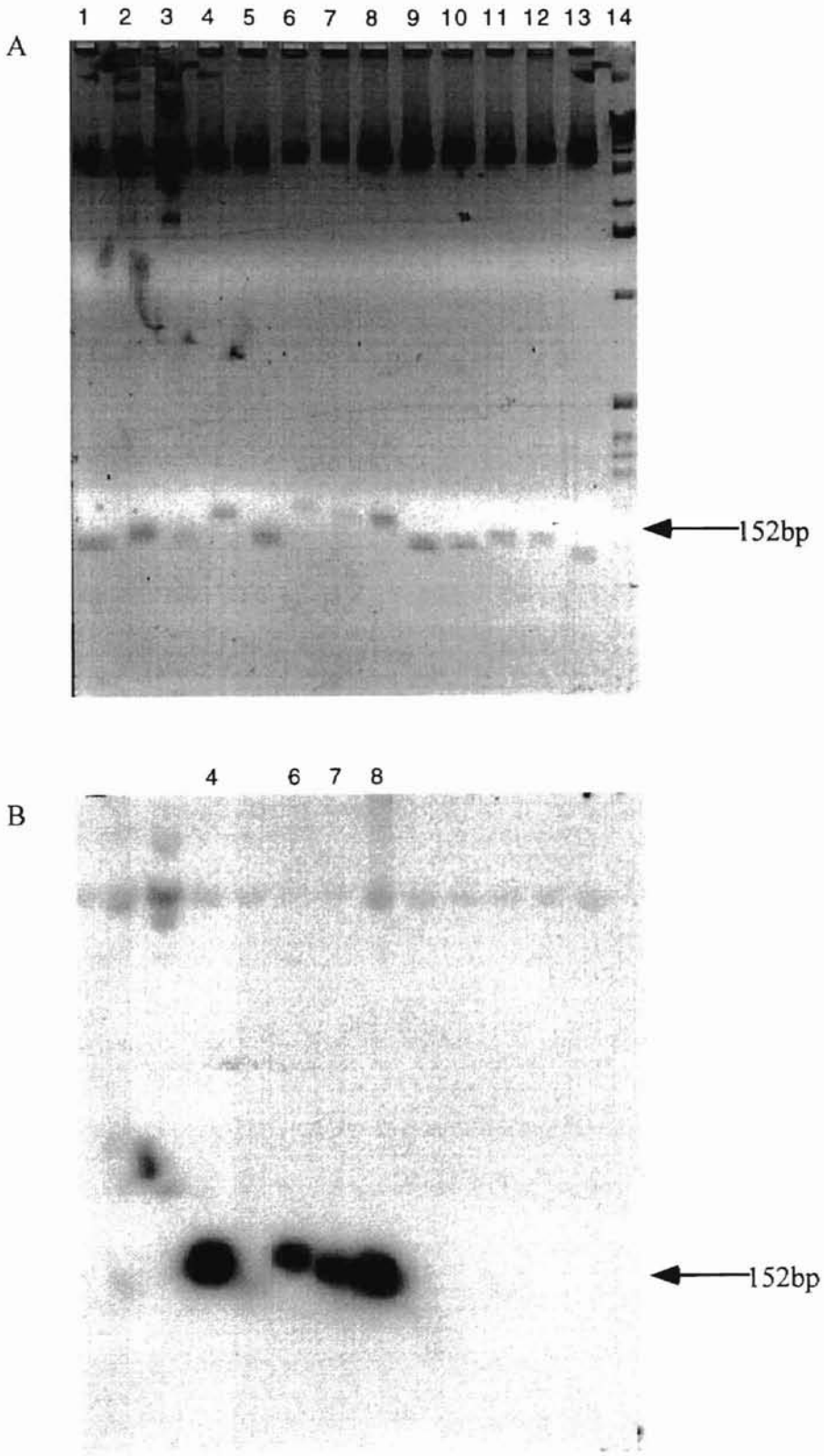
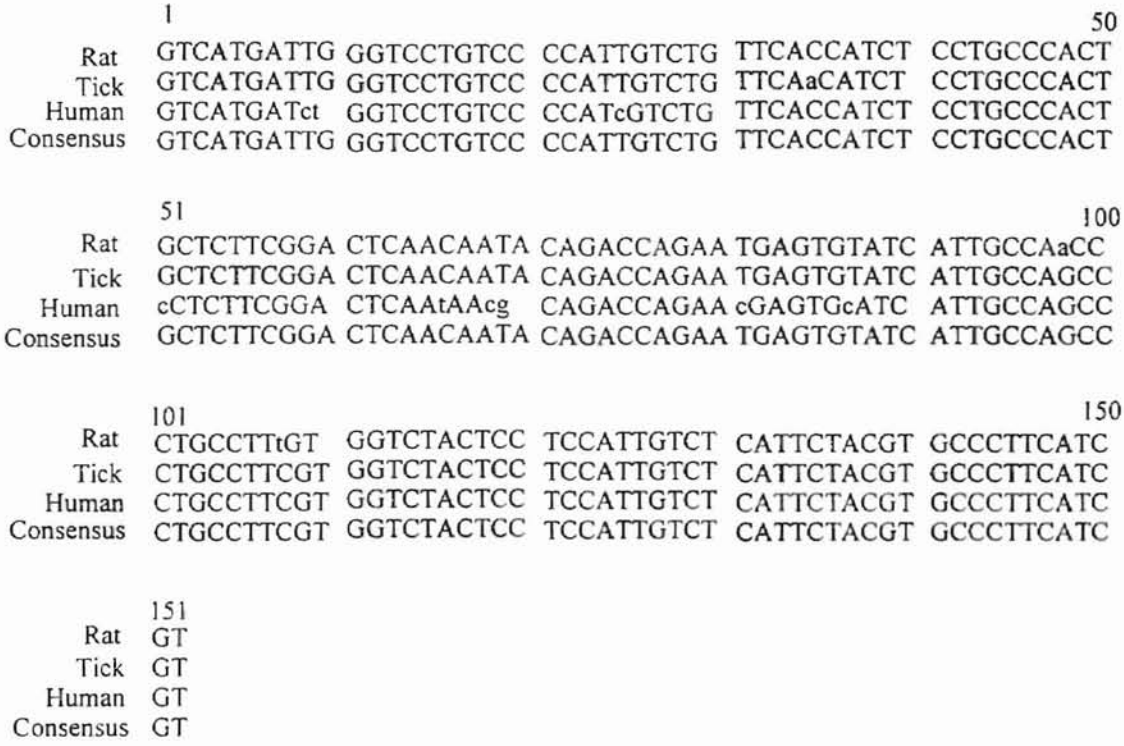

