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# The Spatial Distribution of a Transcript of a Drosophila Octopamine/Tyramine Receptor.

Laura M. McKay

# The Spatial Distribution of a Transcript of a *Drosophila* Octopamine/Tyramine Receptor

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by

Laura M. McKay

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789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 - 1346

# THESIS ACCEPTANCE

Acceptance for the faculty of the Graduate College, University of Nebraska, in partial fulfillment of the requirements for the degree Masters of Arts, University of Nebraska at Omaha.

Committee

| Name | Department |
|---|---|
| Jean A Deupree | Pharmacology, UNMC |
| Jeffrey A. Frank | Psychology, UNO |
| Edward B Byleen d. | Pharmacology, UNMC |
| | |
| | |

Chairperson Bruce A. Clare

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# ABSTRACT

Two specific receptor subtypes for octopamine, a biogenic amine found in both vertebrates and invertebrates, have been pharmacologically defined in insects. These receptors mediate reponses involved in the regulation of movement, feeding and mating behaviors by inducing a rise in intracellular cAMP levels via adenylate cyclase stimulation or by increasing intracellular calcium levels via an undetermined mechanism. Recent studies on a putative octopamine/tyramine *Drosophila* receptor cDNA permanently expressed in mammalian cells have suggested the existance of an additional novel octopamine receptor subtype. This receptor binds multiple agonists (i.e. octopamine and tyramine) and can differentially couple to two different secondary messenger systems (i.e. $G_i$ and $G_q$). Unlike classical octopamine receptor subtypes (i.e. octopamine$_1$ and octopamine$_2$), this receptor attenuates adenylate cyclase activity.

To study further this receptor in *Drosophila*, the spatial distribution of its mRNA was determined by *in situ* hybridization. The probe was a $^{35}S$-labeled riboprobe derived from a 596 bp cDNA subclone encoding the third intracellular loop of this receptor. The expression sites for mRNA

transcripts were identified in intact tissue sections (8-10μ) of larvae and adult flies using tissue autoradiography and analyzed under brightfield and darkfield microscopy. mRNA transcripts were localized in neuronal tissues of the larvae and adult fly, as well as in non-neuronal tissues of the larvae. This localization is consistent with the proposed roles for octopaminergic systems in *Drosophila*.

***With love to my husband and best friend James.***

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I would like to thank my husband James for his patience and love during this period; Dr. Laurence De'Boer for his consistent encouragement and understanding; and my committee members: Dr. Dave Bylund, Dr. Jean Deupree and Dr.Jeffrey French, for their time and supportive advice.

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# INTRODUCTION

Octopamine *(Figure 1)*, a biogenic amine found in both vertebrates and invertebrates (see review by David and Coulon, 1985), is thought to play an important role in the modulation of neurochemical responses regulating movement (Ramirez and Pearson, 1991), feeding (Braun and Bicker, 1992), and mating behaviors in insects (Ramaswamy, et al., 1984). It has been shown to act as a neurotransmitter, neuromodulator, and neurohormone in the nervous system of insects (Axelrod and Saavedra, 1977; Evans, 1980), where it has comparable functions to that of adrenergic amines in mammals (See review by Evans, 1993).

Receptors binding octopamine have been identified in a variety of insect tissues (see review by David and Coulon, 1985). Two pharmacologically distinct octopamine receptor subtypes have been defined in insects, octopamine$_1$ and octopamine$_2$, as well as two receptor subclasses, octopamine$_{2A}$ and octopamine$_{2B}$. Receptor subtypes for octopamine were first identified during early studies in the locust to explain differences in pharmacological profiles and physiological responses to octopamine (Evans, 1977). These receptors mediate octopamine's action in tissue by increasing the levels of intracellular calcium or by increasing the level of adenylate cyclase in target cells. The octopamine$_1$-

receptor subtype is known to mediate a cellular response by inducing a transient rise in cystosolic calcium levels; while the octopamine$_2$-receptor subtype is known to induce a cellular response by increasing cyclic adenosine monophosphate (cAMP) levels via adenylate cyclase stimulation (Evans 1984).

Recent studies on a cloned *Drosophila* octopamine/tyramine receptor cDNA, suggest that an additional, novel octopamine-receptor subtype exists in insects (Arakawa, et. al; 1990; Saudou et al., 1990). This receptor is a member of the G-protein coupled receptor family and exhibits significant structural homology to adrenergic receptors in mammals. Like $\beta_2$-adrenergic receptors *(Figure 2)*, it has seven transmembrane regions with conserved amino acid residue sites in both the second and third intracellular loops (ICL); as well as an extracellular amino-end terminus and intracellular carboxyl-end terminus. Unlike either of the profiles established for the octopamine$_1$ and octopamine$_2$-receptor subtypes, the cloned receptor, when expressed in mammalian cells, can differentially couple to two different second messenger systems and induce two separate cellular responses (Robb et al., 1994). As with the octopamine$_1$-receptor subtype, it can mediate a cellular response via a mechanism inducing a transient rise in intracellular calcium levels, but unlike the octopamine$_1$ and octopamine$_2$-receptor subtypes, it can also attenuate adenylate cyclase activity. These responses

mediated (the elevation of intracellular calcium and the attenuation of adenylate cyclase) are known to occur via separate G-protein coupled pathways ($G_i$ and $G_q$).

Definitive classification of this receptor as an octopamine receptor has been difficult due to its capacity to bind multiple ligands. *In vitro* binding studies in membrane preparations from mammalian cells permanently expressing the octopamine/tyramine receptor have also shown it to bind tyramine, the non-β-hydroxylated precursor of octopamine *(Figure 3)*. Skepticism as to the endogenous ligand for this receptor originates from studies which show that tyramine binding is more specific and effective than octopamine (Saudou et al., 1990), and can also induce differential coupling of the cloned receptor to two separate secondary messenger systems (Evans, 1994). Currently, the status of the classification of this receptor remains unresolved and has been solely based on *in vitro* studies measuring pharmacological and physiological responses of this receptor to either octopamine or tyramine. The interaction of this receptor with these amines *in vivo* or its physiological role in *Drosophila* has not been examined.

Based on the physiological roles of octopamine receptors, in the regulation of locomotion, feeding and mating behaviors, we would expect the putative octopamine/tyramine receptor to be expressed in both the peripheral and central nervous system of the fruit fly. The presence of octopamine and its receptors in the head of the

adult fly has been established (Dudai and Zvi, 1984). Traces of octopamine have been identified in whole body homogenates of adult flies (Watson et al., 1993) and octopaminergic neurons have been identified in both the larvae and adult fly nervous system (Monastrioti, 1995). Additionally, octopamine dependent behaviors have been described in *Drosophila* (O'Dell, 1993). Although traces of tyramine, (lower in concentration than octopamine) have been found in whole body homogenate preparations of adult flies (Watson et al., 1993), the presence of tyramine receptors *in vivo* has not been established in *Drosophila*.

Identifying the cellular locations of this receptor in intact tissue preparations could be useful in defining its role and classification. Presently, the lack of specific antibody probes to label sites with receptor has made its direct detection rather impossible (Robb et al., 1994). However, methods to detect its messenger ribonucleic acid (mRNA), using radioisotopic or fluorescent labels provide an alternate approach to give an indication of potential receptor distribution. *In situ* hybridization with a riboprobe has been shown to be both sensitive and effective as a tool for the detection and localization of mRNA in intact tissues. Although this method does not detect or localize the receptor per se, it can serve as an indirect estimate as to where the receptor might be expressed. In many instances, mRNA expression has shown to be equivalent to the expression of a protein in a tissue. Based on this

premise, the detection of receptor mRNA in a tissue can be indicative of a receptor's location in that tissue.

To study further the octopamine/tyramine *Drosophila* receptor, *in situ* hybridization was performed to detect and localize its mRNA in intact tissue preparations of larvae and adult flies. $^{35}S$-labeled sense and antisense RNA probes synthesized from a 596 bp cDNA subclone (encoding the third intracellular loop of this receptor) was used to detect the receptor mRNA in the tissues. The information gathered from these studies was used to make inferences about the location and potential function of this receptor in the nervous system of the fruit fly.

# MATERIALS AND METHODS

## SPECIMEN COLLECTION AND PREPARATION:

Wildtype animals (*Canton-S* strain) were raised on standard cornmeal/sucrose/dextrose/yeast media at 25° C. Prior to their collection, the animals were staged and sexed. Mid-third instar larvae and newly eclosed adults (< 24 hr.) were collected. Studies were also attempted on the *inactive* (*iav*) mutant allele in a *Novabrisk* genetic background, but as these experiments, for technical reasons, did not produce informative and reliable results, they will not be discussed. The *Novabrisk* strains were kindly provided by Kevin O'Dell of the Institute of Genetics at the University of Glasgow at Scotland, United Kingdom.

### *Collection of First and Third Instar Larvae*

Adult wild-type flies were placed in a breeding chamber and allowed to lay eggs on yeasted grape-juice plates (Ashburner, 1989). Plates were changed frequently and staged for larvae collection. Mid-third instars were collected 48-60 hr. after egg laying. Larvae were washed to free from food by floating in 50% glycerol in water and rinsed in *Drosophila* Ringer's Solution (0.128 M NaCl, 0.046 M KCl, 0.019 M $CaCl_2$). A small incision was introduced in

the larval wall body prior to fixation, to allow penetration of the fixative.

*Collection of Adults*

Adult flies were first anesthetized using moist $CO_2$ and sexed while viewed under a dissection macroscope (Carl Zeiss, #475002, West Germany) with fiber-optic illumination (Fiber-lite, Series 180, Dolan Jenner Industries Inc.). The animals were transferred into depression plates, where they were dissected in fixative. Mouthparts, wings and legs were carefully removed with fine forceps (Dumont #5-Biology) to ensure penetration of fixative. Heads and bodies were collected separately. For some animals, a sharp razor blade was used to separate heads from bodies.

*Fixation and Embedding of Specimens*

Procedures used for fixing and embedding fly heads, bodies, and whole animals were adapted from a protocol provided by Paul Ingam.

To preserve tissue morphology and maximize target retention in the sections, specimens were immersed in freshly prepared Carnoy's Fixative Solution (6 parts isopropanol, 3 parts chloroform, 1 part formic acid) (Humason, 1972) for 90 min. immediately after their collection. Following fixation, specimens were dehydrated through a graded ethanol series (20 min. each in 50%, 70%, 95%, 2x100%), cleared twice in xylene and transferred into

embedding molds containing molten paraffin at 60° C. During the dehydration of larvae, 0.1% eosin-Y was sometimes added to the 95% ethanol to stain larvae and facilitate their handling.

Specimens were infiltrated with paraffin overnight at 60° C and oriented for sectioning on the subsequent day. Animals were oriented in molten paraffin using a warm dissecting needle while viewed under the dissecting microscope with fiber-optic illumination. After orienting the specimens, the paraffin was allowed to harden at room temperature. When solid, the blocks were released from the molds, trimmed with a fresh single-edge razor blade, re-oriented if necessary and mounted onto wooden pegs so that saggital sections of the specimens could be obtained. Serial 8-10 μm thick sections were prepared using a microtome (AO-820 Spencer) fitted with stainless steal razor blades. Sections were floated onto droplets of diethylpyrocarbonate-treated water (DEPC-$H_2O$) placed on poly-L-lysine-treated slides and allowed to expand on a slide warmer (Chicago Surgical & Electrical Company, Melrose Park, Illinois) at 42° C for 2-3 min. After expansion of the sections, excess water was removed with a syringe to avoid damage by bloating of the sections. The slides were left to dry overnight on the slide warmer at 42° C.

*Pretreatment of Slides with Poly-L-lysine*

To prevent detachment of sections during hybridization and washing procedures, slides were coated with a poly-L-lysine slide adhesive solution (M.W. > 250,000; Catalog #P8920, Sigma Diagnostics, St. Louis). Based on prior experiments, coating of slides with poly-L-lysine worked best to retain tissue sections embedded in paraffin. Attachment of sections using a silane solution (1 mL gamma-methacryloxypropyltrimethoxysilane, 200 mL ethanol, and 6 mL 10% acetic acid) worked, when combined with egg albumin (See H.M. Dyanov and S.G. Dzitoeva, 1995). Fisher-brand Color Frost-Plus slides were also tried, but did not retain the sections well.

Before coating slides with poly-L-lysine, the slides were thoroughly cleansed to remove grease and other particulates. To clean, slides were placed in metal racks, soaked in a detergent solution (Micro Laboratory Cleaning Solution, International Products Corp., Trenton, New Jersey or Alconox) for 2 hr. and rinsed in running-tap water for 1 hr. After rinsing in water, the slides were soaked in ethanol (2 x 100%, 10 min. each), rinsed in acidic alcohol (1% HCl in 70% ethanol) and baked for >4 hr. at 350° C to eliminate nuclease activity. After cooling to room temperature, an adhesive coating was applied to each slide by immersing racks into a poly-L-lysine solution (Sigma, diluted 1:10 in $H_2O$) for 5 min. Following treatment, the slides were set to dry in an oven for 1 hr. at 60° C,

covered with foil to protect from dust, and stored at room temperature until needed.

*Dewaxing and Dehydration of Tissue Sections*

Mounted sections were dewaxed by incubating the slides in two changes of xylene, 15 min. each, and rehydrated through a graded ethanol series (2 x 100%, 95%, 70%, 50%, 15 min. each) and twice in DEPC-$H_2O$, 10 min. each. Following rehydration, the slides were soaked in 0.2 M HCl for 20 min., rinsed in DEPC-$H_2O$ for 5 min., and equilibrated in 2X SSC buffer (0.15 M NaCl, 0.015 M tri-sodium citrate) for 30 min. at room temperature. After rinsing in DEPC-$H_2O$ for 5 min., the sections were treated with proteinase K (0.125 mg/mL) diluted in TE (0.05 M Tris-HCl, 0.005 M EDTA, pH 7.5) for 10 min. at room temperature. Proteinase K activity was stopped by immersing the slides in 0.2% glycine in 1X PBS (0.13 M NaCl, 0.007 M $Na_2HPO_4$, 0.003 M $NaH_2PO_4$) for 30 sec. followed by two rinses in 1X PBS, 30 sec. each. To prevent diffusion of target RNA, the sections were post-fixed in freshly prepared 4% paraformaldehyde in PBS for 20 min. at room temperature, equilibrated in 1X PBS and acetylated to reduce non-specific binding of the probe.

*Acetylation of Tissue*

The slides were incubated in freshly prepared 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min. at room temperature. Following acetylation, the slides

were rinsed in 1X PBS, dehydrated through a graded ethanol series (30%, 60%, 80%, 95%, 100%, 2 min. each) and air dried.

**PREPARATION OF RNA PROBES:**

Non-radiolabel *in situ* hybridization with digoxigenin-labeled riboprobes was initially attempted, but did not produce reliable results with a high enough signal to noise ratio, due to the low levels of this receptor mRNA in the fly. A more viable procedure using [$^{35}$S]-UTP labeled riboprobes demonstrated clearer signal expression in control experiments. As a result, [$^{35}$S]-UTP labeled riboprobes were selected for *in situ* hybridization.

All riboprobes were synthesized from DNA templates using an *in vitro* transcription kit (Catalog #1326, T7/T3 MAXIscript Kit, Ambion Inc., Austin, Texas). [$^{35}$S]-UTP labeled riboprobes were transcribed from a 596 bp subcloned cDNA template (encoding the third intracellular loop of the octopamine receptor). The 596 bp cDNA fragment *(See APPENDIX II)* was subcloned into pBluescript *(Figure 4)* which allowed for the generation of single-stranded sense and anti-sense riboprobes from the T3 and T7 promoter sites in this vector. The 596 bp fragment was obtained from a 2.2 Kb cDNA fragment subcloned into pBluescript.

