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A molecular analysis of an octopamine receptor gene in Drosophila melanogaster.

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A MOLECULAR ANALYSIS OF AN OCTOPAMINE RECEPTOR GENE IN *DROSOPHILA MELANOGASTER*

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By

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THESIS ACCEPTANCE

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ABSTRACT

Octopamine is a biogenic amine. It plays an important role as neurohormone, neuromodulator and neurotransmitter in *Drosophila melanogaster*. These physiological functions of octopamine are essential in feeding, mating and fight-or-flight behavior. Octopamine receptors are structurally and functionally similar to the vertebrate adrenergic receptors. A *Drosophila* gene encoding an octopamine/tyramine receptor was previously identified by probing a *Drosophila* genomic library with a human brain β_2 adrenergic receptor cDNA clone. After identifying a 367-bp fragment of a *Drosophila* genomic DNA, a cDNA library prepared from head mRNA was screened and a 3.3 kb octopamine/tyramine cDNA cloned. A 2.2 kb fragment of this 3.3 kb cDNA, containing the inferred protein coding region of the cDNA, was subcloned and after screening a *Drosophila* genomic library, eight genomic clones were isolated.

In order to begin to understand how the gene for this cloned octopamine receptor is transcriptionally controlled, it is necessary to define its genomic structure and its transcription initiation site. To do this, I have characterized previously isolated genomic clones and generated a restriction map for this genomic region by method of restriction mapping and Southern blotting, and have used primer extension analysis to define the location of the transcription iniation site of this gene by primer extension analyses. This research work also sets the stage for comparing the cDNA restriction map and genomic restriction maps of this gene to assess whether introns are present in its primary transcript.

THIS RESEARCH WORK IS

DEDICATED TO MY *AAI* AND *BABA*

(MOTHER AND FATHER)

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Introduction

Octopamine, a phenolic analogue of noradrenaline (Figure 1), is a biogenic amine. It was first identified in the posterior salivary gland of *Octopus vulgaris* and therefore named as octopamine, by Erspamer in 1951. Octopamine occurs in large amounts in the nervous systems of species representing the phylum Arthropoda including the classes Insecta and Crustacea (Robertson *et. al.*, 1976), as well as in vertebrates. The earliest suggestion that octopamine may function as a neurotransmitter was made by Carlson in 1968. Following the development of a suitable assay procedure, octopamine was found to be present in insects and at a concentration much greater than catecholamines (Robertson and Steele, 1974).

That octopamine might play a specific role in insects was suggested following the discovery of octopamine and related amines such as synephrine and tyramine (Figure 1) in the insect nervous system. Each of these amines exhibited a powerful stimulatory effect on level of activity of the enzyme glycogen phosphorylase in the ventral nerve cord of the cockroach *Periplaneta americana* (Robertson and Steele, 1970; 1972). Octopamine, but not dopamine, noradrenaline, or adrenaline, produced a significant decrease in nerve cord glycogen levels and increase in phosphorylase activity. These effects of octopamine were mimicked by cyclic adenosine-3',5'monophosphate (cAMP) (Robertson and Steele, 1973).

Many neurons communicate with their target cells at specialized contact areas called synapses. The synaptic cleft separating pre- and post-synaptic region of cells is narrow, around 15-50 nm. Information between cells is transferred by quantal release of

small amounts of neurotransmitters. Some of these chemical messengers act almost immediately and are rapidly inactivated. Thus, these chemical messengers are responsible for carrying highly specific short-term messages.

In order to be a neurotransmitter, a chemical substance should be released from the pre-synaptic neurons and it should be antagonized by a competitive blocking agent and stimulation of the nerves should cause release of the substance in physiologically significant amounts. Several lines of evidence suggest that octopamine plays a role as a neurotransmitter in the insect nervous system. These derive from experiments performed on the firefly light organ (Nathanson, 1979) and the glandular cells of the corpus cardiacum of the locust (Orchard, 1982). In the firefly, the timing of light flashing is under the control of the nervous system. It was observed in an *in vitro* preparation that a specific octopamine sensitive-adenylate cyclase causes synthesis of large amounts of adenosine 3', 5'- monophosphate in the adult as well as in larval lanterns. The adenylate cyclase activity of broken cell preparations from isolated light organs was stimulated 20-fold by 10^{-5} M octopamine while the surrounding non-lantern cells were stimulated less than 3-fold. The only amine as effective as octopamine was synephrine, one not detected in the lantern cells (Nathenson, 1979). Furthermore, additional experiments in which an effective concentration of each amine was tested alone and in combination revealed that these amines were acting on a single receptor. These receptors were blocked by the α-adrenergic blocking agent, phentolamine (Figure 2). From these experiments, octopamine meets a few of the criteria to be a neurotransmitter (Orchard, 1982).

Other neurons, neurosecretory cells, release relatively large amounts of chemical messengers, called neurohormones, into the circulatory system. Once there, they are carried some distance where they alter the physiological activity of their target site(s). Neurohormones are generally slow-acting and long-lasting. A role for octopamine as a neurohormone was suggested after it was found in the haemolymph of the locust (Orchard and Loughton, 1981). It was observed that excitation (by handling) of a locust which had previously been fed and left undisturbed for 16 hours induced a rapid rise of both octopamine and lipid levels in the hemolymph. This excitation-induced hyperlypemia could be reduced by injection of the α-blocker phenoxybenzamine (Figure 2) and again stimulated by injection of octopamine. In support of this observation, an *in vitro* experiment showed that low concentrations of octopamine could stimulate release of lipids from the fat body (Orchard and Loughton, 1981). Thus, in invertebrates, octopamine is not only a likely neurotransmitter, but also a likely neurohormone.

The term neuromodulator was introduced in early 1980s. Neuromodulaters are the chemical messengers that alter the quality of synaptic information or changes the spontaneous activity of a receptive neuron or a muscle cell (Evans, 1980). The neuromodulator can be released either into the circulatory system for transport to the target cell or within the vicinity of the target site. Key evidence suggesting that octopamine serves as a neuromodulator comes from experiments on a defined set of neurons called the Dorsal Unpaired Median (DUM) neurons. These neurons innervate the extensor tibiae muscle. In these experiments, stimulation of DUM neurons at 1 Hz reduced the frequency and amplitude of myogenic contractions, while perfusion of

extensor tibiae muscle with octopamine mimicked the action of DUM neurons. This response was dose-dependent, and blocked by phentolamine and phenoxybenzamine (α-blockers) (Figure 2), but not by propranolol or dichloroisoproterenol (β-blocker). Thus, in addition to its other roles, octopamine serves as a neuromodulator (Orchard, 1982).

Further experiments on octopamine suggested that it mediates a wide range of responses including the modulation of visceral muscle contraction described earlier, the release of peptide hormones from neuroendocrine tissues and the stimulation of glycogenolysis in both the fat body and nerve cord. All these observations suggest that octopamine plays a vital role in the invertebrate nervous system (Orchard, 1982).

In *Drosophila,* octopamine is involved in the modulation of visceral muscle contraction, release of peptide hormones from neuroendocrine tissue, and the stimulation of glycogenolysis in fat body and nerve cord (Evans, 1993). It has also been suggested that octopamine plays a role in mating behavior (O'Dell, 1992). *Drosophila* mutants carrying the *inactive* (*iav*) mutation, have reduced octopamine levels and exhibit low locomotor activity and poor mating success. These mutant flies are highly susceptible to the octopamine analogue *p*-Cresol (Figure 1). Octopamine also has a physiological role in insect vision and olfaction (Roeder and Nathanson, 1993).

Most of the functions described for octopamine are activated under stressful conditions to prepare the insect for intense activity (Evans, 1984). This suggests that in insects, octopamine carries out functions similar to those associated with noradrenaline and adrenaline in the sympathetic nervous system of vertebrates. This has led some

authors to refer to octopamine as an invertebrate counterpart of norepinephrine (Robertson *et. al.*, 1976).

In this sense, octopamine has been suggested to be the "fight and flight" hormone in insects (Orchard *et. al.*, 1982). Recent experiments by Adamo *et.* al., (1995) on the cricket *Gryllus bimaculatus* suggest that octopamine contributes to some part of the insect arousal system. It may function as a neurohormone and prepare the animal for a period of extended activity or help it recover from increased energy demands (Adamo *et. al.*,1995).

Receptors for octopamine are widely distributed in the insect nervous system (Evans, 1980). In particular, several pharmacologically distinguishable subtypes of octopamine receptors are present in the locust nervous system (Evans and Robb, 1993). The original classification of octopamine receptors into different subtypes with different modes of action was based on work performed on the octopaminergic response obtained from the extensor-tibiae muscle preparation of the hindleg of the locust (Evans, 1981).

Octopamine receptors present on the myogenic bundle are called OCTOPAMINE$_1$ receptors and mediate their action by a mechanism that elevates the levels of intracellular calcium in this muscle (Evans, 1984). A different class of receptors mediating neuromuscular transmission are called OCTOPAMINE$_2$ receptors. This subclass is further classified into OCTOPAMINE$_{2A}$ and OCTOPAMINE$_{2B}$ receptors. The receptors on the presynaptic terminals of the slow motor neurons are type- 2A receptors, while those mediating postsynaptic effects on muscle are type- 2B receptors. While OCTOPAMINE$_1$ receptors mediate their response by increasing intracellular calcium levels,

$OCTOPAMINE_2$ receptors mediate their response by increasing the levels of adenylase cyclase activity (Evans and Robb, 1993).

The distinction between $OCTOPAMINE_1$ and $OCTOPAMINE_2$ receptor subclasses is defined pharmacologically by selective agonists and antagonists. For example, metoclopramide (Figure 3) blocks $OCTOPAMINE_2$ but not $OCTOPAMINE_1$ receptors while yohimbine (Figure3) blocks $OCTOPAMINE_1$ and not $OCTOPAMINE_2$ receptors. Similarly clonidine (Figure 4) is a better agonist for $OCTOPAMINE_1$ receptors than naphazoline (Figure 4), which is a better agonist for $OCTOPAMINE_2$ receptors. The distinction between 2A and 2B receptor subtype relies on differential potency of drugs such as mianserin and cyproheptadine. These are more potent on the 2A than the 2B subclass of receptors (Evans and Robb, 1993). Studies on $OCTOPAMINE_2$ receptor-mediated responses showed similarities in the pharmacological profiles obtained in different tissues, although their was an element of tissue specific variation of this receptor subclass. This variation was observed to be larger for antagonists than for agonists (Evans and Robb, 1993).

$OCTOPAMINE_1$ and $OCTOPAMINE_2$ receptors belong to the family of receptors that interact with guanine nucleotide binding proteins (G-proteins) and are therefore called G-protein coupled receptors. The receptors belonging to the G-protein coupled receptor family are integral membrane proteins with an amino acid sequence that contains seven hydrophobic regions of 20 to 28 residues, suggesting that the protein transverses the lipid bilayer seven times. The receptor's amino terminus is extracellular and its carboxyl terminus is intracellular. One or more sites for asparagine linked glycosylation are found

in the amino terminal domain. The cytoplasmic domain often contains potential sites for phosphorylation by one or more cytosolic kinases. After binding of the ligand to the receptor's surface outside the plasma membrane; there is a stabilization of a conformational change in the receptor, including a change in a domain located inside the plasma membrane. This structural change promotes an interaction with the second protein in the signal transduction pathway, a GTP-binding protein. G-proteins bind and hydrolyze GTP.

Several classes of G-proteins have been described. They include G_s (stimulatory) and G_i (inhibitory) which regulate the enzyme adenylyl cyclase, G_p activates phospholypase C and G_k and G_{ca} which regulate potassium and calcium channels respectively. Other G-proteins (G_O) also have been described (Koblika, 1992, O'Dowd *et al.*, 1989, Dohlman *et. al.*, 1991.)