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14
152bp
B
4 6 7 8
152bp

Figure 5. Alignments of the tick-derived 152 bp DNA sequence with the rat D2 (GenBank accession No. X17458) and human retinal D2 (GenBank accession No. S69899) dopamine receptor sequences in the same area by Pileup and Pretty programs of the GCG package.


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

sequence analysis software package. The result showed that the 152 bp fragment was 99% identical with the rat and 91% with the human dopamine D2 receptor (Fig. 5).

The sequence data indicates an unusually high similarity between the 152 bp fragment and the rat dopamine D2 receptor. To rule out the possibility of DNA contamination, the following experiments were designed. The 152 bp region was used to design three specific primers. Primer set 1394 and 1396 were used in a PCR reaction, while primer 1395 was used for an internal probe. Contamination could occur during tick feeding when ticks have the opportunity to directly come in contact with cellular components of the vertebrate host. RNA from tick embryos believed to be much less likely to be contaminated with host tissue was used as PCR template. Different amount of the RNA, together with a negative control containing no reverse transcriptase were used in PCR reactions. Southern blotting was followed to confirm PCR products. The results showed that the probe did not hybridize with the negative control without reverse transcriptase. However, the probe hybridized to bands in other reactions except lane 3 which was thought the experimental error (Fig. 6).

RT-PCR results indicated that the 152 bp gene is expressed in all tick feeding stages (Fig. 7).