## A. PREPARATION AND ISOLATION OF PLASMID DNA WITH THE OCTOPAMINE 2.2 Kb cDNA INSERT:

*Cloning of the 2.2 Kb Octopamine-Receptor cDNA*

Subcloning of a *Drosophila* 2.2 Kb octopamine/tyramine-receptor cDNA into pBluescript (by Bruce Chase) was as follows: The 2.2 Kb cDNA insert, previously cloned into a pSVL vector as described by Shoji Arakawa et al. (1991), was kindly provided by Claire M. Fraser. Briefly, the 2.2 Kb insert was released from pSVL following a XbaI and BamHI digest and directionally cloned into the XbaI and BamHI sites of the pBluescript vector. Recombinant plasmids (pBluescript plasmids carrying the 2.2 Kb cDNA insert) resulting from the cloning reactions were used to transform competent XL-1 Blue *E. coli* cells (Stratagene). Plasmid DNA isolated from transformed cells was verified by restriction digest analysis and used in the preparation of DNA templates for probe synthesis.

*Transformation of E. coli Competent Cells*

Frozen *E. coli* cells (XL-1 competent) were thawed on ice and distributed in 200 µl volumes into sterile polypropylene tubes (Falcon 2059, 17 mm x 100 mm). Tubes were allowed to chill on ice for 10 min. before the addition of the DNA. About 5 µl of DNA (0.2 µg/µl) were added to each tube and mixed with the cells. To assess the efficiency of both the cloning and transformation reactions, four mock

transformations were prepared as controls. Three different negative controls were prepared by mixing cells with 5 µl of TE, 1 µg of phosphatased and non-phosphatased vector. A positive control was prepared by adding 1 µg of supercoiled-vector DNA to one group of cells.

DNA was allowed to adhere to the cells by chilling the tubes on ice for 30 min., when chilled, the cells were shocked by heating them in a preheated waterbath (42° C) for 90 sec. and then chilling in an icebath for 2 min. After shock, the cells were allowed to "recuperate" and express the ampicillin resistance gene, by adding 800 µl of prewarmed (37° C) SOC medium and incubation in a shaking waterbath at 37° C for 45 min. The cells were plated in volumes of 10 µl, 100 µl and 895 µl over ampicillin-resistant (100 µg/ml) agar plates, pre-spread with 40 µl of X-gal (20 mg/ml) and 4 µl of IPTG (200 mg/ml). Transformant clones were allowed to grow overnight by incubating plates at 37° C. On the following day (15-18 hr.), potentially positive clones (white colonies) were identified and used to inoculate 1.5 ml of LB medium. Saturated-bacterial cultures were obtained after growth overnight in a shaking waterbath at 37° C and used for small-scale plasmid-DNA preparation. These were used to verify the presence of the cloned insert in $pSK^+/-$ Bluescript.

*Small-scale Plasmid-DNA Preparation*

Saturated bacterial cultures (~3 ml) were transferred into microfuge tubes and centrifuged for 30 sec. to collect the cells in a pellet. After discarding the supernatant, cells were resuspended in 100 µl STET buffer (8% Sucrose, 0.5% Triton-X100, 50 mM EDTA pH 8.0, 10 mM Tris-HCl pH 8.0), lysed with 100 µl of lysosyme (2 mg/ml) in STET and incubated at room temperature for 5 min. Following lysis, the microfuge tubes were heated for 1 min. in a boiling waterbath to inactivate the lysosyme and denature the protein-chromosomal DNA complexes, and centrifuged for 10 min. to pellet cell debris. The pellets were removed with toothpicks, leaving plasmid DNA in solution. Plasmid DNA was precipitated from the supernatant using 30 µl of 4M Ammonium Acetate and 250 µl of Isopropanol. The DNA precipitate was pelleted by centrifugation, washed with 70% ethanol, air-dried and then dissolved in 50 µl of TE. Purity and concentration for each of the DNA solutions were determined. Plasmids carrying the 2.2 Kb cDNA insert were identified by restriction digest and gel electrophoresis analysis.

*Calculating DNA concentration and purity*

DNA concentration (µg/ml) was measured by ultraviolet absorbance spectrophotometry at 260 nm using a spectrophotometer (Perkin-Elmer, Coleman 572). DNA concentration was calculated by assuming that double stranded

DNA absorbs 1.0 $OD_{260}$ when at a concentration of 50 µg/ml. DNA purity was evaluated by determining the $ABS_{260}/ABS_{280}$ ratio. Ratios ($ABS_{260}/ABS_{280}$) less than 1.8 indicated either protein or phenol contaminants in the sample.

*Identification of Bacterial Clones with Recombinant Plasmids*

To verify that the correct subclones were recovered, single restriction enzyme digests with endonucleases EcoRI, HindIII, SalI, XbaI, and XhoI, and a double-digest with BamHI and XbaI were performed. For each digest, 1 µg of DNA (dissolved in a 20 µl reaction volume: 1X Restriction Enzyme Buffers from Promega, 10 µg DNase-free RNase A), was cleaved with 3 units of restriction enzyme. Digest reactions were set on ice in 1.5 ml microfuge tubes, transferred into foam racks, floated in a waterbath at 37° C and incubated for 2 hr. Following incubation, endonuclease activity was stopped by heating at 65° C for 2 min. and a 1/6 final volume of Sample Application Buffer (60 mM EDTA, 15% Ficoll Type 400, 0.25% Bromophenol Blue, 0.25% Xylene Cyanol) were added to each digest. Samples were loaded on a 1.2% agarose gel in 1X TPE (0.08 M Tris-phosphate, 2 mM EDTA pH 7.8), pre-stained with 0.4 µg/ml Ethidium Bromide. Lambda HindIII reference size markers were also loaded at this time. DNA fragments were separated overnight by gel electrophoresis at +20 Volts in 1X TPE Buffer. On the following day, the gel was photographed under UV trans-illumination and analyzed to determine the size of the fragments and derivation of a

restriction map. Size of digest fragments were analyzed using a restriction map obtained from the GCG DNA Sequence Analysis Program at the University of Nebraska Medical Center (GenBank, #M26181).

*Preparation of Saturated Bacterial Cultures*

Saturated cultures for positive recombinant clones (carrying the 2.2 Kb cDNA insert) were grown overnight in 100 ml of LB medium in a shaking waterbath at 37° C. Plasmid DNA isolated from these cultures was purified by polyethylene glycol (PEG) precipitation.

*Isolation and PEG Purification of Plasmid DNA*

DNA was isolated from overnight cultures as follows: 50 ml of each of the saturated cultures were centrifuged (5000 RPM) for 5 min. in an HS4 rotor to pelletize cells. Cells were then resuspended in 0.6 ml of a hypertonic solution (Glucose, Tris-Cl pH 8.0, EDTA) to swell, transferred into 15 ml tubes containing lysosyme (~1 mg), and placed on ice for 10 min. Proteins and other high density residuals were precipitated with 2.4 ml of Solution III (NaOH, SDS) and placed on ice for 10 min. After treatment with a strong base, the supernatant was neutralized by adding 2.0 ml of Solution III (KAc, glacial acetic acid), then incubated on ice for 10 min. Precipitates were collected in a pellet after 30 min. of centrifugation (10,000 RPM) in an HB4 rotor. The supernatant was transferred into a fresh 15 ml tube,

treated with 30 µl of RNase A (2 mg/ml) to destroy any single stranded RNA and incubated for 30 min. at 37° C. Nuclease activity was terminated by adding proteinase K (100 mg/ml) and incubating for 30 min. at 50° C. Plasmid-DNA extraction by phenol:chloroform followed and DNA was precipitated in 0.6 volumes of isopropanol. The DNA precipitate was collected in a pellet after 10 min. of centrifugation (7000 RPM) and the supernatant was discarded. DNA pellets were washed with 70% ethanol, air-dried, and resuspended in 0.4 ml of TE.

To purify DNA, 85 µl of 5M NaCl and 0.5 ml 13% PEG 6000 were added to each DNA solution and mixed in 1.5 ml microfuge tubes. The tubes were chilled on ice for 60 min. and centrifuged for 10 min., forming pellets. After discarding of the supernatant, the pellets were wash with 70% ethanol, air dried, and resuspended in 200 µl of TE. DNA purity and concentration were determined.

*Phenol/Chloroform Extraction of DNA.* DNA solutions were mixed with 1 volume of phenol/chloroform mixture by gentle immersions and centrifuged for 10 min. The aqueous phase was carefully removed and transferred into a new microfuge tube where it was once again extracted with 1 volume of a phenol/chloroform mixture.

*Ethanol Precipitation of DNA.* Linearized DNA was precipitated overnight in 1/10 volumes of 3 M Sodium Acetate, 1 µg of carrier tRNA and 3 volumes of 100% ethanol at - 20° C. 15-18 hr. later, the precipitate was recovered in a pellet after 10 min. of centrifugation, washed in 70%

ethanol, air dried, and diluted in 10 µl of TE (0.5 µg/µl). Linearized template DNA was stored as an ethanol precipitate at -20° C until ready to use.

## B. PREPARATION OF [$^{35}$S]-UTP LABELED RIBOPROBES:

*Subcloning of the 596 bp cDNA fragment*

The 596 bp cDNA fragment was excised from the 2.2 Kb octopamine receptor cDNA insert *(Figure 5b)*. Following a HindIII and SpeI digest, the released fragment was directionally cloned into the HindIII and SpeI sites of the pBluescript vector *(Figures 5c & 5d)*.

To exicise the 596 bp cDNA fragment from pBluescript, 10 µg of plasmid DNA (carrying the 2.2 Kb octopamine-tyramine receptor cDNA insert) were digested overnight (37° C) with 30 units of HindIII and SpeI. In addition, vector DNA with complementary ends to the insert was also generated by digesting 10 µg of pSK+/- Bluescript with 30 units of HindIII and SpeI. Each digest reaction was prepared in a 100 µl volume containing 1X Enzyme Buffer B (Promega) and 1.0 µl RNase A. Both the insert and linearized vector were separated from the digests reactions by gel electrophoresis on a 1.0% agarose gel and recovered by gel electroelution onto a NA-45 membranes (Sambrook et al., 1989). Membranes were washed in low salt buffer (0.15 M NaCl, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA) and incubated in high salt buffer (50 mM Arginine, 1 M NaCl) for 30 min. (65° C) to elute the DNA. The DNA was phenol/chloroform extracted from the high salt

buffer solution, precipitated in ethanol, washed with 70% ethanol and diluted with 10 µl of TE (~0.1 µg/µl of DNA). To subclone the 596 bp fragment into pBluescript, an equimolar mixture of vector DNA (pSK+/- Bluescript) and insert, diluted in 1X T4 DNA ligase buffer, was incubated for 4 hr. in a thermocycler at 16° C with 0.5 µl of T4 DNA ligase.

*Preparation of plasmid DNA.*

Plasmid DNA was isolated from overnight grown cultures and purified by PEG precipitation as previously described. A plasmid DNA purification kit (Perfectprep™, 5 Prime --> 3 Prime, Inc.) was tried, but failed provide sufficiently high yields of plasmid DNA to generate probes.

*Verifying Insert Sequence and Orientation*

The sequence and orientation of the 596 bp insert in pBluescript was verified by DNA sequence analysis using a DNA Sequenase® sequencing Kit from United States Biochemical (USB). For each reaction, plasmid DNA was denatured by alkaline denaturation and annealed to a M13 reverse primer (which flanked the 3'-end of the cDNA insert allowing for the transcription of the antisense strand). [$\alpha^{32}$P]-UTP was used as a label during primer extension. Di-deoxynucleotides were used to termination the reactions. Products from the sequencing reactions were separated by gel electrophoresis (1200 Volts) on a 6% denaturing polyacrylamide gel at 45° C for 3 hr.. The gel was fixed (15% methanol:5% acetic acid

%V:%V; 15 min.) blotted on blotter paper, dried in a gel drier (80° C) for 45 min. and placed over X-ray film in a film cassette. After a 72 hr. of exposure at -80° C, the film was developed. The sequences for each clone were examined and analyzed using sequence information compiled from the GCG data base program at the University of Nebraska Medical Center.

*Growing Positive Clones*

Saturated bacterial cultures were grown for all clones carrying the 596 bp insert. Plasmid DNA isolated from these cultures was PEG purified, phenol/chloroform extracted, and resuspended in 200 µl of TE buffer. Plasmid DNA concentration and purity were determined by ultraviolet absorbance readings at 260 and 280 nm.

*Preparation of DNA Templates*

Plasmid DNA was linearized through cleavage at either the HindIII or SpeI sites. For each reaction 10 µg of plasmid DNA were cleaved with 30 units of enzyme in a 200 µl reaction volume. Plasmid DNA was cleaved with HindIII to generate a DNA template with a T3 promoter (transcribes the sense strand) and SpeI to generate a DNA template with a T7 promoter site (transcribes the antisense probe) *(Figure 6)*. The DNA mixtures were treated with proteinase K (10 mg/ml) for 30 min. at 50° C. The DNA was phenol/chloroform extracted and precipitated in ethanol. DNA precipitate was

pelleted by centrifugation, washed in 70% ethanol and resuspended in 10 µl of TE.

*Riboprobe Synthesis*

Riboprobes were generated according to kit (Maxiscript *In vitro* transcription kit , Ambion) specifications using [$^{35}$S]-UTP as a label. Each riboprobe was transcribed in a separate 1.5 ml reaction tube. The kit components were mixed, at room temperature, with 2.0 µl $dH_2O$ in the following order: 2 µl of 10X transcription buffer; 1 µl of 200 mM DTT; 1.0 µl of 10 mM ATP; 1.0 µl of 10 mM CTP; 1.0 µl of 10 mM GTP; 1 µl of RNase Inhibitor; 5.0 µl of linearized DNA (0.2 µg/µl); 5.0 µl of [$^{35}$S]-UTP, 1000 Ci/mmol (20 mCi/ml); and 1 µl RNA polymerase (10 U/µl), for a total reaction volume of 20 µl. Sense probes were transcribed from DNA templates (cleaved with HindIII) by adding 1 µl of T3 RNase polymerase and antisense probes were transcribed from DNA templates (cleaved with SpeI) by adding 1 µl of T7 RNA polymerase. The reactions were incubated in a waterbath for 1 hr. at 37$^{o}$ C. Template DNA was degraded by adding 1 µl of RNase-free DNase I (2 U/µl) to each reaction and incubation for 15 min. at 37$^{o}$ C.

*Calculation of Yield and Probe Specific Activity*

For each probe synthesized, two small Whatman DE81 paper filters were spotted with 1.0 µl of a 1:20 dilution of the probe mixture. One of the filters was sequentially

washed 5x in 0.5 M $Na_2HPO_4$ (2-3 min.), 2x in DEPC-$H_2O$ (3-4 min.), and 2x in 95% Ethanol (3-4 min.). Both filters were air-dried and counted separately in 5 ml of Optima-Gold scintillation fluor. Counts per minute (cpm) were measured with a Beta Counter (Beckman).

The percent and total of $^{35}S$-UTP-label incorporated into the RNA transcripts, moles of $^{35}S$-UTP in the reaction, number of RNA nanograms (ng) synthesized and total cpm incorporated into the RNA product were calculated using formulas provided in the MAXIscript *in vitro* transcription kit manual *(See APPENDIX I for Formulas)*. The specific activity for each probe was obtained by dividing the total incorporated cpm by the number of RNA transcripts synthesized in ng. The amount of RNA transcripts was calculated by dividing the total sum of the molecular weights of the four nucleotides ($1320 \times 10^9$ ng) by $10^9$ nmoles.

The probes were diluted in hybridization buffer (50% de-ionized formamide, 10% dextran sulfate, 0.3 M NaCl, 10 mM Tris-HCl, 6 mM $NaH_2PO_4$, 5 mM $Na_2HPO_4$, pH 6.8, 5 mM EDTA, 1x Denhardt's, 10 mM dithiothreitol (DTT) & 1.0 mg/mL tRNA) and prepared 5X concentrated *(See Table III)*. Probes were stored at -20° C until needed.