G-proteins consists of three polypeptide subunits, α, β and γ. If the α subunit contains bound GDP, it exists in an inactive state. Ligand binding to the receptor causes activation of the G-proteins and GDP is replaced by GTP. The αG-GTP complex dissociates from the $\beta\gamma$-dimer and acts on its target protein. αG-GTP has intrinsic GTPase activity that leads to the formation of the inactive αG-GDP complex. This complex reassociates with the $\beta\gamma$-dimer to form inactive trimer that can be activated again (Duzic *et.al.*, 1992, Kaziro *et. al.*, 1991).

One major signal transduction pathway involving G-proteins uses cAMP as an effector molecule. Ligands that bind to stimulatory receptors activate adenylate cyclase through the G_s class of G-proteins and raise intracellular cAMP levels, while ligands that

bind to inhibitory receptors through the G_i class of G-proteins inhibit adenylate cyclase activity. Binding of the ligand causes the receptor to catalyze the displacement of the GDP bound to inactive G_s by GTP; this converts G_s to its active form. As this occurs, the β and γ sub-units dissociate from the α unit; $G_s\alpha$, then moves in the plane of the membrane from the receptor to a nearby molecule of adenylate cyclase. Adenylate cyclase is an integral protein of the plasma membrane with its active site facing the cytoplasm. The association of active $G_s\alpha$ with adenylate cyclase converts the cyclase to its catalytically active form and the enzyme catalyzes the production of cAMP from ATP. In this way, the intracellular level of cAMP is raised. Cyclic AMP activates protein kinase A, which catalyzes the phosphorylation of inactive phosphorylase B kinase to yield its active form. This phosphorylation of cellular proteins in turn results in a response to the ligand (Koblika, 1992, O'Dowd *et al.*, 1989). Ligand-binding to the receptors which inhibits adenylate cyclase utilize G_i proteins. The activated receptor promotes the exchange of GTP for GDP, and the αG_i-GTP binds and decreases adenylate cyclase activity. The inhibitory process is terminated by GTPase activity of αG_i (Yoshikuni and Nagahma, 1994, Kaziro *et. al.*, 1991).

Another major signal transduction pathway involves the second messenger inositol triphosphate (IP_3) and diacylglycerol (DAG). Once the ligand is bound to the receptor, the signal is transduced through a G-protein, G_p, which undergoes conversion to the GTP-bound form. G_p subsequently activates the enzyme phospholypase C. This enzyme is bound to the cytoplasmic surface of the cell and catalyzes the hydrolysis of phosphatidylinositol 4,5-biphosphate to two products: inositol 1,4,5-triphosphate (IP_3)

and DAG. IP_3 is then released from cell membrane and diffuses through the cytoplasm to the endoplasmic reticulum. In this location it binds to IP_3 receptor, a ligand-gated calcium channel. IP_3 binding opens the channel releasing Ca^{2+} from the endoplasmic reticulum to the surrounding cytoplasm. Calcium released by IP_3 acts as a co-activator (with DAG) of protein kinase C. The activated protein kinase C then phosphorylates target proteins. Ca^{2+} released by endoplasmic reticulum by IP_3 may also function directly as a cellular regulator (Kaziro *et. al.*, 1991).

A *Drosophila* gene encoding an octopamine receptor was initially identified by probing a *Drosophila* genomic library with a human brain β_2 adrenergic receptor cDNA clone (Arakawa *et. al.*, 1990, Saudou *et. al.*, 1990). A 367-bp fragment of a *Drosophila* genomic DNA was identified and used to screen a cDNA library prepared from head mRNA. The nucleotide sequence of one cDNA clone obtained contains 3336 base pairs and has a large open reading frame that could encode a polypeptide of 601 amino acids with a calculated molecular mass of 64.7 kDa. Since in a Northern analysis, this cDNA hybridized with a 3.6 kb poly(A^+) RNA, the cloned cDNA appears to be nearly full length (Arakawa *et. al.*, 1990). The inferred polypeptide belongs to the adrenergic and muscarinic receptor family. In particular, comparison of the amino acid sequence of this polypeptide with known sequences of mammalian receptors, showed significant homology with α_2-adrenergic receptors (Arakawa *et al.*, 1990). When this clone was expressed in Chinese hamster ovary (CHO-K1) cells, a polypeptide with pharmacological characteristics of an octopamine/tyramine receptor was seen. Its binding properties were examined in isolated membranes using [^{3}H]yohimbine (Figure 3), an α-adrenergic

receptor antagonist. Octopamine inhibited [^{3}H]yohimbine binding better than dopamine and adrenaline, suggesting pharmacological similarities between octopamine and α_2-adrenergic receptors. Thus, this invertebrate octopamine receptor has a strong similarity with mammalian adrenergic receptors.

When the same *Drosophila* receptor was independently cloned by a different group (Saudou *et. al.*, 1990), they obtained an exactly identical sequence except for the presence of a guanine instead of an adenine at position 413. This change transforms a serine at position 34 to an asparagine. When their clone was expressed in Cos-7 cell line, the most potent inhibitor of [^{3}H] yohimbine binding to membranes was tyramine. Both groups showed that this receptor was capable of inhibiting the forskolin-induced accumulation of cAMP levels when expressed in either CHO-K1 cells (Arakawa *et. al.*, 1990) or in mouse NIH 3T3 cells (Saudou *et. al.*, 1990).

Furthermore, when expressed in mammalian cell lining, this cloned *Drosophila* receptor appears to be able to couple to more than one second messenger system. That is, it can inhibit adenylase cyclase activity (perhaps by coupling to G_i class of G-proteins) as well as lead to the elevation of intracellular calcium (Robb *et. al.*, 1994). This suggests that the cloned *Drosophila* receptor may be coupled *in vivo* to multiple effector pathways via different G-proteins.

Pertusis toxin is a toxin produced by *Bordetella pertussis* which catalyzes the ADP-ribosylation of a specific cysteine in αG_i. It results in αG_i being unable to exchange GTP for bound GDP, causing αG_i to remain in a permanently inactivated state. Cells thus lose the capability of inhibiting adenylyl cyclase under physiological conditions and signal

transduction is impaired (Honner *et. al.*, 1992). A pertusis-sensitive G-protein probably couples the receptor to the inhibition of adenylate cyclase and a pertusis insensitive G-protein couples it to a mechanism responsible for the elevation of intracellular calcium levels (Evans, 1993). The cloned receptor therefore may be a multifunctional receptor potentially activated by octopamine as well as at tyramine, depending on the biogenic amine released presynaptically (Robb *et al.*, 1994). Because of this potential, this receptor has been referred to as an "octopamine/tyramine" receptor.

The genetic information within a DNA molecule serves as the transcriptional template for the synthesis of a large number of RNA molecules. The RNA molecule, in turn, serves as a template for the synthesis of a protein. The sequences that control the expression of a gene, its promoter, are usually found 5' to its transcription initiation site. Promotors are the regions recognized by RNA polymerase II and its transcription factors during transcription initiation.

One question of significant interest is how the gene for the cloned octopamine/tyramine receptor is transcriptionally controlled. This is an important question because first, understanding the transcription control of this gene in a model invertebrate system where genetic analyses are feasible will provide a foundation for comparative studies on related vertebrate α-adrenergic receptors. Vertebrate α-adrenergic receptors play critical roles in numerous physiological processes such as hypertension, glaucoma and stroke.

Second, understanding the transcriptional control of this receptor should help illuminate how subtypes of receptors are controlled at a molecular level, particularily how

a particular cell regulates subtype-specific expression. More specifically, an understanding of a transcription control will be helpful to reveal what role of transcriptional activation plays (if any) in receptor-subtype expression.

Third, since the pesticide amitraz acts as an antagonist for the cloned receptor, understanding the transcription regulation of this receptor may have some bearing on strategies using pesticides.

In this thesis, the location of the transcription initiation site of the cloned octopamine/tyramine receptor gene will be described. In addition, this thesis will also describe the extent of the genomic regions that are transcribed as evidenced from restriction mapping and Southern blot analyses of the cloned genomic regions. This work thus sets the stage for comparing the cDNA restriction map (Arakawa *et. al.*, 1990) and genomic restriction maps of this gene to assess whether predict introns are present in its primary transcript.

Materials and methods

CHARACTERIZATION OF LAMBDA CLONES CONTAINING GENOMIC SEQUENCES FOR THE OCTOPAMINE RECEPTOR:

Identification of the genomic clones:

Eight lambda clones containing 11 to 13 kb of *Drosophila* genomic DNA vector Charon 4A were isolated (Chase, unpublished data) by screening a *Drosophila* genomic library with a 2.2 kb fragment of the 3.3 kb cDNA (Arakawa *et. al.*, 1990). This 2.2 kb probe has been inferred to contain the coding region of the octopamine/tyramine cDNA. In Figure 5, the position of this probe is shown with respect to the 3.3 kb cDNA. To examine whether these clones were unique DNA was prepared from high titer phage stocks and analyzed by restriction mapping.

Preparation of high titer phage stock:

A high titer phage stock for these clones was made using a plate lysate method. In this method, bacteria are infected with bacteriophage and cultivated on nutrient agar. After phage infect and lyse the bacterial cells, cellular debris is formed and phage are released. The phage are then eluted and stored.

Escherishia coli strain K802 was cultivated overnight at 37^{O} C with vigorous agitation in LB broth supplemented with 10mM $MgSO_4$. The cells were centrifuged at room temperature for 10 minutes at 1000g and then resuspended in 40% of the original volume of the culture in SM (0.1M sodium chloride, 50mM Tris-HCl pH 7.5, 10mM $MgCl_2$, without gelatin). From 1 ul to 10 ul of phage (Table 1) were mixed with 20 ul of

bacterial cells per 82 mm plate and the final volume adjusted to 120 ul by adding SM and 3 ml molten top LB agar was added, the mixture quickly mixed thoroughly by vortexing and poured immediately on solidified LB agar plates. These plates were incubated upright in a moist chamber at 37^{O} C for 5 to 8 hours or until lysis was evident. To elute the phage, 5 ml of SM without gelatin and a drop of chloroform was added to the plate, and the plates were rotated at 4^{O} C overnight on a slow shaker. The supernatant containing the lambda phage was collected in a sterile flask and stored at 4^{O} C.

Isolation of phage DNA:

DNA isolation from these phage was performed using a method based on that described by Manfioletti and Schneider (1988). In this method, polyanions present in the phage lysate are adsorbed to an insoluble anion exchange resin (TEAE cellulose). Unadsorbed phage particles are then disrupted by using EDTA and proteinase K, and the released DNA is precipitated by addition of the cationic detergent cetyl-trimethyl ammonium bromide (CTAB). The precipitated CTAB-DNA is then exchanged to Na-DNA and ethanol precipitated.

TEAE cellulose (Sigma) was equilibrated in 10mM Tris-HCl pH 7.1, 1mM EDTA and 0.01% sodium azide was added to prevent bacterial growth. The resin was stored as a 50% slurry at 4^{O} C. Before use, the resin washed with LB media.

To remove the bacterial DNA, 22 ul of DNase I (250 ug/ul) was added to 22 ml of phage lysate and the tubes were incubated at 37^{O} C for 25 minutes. An equal volume of 50% TEAE cellulose in LB and DNase I treated- high titer phage stock were mixed by inversion for 10 minutes. The resin was spun out at 1000X g for 1 minute and the cleared

supernatant was filtered through Whatman number 1 filter paper into a fresh 30 ml Corex tube and 917 ul of 0.5M EDTA (pH 8.0) and 110 ul of proteinase K (20 mg/ml) were added and the tubes incubated for 15 minutes at 45^{O} C. CTAB, 385 ul of 5% in 0.5M NaCl was added and the tubes were incubated at 65^{O} C for 10 minutes and then kept on ice for 15 minutes. DNA was pelleated at 4^{O} C by centrifugation in an HB4 rotor at 10,000 rpm for 10 minutes. The supernatant was discarded and the DNA pellet dissolved in 600 ul of 0.3M sodium acetate (pH 5.2), and transferred into a 2.2 ml eppendorf tube and 1.5 ml of 100% ethanol was added to precipitate the DNA the tubes spun at 12,000X g for 10 minutes at 4^{O} C. The DNA pellet was then washed with 70% ethanol, air-dried and re-suspended in TE (10mM Tris HCl, pH 7.5, 1mM EDTA).