Rapid Amplification of cDNA Ends (RACE)

RACE is one of the common methods to extend gene fragments to full length. Gene specific primer 1394 was used in first strand cDNA synthesis in 5' RACE. Nested primer 1395 and an anchor primer were used in the first strand cDNA amplification. Very faint bands were obtained after PCR amplification. Two probes, oligonucleotide 1396 and the full 152 bp fragment were tested in the hybridization reaction. Only one band showed very weak signal (Fig. 8A and 8B). The signal was not strong after cloning (Fig. 9A and 9B).

Figure 6. Confirmation of the tick-derived 152 bp DNA by Southern blot of secondary PCR products using oligonucleotide probe 1395. Tick embryo RNA with different titers served as RT-PCR template using primers 1394 and 1396, negative control which has no reverse transcriptase added was included. lane1: negative control; lane2: 1:1 embryo RNA; lane3: 1:10 embryo RNA; lane 4: 1:50 embryo RNA; lane 5: 1:100 embryo RNA; lane6: 1:500 embryo RNA.

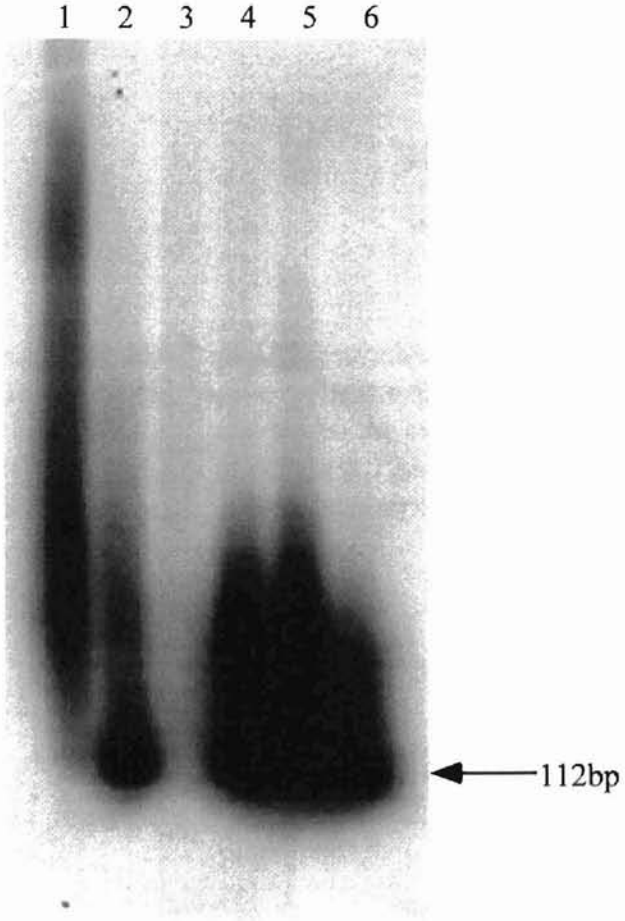

1 2 3 4 5 6
112bp

Figure 7. Expression of the tick-derived 152 bp DNA by Southern blot of secondary PCR products. RNA from tick five feeding stages served as RT-PCR template, primer 1394 and 1396 were for amplification, oligonucleotide 1395 was used as probe. Lane1-4: total RNA from tick salivary glands, Lane1: 20-40 mg, Lane2: 50-100 mg, Lane3: 100-200 mg, Lane4: >250 mg. Lane5: embryo total RNA.

