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Gang Wu

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CHARACTERIZATION OF cDNAs ENCODING POTENTIAL DROSOPHILA ACETYLCHOLINE RECEPTORS

A Thesis

Presented to the

Department of Biology

and the

Faculty of the Graduate College

University of Nebraska

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts

University of Nebraska at Omaha

by

Gang Wu

June 1994
THESIS ACCEPTANCE

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

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ABSTRACT

In the central nervous system (CNS) of insects and other invertebrates, acetylcholine (ACH) functions as a major neurotransmitter and acts upon a number of different types of receptors. One approach to the identification of acetylcholine receptors (AChR) is to use cross-homology between vertebrate and invertebrate AChRs. To this end, monoclonal antibodies (mAbs) to the well-characterized nicotinic vertebrate AChR that cross-react with Drosophila tissues have been developed. One of them, mAb 16.42, was used to screen a lambda-ZAP expression vector library in which Drosophila adult head cDNAs were inserted, and three cDNAs that may encode nAChRs were identified.

Because mAbs identify structural similarities, and the vertebrate AChR is a multifunctional protein complex, a multimeric membrane-spanning, ligand-gated ion channel and hence has a number of functions which might be conserved in other receptors, ion channels or integral membrane proteins, it is possible that this mAb may not have identified AChRs but other proteins having structural and presumably functional similarity to the nicotinic AChR. To determine whether the three cDNAs identified in fact derive from a locus encoding an nAChR or another functionally important protein that acts in
In the invertebrate nervous system, an analysis of them was performed. Southern analyses of the cDNAs have been used to demonstrate that each of the cDNAs is unique. Partial DNA sequence analyses have been used to gain insight into the potential nature of the product of the locus identified by a particular cDNA and has suggested that the cDNAs do not derive from known, previously sequenced genes. *In situ* hybridization of probable "sense" and "antisense" probes derived from the cDNAs with adult tissue sections has been used to relate the pattern of expression of the genes identified by the cDNAs to the pattern of mAb 16.42 cross-reactivity and verify that each cDNA may encode a product that would be found in an appropriate histological site. Finally, the identity of the chromosomal locus from which the cDNA(s) derives has been inferred from *in situ* hybridization to *Drosophila* polytene chromosomes. The results suggest that the three cDNAs identified with mAb 16.42 in a screen of an expression library derive from unique, novel loci that do not encode known *Drosophila* nAChRs but are expressed in patterns in the CNS of *Drosophila* similar to mAb 16.42 cross-reactivity.
To my beloved mother and father, my wife and my daughter
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>THESIS</td>
<td></td>
</tr>
<tr>
<td>ACCEPTANCE</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>iv</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>11</td>
</tr>
<tr>
<td>Excision of cDNAs from lambda-ZAP</td>
<td>11</td>
</tr>
<tr>
<td>Preparation of plasmid DNA</td>
<td>12</td>
</tr>
<tr>
<td>Developing Restriction Map of the cDNAs</td>
<td>13</td>
</tr>
<tr>
<td>Southern blot analysis</td>
<td>14</td>
</tr>
<tr>
<td>DNA Sequencing</td>
<td>17</td>
</tr>
<tr>
<td><em>In situ</em> hybridization to polytene chromosomes</td>
<td>17</td>
</tr>
<tr>
<td>mRNA Localization Using <em>In Situ</em> Hybridization</td>
<td>18</td>
</tr>
<tr>
<td>RESULTS</td>
<td>24</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>30</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>75</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Structural Model of Invertebrate nAChR Subunits</td>
<td>36</td>
</tr>
<tr>
<td>2.</td>
<td>Cross-Reactivity of mAb 16.42 in the <em>Drosophila</em> CNS</td>
<td>38</td>
</tr>
<tr>
<td>3.</td>
<td>Lambda-ZAP Vector Excision</td>
<td>40</td>
</tr>
<tr>
<td>4.</td>
<td>Map of the pBluescript II SK Vector</td>
<td>42</td>
</tr>
<tr>
<td>5.</td>
<td>Restriction Map of cDNAs 4-1, 8-1 and 10-1</td>
<td>44</td>
</tr>
<tr>
<td>6.</td>
<td>Southern Blot Analysis of cDNAs 4-1, 8-1 and 10-1</td>
<td>46</td>
</tr>
<tr>
<td>7.</td>
<td>Spatial Expression of the Locus Identified by cDNA 4-1</td>
<td>48</td>
</tr>
<tr>
<td>8.</td>
<td>Spatial Expression of the Locus Identified by cDNA 8-1</td>
<td>50</td>
</tr>
<tr>
<td>9.</td>
<td>Spatial Expression of the Locus Identified by cDNA 10-1</td>
<td>52</td>
</tr>
<tr>
<td>10.</td>
<td>Chromosomal Location of the Locus Identified by cDNA 4-1</td>
<td>54</td>
</tr>
<tr>
<td>11.</td>
<td>Chromosomal Location of the Locus Identified by cDNA 8-1</td>
<td>56</td>
</tr>
<tr>
<td>12.</td>
<td>Chromosomal Location of the Locus Identified by cDNA 10-1</td>
<td>58</td>
</tr>
<tr>
<td>13.</td>
<td>Partial (T7-end) DNA Sequence of cDNA 4-1</td>
<td>60</td>
</tr>
<tr>
<td>14.</td>
<td>Partial (T3-end) DNA Sequence of cDNA 4-1</td>
<td>62</td>
</tr>
<tr>
<td>15.</td>
<td>Partial (T7-end) DNA Sequence of cDNA 8-1</td>
<td>64</td>
</tr>
<tr>
<td>16.</td>
<td>Partial (T3-end) DNA Sequence of cDNA 8-1</td>
<td>66</td>
</tr>
<tr>
<td>17.</td>
<td>Partial (T7-end) DNA Sequence of cDNA 10-1</td>
<td>68</td>
</tr>
<tr>
<td>18.</td>
<td>Partial (T3-end) DNA Sequence of cDNA 10-1</td>
<td>70</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table | Page
--- | ---
I. Known *Drosophila* nAChR Loci | 71
II. Characterization of Cross-Reacting mAbs Directed Against The Vertebrate nAChR | 72
III. Position of Known *Drosophila* nAChR Genes Compared To cDNAs | 73
IV. Previously Characterized Genes In The Cytological Regions Of Loci Identified With mAb 16.42 | 74
INTRODUCTION