Quantitation of DNA:

To measure the concentration and estimate the quality of the DNA, a 1:100 dilution of the DNA solution was made in TE and the optical density measured at 260 nm and 280 nm. Concentrations of phage DNA were between 0.035 and 0.150 ug/ul.

Restriction digests of phage DNA:

Restriction digests of the purified DNA were performed using the following restriction enzymes: *Eco*RI, *Hin*dIII, *Kpn*I, *Bam*HI and *Sac*I. 2 ug of DNA from each phage was cut in single as well as double digests. Lambda DNA cut with *Hin*dIII was used as a size marker. After digestion (using 3 units of enzyme/ug DNA), 6X stop/loading buffer (69 mM EDTA, 15% Ficoll type 400, 0.25% bromophenol blue, 0.25% xylene cyanol) was added to the tubes, and they were heated at 65^{O} C for 10 minutes to melt the *cos* sites and immediately stored on ice.

Agarose gel electrophoresis:

DNA fragments were separated by size by using a 0.7 % agarose gel containing 0.5 ug/ul ethidium bromide, 1X Tris-Phosphate-EDTA (1X TPE) buffer, run overnight at 30 volts. The next morning, the gel was removed from the gel apparatus and photographed while being transilluminated with 300 nm ultra violet light.

Southern blot analysis:

Southern blot analysis was performed on the genomic DNA clone using the 2.2 kb cDNA fragement as a probe to determine the relationship(s) between these DNAs. In Southern blot analysis, the size-separated DNA fragments are transferred in single-stranded form to positively charged nylon membrane which is then hybridized with a radiolabelled DNA probe. Autoradiography is used to determine the location of probe hybridization.

The gel was treated with a denaturation buffer, in which double stranded DNA get separated into single strands, containing 0.5M sodium hydroxide and 1.5M sodium chloride, twice for 20 minutes each with slow shaking. The gel was then immersed in neutralization buffer containing 1M Tris-Hydrochloric acid pH 8.0, 1.5M sodium chloride twice for 20 minutes each with slow shaking. Treatment with neutralizing buffer returns the pH of the gel to near neutral, which is important for efficient binding of the DNA to the membrane. The gel was placed on a top of a thick filter paper (Whatman 3 MM) pre-wetted with 20X SSPE, laid over a glass plate between two reservoirs of 20X SSPE. A nylon filter (soaked in nanopure water for 10 minutes and then blotting buffer (20X SSPE) for 5 minutes) was laid over the gel, covered first with Whatman 3 MM paper and then a

stack of paper towels that acts to draw up buffer up through the filter paper, gel, and nylon filter. DNA fragments from the gel were carried up and bound to the filter. Gels were blotted overnight to ensure the complete transfer of DNA from the gel to the filter. The DNA-side of the filter was exposed to UV-light using a Stratalinker to crosslink the DNA fragments. The filters containing DNA fragments were dried and stored at room temperature until hybridized.

Preparation of DNA probe for hybridization :

A ^{32}P-labeled 2.2 kb fragment of a cloned octopamine/tyramine receptor cDNA was used as a probe in Southern blot analysis. This fragment contains the inferred coding region from the previously cloned 3.3 kb cDNA. This 2.2 kb fragment was excised from the 3.3 kb cDNA by sequential enzyme cuts by Arakawa *et. al.* (1990). Initially they linearized the vector with *Cla*I and generated blunt ends with DNA polymerase. Then 2.2 kb fragment was released from the vector by cutting it with the enzyme *Alw*NI, whose site was within the defined open reading frame. Finally the 5'-end of the gene was reconstructed by an appropriate synthetic oligonucleotide was ligated to the 2.2 kb fragment, and the resulting DNA ligated into pSVL (Pharmacia), cleaved with the enzymes *Xba*I and *Sma*I. The synthetic oligonucleotide reestablished the 5'end of the gene by destroying the *Alw*NI site with a silent mutation and adding a 5' *Xba*I (Arakawa *et. al.*, 1990). This 2.2 kb fragment was excised (Chase unpublished) using *Xba*I and *Bam*HI and subcloned into *Xba*I, *Bam*HI cleaved-pSK$^+$ Bluescript (Stratagene). Prior to labeling using a random-primer method, the plasmid was linearised by cutting with the enzyme *Kpn*I.

The probe was radiolabelled with ^{32}P by the method of random priming (Feinberg and Vogelstein, 1983) using a Decaprime II kit (Ambion). In this method, double-stranded DNA is separated into single strands using heat. Ten base long sequences of oligonucleotides (DNA decamers) were annealed to this single-stranded DNA at random sites. The Klenow fragment of DNA polymerase was then added along with 2'-deoxynucleoside triphosphates (dNTP) and α–^{32}P-deoxyadenosine triphosphate. In this reaction the nucleotides are incorporated via a DNA polymerization reaction resulting in DNA having radiolabelled ^{32}P. The resulting double-stranded probe was denatured to separate the single strands and for use as a probe.

Hybridization Reaction:

The 2.2 kb fragment was labeled to specific activity of about 10^8 cpm/ug. The blots were pre-hybridized and hybridized in 50% formamide, 5X SSPE, 0.1% SDS, 1X Denhardt's and sheared salmon sperm DNA (100ug/ml) at 42^{O} C and washed in 0.1X SSPE, 0.1% SDS at 50^{O} C, three times for 20-30 minutes each. The filters were exposed to Kodak XRP film for signal detection. Several exposures of up to three days were taken to detect potentially weak signals.

PRIMER EXTENSION ANALYSIS:

Primer extension analysis using *Drosophila* head mRNA was performed to identify the transcription initiation site(s) of this gene. Primer extension is a technique used to map the 5' terminus of mRNA (Calzone *et al.*, 1987). In it, the mRNA is hybridized with an excess of a single-stranded DNA primer, radiolabeled at its 5'-terminus. The enzyme

reverse transcriptase is used to extend this primer to produce DNA complementary to the RNA template. The extension product(s) from this reaction is (are) separated by size on polyacrylamide gel, and the size is used to identify the location of the transcription initiation site of this gene.

Cultivation and collection of flies:

Drosophila melanogaster Canton Special flies were cultivated on a standard fly medium under uncrowded condition. Flies were anaesthetized using carbon dioxide and collected. The flies were quick-frozen by immersing in liquid nitrogen and then stored at -70^{O} C until used. When enough flies had been collected, heads were separated from bodies by vigorously striking the tube containing flies against a hard surface. Then, working over dry ice, the decapitated flies were shaken over a stainless steel sieve, which allowed heads (being small) to pass through the sieve. Heads were collected in a fresh tube.

Isolation of RNA from fly heads:

To collect total RNA from heads, the method of Chomczynski and Nicoletta (1987) was used with modifications. In this method proteins present in the fly heads are denatured by guanidinium thiocyanate, also a strong inhibitor of ribonucleases. RNA is then extracted using phenol-chloroform.

Particular care was taken to insure that glassware and the chemicals were not contaminated with the enzyme Rnase, which degrades RNA. All glassware was sterilized in an autoclave for 20 minutes and then baked at 350^{O} F for at least five hours to destroy the enzyme Rnase. The solutions were treated with 0.1% diethylpyrocarbonate (DEPC),

which inactivates Rnases by covalent modification. To do this, DEPC was added to a volume of 0.1%, the solutions stirred overnight, autoclaved, stirred again for 1 hour, and then re-autoclaved. As solutions containing Tris cannot be DEPC-treated (as Tris inactivates DEPC) these solution (minus Tris) were DEPC treated before Tris was added, and the solution was re-autoclaved after addition of Tris.

To prepare RNA, fly heads were homogenized in a Model Pro 200 homogenizer and a 7 mm X 150 mm generator (Pro Scientific, Inc.) at 20,000 to 30,000 rpm; 10 ml of solution A per gram of was used. Solution A had 1 part water-saturated phenol, 1 part solution B (see below), 0.1 part 2M sodium acetate (pH 4.0) and 720 ul mercaptoethanol per 100 ml solution B was placed over the fly heads (Solution B contains 3.33 ml of 0.75M sodium citrate (pH 7.0), 47.27 gm of guanidine thiocyanate, DEPC in 100 ml). Following homogenization at room temperature, the homogenate was stored at room temperature for 5 minutes to completely dissociate nucleoprotein complexes. Chloroform, 20% of the volume was added to the tube and the contents were mixed thoroughly. After sitting at room temperature for 2-3 minutes, the homogenate was centrifuged at top speed (~ 2000X g) for 5 minutes at 4^{O} C in a Hermel Z230 rotor. After centrifugation, the aqueous phase was transferred to a fresh tube, and extracted with phenol-chloroform (containing equal volumes of nucleic acid grade phenol and chloroform equilibrated with Tris HCl to pH 6.6) until no interphase appeared. RNA in the remaining aqueous phase was precipitated by adding 0.5 ml of isopropanol per ml of solution A and centrifugation at 10,000 g for 10 minutes at 4^{O} C. The supernatant was removed, the RNA pellet was washed with 75% ethanol, dried and dissolved in DEPC water.

Quantitation of RNA:

To measure the concentration of RNA, a 1:100 dilution of the RNA solution was made in DEPC water and its optical density measured at 260 nm and 280 nm. Following quantiation, the RNA was stored at -70^{O} C until used.

Isolation of poly-(A^+) RNA:

Primer extension reactions are usually carried out with poly-(A^+) RNA because poly-(A^+) RNA displays lower background reverse transcription and has less incomplete chain elongation (Calzone *et al.*, 1987). Poly-(A^+) RNA were isolated from total cellular RNA. Most eukaryotic mRNA carry a poly-(A) tail, which can be utilized to purify the mRNA fraction from the bulk of cellular RNA. Cellular RNA is passed over a column consisting of an inert material, cellulose, to which oligonucleotides consisting entirely of deoxythymidine (dT) residues have been attached. The poly-(A) tails hybridize to this oligo(dT) causing the mRNA to bind to the column, while poly-(A^-) RNA passes through. The column is then washed with high ionic strength salt buffer to remove the traces of contaminating RNA. The column is then washed with low salt buffer. Under these conditions the poly(A)-oligo(dT) hybrids dissociate, and the purified Poly-(A^+) RNA elutes from the column.

An mRNA isolation kit manufactured by Clonetech was used to isolate Poly-(A^+) RNA from total RNA. The pre-packed oligo(dT)-cellulose was resuspended by inverting the spin column several times. After the top and the bottom closures were removed, the column was placed upright in a 50 ml centrifugation tube and the storage buffer eluted and discarded. One ml of high salt buffer, provided in the kit, was added to the column and

allowed to drain by gravity through column. This procedure was repeated once, spinning the column at 350X g to prepare it for use.

A day before poly-(A^+) RNA isolation, total cellular RNA was prepared and then stored as an ethanol precipitate at -70^O C. After pelleting the RNA at 12,000 g in a microfuge at 4^O C the supernatant was discarded and RNA pellet partially dried. The pellet was resuspensed in 1 ml elution buffer provided in the kit. The RNA was heated to aid dissolution at 68^{O}C for 5 to 10 minutes, placed on ice and 0.2 ml sample buffer added. The RNA was loaded onto the oligo(dT) column, which was centrifuged at 350X g for 2 minutes in the Hermel Z230 centrifuge. The elute was discarded and 0.25 ml of high salt buffer was added to wash the column, again spinning it at 350X g for two minutes. The column was washed one more time with high salt buffer followed by two washes with a low salt buffer. Elution buffer was warmed to 65^O C and 0.25 ml added onto the column. The column spun at 350X g for two minutes and the eluted poly-(A^+) RNA were collected in a 1.5 ml Eppendorf tube. This step was repeated twice to ensure complete elution of the poly-(A^+) RNA from the oligo(dT) column. The purified RNA quantified by optical density measurements and stored at -70^{O}C until used.