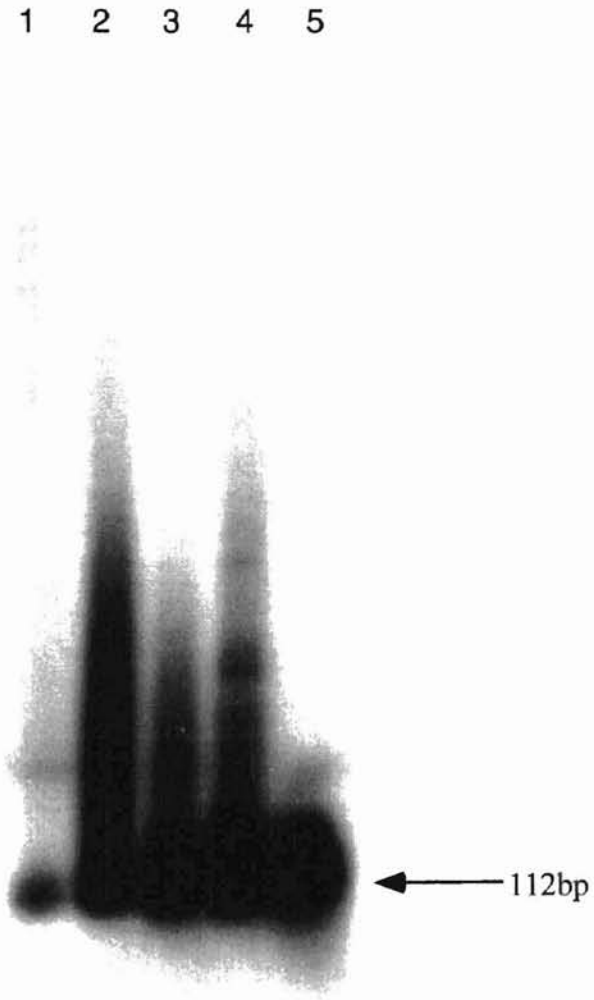
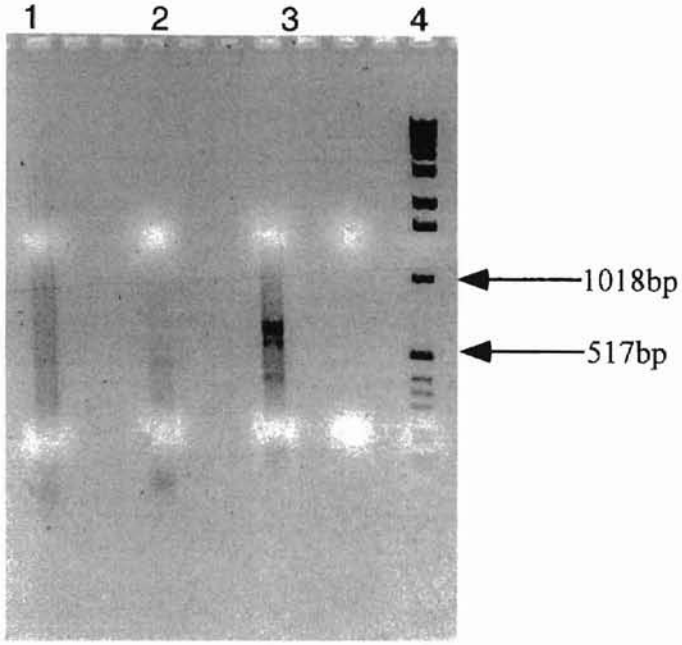

1 2 3 4 5
112bp

Figure 8. 5' RACE results. A. Secondary PCR products using primer 1394 for first strand cDNA synthesis, primer 1395 and anchor primer for first and secondary cDNA amplification. Lane1: feeding stage 50-100 mg; Lane 2: feeding stage 100-200 mg; Lane 3: embryo. B. Southern blot of 5'RACE products using oligonucleotide probe 1396.