*Probe Hydrolysis*

The probes were hydrolyzed by alkaline hydrolysis to reduce their size to ~200 bp. Reduction in base pair length increased the efficiency of probe penetration into the

tissues. A hydrolysis reaction was set for each probe. The probes were incubated in a 0.1 M Sodium Carbonate Buffer (0.1 M $NaCO_3$, pH 10.2, 10 µl 1 M DTT and 89 µl of $H_2O$) at 60° C. The duration of incubation time was calculated *(See Table I for Formula)*, which varied according to probe length. The hydrolysis reactions were stopped by adding 7.0 µl of a 3M NaOAc solution in 15% glacial acetic acid. $CO_2$ was cleared from the samples by brief centrifugation and adding 2.5 volumes 100% ethanol.

## C. PREPARATION OF ANTISENSE AND SENSE *elav* CONTROLS

[$^{35}$S]-UTP labeled antisense and sense riboprobes for *elav*, a highly expressed protein in *Drosophila* tissues (Vincent III and White, 1988), were synthesized. These served as a suitable controls for evaluating the functionality of the developed method serving both as positive (antisense probe) and negative (sense) controls during the development and execution of *in situ* hybridization assays. Recombinant plasmids (*elav* insert in pGEM-1; *See APPENDIX III & IV*) were kindly provided by Dr. Marie Laure-Samson (Biochemistry, University of Nebraska Medical Center). A digest using NotI used to generate a linear DNA template with a T7 promoter site (antisense probe) and a SP6 promoter site (sense probe) *(See Figure 6)*. Riboprobes for *elav* were generated using a MAXIscript *in vitro* transcription kit from Ambion as described above. The probes were hydrolyzed by alkaline hydrolysis at 60° C *(See Table I for*

*length of hydrolysis).* Probe yields and specific activity were determined for each probe *(Table II).* Probes were stored at - 20° C 5X concentrated *(See Table III).*

## *In Situ* Hybridization

*In situ* hybridization and autoradiographic signal detection were adapted from Markopoulou and Artavanis-Tsakonas (1989).

### *Hybridization Conditions*

The specific activity and concentration varied for each probe *(See Tables II & III).* Before their use, each probe was diluted in hybridization buffer *(See Table III),* heated for 30 sec. at 80° C, and centrifuged for 30 sec. *Elav* control slides were overlayed with 25 µl of probe, while the "octopamine" slides were overlayed with 35 µl of probe. Due to the greater number of slides overlayed with the sense and antisense "octopamine" riboprobes, probes were diluted in a larger volume of hybridization buffer to yield sufficient amount of probe for each slide *(See Table III).* After overlaying of the probes, the slides were covered with siliconized coverslips and edges sealed with rubber cement to protect against evaporation. The coverslips were siliconized by soaking in a 5% di-chlorodimethysilane in chloroform. The slides were incubated (55° C) overnight in a sealed-humid chamber saturated with wash buffer (50% de-ionized formamide,

0.3 M NaCl, 10 mM Tris-HCl, 6 mM $NaH_2PO_4$, 5 mM $Na_2HPO_4$, pH 6.8, 5mM EDTA, 10 mM DTT).

*Washes*

After overnight incubation, the slides were washed repetitively in buffer solutions and treated with RNase A. Treatment with RNase A reduced nonspecific binding, which reduced potential background signaling. The slides were transferred into a wire slide rack, dipped into wash buffer to remove the coverslips, washed in wash buffer (55° C) for 1 hr and incubated (55° C) in wash buffer for 5 hr. After a 5 min. incubation (37° C) in NTE (0.5 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 10 mM DTT), the slides were treated with RNase A (20 $\mu g$/mL) in NTE for 30 min. (37° C) and washed for 1 hr. in five changes of 0.5 NTE (37° C) and incubated overnight in wash buffer (55° C). Following overnight incubation, the slides were dehydrated through a graded ethanol series (30%, 60%, 80%, 95%, 2X 100%) prepared in 0.3 M ammonium acetate and allowed to air-dry.

*Autoradiography*

A method adapted for Kodak NTB-2 emulsion (Cat# 165-4433) was used at first. Due to high background on the developed slides, a method using Amersham emulsion (LM-1 for Light Microscopy; Code #RPN 40) was used instead. After, some slides dipped in Amersham emulsion were also found to have high background. It was determined that background

problems were largely due to light leaks in the darkroom used for dipping and developing the slides. Unfortunately, this problem compromised a significant amount of the data gathered, before being resolved.

*Autoradiography using Amersham Emulsion.* The slides were individually dipped for 5 sec. into the emulsion (42° C), vertically drained for 5 sec. and placed to dry for 5 min. at room temperature. The slides were exposed at 4° C in "light-tight" boxes, sealed with tape . The exposure time varied, some slides were exposed for 72 hr., while others were exposed for longer periods (up to 240 hr.). The slides were placed on a wire rack and developed in emulsion (18-20° C) for 10 min., dipped in Kodak Stop Buffer for 30 sec. (18-20° C), fixed in Kodak Rapid Fix (18-20° C) for 5 min., and rinsed in running tap water (18-20° C) for 15 min.

*Staining and Mounting of Slides*

The slides were stained in 0.01% toluidine blue, 0.05% methylene blue, 0.05% borax for 5 min. After staining, the slides were dehydrated through a graded ethanol series (30%, 60%, 80%, 95%, and 2x100%, 2 min. each), cleared twice in xylene and mounted in Permount.

The slides were viewed under brightfield and darkfield optics using 10X ocular and 10X, 20X and 40X objectives on a Zeiss Standard or Olympus System Microscope (Model BX 40, Japan). Photomicrographs were taken using 400 FujiFilm Super

G Plus film (for 35 mm color prints) with a blue filter to correct for color changes with tungsten filament illumination.

# RESULTS

Several different methods were employed in an attempt to determine the distribution of mRNA transcripts of a putative *Drosophila* octopamine/tyramine receptor by *in situ* hybridization to larval and adult sections. The most successful approach to detect and localize the mRNA transcripts for this receptor utilized antisense $^{35}$S-UTP labeled riboprobes generated from a 596 bp cDNA subclone that encodes this receptor's third intracellular loop. Similarly, antisense $^{35}$S-UTP labeled riboprobes derived from an *elav* cDNA clone were highly effective positive control probes for these assays.

*Characterization and Verification of cDNA Subclones*

Restriction digest analyses to verify that the initial 2.2 Kb fragment of the 3.3 Kb cDNA, previously described (Arakawa et al., 1990), was correctly subcloned into pBluescript were performed. Plasmid DNA was prepared from potentially positive bacterial clone (white colonies) cultures grown overnight and digested with restriction enzymes. Restriction maps *(data not shown)* derived for several independently isolated clones indicated each was the correct 2.2 Kb subclone from the previously published 3.3 Kb octopamine/tyramine receptor cDNA. A single restriction enzyme digest with XbaI indicated the loss of this site

during the cloning of the 2.2 Kb cDNA insert into pBluescript.

A 596 bp SpeI and HindIII fragment of this 2.2 Kb cDNA subclone was directionally cloned into the SpeI and HindIII sites of the pBluescript vector, so that transcription of a linearized plasmid from T3 and T7 promoters would respectively result in either sense or antisense transcripts *(Figure 6)*. DNA sequence analysis was used to directly verify that this 596 bp fragment was inserted in the 5' to 3' direction *(Figure 7)*, with the SpeI site situated ~29 bp downstream from the T3 polymerase promoter site. This orientation was also supported by restriction digest analyses of the recombinant plasmid which demonstrated that the fragment was inserted in the HindIII and the SpeI sites of the vector: a double digest with SpeI and HindIII confirmed the size of the insert to be ~ 600 bp, while single restriction digests with SpeI or HindIII linearized the plasmid. From these data, the HindIII site was estimated to be ~623 bp downstream from the SpeI site and ~47 bp upstream from the T7 polymerase promoter site. These analyses of the 596 bp subclone indicate that it can be used generate appropriate templates for the synthesis of sense and antisense probes.

*Characterization of the $^{35}$S-UTP Labeled Riboprobes*

Two critical factors for the success of *in situ* hybridization are the specific activity and the concentration

of the $^{35}S$ labeled probe in the hybridization solution. For rare mRNAs it is especially important to use highly labeled probes at sufficient concentration ratios (~ 500 pg/µl) to obtain a reliable signal to noise ratio (Amersham International, 1994). The percentage of incorporated $^{35}S$-UTP in the riboprobes, the specific activity, and the probe concentration are presented in Tables II and III. The percent incorporation calculated for each probe was greater than 50% indicating good incorporation of the [UTP]-$^{35}S$ label. Generally this value is ~ 50% (See MAXIscript *In vitro* transcription manual). The probe concentration and specific activity for the "octopamine" riboprobes were lower than that for the *elav* probes.

Distribution of *elav mRNAs*

Because of its widespread an abundant distribution in neuronal cells (Vincent and White, 1988), the spatial profile of *elav* transcription was assessed as a control for assay function.

Sections of third instar larvae (8-10 µm) were hybridized either with *elav* antisense (positive control) or sense riboprobes. Analyses of the sections using brightfield and darkfield illumination revealed an intense signal in those sections hybridized with the *elav* antisense probe *(See figure 8)*. As expected, *elav* transcripts are abundant in the neuronal tissues of the fly and are widespread. An intense signal is primarily detected in the cellular cortex

of the brain and optic lobes. Slight signal is evident in the neuropil, imaginal disks and salivary glands. In contrast, sections hybridized with the sense *elav* probe showed no significant labeling *(data not shown)*. These data thus indicate that the *in situ* hybridization assay used in this work was able to detect relatively abundant mRNA species in all neuronal tissues.

*Localization of octopamine/Tyramine-Receptor mRNA in the Third Instar Larvae*

*In situ* hybridization to tissue sections with sense and antisense $^{35}$S-UTP labeled riboprobes suggested that mRNA transcripts for the *Drosophila* octopamine/tyramine receptor are distributed throughout the central nervous system of the larvae *(Figure 9)*. Transcripts appear to be present predominantly in the CNS cellular cortex, albeit at a much lower level than *elav* transcripts *(Figure 8)*. Sparse signal is detected over the centrally located neuropil of the larvae brain hemispheres, albeit less than in the cortical regions. In addition to the signal detected in the CNS (neuronal tissues), other areas such as the imaginal disks and salivary glands, showed relatively abundant signal *(See Figure 9 & 10)*. Analysis of signal using dark field illumination allowed for the most sensitive detection of silver grains. Sections hybridized with the sense riboprobe for this receptor showed no significant labeling *(data not*

*shown)*. Background levels were fairly low when compared to signal detected over the tissues, as shown in *Figure 10*.

*Localization of octopamine/Tyramine-Receptor mRNA in the Adult Fly*

*In situ* hybridization to tissue sections (8-10 μm) of heads from male adult, using sense and antisense $^{35}$S-UTP labeled riboprobes, suggested that mRNA transcripts for the *Drosophila* octopamine/tyramine receptor are not abundant in this tissue. The signal that is observed is widely and unevenly distributed. It can be seen over most of the tissues, but with higher intensity in specific cortical areas. Analysis of the sections by darkfield microscopy revealed low levels of hybridization signal in the neuropil, subsets of the optic lobes and central brain. A slightly more intense signal is seen over the cell body layers of these regions, such as in the lamina, medulla, lobula and lobula plate of the optic lobes and in the antennal lobes in the central brain *(Figure 11)*. Hybridization is also seen in the retina.

Sections hybridized with the sense riboprobe for this receptor showed no significant labeling. *Figure 12* demonstrates that essentially no signal can be seen over the structures of adult brain. The patterns of signal observed with an antisense probe over the cell body layers of the lamina, medulla, lobula and lobula plate are absent in these sections, even when high background is present *(See*

*Figure 12)*. Comparison of sense and antisense probe signals revealed nonspecific binding of the probe in the adipose tissues and cuticle *(Figures 11 & 12)*.

Analysis of signal in heads of adult female flies and adult bodies revealed no conclusive information. Poor signal reproducibility and inadequate morphology of these sections precluded reliable analysis. In many of these sections, light leaks in the darkroom caused problems with high background that led to poor signal to noise ratios. In other slides, the loss of sections during processing failed to allow any inferences to be made.

In summary, the distribution profile obtained for the mRNA transcripts of the *Drosophila* octopamine/tyramine receptor suggest that this receptor's mRNA is widely distributed in the CNS of the third instar larvae and adult male fly. Although this transcript is present in the CNS, it is relatively discretely distributed when compared to *elav* transcripts. In contrast to *elav* expression in the larvae, this transcript is also found, at relatively high levels, in the imaginal disks and salivary glands.

# DISCUSSION

The spatial distribution of the mRNA for a putative *Drosophila* octopamine/tyramine receptor was examined by *in situ* hybridization to intact tissue preparations of larvae and adult flies. Analysis of the spatial patterns of transcription indicated this gene's mRNA expression to be non-uniform and distributed throughout CNS tissues, albeit quite rare when compared to that of *elav*. In some sections an intense signal could be observed over some cortical regions. Transcripts for this receptor were found not only in areas of the CNS in both the larvae and adult fly, but also in non-neuronal tissues in the larvae. If the distribution of mRNA for this receptor reflects its cellular distribution, these findings suggest for this receptor to be widely utilized. It serves functions in the brain hemispheres, salivary glands and imaginal disks of the larvae, and in the central brain and optic lobes of the adult (male) fly.

The abundance of the transcripts for this receptor in both the adult and larvae tissues was low, in clear contrast to what was seen for *elav* transcripts *(Figures 8 & 9)*. This level may suggest that there is tight regulation of this receptor at the level of transcriptional or transcript stability (i.e. a lower mRNA half life than *elav*). From these experiments alone, it is not possible to infer whether

if one or both factors contributes to the low levels of mRNA for this gene.

From an experimental perspective, factors such as low probe concentration, low specific activity of the label, and short lengths of autoradiographic exposure, can result in low hybridization signal (Amersham Life Sciences, 1994). In these experiments it is possible that the low hybridization signal in some of the tissue sections hybridized with the octopamine/tyramine-receptor antisense riboprobe may be attributed to insufficiently high probe concentration (*See Table III*). Usually probe concentrations of 0.5 pg/µl are recommended for *in situ* hybridization with RNA probes (Amersham Life Sciences, 1994); the antisense riboprobe used in the detection of octopamine/tyramine receptor mRNA transcripts was ~2.4X more diluted than the *elav* antisense (positive) control probe *(See Table III)*.

Not withstanding these concerns, the results of the *in situ* analyses in the larvae tissues suggest multiple roles for this receptor in neuronal and non-neuronal tissues of the larvae. First, the direct localization of transcripts over the cytoplasm of cells in the salivary glands may suggest a role for this receptor in the functions of this gland in digestion. For example, binding of ligands to this receptor may induce a response leading to the secretion of digestive enzymes from secretory vesicles. Second, transcriptional activity in the imaginal disks may suggest potential roles for this receptor in cellular processes controlling

metamorphosis of the larvae into the adult fly. The imaginal disks are characteristic of insects undergoing metamorphosis. These are composed of a group of undifferentiated cells which are mitotically active. It is believed that in response to hormonal stimulation, these cells differentiate to give rise to specific adult structures such as the wings, legs and antenna in the fly (Russell, 1996). Binding of hormones to this receptor may mediate cellular responses regulating cell differentiation in the imaginal disks in the larvae. Third, detection of transcripts in the larval brain hemispheres may suggest roles for this receptor in processing of sensory information.

Transcriptional activity in the head of the male adult fly may suggest functions for this receptor in specific cell-types in the optic lobes and central brain. The location of silver grains over some structures of the optic lobes and retina (i.e. the lamina, the outer medulla, inner medulla, lobula, and lobula plate) may suggest regulatory functions in sensory perception, motion detection and the regulation of movement. The distribution of transcripts, particularly over the lobula and lobula plate are supportive of these functions. In larger flies (i.e. *Calliphora* and *Phaenicia*), for example, the lobula plate has been implicated with the detection of motion (FlyBrain Atlas, 1995); and the lobula and lobula plate are known to provide major sensory input into descending neurons supplying motor control centers in the thoracic-abdominal (FlyBrain Atlas, 1995). The

localization of transcripts in the central brain also suggest roles for this receptor in learning and memory processes. Neurons descending from the central brain, like in the lobula and lobula plate, carry sensory information to motor centers in the thoracic abdominal ganglia. The localization of silver grains in areas containing the cell bodies of nerve cells indicate potential roles for this receptor in signal transduction in the CNS.