Designing the oligonucleotide primer for Primer Extension :

The following factors were taken into consideration in designing the oligonucleotide primer. We sought to design a single-stranded DNA primer, 30 to 40 nucleotides in length that would not form internal DNA:DNA hybrids as secondary structures. The primer was designed to bind to a target sequence located within 100 nucleotides of 5' terminus of the published cDNA sequence, as primers that hybridize to

more distal sites can generate heterogeneous extension products because reverse transcriptase may stop or pause in regions of high secondary structure in the template RNA. The primer was designed so to have about 60% GC content.

An oligonucleotide primer of 31 bases was designed that was complementary to a region 29 bases downstream of the 5' end of the published octopamine/tyramine cDNA sequence (Arakawa *et al.*, 1990), and had the sequence 5' GCC TGA GTG GAC TGC AAG TTA TAA TTG TTG G 3'. The oligonucleotide was synthesized and HPLC purified by Operon Technologies, Inc. (1000 Atlantic Avenue, Alameda, CA 94501). The oligonucleotide was resuspended in sterile distilled water and stored at -20^{O} C until used. Some of the oligonucleotide was dried down using a Speed Vac, and stored at -20^{O} C.

Radiolabelling the oligonucleotide probe:

To a microcentrifuge tube, 2 ul of primer (10 pmol), 3 ul of nuclease free water, 1 ul of 10X forward exchange buffer (contains 500mM Tris-HCl, pH 7.5, 100mM $MgCl_2$, 50mM DTT, 1mM spermidine), 3 ul [γ-^{32}P]ATP (3000 or 6000 Ci/mmol), and 1 ul (100 units) of T4 polynucleotide kinase, were added. Nuclease-free water, forward exchange buffer, T4 polynucleotide kinase were provided in a kit from Promega Corporation. In a parallel reaction, 10 pmol of control primer, for a positive control Kanamycin resistant gene RNA, was also labeled. In an another parallel reaction, 5 ul (10 pmol) of dephosphorylated X174 *Hin*fI DNA was labeled (X174 *Hin*fI DNA used as a size marker). The tubes were incubated at 37^{O} C for 10 minutes, heated to 90^{O} C for 2 minutes to inactivate the T4 polynucleotide kinase and 90 ul of nuclease free water added to adjust the final concentration of the probe to 100 fmol/ul. The labeled primers were stored at -

20^{O} C. A 1.2 kb positive control Kanamycin resistant gene RNA was provided in the kit at a concentration of 10 ng/ul. This RNA was diluted to 1 ng/ul by adding nuclease-free water.

Primer extension reaction:

The most critical consideration in the primer extension reaction experiments was the temperature to be used for annealing the primer to the RNA template. Many trial experiments were performed to determine the appropriate annealing temperature. Optimum annealing was found after incubation at 58^{O} C for three hours.

To an Eppendorf tube, 1.64 ug of poly-(A^{+}) RNA, 2.6 ul of labeled primer and 5 ul of AMV primer extension 2X buffer (containing 100mM Tris-HCl, pH 8.3 at 42^{O} C, 100mM KCl, 20 mM $MgCl_2$, 20mM DTT, 2mM each dNTP, 1mM spermidine) were added and mixed well by pipetting. A parallel reaction contained 5 ul of the positive control Kanamycin resistant gene RNA, 1 ul of 10 pmol control labeled primer, 5 ul of AMV primer extension 2X buffer. Both the tubes were stored at 58^{O} C for three hours for primer annealing.

After annealing, reverse transcription was performed by adding 5 ul of AMV primer extension reaction buffer, 1.4 ul of 40 mM sodium pyrophosphate, 1 ul of AMV reverse transcriptase and 1.6 ul of nuclease free water. The extension reactions were done at 40^{O} C for 30 minutes, then 20 ul of loading dye containing 98% formamide, 10 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue was added and then the tubes were heated at 90^{O} C for 10 minutes and 20 ul of the reaction loaded onto a 8% polyacrylamide 7M urea gel to separate the reaction products by size.

Preparation of 8% polyacrylamide 7M urea gel:

To make 500 ml of acrylamide stock solution, 38 gm of acrylamide, 2 gm of bis-acrylamide, 210 gm of urea, 50 ml of 10X tris borate EDTA buffer and 200 ml water were mixed and slowly stirred. The final volume was adjusted to 500 ml by adding water, the solution filtered through number 1 Whatman filter and stored at 4^{O} C.

Acrylamide solution, 100 ml, was mixed with 1 ml of 10% ammonium persulfate (0.1gm/ml) and 30 ul of TEMED. The gel solution cast and allowed to set for at least 2 hours. After the gel was pre-run for 30 minutes at 45 volts, the samples and a DNA size marker were loaded and then the gel was run at 45 volts until the blue dye (bromophenol blue) was 2 inches from the bottom of the plates. The gel was fixed for 15 minutes in a fixative containing 15% methanol and 5% glacial acetic acid, blotted onto a filter paper and dried at 80^{O} C in a BioRad gel drier for two hours. The dried gel was exposed to Kodak XRP film for signal detection, with several exposures (up to seven days) taken to detect potentially weak signals.

Results

GENOMIC CLONE IDENTIFICATION RESULTS:

Eight potentially different phage containing genomic DNA were identified in a screen of a genomic library with the 2.2 kb octopamine receptor gene cDNA library. To identify the unique and overlapping clones, phage DNA was isolated, digested with the restriction enzymes *Eco*RI, *Hin*dIII, *Kpn*I, *Bam*HI, *Sac*I and the resulting DNA fragments were separated by size using agarose gel electrophoresis. Representative photographs of the restriction enzyme digests of these phage are shown in figures 6, 8, 10. Based on the pattern of bands seen in these digests, phage 1 is unique, phage 9, 10, 18 are identical and phage 15 and 8 are identical. Phage 12 and 17 did not show strong homology to the 2.2 kb cDNA in the Southern blot analysis and therefore are not considered for further analysis. Thus, of the original eight phage identified from screen, three unique phage were found.

To identify the genomic regions with homology to the 2.2 kb cDNA, the gels used for restriction enzyme analyses were blotted onto a nylon membrane and the membranes were hybridized with a ^{32}P-radiolabeled 2.2 kb fragment of the 3.3 kb octopamine/tyramine receptor cDNA. Autoradiography of the blots revealed the segments having homology between the 2.2 kb cDNA and the cloned octopamine/tyramine genomic DNA.

Figure 6a shows a photograph of a agarose gel on which were separated the restriction fragments of the phage 1. A partial restriction map of this phage is shown in

the line drawing of Figure 7. In Figure 6b, an autoradiogram of a Southern blot of the gel is shown with the dark bands on the autoradiogram identifying regions of homology between the restriction fragments of the phage genomic DNA and the 2.2 kb cDNA primer. In this phage, a 1 kb fragment of *Eco*RI and *Hin*dIII shows strong hybridization.

In a Figure 8a, photograph of agarose gel of the phage 10 (which is identical to 9 and 18) is shown. The 1.3 kb and 0.9 fragments of *Eco*RI and *Hin*dIII are identical in relative position to the same fragments seen in phage 1, therefore, these phage overlap. In Southern blot analysis, the 1.4 kb *Eco*RI and *Hin*dIII fragment of the phage 10 shows strong homology with the 2.2 kb cDNA. There is also some homology with the 3.4 kb *Hin*dIII fragment. A partial restriction map of this phage is shown in a line drawing in Figure 9.

Figure 10a shows a photograph of the gel on which restriction fragments of phage 15 were separated. The restriction map generated for this phage places the 0.9 kb, 1.3 kb and 1.4 kb fragments in the same relative order as in phage 1 and 10, suggesting these phage overlap. This overlap is confirmed by Southern blot analysis (shown in Figure 10b) where a homology between the cDNA probe and the phage can be seen in the 1.4 kb and also in the 4.2 kb *Eco*RI and *Hin*dIII fragments. Figure 11 shows a partial restriction map of phage 15. *BamH*I and *Kpn*I sites are not shown in the restriction map because these enzymes sites are not present in the published 3.3 kb cDNA sequence but these enzyme sites are present in the vector.

There were some restriction fragments whose location could not be determined unambiguously, as they could not be uniquely ordered from the restriction digests

performed. Since these fragments did not show any homology with the 2.2 kb cDNA these fragments were not further pursued. Considering the restriction maps of the individual phage, and the regions of relative restriction fragment position and homology to the 2.2 kb cDNA, these phage can be aligned, as shown in the composite Figure 12. The phage span a total amount of cloned genomic DNA equal to 17.8 kb.

RESULTS OF PRIMER EXTENSION ANALYSIS:

A primer extension analysis using *Drosophila* head mRNA was performed to identify the transcription initiation site(s) of the octopamine/tyramine gene. In a primer extension reaction, the RNA is hybridized with an excess of a single stranded DNA primer, radiolabeled at its 5'-terminus. The enzyme reverse transcriptase is then used to extend this primer to produce a single stranded copy of DNA complementary to the RNA template. A primer extension reaction containing a Kanamycin resistance gene RNA (an *in vitro* transcription product), was performed as a positive control for the primer extension analysis. The Kanamycin resistance gene RNA was hybridized with a control primer provided in the kit. In Figure 14 lane A shows the octopamine/tyramine primer extension product and lane B contains primer extension reaction product of the positive control, Kanamycin resistant gene RNA.

In lane B containing the positive control RNA, the length in bases is indicated next to the corresponding band on the autoradiogram. The upper band is the major cDNA extension product which is 87 bases. The lower band is 25 bases, the size of the ^{32}P-labeled control primer.

Lane A is the primer extension products seen with the primer for the octopamine/tyramine receptor gene. The size of the extension product is observed to be 58 bases (which is represented in the upper band). A lower smeared band is the ^{32}P-labeled oligonucleotide primer. In Figure 15 the results of this primer extension reaction are diagrammed.

From these results, the site of transcription initiation can be predicted. Consider that oligonucleotide primer is 31 bases long and located at 29 bases downstream of the published cDNA sequence; and the first six bases (5'-GAATTC-3') of the cDNA constitute artificial *Eco*R I site constructed to clone the cDNA into the vector. Given an observed primer extension product of 58 bases, this would indicate that there are four bases at the 5' end of the mRNA transcript [58-(31+29-6) = 4] in addition to those in the published cDNA sequence.

Discussion

From the phage restriction digests used to draw the restriction map, it became evident that the phage overlap. Southern blot analysis confirmed this overlap and localized the sites of homology between the 2.2 kb cDNA fragment and the octopamine/tyramine receptor gene's genomic DNA.

When the restriction map of this genomic region (shown in Figure 13) is compared with a restriction map generated from the published 3.3 kb cDNA sequence (Arakawa *et. al.*, 1990, Saudou *et. al.*, 1990) it can be seen that the sizes of the restriction fragments are not identical. While some of the differences may be due to restriction site polymorphism (since the cDNA library was made from a different wild type strain than that of the genomic DNA library), this also raises the possibility of intronic sequences in this gene. Since a cDNA is a DNA copy of mRNA, in which the introns are spliced out, introns present in the primary transcript of this gene would not be present in the mature transcript or cDNA copy. Several fragments of this cDNA restriction map of the octopamine/tyramine receptor are much shorter than similarly positioned fragments in the genomic DNA map (shown in Figure 13). This may suggest that introns are present in the genomic DNA.