A



B

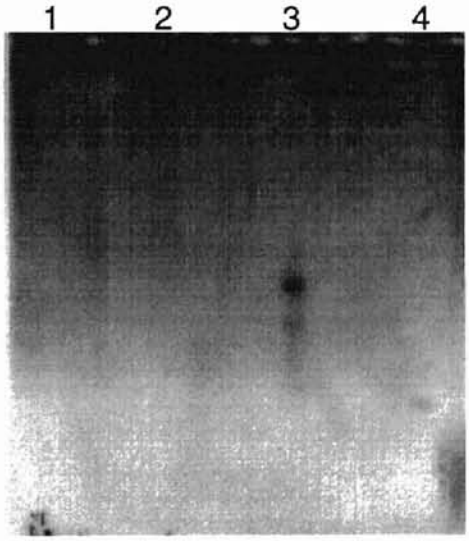
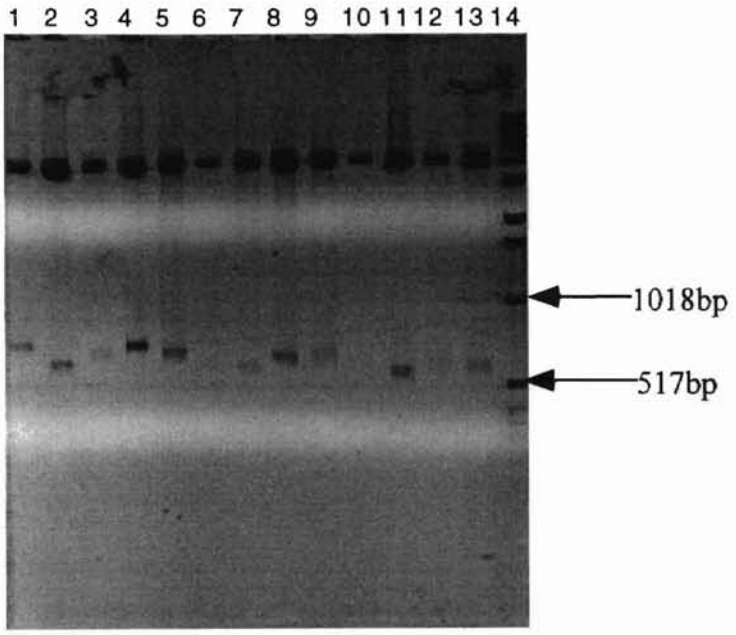
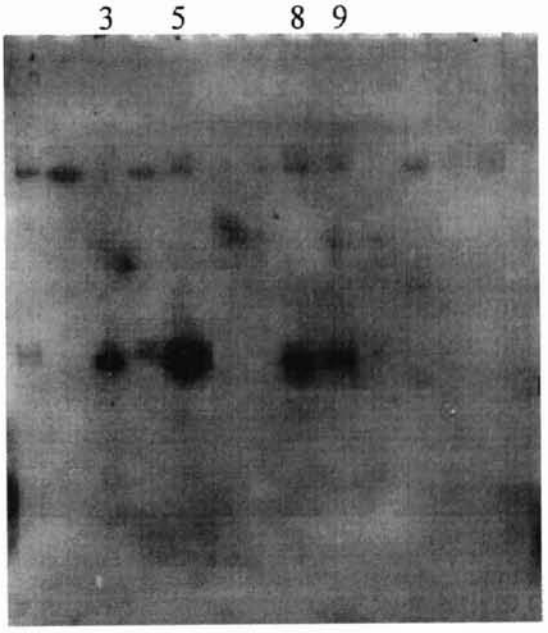


Figure 9. Southern blot of the 5'RACE products with oligonucleotide probe 1396 after TA cloning. A. confirmation of the secondary PCR products after TA cloning and Wizard Miniprep by EcoRI digestion. B. Southern blot of the secondary PCR DNA with oligonucleotide probe 1396.

A



B



Gene specific primer 1396 was used to amplify the 3' end of dopamine D2 receptor. Oligonucleotide 1395 and the full length of 152 bp fragment served as probe. Again, very weak signals were obtained.

With several different gene specific primers, little was improved. One band from each RACE was isolated and cloned. Southern blotting was used to check the insert again. Very weak signals were shown on the film. One sample was sequenced. The sequence showed no relation to dopamine receptors at all (data not shown).

cRACE, one of the modified RACE techniques was employed to elongate the 5' end. However, the PCR products did not hybridize with the probe at all (data not shown).

Southern Blot of Tick Genomic DNA

Due to the high similarity between the 152 bp sequence and the rat dopamine D2 receptor, and failure of extension, it seemed necessary to demonstrate the presence of the 152 bp in the tick genome. One band was shown in bovine at size of 2 kb, but no signals appeared in ticks and the positive control of mouse (data not shown). It is unclear why genomic DNA from the mouse positive control did not hybridize to the probe.

Northern Blot

Northern blotting is a common way to confirm that a target gene is expressed in a certain tissue. An RNA probe derived from the 152 bp DNA fragment was used to hybridize RNA samples from different feeding stages of the tick, but none of the autoradiograms showed promising bands (data not shown).