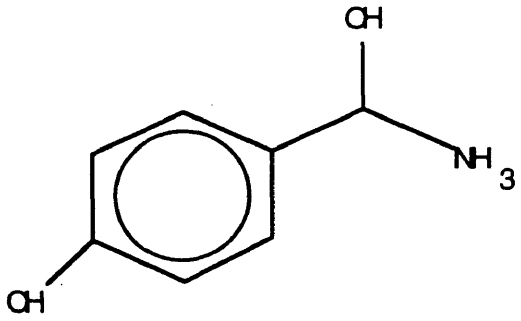
Multiple roles, as well as ligand types (i.e. neurohormones, neurotransmitters and neuromodulators) have been suggested for this receptor. The widespread, but non-uniform distribution of the mRNA transcripts for this putative octopamine/tyramine receptor, in a variety of tissues (i.e. glandular, nervous, and non-nervous tissues) may reflect its functional diversity. Based on its (*in vitro*) pharmacological profile, this receptor may bind to multiple ligands in different cellular locations or bind specifically to one multifaceted ligand, such as octopamine

Although the potential roles and ligands described here for this receptor are characteristic to that of octopaminergic systems in insects, results from these studies cannot determine with certainty if this putative octopamine/tyramine receptor is exclusively an octopamine receptor. The full classification of this receptor, as disscussed (Robb et al., 1994), must await the identification of the endogenous ligand at specific tissue sites. Nevertheless, the distribution profile presented and

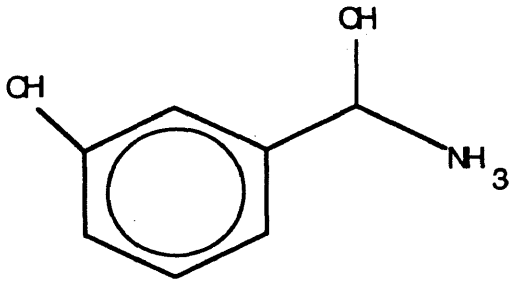
the roles described here have shed some light on the identity of this receptor. Additional studies defining the distribution of this octopamine/tyramine-receptor protein in *Drosophila* tissues would extend on this analysis and provide more accurate information as to expression levels of this receptor in tissues of the fly.

# Figure 1

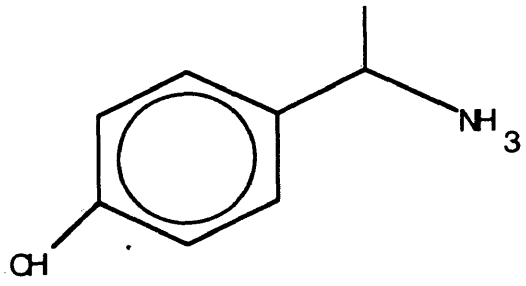
Molecular Structures of Octopamine and Tyramine



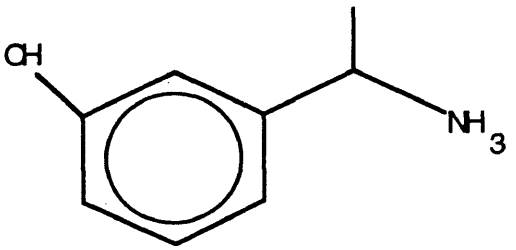

*para*-octopamine




*meta* -octopamine




*para*-tyramine

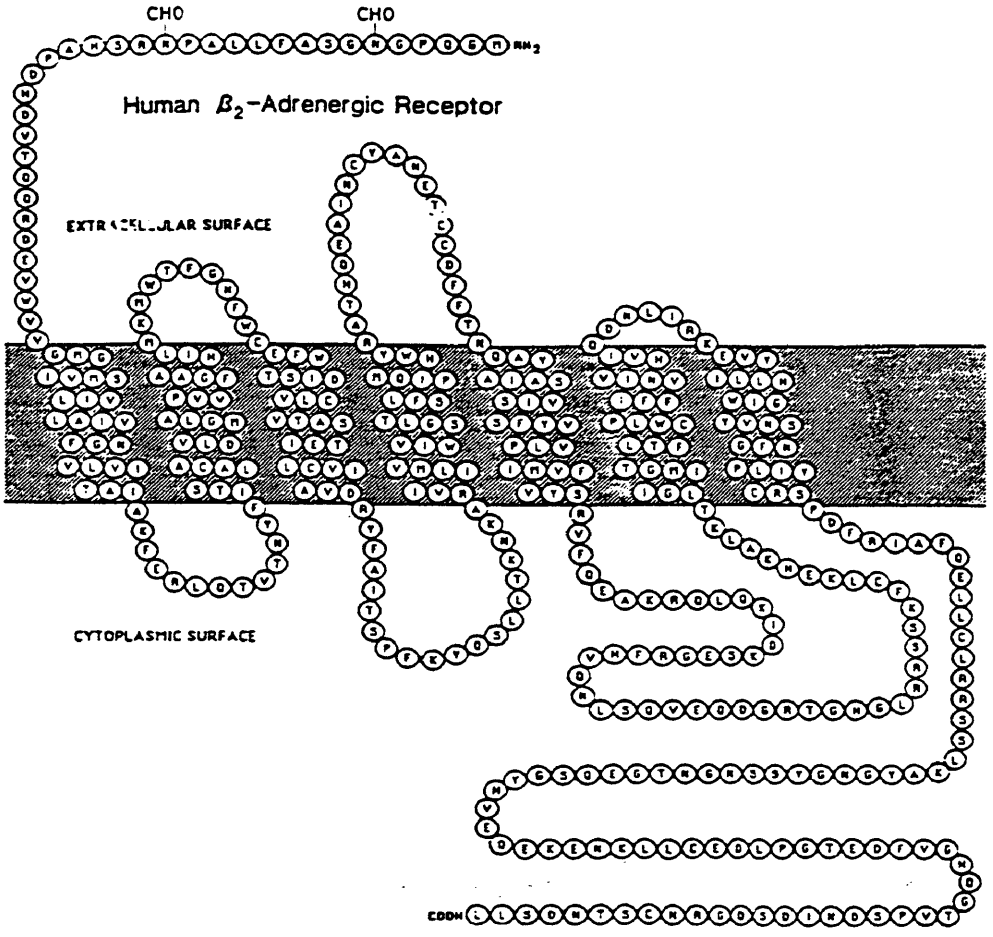



*meta*-tyramine

## Figure 2

Mammalian $\beta_2$-Adrenergic Receptor ($\beta_2$AR).

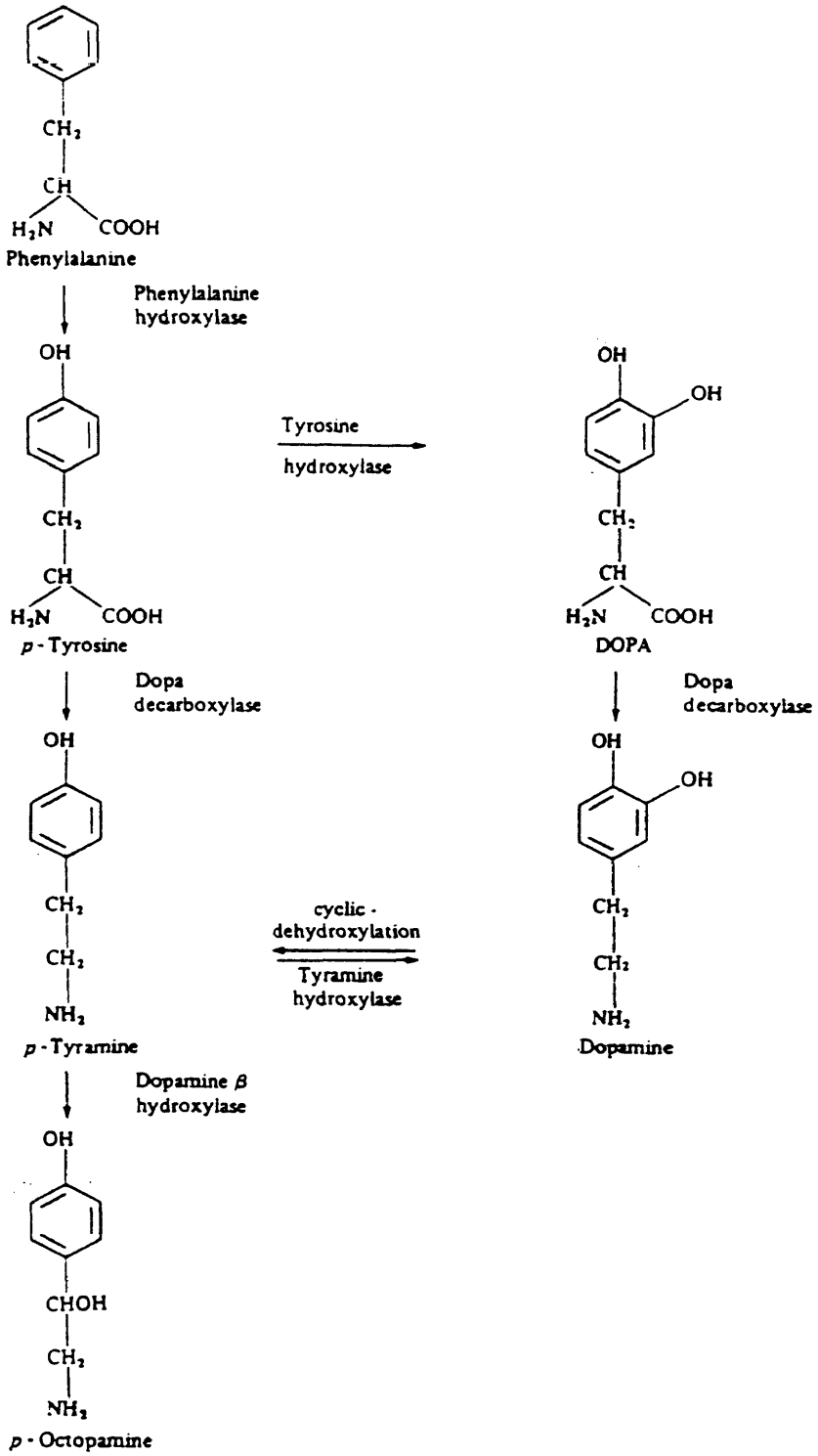
This receptor is composed by a polypeptide chain with seven hydrophobic regions, each spanning the membrane. These create intracellular and extracellular loops, as well as an extracellular amino-terminus and a cytoplasmic carboxyl terminal region. *(From Cooper et al., 1991)*


CHO
CHO
Human $\beta_2$-Adrenergic Receptor
EXTRACELLULAR SURFACE
CYTOPLASMIC SURFACE

# Figure 3

Biosynthesis of Octopamine from Tyrosine via Tyramine

*(From David and Coulon, 1985).*


Phenylalanine
Phenylalanine hydroxylase
p-Tyrosine
Tyrosine hydroxylase
DOPA
Dopa decarboxylase
p-Tyramine
cyclic-dehydroxylation
Tyramine hydroxylase
Dopamine
Dopamine β hydroxylase
p-Octopamine

# Figure 4

Map of the pBluescript II SK +/- Vector

*(From Stratagene)*

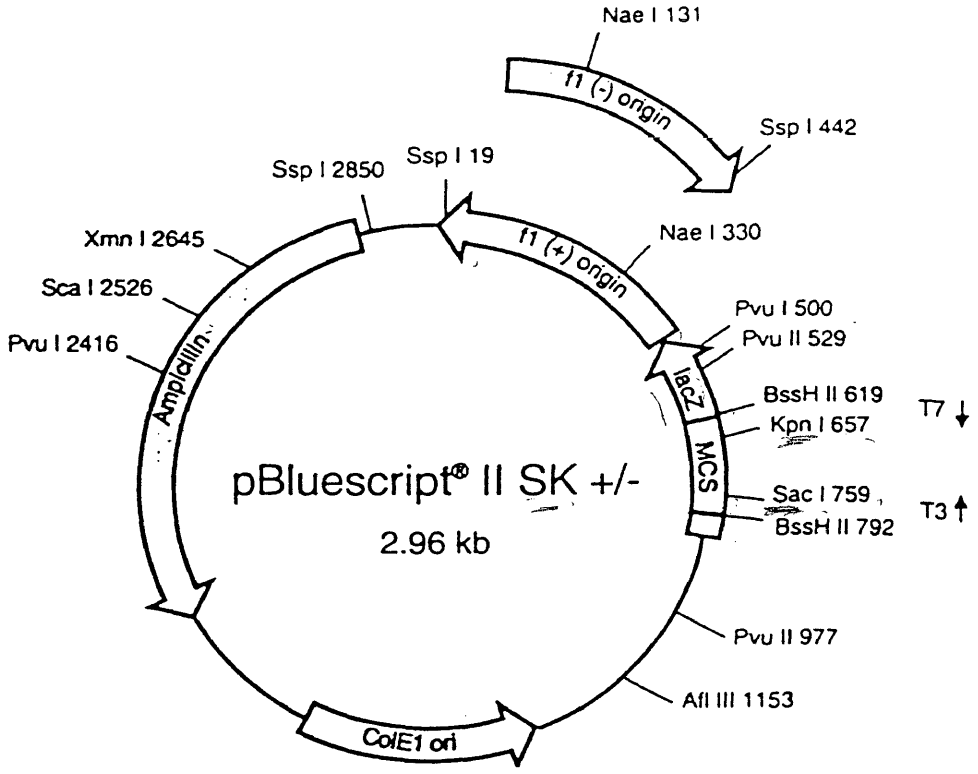
The pBluescript II SK +/- vector is a 2961 base pair phagemid derived from pUC19. The SK designation indicates the polylinker is oriented such that the *lac Z* transcription proceeds from Sac I to Kpn I.

***Lac Z:*** (lac promoter: 816-938 bp) This portion of the *lac Z* gene provides α-complementation for blue/white color selection of recombinant phagemids. An inducible lac promoter upstream from the *lac Z* gene permits fusion protein expression with the β-galactosidase gene product.