In the central nervous system (CNS) of insects and other invertebrates, acetylcholine (ACh) functions as a major neurotransmitter and acts upon a number of different types of receptors (Callec, 1974; Gerschenfeld, 1973; Kehoe et al., 1976; Pitman, 1971). As early as 1914, Dale reported that in vertebrates, acetylcholine acts at two distinct receptors. These receptors are differentiated by the spectrum of pharmacological agents that are able to act on them as agonists and antagonists. In particular, the ability of one class of receptor to bind nicotine and another to bind muscarine has led to their designation as nicotinic and muscarinic receptors, respectively. Modern biochemical and molecular biological techniques have allowed intense study of the molecular biology, synthesis, regulation, structure and function of these receptors. Indeed, extensive studies pioneered by Changeux and colleagues (Changeux, 1992) on the nicotinic acetylcholine receptor of Torpedo have allowed it to be among the best characterized neurotransmitter receptors.

Nicotinic acetylcholine receptors (nAChR) belong to a class of ligand-gated ion channel neurotransmitter receptors that are members of the superfamily of neurotransmitter receptors that also include the GABA-A and glycine receptors.
(Barnard et al., 1987). These receptors are composed of multiple homologous subunits. At the vertebrate neuromuscular junction, where nAChRs have been best characterized, the subunits of the nicotinic receptors have been designated ∈, β, γ and δ (Conti-Tronconi et al., 1982). The native Torpedo receptor is a pentamer composed of two ∈, one β, one γ and one δ subunits. Each subunit possesses four transmembrane domains, as well as glycosylated extracellular and intracellular domains. When a molecule of acetylcholine binds to the extracellular domain of each of the ∈-subunits, a conformation change in the receptor occurs, opening an ion channel formed by homologous transmembrane domains of each of the subunits. In this way, extracellular Na⁺ passes into and intracellular K⁺ out of the cell.