Adenylate Cyclase Activity Assay

To further elucidate the possible presence of multiple dopamine receptors, membranes isolated from tick salivary glands were incubated with SCH 23390, a highly

specific D1 antagonist in mammalian, and the D2 antagonists spiperone and sulpiride (Fig. 10). SCH 23390 decreased cAMP about 15% and 11%, at concentrations of 10^{-5} M and 10^{-7} M, respectively. Spiperone increased cAMP about 14% and 8%, sulpiride increased cAMP about 11% and 7%, at concentrations of 10^{-5} M and 10^{-7} M, respectively. When 10^{-8} M dopamine was added, D2 antagonists showed effects close to zero at both concentrations except 10^{-7} M sulpiride decreased cAMP 18%. SCH 23390 decreased cAMP 50 % at two concentrations (Fig. 11).

Cyclic-AMP dependent Protein Kinase

A partial sequence of the tick catalytic subunit of cAMP-dependent protein kinase was obtained previously by RT-PCR and 5' RACE. 3' RACE was not successful (McSwain et al., unpublished). So, I tried to obtain the whole sequence by screening a library. Two screening methods (GeneTrapper™ cDNA positive selection and plaque hybridization) and two libraries (3-day feeding tick salivary gland cDNA library and tick embryo library) were tested.

GeneTrapper™ cDNA Positive Selection

A three-day feeding tick salivary gland cDNA library was first chosen to do screening. Oligonucleotide probe 1490 was synthesized and gel purified by OSU core facility. After one time amplification of the λ phage library, single strand DNA which contained the insert and pBluescript vector was excised. The capture and repair steps were performed according to the protocol. Nothing grew on the LB/ampicillin plates after transformation indicating no DNA was captured by the magnetic beads.

In order to determine if the library contained the gene for the catalytic subunit of the cAMP-dependent protein kinase, PCR was performed in which the cDNA library served as template . Two libraries, a 3-day feeding tick salivary gland cDNA library and a tick embryo library were tested. Primers 1244 and 1245 were used in PCR amplification,

Figure 10. Percent change in cAMP produced by membranes incubated in 10^{-5} M and 10^{-7} M SCH 23390, spiperone and sulpiride without added dopamine. Each reaction was triplicated. The activity of adenylate cyclase was 60.6±1.6 pmols cAMP formed/mg protein/min for basal, 69.2±0.2 for 10^{-5} M spiperone, 65.4±1.4 for 10^{-7} M spiperone, 66.8±1.8 for 10^{-5} M sulpiride, 64.6±1.5 for 10^{-7} M sulpiride, 51.6±2.1 for 10^{-5} M SCH 23390, and 54.2±0.4 for 10^{-7} M SCH 23390.