**MCS:** (657-759 bp) Multiple cloning site flanked by T3 and T7 promoters.

**Ampicillin:** (2832-2961 bp) Ampicillin resistance gene for antibiotic selection of the phagemid vector.

*GenBank # 52325 (SK+), 52324 (SK-).*


Nae I 131
f1 (-) origin
Ssp I 442
Ssp I 2850
Ssp I 19
Xmn I 2645
f1 (+) origin
Nae I 330
Sca I 2526
Pvu I 500
Pvu I 2416
Pvu II 529
Amp/ampR
lacZ
BssH II 619
T7
Kpn I 657
MCS
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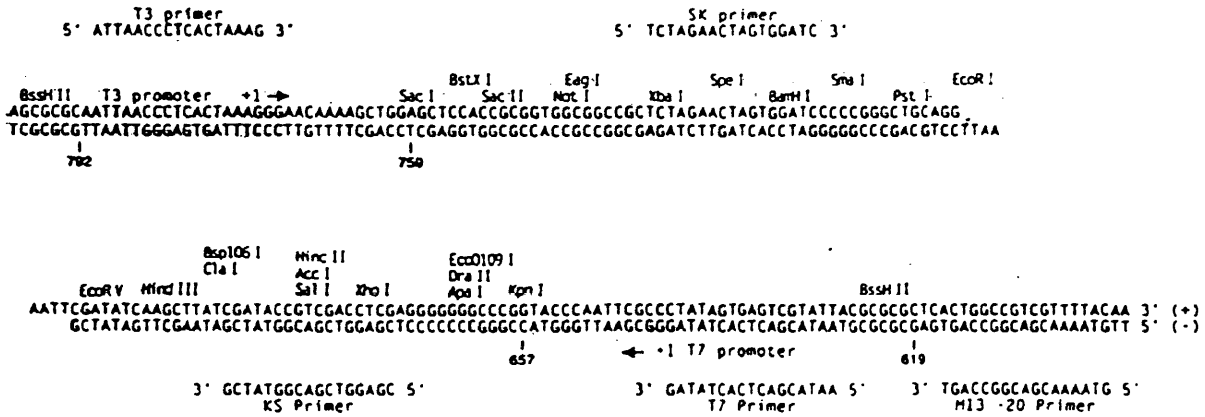
T3
BssH II 792
Pvu II 977
AfI III 1153
ColE1 ori


T3 primer
5' ATTAACCCTCACTAAAG 3'
SK primer
5' TCTAGAACTAGTGGATC 3'
BssHII T3 promoter +1
BstX I
Eag I
Spe I
Sma I
EcoR I
Sac I
Sac II
Not I
Xba I
BamH I
Pst I
AGCGCGCAATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGG
TCGCGCGTTAATTGGGAGTGATTTCCCTTGTTTTCGACCTCGAGGTGGCGCCACCGCCGGCGAGATCTTGATCACCTAGGGGGCCCGACGTCCTTAA
782
750
Bsp106 I
Cla I
Hinc II
Acc I
Sal I
Xho I
Eco0109 I
Dra II
Apa I
Kpn I
EcoR V
Hind III
BssH II
AATTCGATATCAAGCTTATCGATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAATTCGCCCTATAGTGAGTCGTATTACGCGCGCTCACTGGCCGTCGTTTTACAA 3' (+)
GCTATAGTTCGAATAGCTATGGCAGCTGGAGCTCCCCCCCGGGCCATGGGTTAAGCGGGATATCACTCAGCATAATGCGCGCGAGTGACCGGCAGCAAAATGTT 5' (-)
657
+1 T7 promoter
619
3' GCTATGGCAGCTGGAGC 5'
KS Primer
3' GATATCACTCAGCATAA 5'
T7 Primer
3' TGACCGGCAGCAAAATG 5'
M13 -20 Primer

# Figure 5

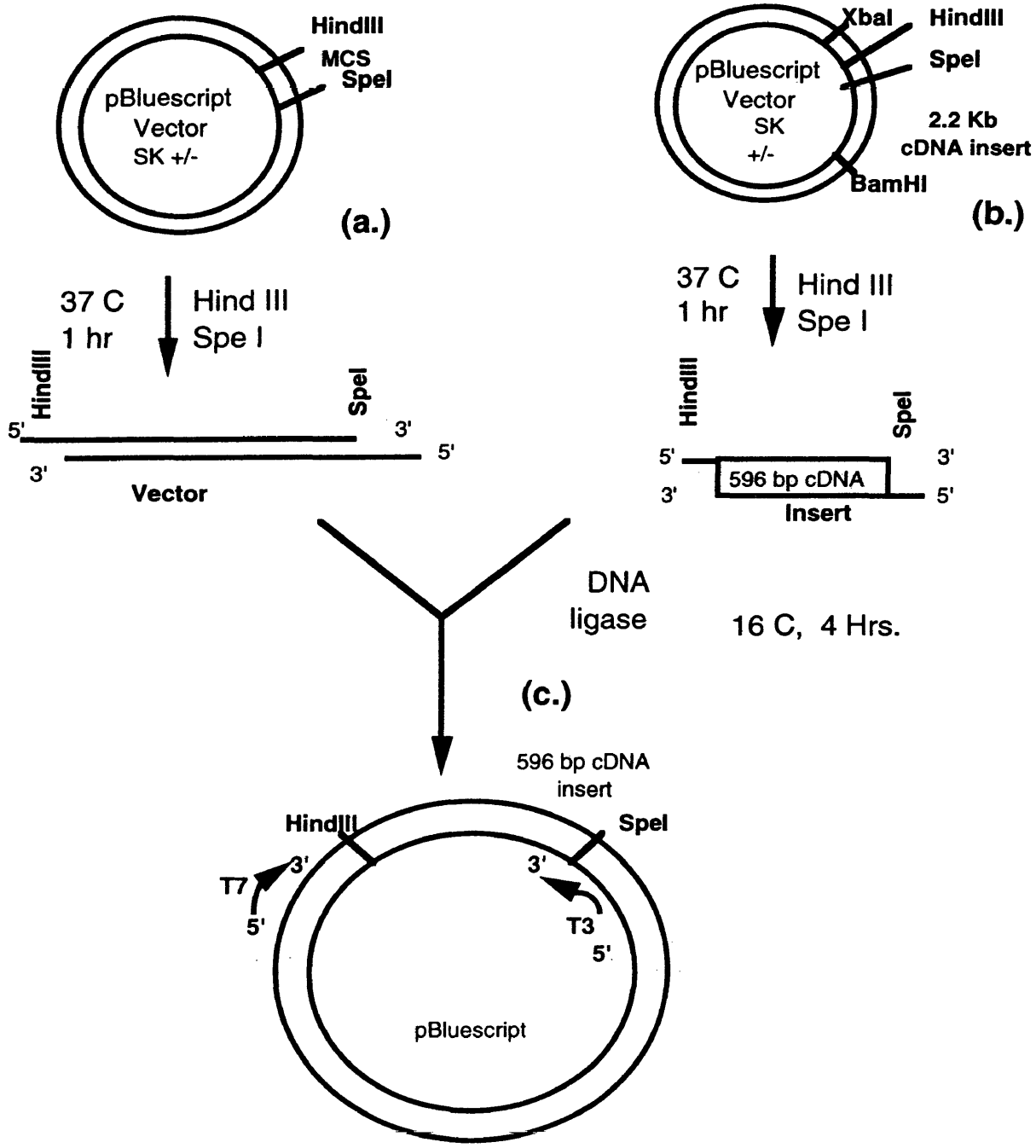
Subcloning of the 596 bp cDNA Fragment into pBluescript

a) Cleavage of vector with restriction enzymes HindIII and SpeI.

b) Release of the 596 bp cDNA fragment from the HindIII and SpeI sites of the 2.2 Kb *Drosophila* octopamine/tyramine-receptor cDNA, previously cloned into pBluescript.

c) Ligation of the vector and cDNA fragment with DNA ligase.

d) End product. Recombinant plasmids carrying the 596 bp cDNA fragment.


HindIII
MCS
SpeI
pBluescript
Vector
SK +/-
(a.)
XbaI
HindIII
SpeI
pBluescript
Vector
SK
+/-
2.2 Kb
cDNA insert
BamHI
(b.)
37 C
1 hr
Hind III
Spe I
HindIII
SpeI
5'
3'
3'
5'
Vector
37 C
1 hr
Hind III
Spe I
HindIII
SpeI
5'
3'
596 bp cDNA
3'
5'
Insert
DNA
ligase
16 C, 4 Hrs.
(c.)
596 bp cDNA
insert
HindIII
SpeI
T7
3'
5'
3'
T3
5'
pBluescript

# Figure 6

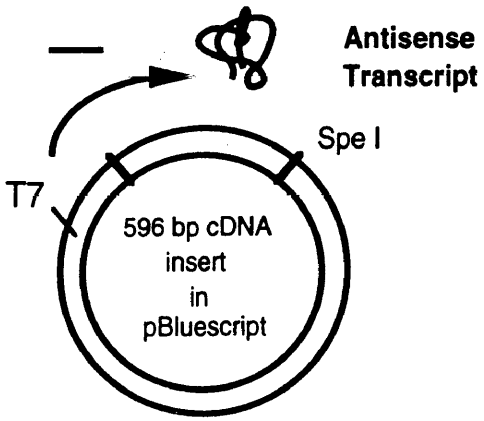
Transcription of the Riboprobes

a) An antisense RNA probe used in the detection of receptor mRNA was generated from a DNA template linearized with SpeI and synthesized with a T7 polymerase from the T7 promoter site of the vector.

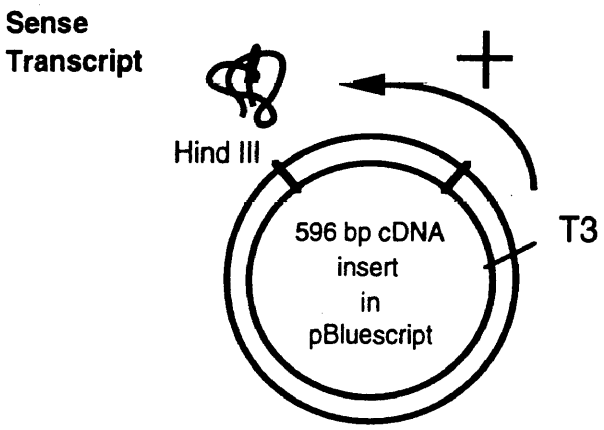
b) A sense RNA probe (negative control) was generated from a DNA template linearized with HindIII and synthesized with a T3 polymerase from the T3 promoter site of the vector.

c) A sense probe (negative control) for *elav* mRNA detection was generated from a DNA template linearized with NotI and synthesized with a Sp6 polymerase from the Sp6 promoter site of the vector.

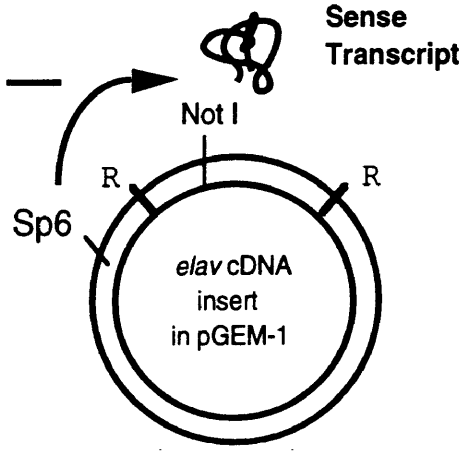
d) An antisense probe (positive control) for the detection of *elav* mRNA was generated from a DNA template linearized with NotI and synthesized with a T7 polymerase from the T7 promoter site of the vector.


—
Antisense
Transcript
Spe I
T7
596 bp cDNA
insert
in
pBluescript

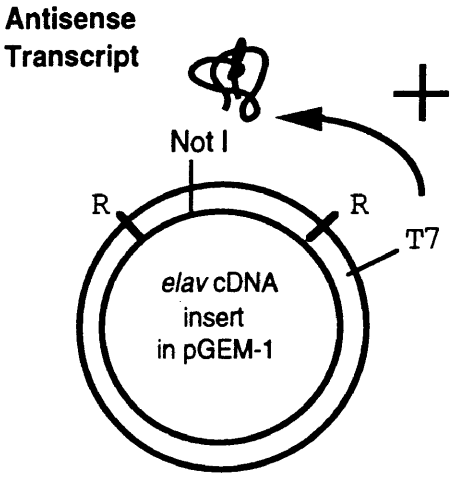
(a.)


Sense
Transcript
+
Hind III
T3
596 bp cDNA
insert
in
pBluescript

(b.)


—
Sense
Transcript
Not I
R
R
Sp6
elav cDNA
insert
in pGEM-1

(c.)


Antisense
Transcript
+
Not I
R
R
T7
elav cDNA
insert
in pGEM-1

(d.)

# Figure 7

Partial (T3-end) DNA Sequence of the 596 bp cDNA Fragment

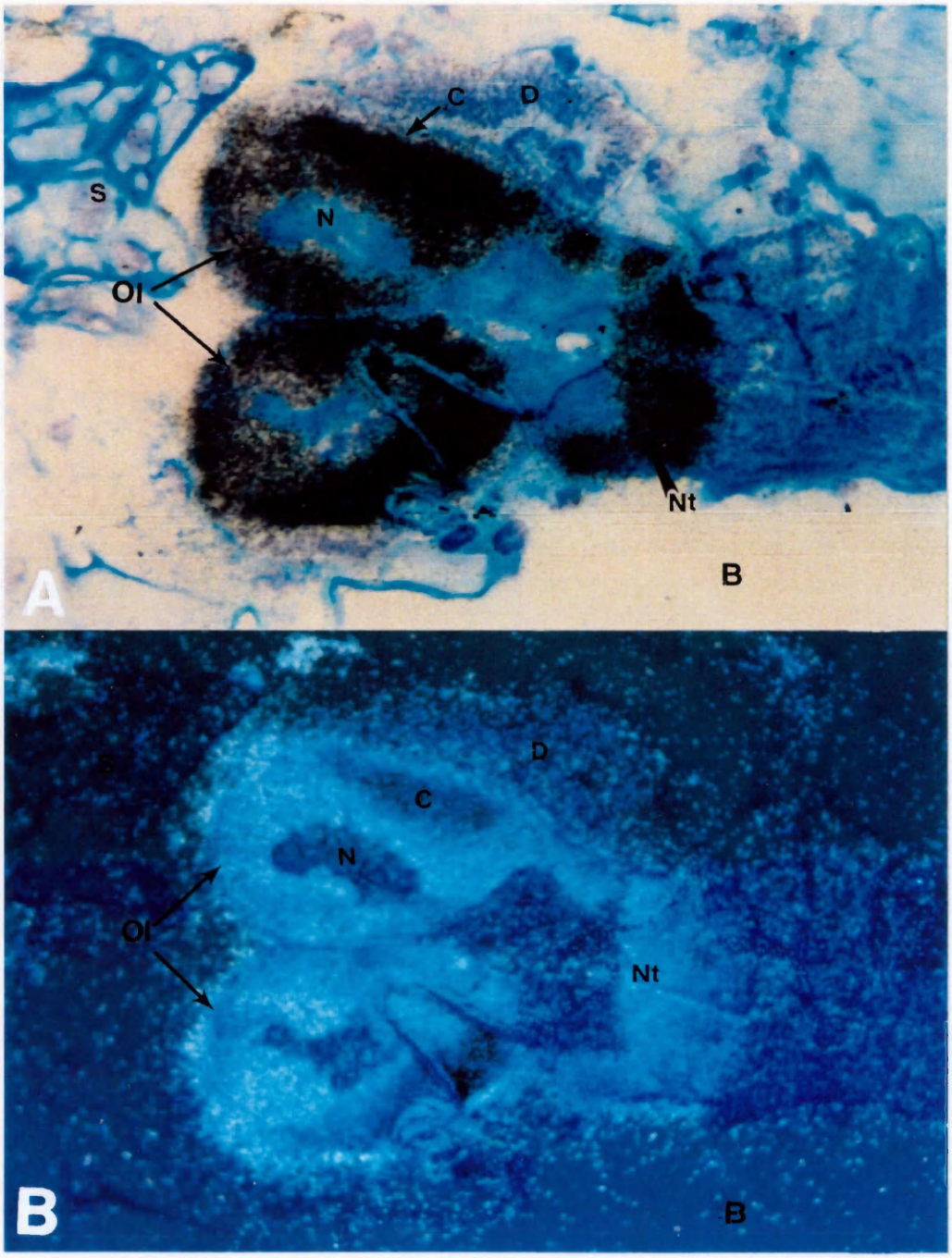
The base labeled as 1 corresponds to base 1838 of the published 3.3 Kb octopamine/tyramine receptor cDNA sequence (Arakawa et al., 1990). See APPENDIX II for the complete sequence.

```
  1   CTAGTCTTCT  TCTGCAACGG  AGTGACAGAA  ACGGCAGCGA  CTCCCGTCGA

 50   GGATTGCGGC  GGGGTCAGCT  TTAGGGACGT  TTGCTCGTCC  GCCTGAGTCA

100   CGCAGACCTG  TATGCAACCT  TTGGGGATCG  C
```