The methods used in this thesis have their own limitations. In gel electrophoresis, fragments less than 0.5 kb were not readily detected. The concentration of agarose used to make the gel (0.7%) precluded accurate analysis of these sized fragments. For example, in the octopamine/tyramine cDNA, a *Sac*I site is only 34 base pairs away form

the only *Hin*dIII site. Considering the limitation of the agarose gel electrophoresis, such *Sac*I site location would not be resolved in the genomic DNA restriction digest.

From the restriction maps and Southern blots it has been established that the genomic clones contain the transcribed region of the octopamine/tyramine gene. The direction of transcription, which might have been inferred if alignment of cDNA and genomic DNA restriction maps had been possible, was not possible to determine in this study. In further research on these genomic clones, it is important to subclone the genomic clones and either generate a finer restriction map or sequence them. Such analyses of the subclones will also help to identify the specific location of possible introns

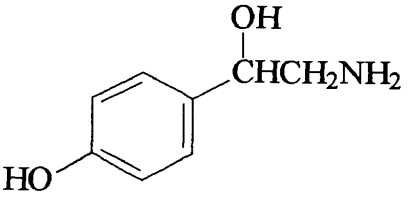
In the primer extension analysis, the four base extension of the single stranded cDNA formed from head mRNA indicate the presence of only a few base pairs beyond the 5'end of the published octopamine/tyramine cDNA sequence. While this result does not give any information about the bases, it does confirm that the 3.3 kb cDNA is nearly a full length cDNA clone, and aids in suggesting where in the genomic DNA clone the promoter should be located.

As described earlier in this thesis, promoters are the regions of transcription initiation, it is likely that sequences 5' to these four bases are critical for the initiation of transcription. To establish this, it is important to further characterized the genomic clones described in this thesis to identify and characterize the promotor region of this gene. Unless there are large introns in the 5' end of this gene's transcription unit, it is most likely that, this region may already been cloned in one of the genomic clones.

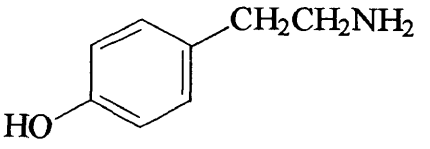
Once a potential promoter sequence has been identified, it can be compared with those of known α_2-adrenergic receptors. Sequences required for the correct transcription of this gene can be confirmed by placing them upstream of *lac* Z gene, generating transgenic *Drosophila*, and assessing whether β–galactoside shows a pattern consistent with expression of this gene (L. Mckay, M. A. thesis).

Figure 1: Octopamine and structurally similar compounds. p-cresol is an octopamine analogue.

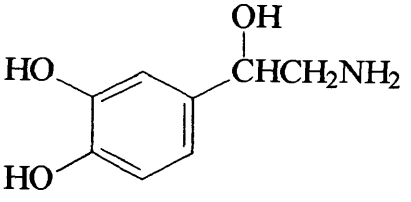
Figure 1



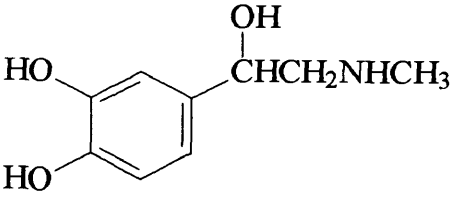
p-octopamine



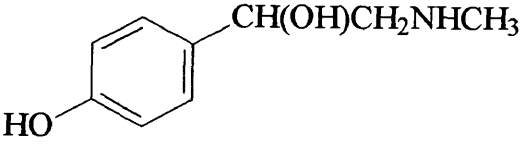
p-tyramine



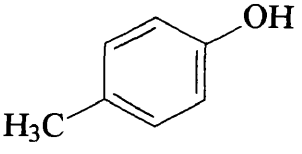
Noradrenaline



Adrenaline



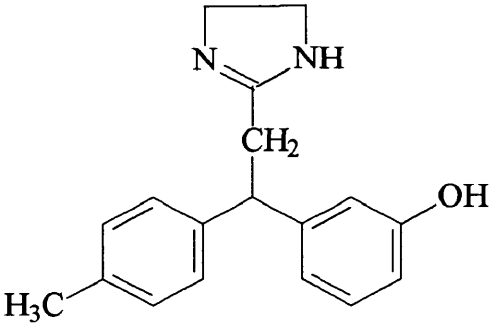
Synephrine



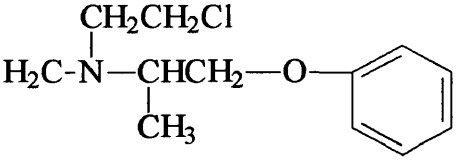
p-cresol

Figure 2: Alpha adrenergic receptor ligands

FIGURE 2



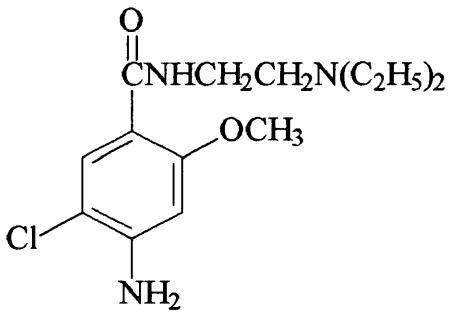
Phentolamine



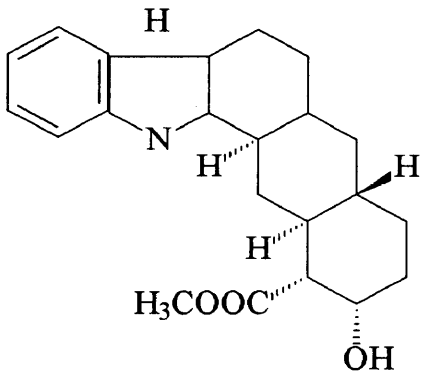
Phenoxybenzamine

Figure 3: Octopamine Receptor Antagonists

Figure 3



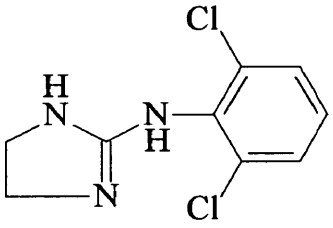
Metoclopramide



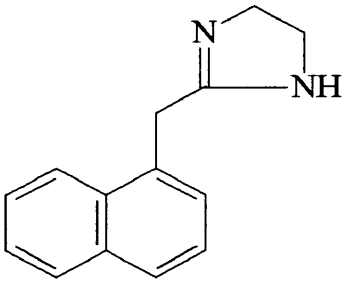
Yohimbine

Figure 4: Octopamine receptor agonists

Figure 4



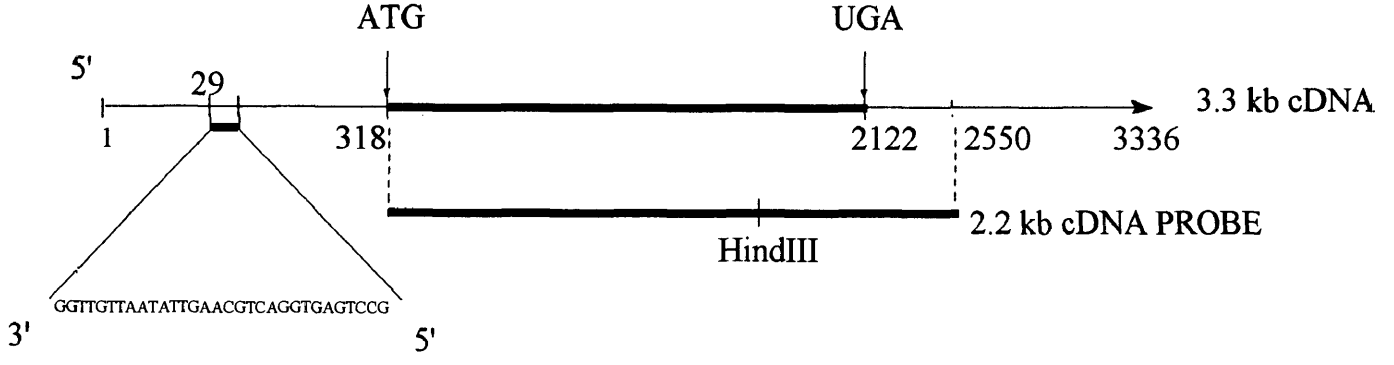
Clonidine



Nephazoline

Figure 5: A line drawing showing the location of 2.2 kb cDNA probe relative to the cloned 3.3 kb octopamine cDNA. The figure also shows the location of the 32 base primer used for primer extension reaction.

Figure 5



31 nucleotide primer for primer extension

Figure 6a: Restriction enzyme digestion of phage 1. Lane 1 and 17 are lambda marker lanes (lambda DNA cut with *Hin*dIII). Lane 2 to 16 contain the restriction fragments of the phage DNA cut with the respective enzymes as indicated at the top of the lanes. Abbreviations for the restriction enzymes used are as follows: R- *Eco*RI, H- *Hin*dIII, K- *Kpn*I, B- *Bam*HI, S- *Sac*I. The sizes of the bands in the marker lane from top to the bottom are-23.130 kb, 9.416 kb, 6.557 kb, 4.361 kb, 2.322 kb, 2.027 kb, 0.564 kb.

Figure 6b: Southern blot analysis of phage 1. Dark bands in the autoradiogram indicate the homology between 2.2 kb cDNA probe and the phage DNA.

Figure 6

FIGURE 6A

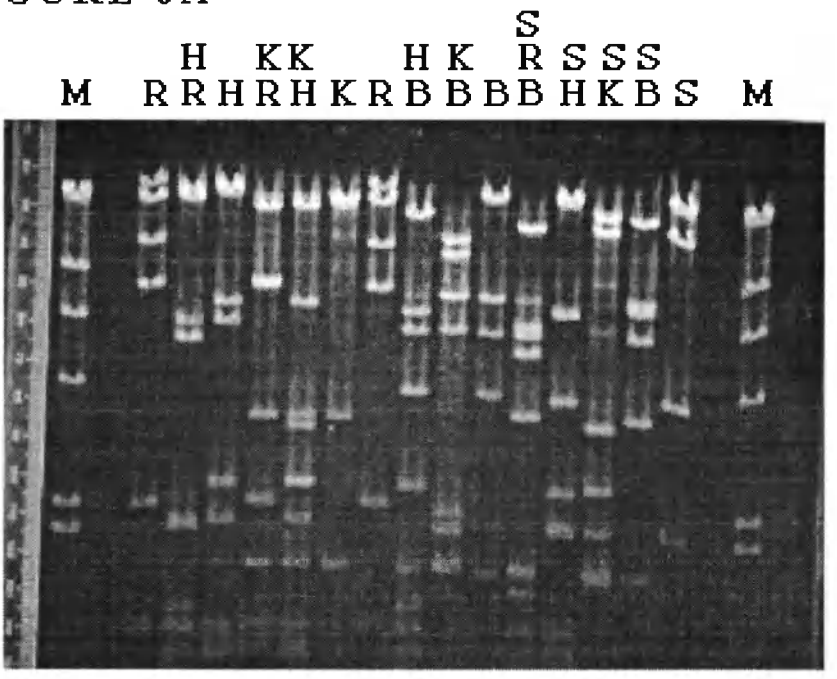


FIGURE 6B

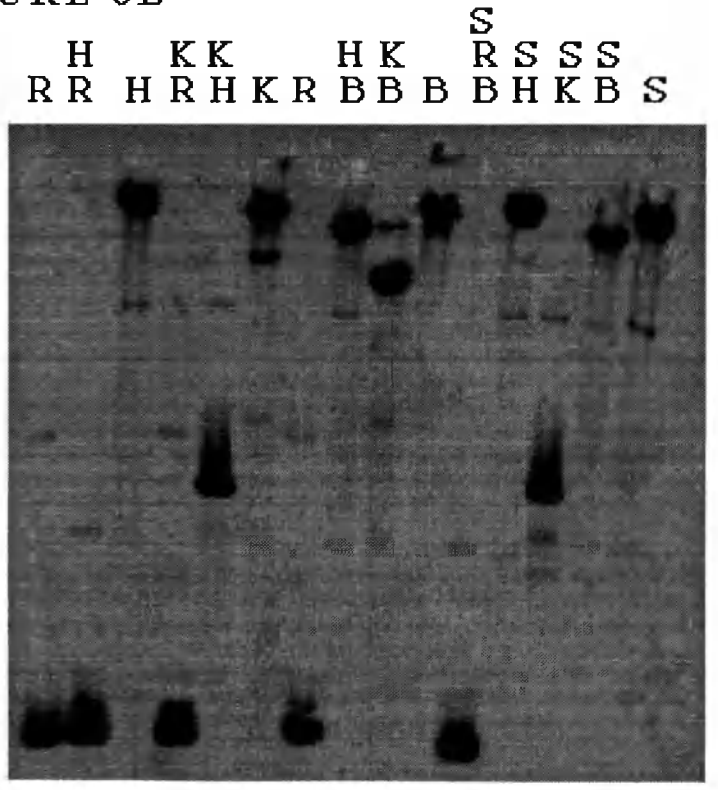


Figure 7: A line drawing showing the restriction map of phage 1.