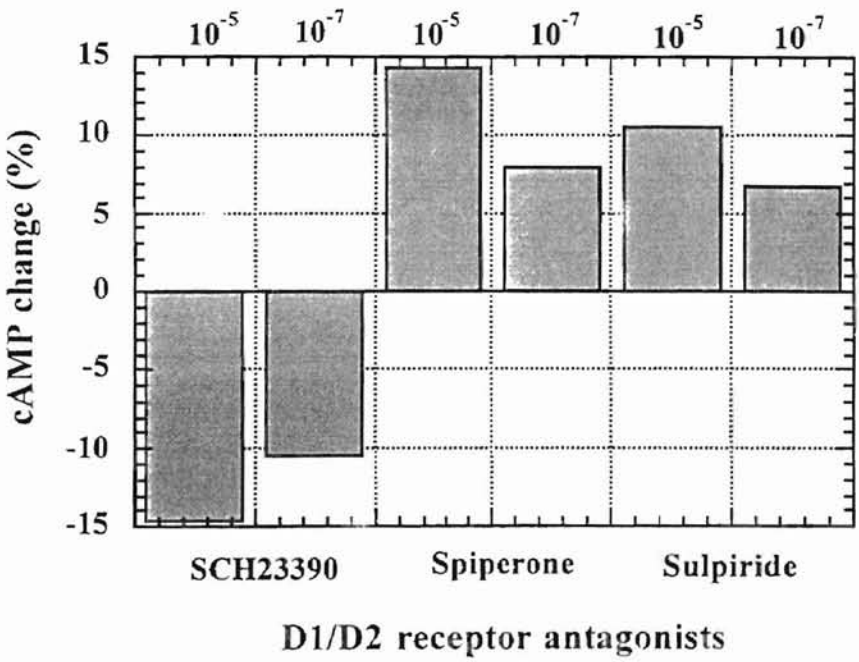
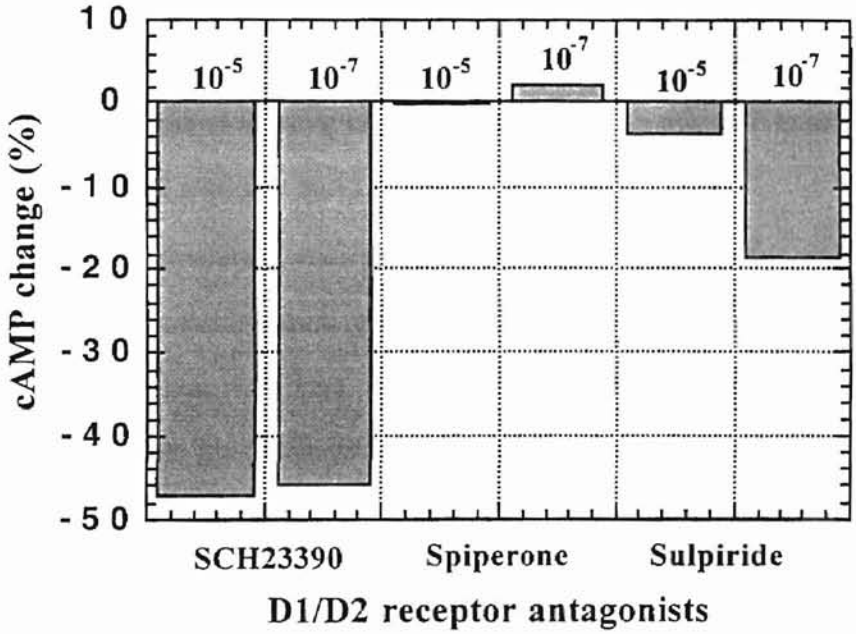

10^-5
10^-7
10^-5
10^-7
10^-5
10^-7
15
10
5
0
-5
-10
-15
cAMP change (%)
SCH23390
Spiperone
Sulpiride
D1/D2 receptor antagonists

Figure 11. Percent change in cAMP in membranes incubated in 10^{-5} M and 10^{-7} M of SCH 23390, spiperone and sulpiride with the presence of 10^{-8} M dopamine. Each reaction was triplicated. The activity of adenylate cyclase was 38.9±1.3 pmols cAMP formed/mg protein/min for basal, 41.6±0.1 for 10^{-8} M dopamine added only, 41.5±0 .4 for 10^{-5} M spiperone, 42.5±0.6 for 10^{-7} M spiperone, 40.1±1.6 for 10^{-5} M sulpiride, 34.0±2.8 for 10^{-7} M sulpiride, 22.0 ± 0.5 for 10^{-5} M SCH 23390, and 22.5±0.4 for 10^{-7} M SCH 23390.


10
0
-10
-20
-30
-40
-50
cAMP change (%)
10-5
10-7
10-5
10-7
10-5
10-7
SCH23390
Spiperone
Sulpiride
D1/D2 receptor antagonists

and oligonucleotide 1491 was used as probe in hybridization. Strong hybridization bands were found in both primary and secondary PCR reactions using the tick embryo library as template. Nothing appeared when the 3-day feeding tick salivary gland cDNA library was served as PCR template. The hybridized PCR product was sequenced by OSU core facility. The sequence of this 230 bp fragment totally matched what was expected.

So I tried to use the tick embryo library to isolate a positive clone. The same procedures were performed as in 3-day feeding tick salivary gland cDNA library. Some colonies grew on one corner of the transformation plates. Seven of them were selected for PCR. No products were evident on the gel. However, the colonies showed signal on the film after colony hybridization using oligonucleotide 1491 as probe. These colonies failed to grow either on liquid media or solid plates.