# Figure 8

Distribution of *elav* Transcripts in the CNS of the Third Instar Larvae

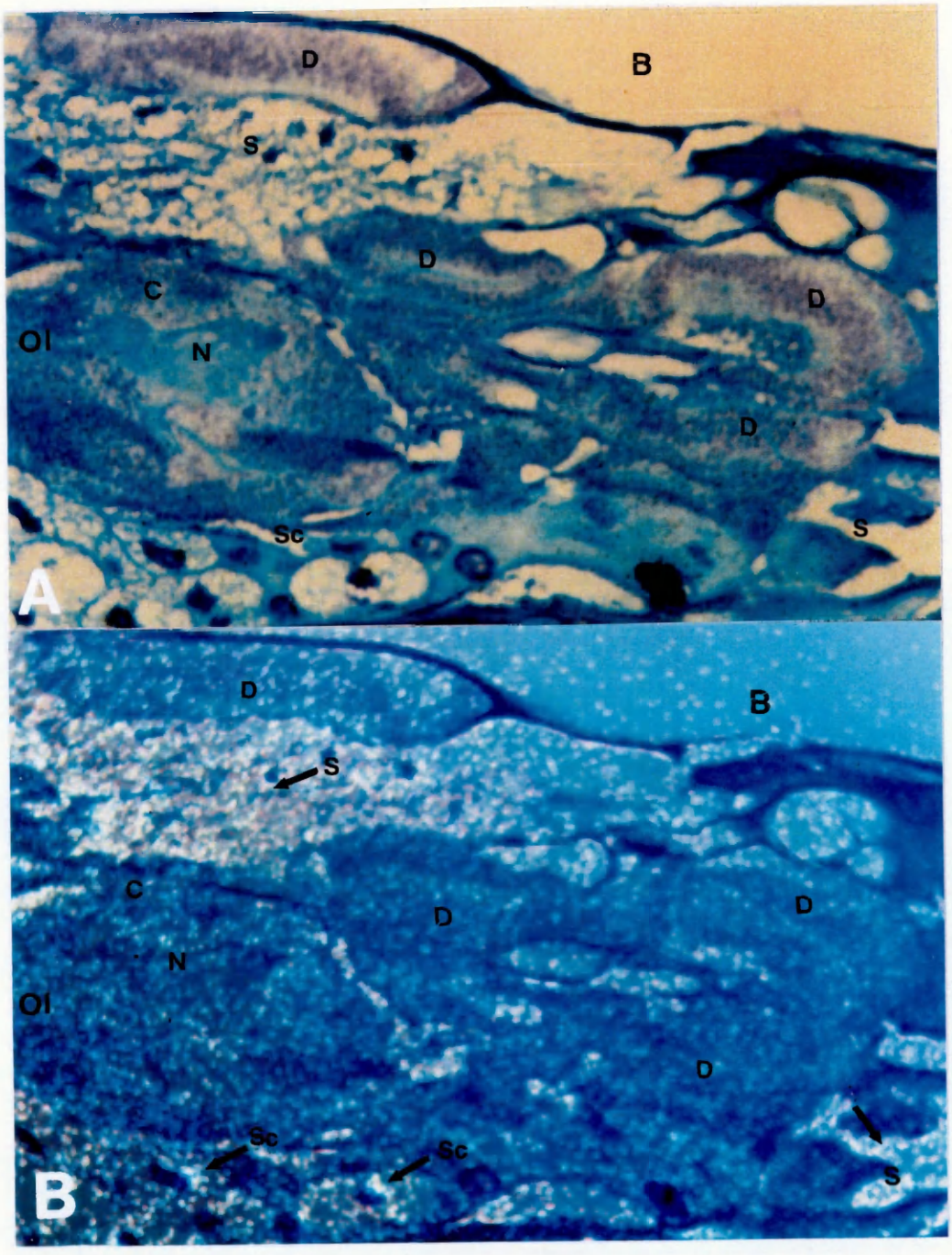
a) Brightfield and b) darkfield microscopy of a horizontal section of a third instar larvae. High levels of hybridization are seen over the areas of the cellular cortex of the brain hemispheres and the developing optic lobes (Ol). Slight signal is observed over the neuropil (N), imaginal disks (Data not shown) and salivary glands (Data not shown). No significant labeling was observed for the sense (negative control) *elav* probe (Data not shown). The background levels (B) were low. Exposure time: 72 Hr.; Magnification: 200X.


C
D
S
N
Ol
Nt
A
B
D
C
N
Ol
Nt
B
B

# Figure 9

Distribution of mRNA Transcripts of the *Drosophila* Octopamine/Tyramine Receptor in the Third Instar Larvae

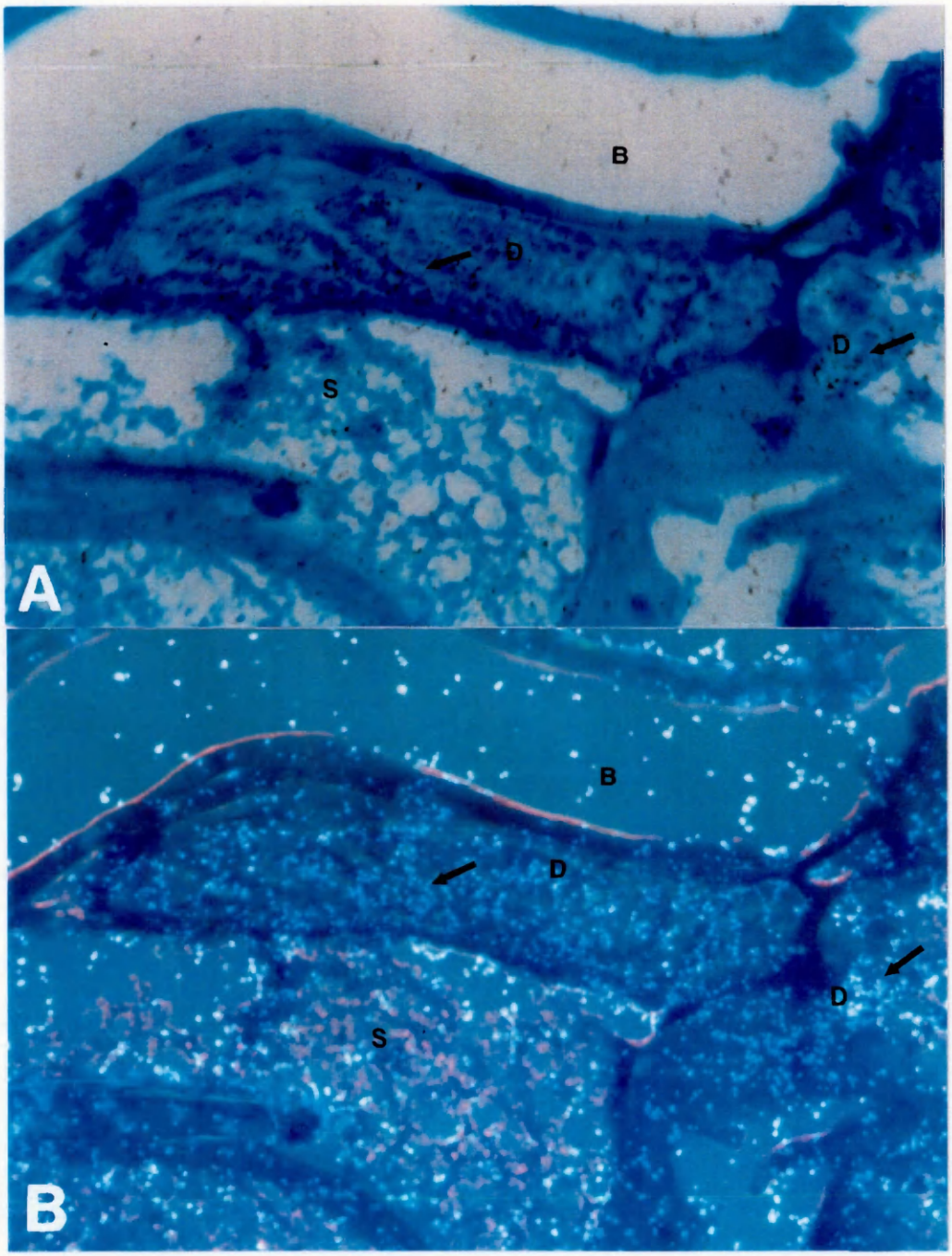
a) Brightfield and b) darkfield microscopy of a horizontal section through a third instar larvae. Low hybridization levels are seen with the antisense octopamine/tyramine receptor $^{35}S$-labeled riboprobe. Signal is predominant in the CNS cellular cortex. Some signal is observed over the areas of the brain hemispheres, optic lobes (Ol) and disks (D); with less signal over the neuropil (N). More intense hybridization signal is observed over the salivary glands (S, black arrow). Signal in the salivary glands appears to be localized directly over the cytoplasm of cells (Sc, black arrow). Background levels (B) are modest, and less than signal over these tissues. Exposure time: 240 Hr.; Magnification: 200X.


D
B
S
D
C
D
Ol
N
D
Sc
S
A
D
B
S
C
D
D
N
Ol
D
Sc
Sc
S
B

# Figure 10

Distribution of mRNA Transcripts of the *Drosophila* Octopamine/Tyramine Receptor in the Third Instar Larvae

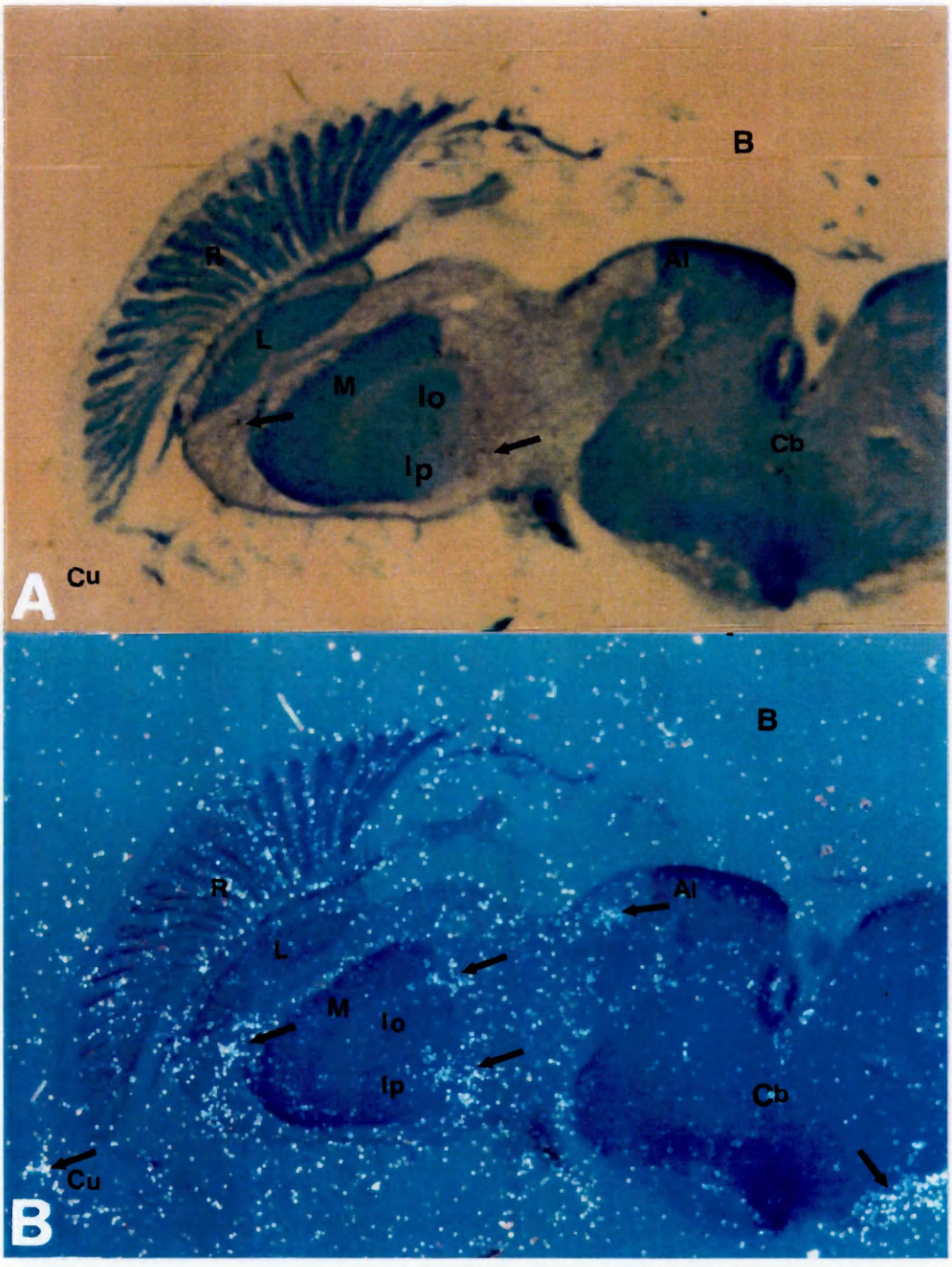
a) Brightfield and b) darkfield microscopy of a horizontal section of a third instar larvae. These photographs compare the intensity and distribution of hybridization signal over the imaginal disks (D) and salivary glands (S) with the background (B) signal for the $^{35}S$-labeled octopamine/tyramine receptor riboprobe. Exposure time: 240 Hr.; Magnification: 400X.


B
D
D
S
A
B
D
D
S
B

# Figure 11

Distribution of mRNA Transcripts for the *Drosophila* Octopamine/Tyramine Receptor in an Adult Male Head

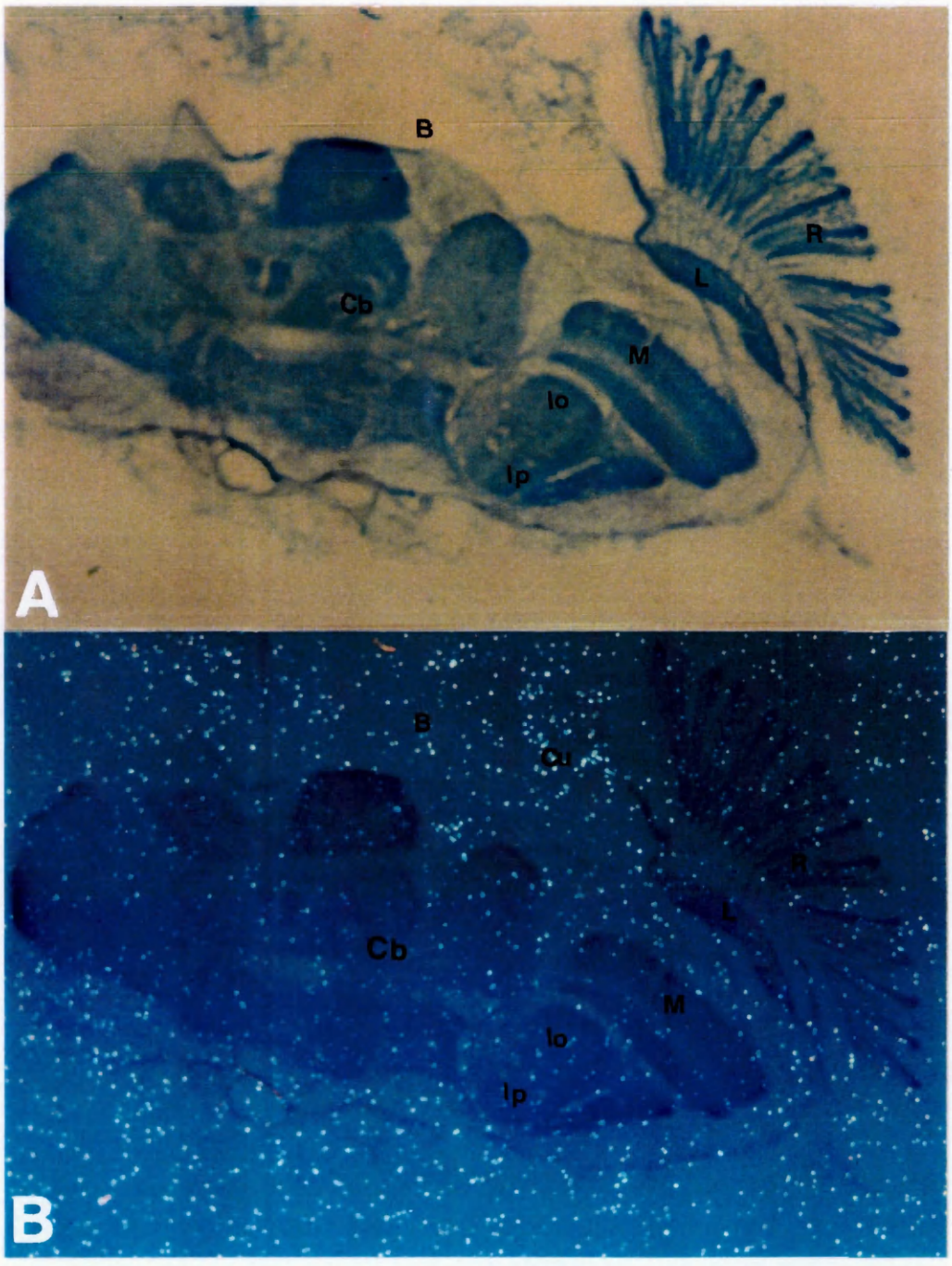
a) Brightfield and b) darkfield microscopy of a horizontal section through the head of an adult male probed with an $^{35}S$-labeled antisense octopamine/tyramine receptor riboprobe. Hybridization was widely, but unevenly distributed. Low levels of hybridization signal are seen in the central brain (Cb), the neuropil and subsets of the optic lobes (Ol) . A slightly more intense signal is observed over the cell body layers of these regions, especially in the antennal lobe (Al), lamina (L), medulla (M), lobula (lo) and lobula plate (lp) (Black arrows), as well as the retina (R). Nonspecific binding, based on comparison with sense-hydridized sections is seen over the cuticle (Cu). Exposure time: 72 Hr.; Magnification: 200X.