Figure 7

Phage 1

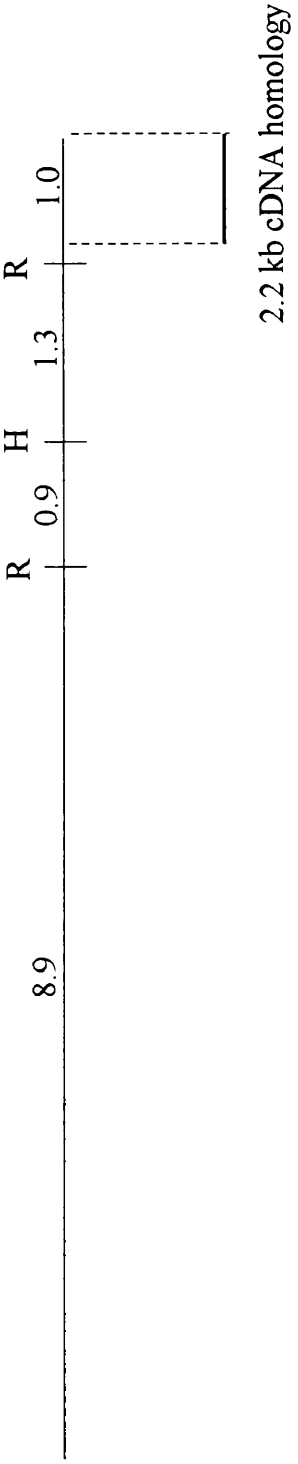


Figure 8a: Restriction enzyme digest of phage 10. Lane 1 and 17 are lambda marker lanes (lambda DNA cut with *Hin*dIII). Lane 2 to 16 contain the restriction fragments of the phage DNA cut with the respective enzymes as indicated at the top of the lanes. Abbreviations for the restriction enzymes used are as follows: R- *Eco*RI, H- *Hin*dIII, K- *Kpn*I, B- *Bam*HI, S- *Sac*I. The sizes of the bands in the marker lane from top to the bottom are-23.130 kb, 9.416 kb, 6.557 kb, 4.361 kb, 2.322 kb, 2.027 kb, 0.564 kb.

Figure 8b: Southern blot analysis of phage 10. Dark bands in the autoradiogram indicate the homology between 2.2 kb cDNA probe and the phage DNA.

Figure 8

FIGURE 8A

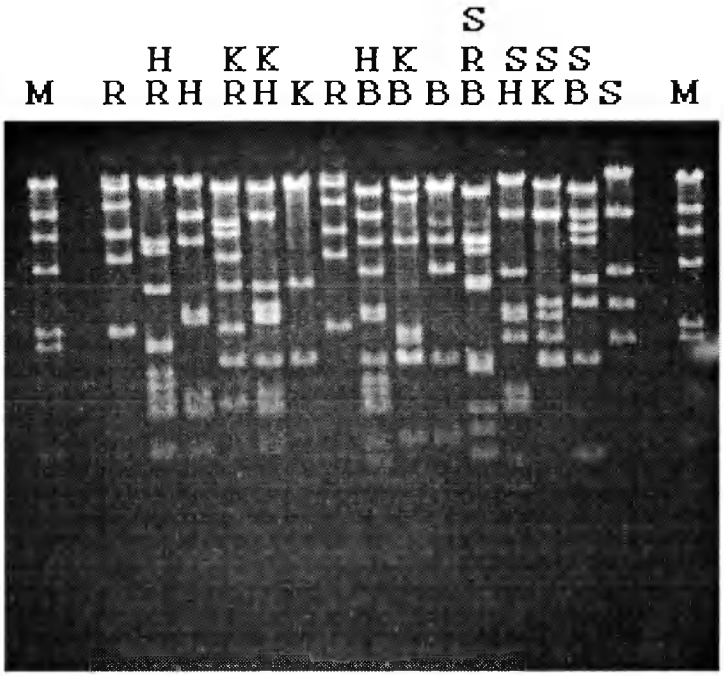


FIGURE 8B

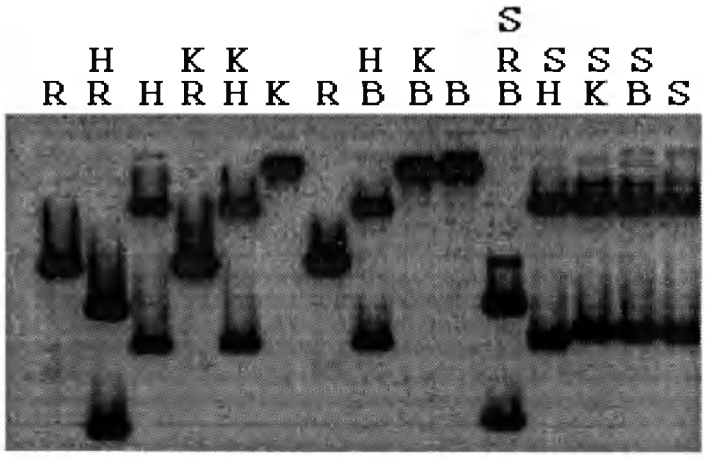


Figure 9: A line drawing showing the restriction map of phage 10.

Figure 9

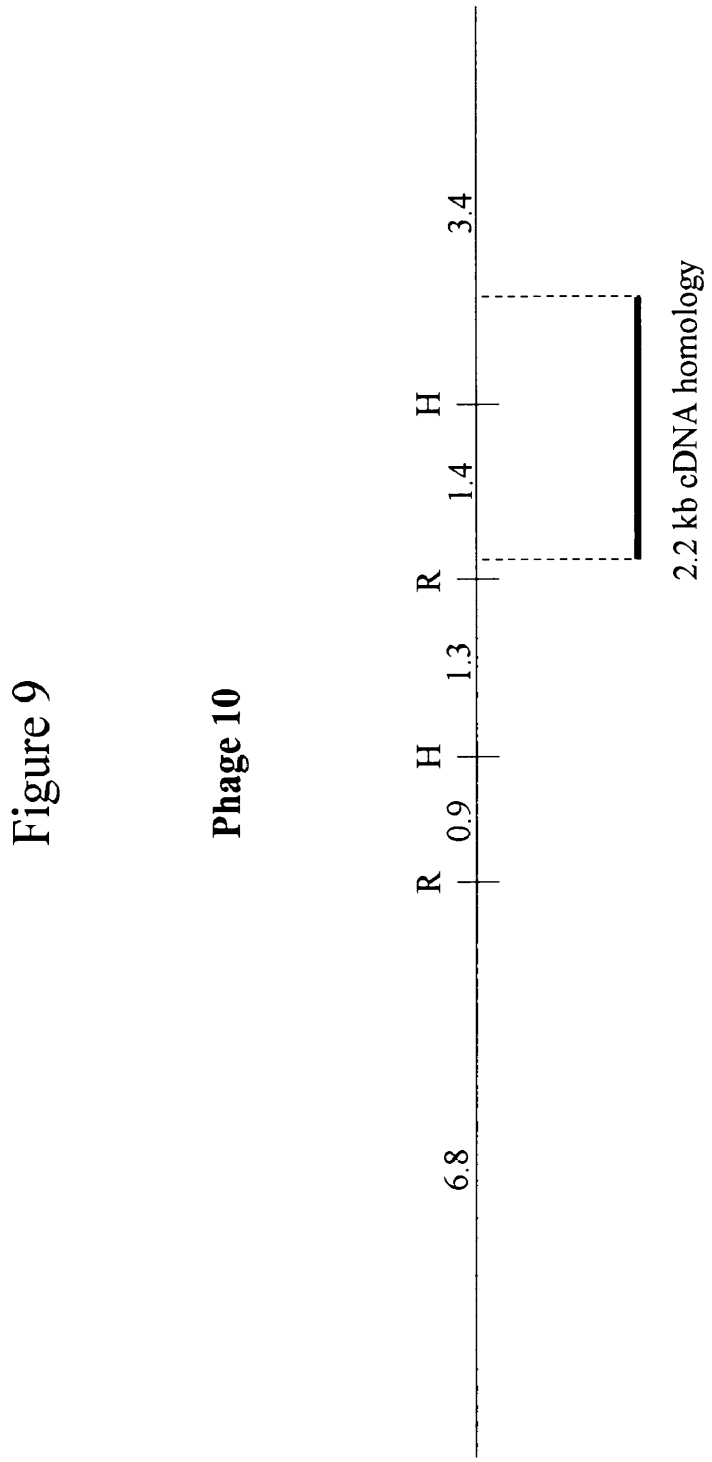


Figure 10a: Restriction enzyme digest of phage 15. Lane 1 and 17 are lambda marker lanes (lambda DNA cut with *Hin*dIII). Lane 2 to 16 contain the restriction fragments of the phage DNA cut with the respective enzymes as indicated at the top of the lanes. Abbreviations for the restriction enzymes used are as follows: R- *Eco*RI, H- *Hin*dIII, K- *Kpn*I, B- *Bam*HI, S- *Sac*I. The sizes of the bands in the marker lane from top to the bottom are-23.130 kb, 9.416 kb, 6.557 kb, 4.361 kb, 2.322 kb, 2.027 kb, 0.564 kb.

Figure 10b: Southern blot analysis of phage 15. Dark bands in the autoradiogram indicate the homology between 2.2 kb cDNA probe and the phage DNA.