Another electroporation was performed using the same captured and enriched DNA. This time the colonies were grown very evenly on the plates. No bands were obtained after PCR. There were very faint bands of about 1200 bp in addition to strong vector bands shown on the gel picture after digestion of the purified plasmid DNA. However, they were not able to hybridize with the probe. Sequencing result showed that only the vector. It was odd that the colonies had white color when LB/ampicillin plate with X-gal was used. Two hundred colonies were grown again with positive control. The hybridization results only showed the signal with control.

Screening Tick Embryo cDNA Library by Plaque Hybridization

Duplicate membranes were made from each plate. All the films had a background that was very low, even after one week exposure. The probe activity was 10^7 cpm per ml hybridization solution. The membranes were just washed two times at 50 °C with 0.1% SSC and 0.1% SDS. Five plaques which appeared positive were picked to do the secondary screening. Nothing showed on the hybridization film.

CHAPTER V

SUMMARY AND CONCLUSIONS

Dopamine receptors from invertebrates are not characterized as well as in vertebrates. RT-PCR, one of the standard approaches in cloning was employed. The primers using in amplification were designed from the sequences of vertebrates. A 152 bp fragment that was obtained showed 99% homology to the rat dopamine D2 receptor gene. It is rare to find so high homology between two genes (Human D2 receptor DNA sequence has 91% homology to rat D2 receptor DNA sequence in this area). Contamination is a concern in RT-PCR since it happens very often, because of the sensitivity of this method. A negative control without reverse transcriptase was performed simultaneously. No band was observed in the negative control. PCR technique is known to be difficult to control. However, contamination is really unlikely since the ticks were feeding on sheep, and contact with a rat seems remote. Furthermore, special attention during PCR had always been paid.

The use of 3'RACE, 5'RACE and cRACE was not successful in extending the 152 bp fragment to its full length. The failure could happen in some cases, since the principles of RACE are almost the same as PCR. Some limitations, for example, the high demand for gene specific primers and misincorporation are common in PCR reactions. In 5' RACE, the efficiency to add T-tail to 5' end by TdT is very low.

Aside from contamination, there could be the other reasons for these negative results, such as that tick has very low expression of this D2 receptor gene. Perhaps for the same reason, the 152 bp fragment did not detectably hybridize with tick RNA.

At this point, it is necessary to do some pharmacological studies to show evidence of a D2 receptor in tick salivary glands. The experiments were done by measuring the synthesis of cAMP. One D1 antagonist SCH 23390 which is expected to block D1 receptor and decrease the synthesis of cAMP, and two D2 antagonists spiperone and sulpiride that would inhibit D2 receptor and increase the synthesis of cAMP were used separately in the assays. The synthesis decreased when membranes were incubated with SCH 23390, a highly specific D1 antagonist in mammalian cells, with or without a low concentration of dopamine. However, the cAMP synthesis only increased when membranes were incubated with the specific D2 antagonists spiperone or sulpiride alone, not in the presence of low concentration of dopamine. There is strong evidence for a dopamine D1 receptor in tick salivary glands. However, for the D2 receptor, it is still unclear based on my and other results. One possibility is that the specificity and effects of antagonists have some differences between vertebrates and invertebrates. Another explanation is that the D2 antagonists may also stimulate some other receptors and produce cAMP without low concentration of dopamine.

For the cloning the cAMP-dependent protein kinase, I could amplify a specific fragment of the gene using the tick embryo library as template, but failed to isolate if from this library using the very specific 667 bp probe. This might be the expression of the cAMP-dependent protein kinase gene is not high enough to be detected by isolating the positive plaque from 1,000,000 plaques. This gene can be ampified by PCR, since PCR is more sensitive than library screening.

After millions of years' evolution, ticks have fully adapted to a parasitic life style in which they feed on a host consistently for up to two weeks. Ticks have the unique prostaglandin synthesis system as well as a unique secretory phospholipase A2. It is very likely that the dopamine receptor system in ticks has its unique characteristics as compared with that in vertebrates. This may be the very reason that the work on dopamine D2 receptors in ticks is so frustrating.

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