B
R
Al
L
M
lo
lp
Cb
A
Cu
B
R
Al
L
M
lo
lp
Cb
Cu
B

# Figure 12

*In situ* Hybridization with the *Drosophila* Octopamine/Tyramine $^{35}S$-Labeled Sense Riboprobe (Head of Adult Male Fly)

a) Bright field and b) dark field microscopy of a horizontal section through the head of an adult male probed with an $^{35}S$-labeled sense octopamine/tyramine receptor riboprobe. These sections did not display significant hybridization signal over the CNS structures, when compared to background (B) signal distribution. Nonspecific binding was seen over the adipose tissues (A). Exposure time: 110 Hr.; Magnification: 200X.


B
R
L
Cb
M
lo
lp
A
B
Cu
R
L
Cb
M
lo
lp
B

# TABLE I

Probe Length (Kb) and Alkaline Hydrolysis Incubation Time (Min.)

| Probe | $L_0$ (Kb) | $L_f$ (Kb) | t* |
|---|---|---|---|
| elav sense (T3) | 0.641 | 0.150 | 46 |
| elav antisense (T7) | 1.971 | 0.150 | 56 |
| 596 bp cDNA Sense (T3) | 0.596 | 0.150 | 45 |
| 596 bp cDNA Antisense (T7) | 0.596 | 0.150 | 45 |

*$t = (L_0 - L_f) / (0.11\ L_0 L_f)$; were t represents the incubation time in min., $L_0$ is the initial length of the transcript, and $L_f$ is the final length of the transcript desired. *(Formula from Amersham Life Sciences)*

# TABLE II

Probe Yields and Specific Activity*

| Probe | %Incorporation (UTP nmoles) | Total Incorporation (UTP nmoles) | CPM Incorporated | # of RNA Synthesized (ng) | Specific Activity (CPM/ng) |
|---|---|---|---|---|---|
| *Negative Controls* | | | | | |
| *elav* Sense(T3) | 86.22 | 0.0862 | $15.2 \times 10^7$ | 114 | $1.33 \times 10^9$ |
| 596 bp cDNA Sense (T3) | 60.90 | 0.0609 | $12.7 \times 10^7$ | 80.40 | $1.58 \times 10^9$ |
| *Positive Control* | | | | | |
| *elav* Antisense (T7) | 104 | ~0.100 | $11.2 \times 10^7$ | 132 | $0.85 \times 10^9$ |
| *Experimental* | | | | | |
| 596 bp cDNA Antisense (T7) | 76.50 | 0.0765 | $14.24 \times 10^7$ | 100.10 | $1.48 \times 10^9$ |

*The specific activity ($10^3$ mCi/mmoles) and concentration (20 mCi/ml) of the $^{35}$S-UTP label, total amount of $^{35}$S-UTP (0.1 nmoles) in the reaction, total of incorporated $^{35}$S-UTP (0.1 ng + % Incorporation) and # of RNA ng synthesized were used in the calculation of these values (See Formulas and Calculations in Appendix). Cold or unlabeled UTP was not used in the synthesis of the riboprobes.

# TABLE III

Probe Concentration and Animals

| Probe | 5X Stock Concentration (ng/ul) | Dilution Volume (uL) | Final Probe Concentration (pg/ul) | # of Slides | Stage and # of animals/slide | Volume of Probe Used Per Slide (ul) |
|---|---|---|---|---|---|---|
| *Negative Controls* | | | | | | |
| *elav* Sense(T3) | 1.50 | 154 | 974 | 4 | 1 slide: 4-5 bodies<br>3 slides: 8-10 larvae | 35 |
| 596 bp cDNA Sense (T3) | 1.22 | 330 | 370 | 12 | 4 slides: 4-5 heads each<br>5 slides: 4-5 bodies each<br>3 slides: 8-10 larvae | 25 |
| *Positive Control* | | | | | | |
| *elav* Antisense (T7) | 1.50 | 352 | 426 | 6 | 2 slides: 4-5 bodies<br>4 slides: 4-5 larvae per 2 slides | 35 |
| *Experimental* | | | | | | |

# APPENDIX I

## Formulas

*(From Ambion)*

1.) % UTP Incorporated into RNA =

$$\frac{\text{In-Counts (cpm)}}{\text{Un-Counts (cpm)} + \text{In-Counts (cpm)}} \times 100$$

Un-Counts = Unincorporated Counts (cpm)

In-Counts = Incorporated Counts (cpm)

2.) # Moles of UTP in the Reaction =

#mCi of UTP = UTP (ml) x Specific Activity (mCi/ml)

#mmoles of UTP = #mCi of UTP/UTP Concentration

3.) UTP Incorporated into the Reaction =

nmoles of UTP x % UTP Incorporated

4.) Nanograms of RNA Synthesized =

$1320 \times 10^9$ ng x UTP Incorporated (cpm)$/10^9$

5.) Specific Activity =

Total Incorporation (cpm) x 400/ng of RNA synthesized

# Appendix II

Sequence of the 596 bp cDNA fragment encoding the third intracellular loop of the octopamine/tyramine receptor cDNA.

*(From GenBank #52325)*

```
     CTGGGCTCCTTCTTTATTCCGCTGGCCATCATGACGATCGTCTACATCGAGATCTTCGTG
1141 ---------+---------+---------+---------+---------+---------+ 1200
     GACCCGAGGAAGAAATAAGGCGACCGGTAGTACTGCTAGCAGATGTAGCTCTAGAAGCAC

     GCCACGCGGCGCCGCCTAAGGGAGCGAGCCAGGGCCAACAAGCTTAACACGATCGCTCTG
1201 ---------+---------+---------+---------+---------+---------+ 1260
     CGGTGCGCCGCGGCGGATTCCCTCGCTCGGTCCCGGTTGTTCGAATTGTGCTAGCGAGAC

     AAGTCCACTGAGCTCGAGCCGATGGCAAACTCCTCGCCCGTCGCCGCCTCCAACTCCGGC
1261 ---------+---------+---------+---------+---------+---------+ 1320
     TTCAGGTGACTCGAGCTCGGCTACCGTTTGAGGAGCGGGCAGCGGCGGAGGTTGAGGCCG

     TCCAAGTCGCGTCTCCTAGCCAGCTGGCTTTGCTGCGGCCGGGATCGGGCCCAGTTCGCC
1321 ---------+---------+---------+---------+---------+---------+ 1380
     AGGTTCAGCGCAGAGGATCGGTCGACCGAAACGACGCCGGCCCTAGCCCGGGTCAAGCGG

     ACGCCTATGATCCAGAACGACCAGGAGAGCATCAGCAGTGAAACCCACCAGCCGCAGGAT
1381 ---------+---------+---------+---------+---------+---------+ 1440
     TGCGGATACTAGGTCTTGCTGGTCCTCTCGTAGTCGTCACTTTGGGTGGTCGGCGTCCTA
     TCCTCCAAAGCGGGTCCCCATGGCAACAGCGATCCCCAACAGCAGCACGTGGTCGTGCTG
1441 ---------+---------+---------+---------+---------+---------+ 1500
     AGGAGGTTTCGCCCAGGGGTACCGTTGTCGCTAGGGGTTGTCGTCGTGCACCAGCACGAC
```

```
      GTCAAGAAGTCGCGTCGCGCCAAGACCAAGGACTCCATTAAGCACGGCAAGACCCGTGGT

1501 ---------+---------+---------+---------+---------+---------+ 1560

      CAGTTCTTCAGCGCAGCGCGGTTCTGGTTCCTGAGGTAATTCGTGCCGTTCTGGGCACCA


      GGCCGCAAGTCGCAGTCCTCGTCCACATGCGAGCCCCACGGCGAGCAACAGCTCTTACCC

1561 ---------+---------+---------+---------+---------+---------+ 1620

      CCGGCGTTCAGCGTCAGGAGCAGGTGTACGCTCGGGGTGCCGCTCGTTGTCGAGAATGGG


      GCCGGCGGGGATGGCGGTAGCTGCCAGCCCGGCGGAGGCCACTCTGGAGGCGGAAAGTCG

1621 ---------+---------+---------+---------+---------+---------+ 1680

      CGGCCGCCCCTACCGCCATCGACGGTCGGGCCGCCTCCGGTGAGACCTCCGCCTTTCAGC


      GACGCCGAGATCAGCACGGAGAGCGGGAGCGATCCCAAAGGTTGCATACAGGTCTGCGTG

1681 ---------+---------+---------+---------+---------+---------+ 1740

      CTGCGGCTCTAGTCGTGCCTCTCGCCCTCGCTAGGGTTTCCAACGTATGTCCAGACGCAC


      ACTCAGGCGGACGAGCAAACGTCCCTAAAGCTGACCCCGCCGCAATCCTCGACGGGAGTC

1741 ---------+---------+---------+---------+---------+---------+ 1800

      TGAGTCCGCCTGCTCGTTTGCAGGGATTTCGACTGGGGCGGCGTTAGGAGCTGCCCTCAG


    GCTGCCGTTTCTGTCACTCCGTTGCAGAAGAAGACTAGTGGGGTTAACCAGTTCATTGAG

1801 ---------+---------+---------+---------+---------+---------+ 1860

    CGACGGCAAAGACAGTGAGGCAACGTCTTCTTCTGATCACCCCAATTGGTCAAGTAACTC
```