Figure 10

FIGURE 10A

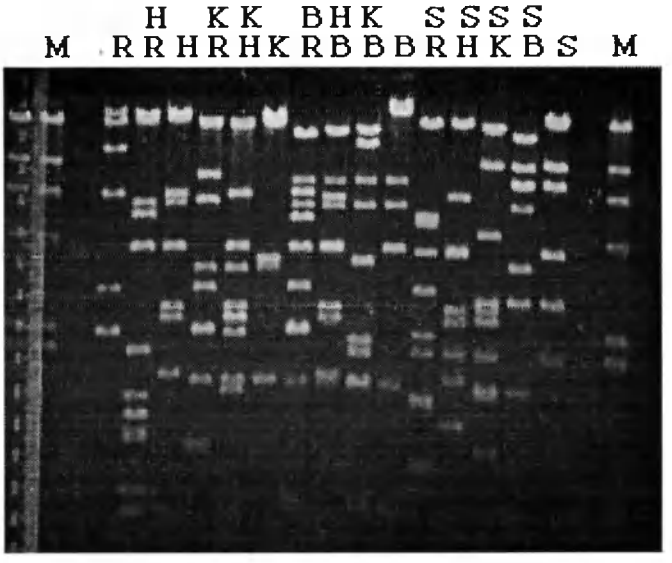


FIGURE 10B

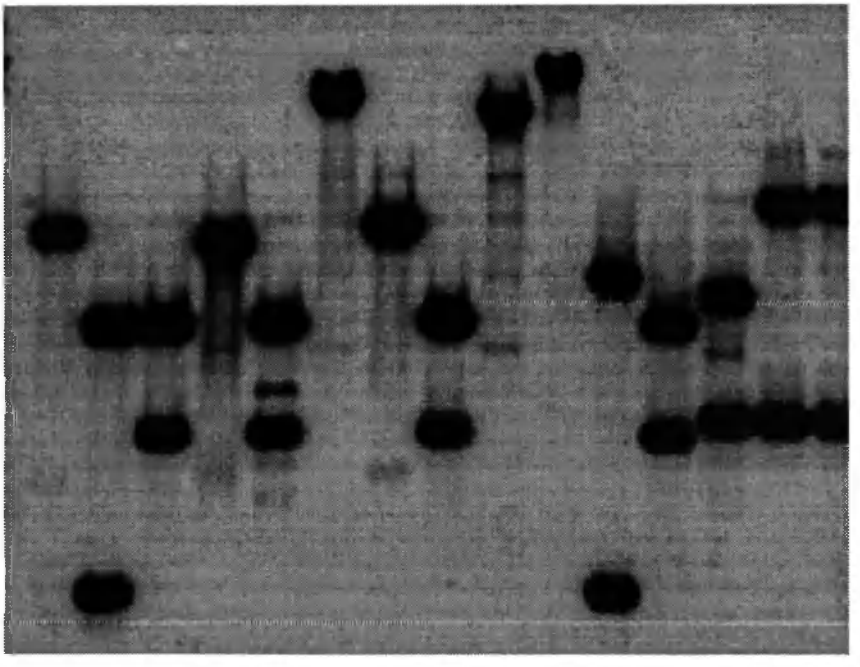


Figure 11: A line drawing showing the restriction map of phage 15.

Figure 11

Phage 15

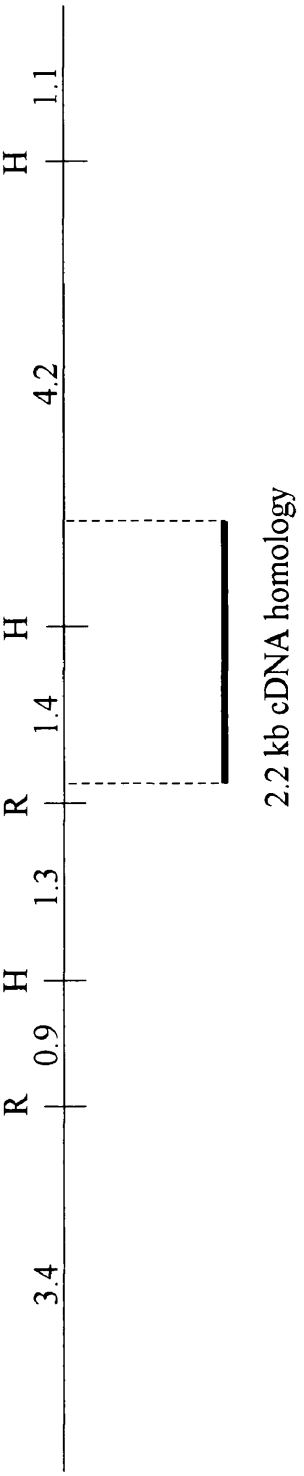


Figure 12: A composite figure of the phage insert restriction maps showing where the genomic clones overlap. The 0.9, 1.3 kb fragments show the same relative position in all three clones. The site of the 2.2 kb cDNA homology is also shown.

Figure 12

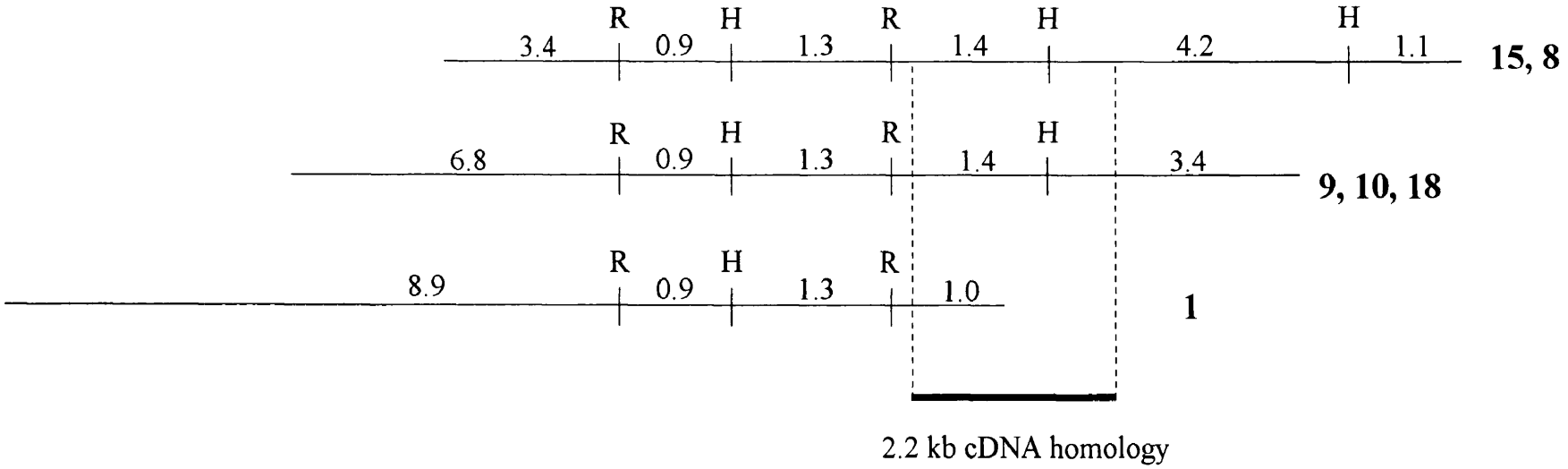

R
H
R
H
H
3.4
0.9
1.3
1.4
4.2
1.1
15, 8
R
H
R
H
6.8
0.9
1.3
1.4
3.4
9, 10, 18
R
H
R
8.9
0.9
1.3
1.0
1
2.2 kb cDNA homology

Figure 13: Correlation of genomic DNA and cDNA restriction maps of the octopamine/tyramine receptor gene. The restriction fragment sizes in genomic DNA restriction map are in kilobases (kb) and in cDNA restriction map the restriction fragment sizes are in bases.

Figure 13

Figure 13A

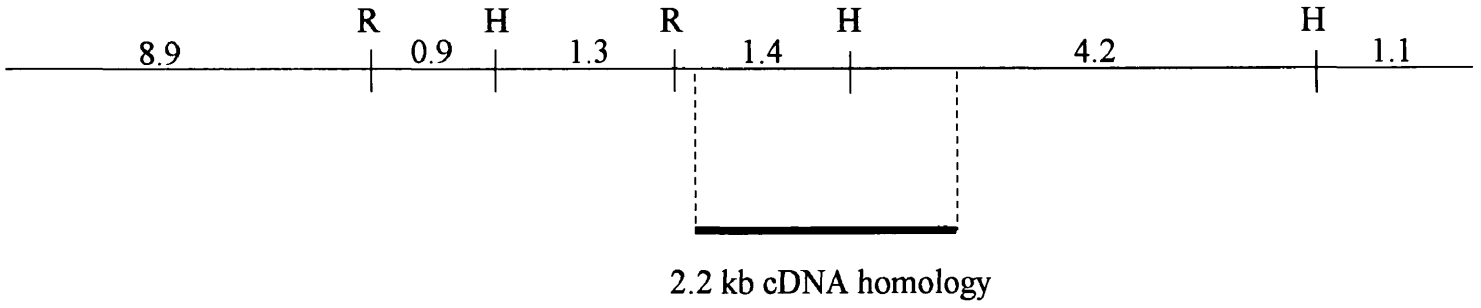


Figure 13B

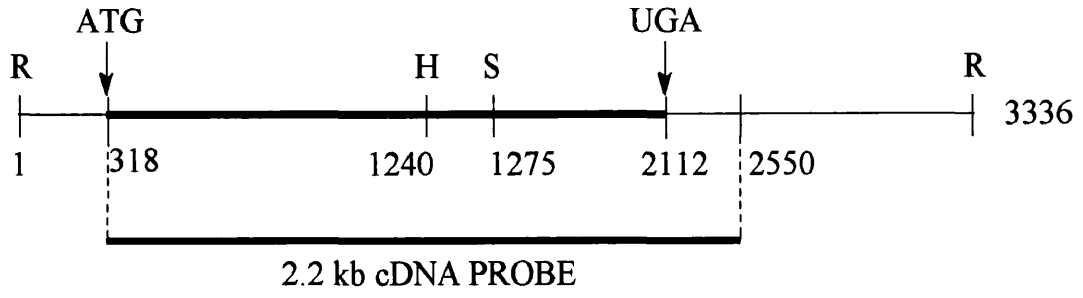


Figure 14: Primer extension analyses, the figure is a copy of an autoradiogram of the signal obtained following polyacrylamide gel electrophoresis of the primer extension reactions. Lane A shows the octopamine/tyramine primer extension reaction product, lane B shows the primer extension product of the positive control Kanamycin resistance gene RNA. The size of the bands are indicated. The octopamine/tyramine receptor primer extension product is 58 bases and the primer extension product for the positive control Kanamycin resistance gene RNA is 82 bases.

Figure 14

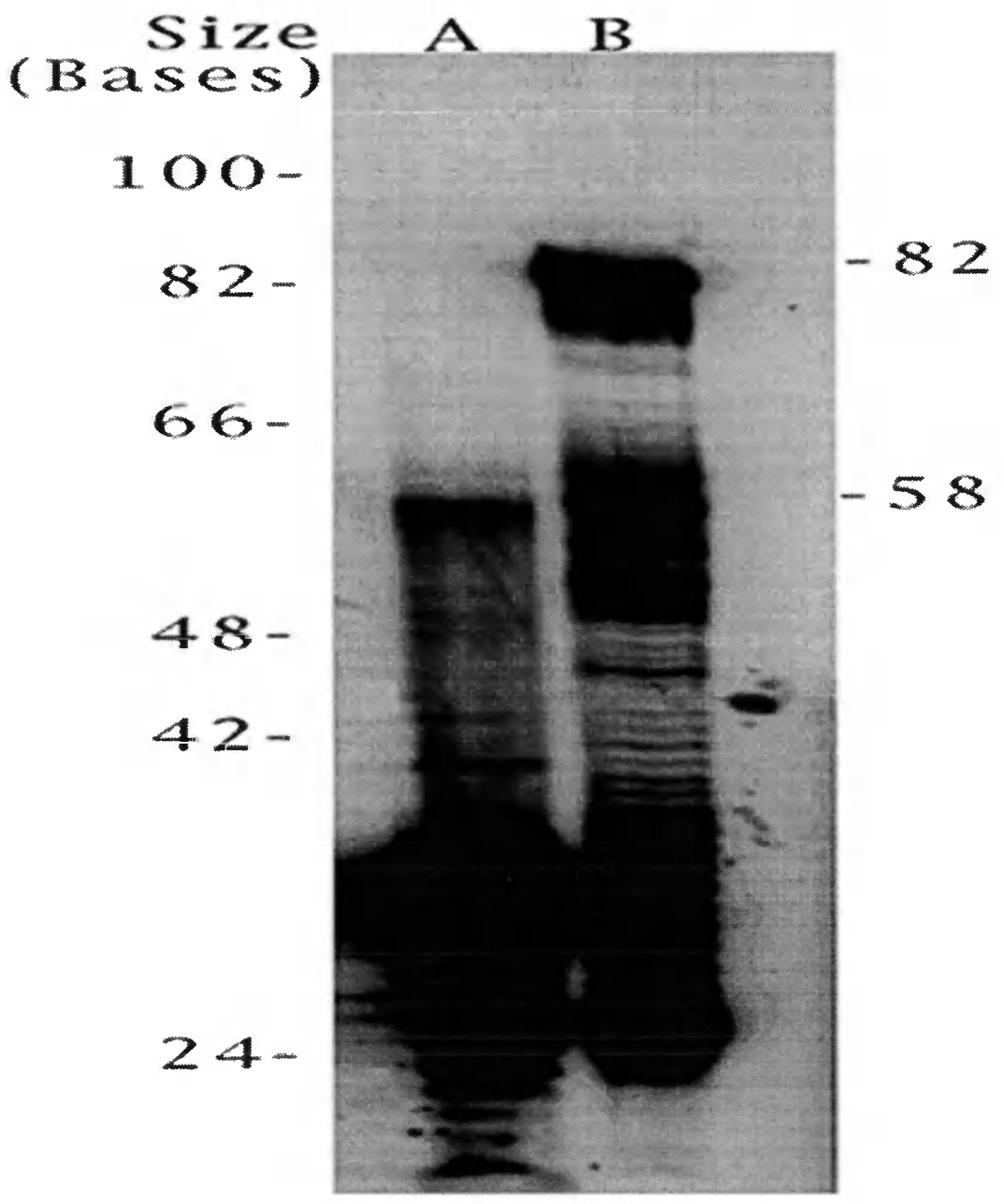


Figure 15: Interpretation of the primer extension analyses. The line drawing shows the relative locations of the primer extension reaction product and the location of primer used for the primer extension reaction with respect to the published 3.3 kb octopamine cDNA.