# Appendix III

Sequence of the *elav* cDNA probe fragment

(GenBank #M21153)

```
      GAAGGGACGCAAGAGCAGCTACAGCGCGCAGCAGTGCCAGAAAGTCCTGCGCTCGCCCCA
6000  +---------+---------+---------+---------+---------+--------- 6059
      CTTCCCTGCGTTCTCGTCGATGTCGCGCGTCGTCACGGTCTTTCAGGACGCGAGCGGGGT

      GAAGCATCCTGTTCCTGTGACGCAGCGGATCTGGTCTCGCGTTCGCTCCACTACCTCCAC
6060  +---------+---------+---------+---------+---------+--------- 6119
      CTTCGTAGGACAAGGACACTGCGTCGCCTAGACCAGAGCGCAAGCGAGGTGATGGAGGTG

      TTCCTACACCTACAGTACACATATCACATGAACTTAAAACTAGTGAACGGATTAATTAAA
6120  +---------+---------+---------+---------+---------+--------- 6179
      AAGGATGTGGATGTCATGTGTATAGTGTACTTGAATTTTGATCACTTGCCTAATTAATTT

      AAGGAATCGCAAAACAAAAGCTCGCTAATCGCGCAGACACAGCAGAAGCTTAAATTCAGA
6180  +---------+---------+---------+---------+---------+--------- 6239
      TTCCTTAGCGTTTTGTTTTCGAGCGATTAGCGCGTCTGTGTCGTCTTCGAATTTAAGTCT

      AAAGTTTAGTTCTTCAGTGCCGAAGGTGTGTTTGCAGAGCTTTGATTTCTAGTGCGCGGG
6240  +---------+---------+---------+---------+---------+--------- 6299
      TTTCAAATCAAGAAGTCACGGCTTCCACACAAACGTCTCGAAACTAAAGATCACGCGCCC

      TGTGGGAAGAGAGGACTAAATAGAGACAGAGAGAGCGGCCGGATGAGAAAGTGGGGCGCT
6300  +---------+---------+---------+---------+---------+--------- 6359
      ACACCCTTCTCTCCTGATTTATCTCTGTCTCTCTCGCCGGCCTACTCTTTCACCCCGCGA

      GGGCGGCGCCTGTTAGCAAAGCTTCGTTGACCAACCGTTATTTCCTGAACAAAGGAATAA
6360  +---------+---------+---------+---------+---------+--------- 6419
      CCCGCCGCGGACAATCGTTTCGAAGCAACTGGTTGGCAATAAAGGACTTGTTTCCTTATT

      CATACATTCATCACTTAATCACCAACTGTTTTTAAATCCACAGAATTATAATTTAGCGTA
6420  +---------+---------+---------+---------+---------+--------- 6479
      GTATGTAAGTAGTGAATTAGTGGTTGACAAAAATTTAGGTGTCTTAATATTAAATCGCAT

      TGATTCAAGATTTGGGAATTGTGCGCACCCAGGATAACCGCGTTTGTGTTTAATTTACAA
6480  +---------+---------+---------+---------+---------+--------- 6539
      ACTAAGTTCTAAACCCTTAACACGCGTGGGTCCTATTGGCGCAAACACAAATTAAATGTT

      ACATGCCCCTAATGTCTATTAAATAAAAGTTCAGCGTCGGAAAATTAAAGAGTCTGGTAA
6540  +---------+---------+---------+---------+---------+--------- 6599
      TGTACGGGGATTACAGATAATTTATTTTCAAGTCGCAGCCTTTTAATTTCTCAGACCATT

      GGATTACAGTATATTTATGCTGTAATATCCTTTAATTGTCCCGGGTCACATTTTTCCGTA
6600  +---------+---------+---------+---------+---------+--------- 6659
      CCTAATGTCATATAAATACGACATTATAGGAAATTAACAGGGCCCAGTGTAAAAAGGCAT

      TCCCCACGCCCTAAAGCATTTTCGTATCCATTAATTAAACAAGTAAAGGTGTAGGCTTAT
6660  +---------+---------+---------+---------+---------+--------- 6719
      AGGGGTGCGGGATTTCGTAAAAGCATAGGTAATTAATTTGTTCATTTCCACATCCGAATA

      ATTATGTTATACAGTCCGCTCTCACAAGCATTATAAGAAATATATTACTTGTCTGTTCAC
6720  +---------+---------+---------+---------+---------+--------- 6779
      TAATACAATATGTCAGGCGAGAGTGTTCGTAATATTCTTTATATAATGAACAGACAAGTG
```

```
     CAAATAGGTAAAAAATATACATATCATATCAAACGATTTAAATAATTGCGCCCTGGGCAG
6780 +---------+---------+---------+---------+---------+--------- 6839
     GTTTATCCATTTTTTATATGTATAGTATAGTTTGCTAAATTTATTAACGCGGGACCCGTC

     ATGTAAATAAAGAAATATAAATTAAAAAATATCGTTAGAATAAACCAACAAATTTTCTAT
6840 +---------+---------+---------+---------+---------+--------- 6899
     TACATTTATTTCTTTATATTTAATTTTTTATAGCAATCTTATTTGGTTGTTTAAAAGATA

     TCAAACCCTAGCTGATTGATAATCAATTAGCCCGTTATAACTACTAAAACCCATAATAAT
6900 +---------+---------+---------+---------+---------+--------- 6959
     AGTTTGGGATCGACTAACTATTAGTTAATCGGGCAATATTGATGATTTTGGGTATTATTA

     ACAAAAAACTACTAACACATCATGCAAATGTTTTAATTTTGACATTTAACTATTCTTTCC
6960 +---------+---------+---------+---------+---------+--------- 7019
     TGTTTTTTGATGATTGTGTAGTACGTTTACAAAATTAAAACTGTAAATTGATAAGAAAGG

     ATGCGCCTACCCCTGCGTTGGTCGTGACAACCAACAATAAACTTCTCGACTCTGCCCGAT
7020 +---------+---------+---------+---------+---------+--------- 7079
     TACGCGGATGGGGACGCAACCAGCACTGTTGGTTGTTATTTGAAGAGCTGAGACGGGCTA

     CCTCTCACCTATCACTCAGTGGACTTTATTATGGCAAATACCGGAGCTGGCGGCGGAGTA
7080 +---------+---------+---------+---------+---------+--------- 7139
     GGAGAGTGGATAGTGAGTCACCTGAAATAATACCGTTTATGGCCTCGACCGCCGCCTCAT

     GACACACAGGCACAGCTAATGCAGAGTGCCGCTGCAGCCGCAGCAGTGGCGGCAACAAAC
7140 +---------+---------+---------+---------+---------+--------- 7199
     CTGTGTGTCCGTGTCGATTACGTCTCACGGCGACGTCGGCGTCGTCACCGCCGTTGTTTG

      N                                   N
      o                                   o
      t                                   t
      I                                   I
     GCGGCCGCCGCTCCCGTACAGAATGCAGCCGCCGTGGCGGCCGCCGCCCAGCTGCAGCAG
7200 +---------+---------+---------+---------+---------+--------- 7259
     CGCCGGCGGCGAGGGCATGTCTTACGTCGGCGGCACCGCCGGCGGCGGGTCGACGTCGTC

     CAACAGGTGCAACAGGCAATCCTGCAGGTGCAGCAGCAGCAGACACAGCAAGCGGTGGCC
7260 +---------+---------+---------+---------+---------+--------- 7319
     GTTGTCCACGTTGTCCGTTAGGACGTCCACGTCGTCGTCGTCTGTGTCGTTCGCCACCGG

      N
      o
      t
      I
     GCGGCCGCTGCCGCAGTGACCCAGCAGCTCCAACAGCAACAGCAGGCTGTCGTGGCCCAA
7320 +---------+---------+---------+---------+---------+--------- 7379
     CGCCGGCGACGGCGTCACTGGGTCGTCGAGGTTGTCGTTGTCGTCCGACAGCACCGGGTT

     CAGGCTGTAGTGCAGCAGCAACAACAGCAGGCGGCGGCAGTGGTGCAACAGGCGGCGGTC
7380 +---------+---------+---------+---------+---------+--------- 7439
     GTCCGACATCACGTCGTCGTTGTTGTCGTCCGCCGCCGTCACCACGTTGTCCGCCGCCAG

     CAACAGGCTGTGGTGCCCCAGCCGCAGCAGGCGCAGCCCAATACGAATGGCAATGCAGGA
7440 +---------+---------+---------+---------+---------+--------- 7499
     GTTGTCCGACACCACGGGGTCGGCGTCGTCCGCGTCGGGTTATGCTTACCGTTACGTCCT
```

```
    TCGGGATCGCAAAATGGCAGCAACGGCAGCACGGAGACGCGCACAAACCTTATTGTCAAC
7500 +---------+---------+---------+---------+---------+--------- 7559
    AGCCCTAGCGTTTTACCGTCGTTGCCGTCGTGCCTCTGCGCGTGTTTGGAATAACAGTTG

    TACTTGCCGCAAACAATGACCGAAGACGAGATCCGTTCGCTCTTCTCCAGCGTCGGCGAG
7560 +---------+---------+---------+---------+---------+--------- 7619
    ATGAACGGCGTTTGTTACTGGCTTCTGCTCTAGGCAAGCGAGAAGAGGTCGCAGCCGCTC

    ATTGAGTCGGTGAAGCTGATACGCGACAAGTCGCAGGTCTACATCGATCCTCTCAATCCG
7620 +---------+---------+---------+---------+---------+--------- 7679
    TAACTCAGCCACTTCGACTATGCGCTGTTCAGCGTCCAGATGTAGCTAGGAGAGTTAGGC

    CAGGCGCCCAGCAAGGGCCAAAGTCTGGGCTACGGCTTTGTTAACTATGTCCGGCCGCAA
7680 +---------+---------+---------+---------+---------+--------- 7739
    GTCCGCGGGTCGTTCCCGGTTTCAGACCCGATGCCGAAACAATTGATACAGGCCGGCGTT

    GATGCCGAGCAGGCTGTTAATGTTCTAAACGGCCTGCGACTGCAGAACAAAACCATAAAG
7740 +---------+---------+---------+---------+---------+--------- 7799
    CTACGGCTCGTCCGACAATTACAAGATTTGCCGGACGCTGACGTCTTGTTTTGGTATTTC

    GTGTCGTTTGCCCGCCCGTCGTCCGATGCCATTAAAGGCGCCAACCTTTATGTGTCGGGG
7800 +---------+---------+---------+---------+---------+--------- 7859
    CACAGCAAACGGGCGGGCAGCAGGCTACGGTAATTTCCGCGGTTGGAAATACACAGCCCC

    CTGCCAAAGACGATGACCCAGCAGGAACTGGAGGCCATCTTCGCACCATTCGGAGCAATA
7860 +---------+---------+---------+---------+---------+--------- 7919
    GACGGTTTCTGCTACTGGGTCGTCCTTGACCTCCGGTAGAAGCGTGGTAAGCCTCGTTAT

    ATCACATCGCGCATTCTGCAGAACGCTGGCAACGATACGCAGACGAAAGGAGTAGGCTTT
7920 +---------+---------+---------+---------+---------+--------- 7979
    TAGTGTAGCGCGTAAGACGTCTTGCGACCGTTGCTATGCGTCTGCTTTCCTCATCCGAAA

    ATTCGATTCGATAAGCGGGAGGAGGCCACAAGGGCTATCATTGCCTTGAACGGTACTACA
7980 +---------+---------+---------+---------+---------+--------- 8039
    TAAGCTAAGCTATTCGCCCTCCTCCGGTGTTCCCGATAGTAACGGAACTTGCCATGATGT

    CCATCCAGCTGCACAGACCCCATAGTGGTAAAATTCTCCAATACGCCCGGCAGCACCAGT
8040 +---------+---------+---------+---------+---------+--------- 8099
        GGTAGGTCGACGTGTCTGGGGTATCACCATTTTAAGAGGTTATGCGGGCCGTCGTGGTCA

    AAGATCATCCAGCCGCAGCTGCCCGCTTTCCTCAATCCCCAACTGGTGCGACGCATCGGG
8100 +---------+---------+---------+---------+---------+--------- 8159
    TTCTAGTAGGTCGGCGTCGACGGGCGAAAGGAGTTAGGGGTTGACCACGCTGCGTAGCCC

    GGCGCCATGCACACGCCAGTTAACAAGGGTCTGGCCCGGTTTTCACCAATGGCGGGCGAC
8160 +---------+---------+---------+---------+---------+--------- 8219
    CCGCGGTACGTGTGCGGTCAATTGTTCCCAGACCGGGCCAAAAGTGGTTACCGCCCGCTG

    ATGCTGGACGTAATGCTGCCCAATGGACTAGGAGCGGCGGCGGCGGCGGCCACAACGCTG
8220 +---------+---------+---------+---------+---------+--------- 8279
    TACGACCTGCATTACGACGGGTTACCTGATCCTCGCCGCCGCCGCCGCCGGTGTTGCGAC

    GCCAGTGGGCCTGGCGGCGCGTATCCCATTTTCATCTACAACTTGGCGCCGGAAACGGAG
8280 +---------+---------+---------+---------+---------+--------- 8339
    CGGTCACCCGGACCGCCGCGCATAGGGTAAAAGTAGATGTTGAACCGCGGCCTTTGCCTC
```

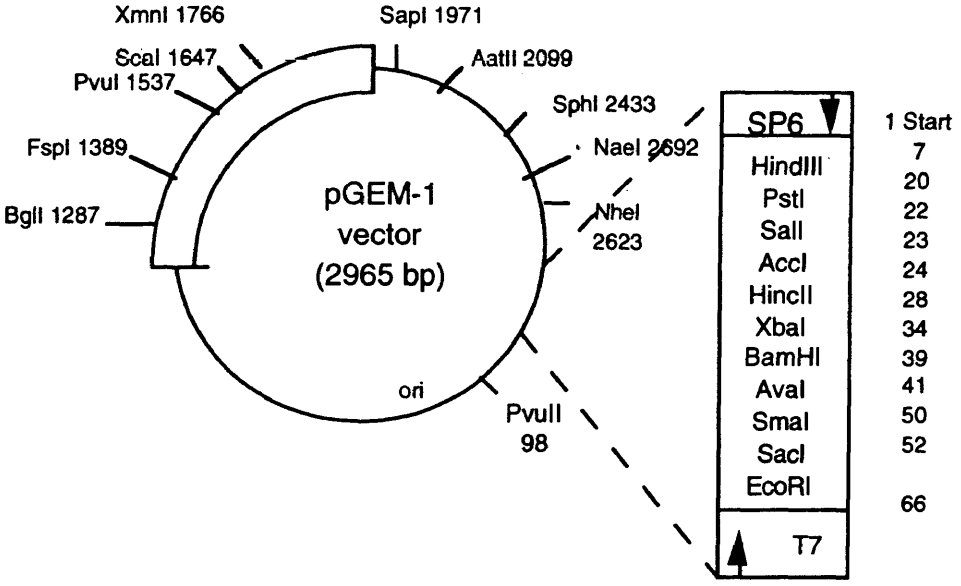
```
    GAGGCTGCCCTGTGGCAGCTGTTTGGTCCATTTGGAGCTGTGCAATCGGTAAAGATTGTC
8340 +---------+---------+---------+---------+---------+---------+---------- 8399
    CTCCGACGGGACACCGTCGACAAACCAGGTAAACCTCGACACGTTAGCCATTTCTAACAG

    AAGGATCCCACAACGAATCAGTGCAAGGGCTACGGCTTCGTTTCGATGACCAACTACGAT
8400 +---------+---------+---------+---------+---------+---------+---------- 8459
    TTCCTAGGGTGTTGCTTAGTCACGTTCCCGATGCCGAAGCAAAGCTACTGGTTGATGCTA

    GAGGCGGCCATGGCCATTCGGGCGCTCAACGGCTACACCATGGGCAATCGGGTGCTGCAG
8460 +---------+---------+---------+---------+---------+---------+---------- 8519
    CTCCGCCGGTACCGGTAAGCCCGCGAGTTGCCGATGTGGTACCCGTTAGCCCACGACGTC

    GTCAGCTTCAAGACCAACAAAGCCAAGTAGAGCGGCCCAAATGGAAGTGGACAAGGACGC
8520 +---------+---------+---------+---------+---------+---------+---------- 8579
    CAGTCGAAGTTCTGGTTGTTTCGGTTCATCTCGCCGGGTTTACCTTCACCTGTTCCTGCG

    AGCGGGAGCACCAGCAACCACAACCCATTATCTTGAATCTAAACCTGACCACACAACACA
8580 +---------+---------+---------+---------+---------+---------+---------- 8639
    TCGCCCTCGTGGTCGTTGGTGTTGGGTAATAGAACTTAGATTTGGACTGGTGTGTTGTGT

    CATATATAAATATGCATGGTATAACGGTAACTAAGCGCAACAAAACAGTATGTGTACAAC
8640 +---------+---------+---------+---------+---------+---------+---------- 8699
    GTATATATTTATACGTACCATATTGCCATTGATTCGCGTTGTTTTGTCATACACATGTTG

    ACACAAGAAAAAAACAAGAAAGTAGCAGGCAGGAGAAAATACTCTTTCCACTAAACGACA
8700 +---------+---------+---------+---------+---------+---------+---------- 8759
    TGTGTTCTTTTTTTGTTCTTTCATCGTCCGTCCTCTTTTATGAGAAAGGTGATTTGCTGT

    ACGAAACGGCGGAAATCCCCCTAAAAACACAAATCTCCTCCCCAAAACCGCCGGCCAAAA
8760 +---------+---------+---------+---------+---------+---------+---------- 8819
    TGCTTTGCCGCCTTTAGGGGGATTTTTGTGTTTAGAGGAGGGGTTTTGGCGGCCGGTTTT

    GCGTTGCAATAGAAAAATTCTTCTTGTTTAGCATTTAAGTAAAAAACCTTAACTAAACGA
8820 +---------+---------+---------+---------+---------+---------+---------- 8879
    CGCAACGTTATCTTTTTAAGAAGAACAAATCGTAAATTCATTTTTTGGAATTGATTTGCT

    AGCGAGCAGAAGAAGTTATAAGATCGGGAGAAGCGAATAGGTAGGGGGTTGAGAAAGAAC
8880 +---------+---------+---------+---------+---------+---------+---------- 8939
    TCGCTCGTCTTCTTCAATATTCTAGCCCTCTTCGCTTATCCATCCCCCAACTCTTTCTTG

    AATGAGATTTTAAGAAATAATATTGCAAAATGAAAACACACTGTGGCAGTGGCAGCTGTG
8940 +---------+---------+---------+---------+---------+---------+---------- 8999
    TTACTCTAAAATTCTTTATTATAACGTTTTACTTTTGTGTGACACCGTCACCGTCGACAC

    AAAGGTCAAAGGTTGGCACAGTCGATCTAGTCACAAAGCAAAACAAAGAAATCAACCAAA
9000 +---------+---------+---------+---------+---------+---------+---------- 9059
    TTTCCAGTTTCCAACCGTGTCAGCTAGATCAGTGTTTCGTTTTGTTTCTTTAGTTGGTTT

    CAAACACAAAACACACAAAAACGAAAACAAATTTAACTTTATTAGCAAAAAAGAAAATGA
9060 +---------+---------+---------+---------+---------+---------+---------- 9119
    GTTTGTGTTTTGTGTGTTTTTGCTTTTGTTTAAATTGAAATAATCGTTTTTTCTTTTACT

    GAAATTTTAAAAATTTATTAACTTTAAGTAAAACGCAGCAATGAAATCTTCCAAACAAAA
9120 +---------+---------+---------+---------+---------+---------+---------- 9179
    CTTTAAAATTTTTAAATAATTGAAATTCATTTTGCGTCGTTACTTTAGAAGGTTTGTTTT
```

```
    GAAAGCAAAACAGCAAATCTGTTTGTTACACTTCAACTTTTTCTACCATAAAAACTTTGA
9180 +---------+---------+---------+---------+---------+--------- 9239
    CTTTCGTTTTGTCGTTTAGACAAACAATGTGAAGTTGAAAAAGATGGTATTTTTGAAACT

                                            E
                                            c
                                            o
                                            R
                                            I
    ATCTTGATACTAAACCTAAAGCAGAATTAAGAAAATAGAGGAATTC
9240 +---------+---------+---------+---------+----- 9285
    TAGAACTATGATTTGGATTTCGTCTTAATTCTTTTATCTCCTTAAG
```

# Appendix IV

pGEM-1 Vector Promoter and Multiple Cloning Sites

*(From Promega)*




**pGem-1 Vector circle map and multiple cloning sites (GenBank Accession Number X65300).**

**Description *(From Promega)*:** The Riboprobe Gemini System pGEM-1 Vector is a plasmid that contains SP6/T7 promoters flanking the pUC13 polylinker. This plasmid carries the gene for ampicillin resistance and is 2865 bp in length.

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