Figure15

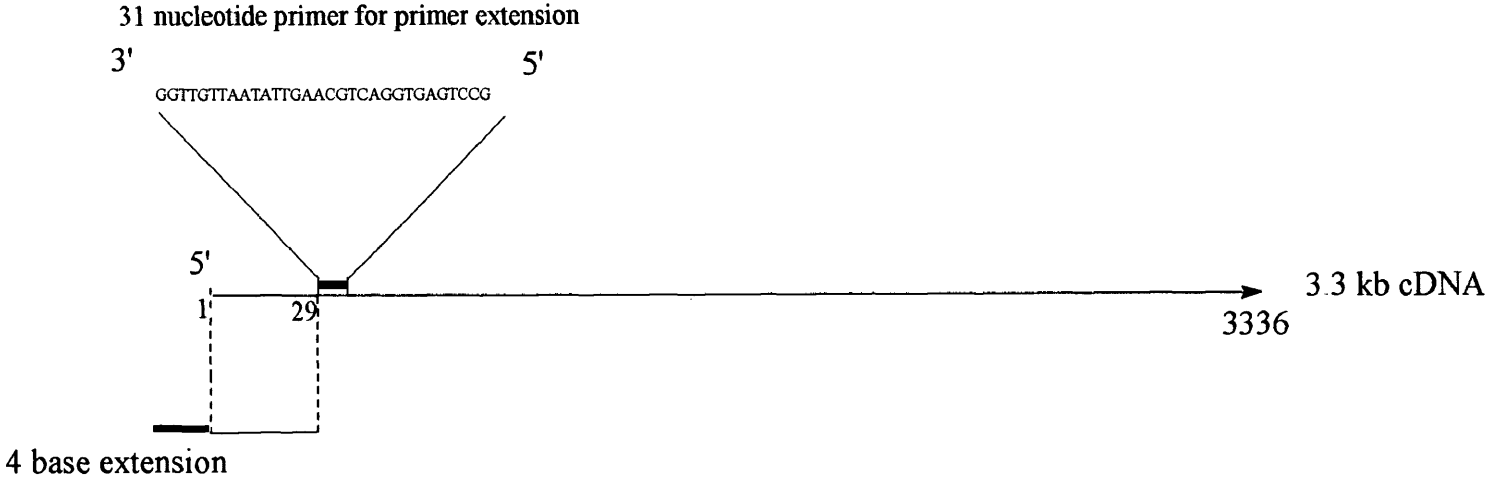

31 nucleotide primer for primer extension
3'
5'
GGTTGTTAATATTGAACGTCAGGTGAGTCCG
5'
1
29
3336
3.3 kb cDNA
4 base extension

Table I: Amount of High Titer Phage Stocks Used To Obtain Confluent Plate Lysis

Phage	Titer of phage stock pfu/ml $X10^{10}$	Amount of dilution used to obtain confluent lysis
1	0.1	10 ul of 10^{-2}
8	0.1	10 ul of 10^{-2}
9	2.4	10 ul of 10^{-2}
10	0.63	10 ul of 10^{-2}
12	0.3	10 ul of 10^{-2}
15	2.0	1 ul of 10^{-2}
17	5.7	1 ul of 10^{-2}
18	0.02	10 ul of 10^{-2}

Table II

Table of Abbreviations

ADP	Adenosine Di Phosphate
ATP	Adenosine Tri Phosphate
cAMP	Cyclic Adenosine-3',5'Monophosphate
Ca^{2+}	Calcium ions
cDNA	Complementary Deoxy Ribonucleic Acid
CHO-K1 Cells	Chinese Hamster Ovary Cells
Ci/mmol	Curies per Millimole
cpm/ug	Counts per Minutes per Microgram
CTAB	Cetyl-trimethyl ammonium bromide
DAG	Diacylglycerol
dNTP	Deoxynucleoside Tri Phosphates
dT	Deoxy Thymidine
DTT	Dithiothaeotol
DUM	Dorsal Unpaired Median
EDTA	Ethidium Diamino Tetra Acetic Acid
fmol/ul	Fenta mol per Microliter
G-proteins	Guanine Nucleotide Binding Proteins
GDP	Guaninenucleotide Di Phosphate

GTP	Guaninosine Tri Phosphate
Hz	Hertz
IP_3	Inositol Tri Phosphate
kb	Kilobases
kDa	Kilodalton
LB media	Lauria-Bertoni Media
M	Mole
$MgSO_4$	Magnesium Sulfide
mg/ml	Milligram per Milliliter
ml	Milliliter
mRNA	Messenger Ribonucleic Acid
mM	Millimolar
mm	Millimeter
NaCl	Sodium Chloride
nm	Nanometer
ng/ul	Nanogram per Microliter
^{32}P	Radiolabelled Phosphorous
pmol	Picomole
RPM	Revolutions per Minute
SDS	Sodium Doedocyl Sulfate
SSPE	Sodium Chloride, Sodium Phosphate, EDTA

TE	Tris Ethidium Diamino Tetra Acetic Acid
TEMED	N, N. N', N'- Tetra Methyl Ethylene Diamine
TPE	Tris Phosphate EDTA
U	Units
ug/ul	Microgram per Microliter
ul	Microliter
UV-light	Ultra Violet light

REFERENCES

Adamo, S. A., Linn, C. E., Hoy, R. R. (1995) The role of neurohormonal octopamine during 'Fight or Flight' behavior in the field cricket *Gryllus bimaculatus*. Journal of Experimental Biology, 198:1691-1700.

Arakawa, S., Gocayne, J., McCombie, W. R., Urquhart, D. A. (1990) Cloning, localization, and permanent expression of a *Drosophila* octopamine receptor. Neuron, 2:343-354.

Calzone, F. J., Britten, R. J., Davidson, E., H. (1987) In Methods in enzymology, Mapping of gene transcript by nuclease protection assays and cDNA primer extension. Editors: S. Berger and A. R. Kimmel, New York: Academic press, pp.611-632.

Dohlman, H.G., Thorner, J., Caron, M.G., Lefkowitz, R.J. (1991) Model systems for the study of seven-transmembrane-segment receptors. Annual Review of Biochemistry, 60:653-688.

Duzic, E., Coupry, I., Downing, S., Lanier, S.M. (1992) Factors determining specificity of signal transduction by guanine nucleotide-binding protein-coupled receptors. Coupling of alpha 2-adrenergic receptor subtypes to distinct G-proteins. Journal of Biological Chemistry, 267(14):9844-9851.

Evans, P. D. (1993) Molecular studies on insect octopamine receptors. In Pichon (ed.): Comparative Molecular Neurobiology. Boston: Verlag Birkhauser, pp. 286-296.

Evans, P. and Robb, S. (1993) Octopamine receptors subtypes and their modes of action. Neurochemical Research, 18: 869-874.

Evans, P. D. (1984) Studies on the mode of action of octopamine, 5-hydroxytryptamine and prolactin on a myogenic rhythm in the locust. Journal of Experimental Biology, 110:231-251.

Evans, P. D. (1981) Multiple receptor types for octopamine in locust. Journal of Physiology, 318:99-122.

Evans, P. D. (1980) Biogenic amines in the insect nervous system. Advance Insect Physiology, 15: 317-473.

Feinberg, A. P., Vogelstein, B. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Analytical biochemistry, 137:6-13

Kaziro, Y., Itoh, H., Nakafuku, M., Satoh, T. (1991) Structure and function of signal transducing GTP-binding proteins. Annual Review of Biochemistry, 60:349-400.

Koblika, B. (1992) Adrenergic receptors as model for G-protein- coupled receptors. Annual Review of Neuroscience, 15: 87-114.

Manfioletti, G., Schneider, C. (1988) A new and fast method for preparing high quality lambda DNA suitable for sequencing. Nucleic Acid Research, 16:2873-2884.

Nathanson, J. A. (1979) Octopamine receptors, adenosine 3', 5'- monophosphate, and neural control of firefly flashing. Science, 203:65-68.

O'Dell K. M. C. (1993) The effect of the inactive mutation on longevity, sex, rhythm and resistance to p-Cresol in *Drosophila melanogaster*. Heredity, 70: 393-399.

O'Dowd, B. F., Lefkowitz, R. J., Caron, M. G. (1989) Structure of the adrenergic and related receptors. Annual Review of Neuroscience, 12: 67-83.

Orchard, I. (1982) Octopamine in insects: neurotransmitter, neurohormone, and neuromodulator. Canadian Journal of Zoology, 60:659-669.

Orchard, I., Loughton, B. G. (1981) The control of release of hyperlipaemic hormone from corpus cardiacum of Locasta migratoria. Comp. Biochem. Physiol., 68A:25-30.

Robb, S., Cheek, T. R., Hann, F. L., Hall, L. M., Midgley, J. M., Evans, P. D. (1994) Agonist-specific coupling of a clone *Drosophila* octopamine/ tyramine receptor to multiple second messenger systems, The EMBO Journal, 13: 1325-1330.

Robertson, H. A., Jurio A. V. (1976) Octopamine and some related noncatecholic amines in invertebrate nervous system. International Review of Neurobiology, 19:173-224.

Robertson, H. A. (1975) The innervation of the salivary gland of the moth, *Manduca sexta*: evidence that dopamine is the transmitter. Journal of Experimental Biology, 63:413-419

Robertson, H. A. Steele, J. E. (1974) Octopamine in insect central nervous system: distribution, biosynthesis, and possible physiological role. Journal of Physiology, 237:34P-35P.

Robertson, H. A. Steele, J. E. (1972) Activation of insect nerve cord phosphorylase by octopamine and adenosine 3', 5' -monophosphate. Journal of Neurochemistry, 19:1603-1606.

Roeder, T., Nathanson, J. A. (1993) Characterization of insect neuronal octopamine receptors (OA_3 receptors). Neurochemical Research, 18: 921-925.

Saudou, F., Amlaiky, N., Plassat, J. L., Borrelli, E. (1990) Cloning and characterization of a *Drosophila* tyramine receptor. The EMBO Journal, 9: 3611-3617.

Yoshikuni, M., Nagahma, Y. (1994) Involvement of an inhibitory G-protein in the signal transduction pathway of maturation-inducing hormone (17 alpha, 20 beta-dihydroxy-4-pregnen-3-one) action of rainbow trout (Oncorhynchus mykiss) oocytes. Developmental Biology, 166(2):615-622.