α-Bungarotoxin (α-Btx) is a component of the venom of the snake Bungarus multicinctus and acts as a highly potent antagonist to vertebrate muscle nAChRs. Because it has the property of binding the α-subunit of the nAChR essentially irreversibly, it has been instrumental in allowing the initial purification of the vertebrate nAChR at the neuromuscular junction, as well as the initial pharmacological characterization of putative nAChRs in the vertebrate CNS and in invertebrates (Dudai, 1977, 1978; Hall et al., 1975; Rudloff, 1978). In insects, neuronal nAChRs are blocked by α-
Btx (see Breer and Satelle, 1987 for a review) and high levels of α-Btx binding sites are found throughout the ganglionic CNS (Dudai and Amsterdam, 1977). The existence of high-affinity α-Btx binding sites with nicotinic specificity suggests that α-Btx in insects bind to neuronal nAChRs (Rudloff, 1978). However, there is also some evidence that α-Btx can bind to other, non-nAChR proteins in both vertebrates and invertebrates. Therefore, there is the formal possibility that not all α-Btx binding proteins are nAChRs and it has been shown that not all nAChRs bind α-Btx (Bossy et al., 1988), including some in *Drosophila*.

Invertebrate nAChRs remain much less well characterized than vertebrate nAChRs. Molecular characterization of the genes for invertebrate subunits has allowed their classification into two different types of subunits: α-like and β, or non-α-like (Whiting et al., 1987; Cooper et al., 1991). The α-like ligand-binding subunits are characterized by two consecutive cysteines preceding the first transmembrane region (M1), while structural subunits lacking the two cysteines are named β-like or non-α subunits. Like the vertebrate receptor, the invertebrate receptor is thought to be composed of five subunits that are oriented around a central cation channel. All known subunits are membrane-spanning glycoproteins, show significant sequence similarity
with each other and appear to have a similar structural organization. Each of the subunits contain four hydrophobic membrane spanning domains (M1, M2, M3, M4) of which M2 is the most highly conserved and lines the cation channel. For each subunit, the N-terminus is extracellular, and the short extracellular C-terminal tail is highly variable both in sequence and in length (Figure 1; see Gundelfinger et al., 1992 for review).

The existence of multiple classes of nAChR subunits in insect nervous systems, as in their vertebrate counterparts, suggest that these receptors are composed of a highly heterogeneous group of proteins. The use of DNA probes from vertebrate nAChR subunits to screen Drosophila genomic and cDNA libraries has led to the isolation of a family of five different putative subunits of Drosophila nAChRs (Table I). The cDNA and the gene for the first non-α-like subunit from Drosophila (referred to as the AChR protein of Drosophila, abbreviated ARD), was identified by its cross-hybridization with the cDNA encoding the γ-subunit of the nAChR from the electroplax of the Torpedo californica (Hermans-Borgmeyer et al., 1986). The ARD possess 33-47% homology with vertebrate muscle and neuronal nAChR polypeptides and exhibits the structural features of a non-ligand binding subunit. The corresponding ard gene has seven exons whose exon-intron
boundaries correspond to those found in vertebrate nAChR genes (Sawruk et al., 1988). The chicken neuronal α2-subunit gene was used to isolate the gene of the first α-like subunit (ALS) (Bossy et al., 1988). Later, another two α-like subunits [(Drosophila α-like subunits 2 and 3 (Da2 and Da3)] were identified by Schmitt and co-workers and Gundelfinger and co-workers (Baumann et al., 1990; Jonas et al., 1990; Sawruk et al., 1990; Mülhardt et al., unpublished results). One additional structural nAChR subunit from the Drosophila nervous system [the second β-like subunit of Drosophila (SBD)] was reported by Sawruk and co-workers (Sawruk, 1990). This identification of multiple genes encoding nAChR-like proteins in Drosophila suggests that there is a remarkable heterogeneity for these receptors in invertebrates. This is in accord with the detection of additional nAChR subtypes in insects at both the pharmacological and physiological level (Gundelfinger, 1992).

The heterogeneity of subunits for putative nAChRs in invertebrates has led to speculation as to whether the invertebrate nAChRs are more "primitive" than the vertebrate nAChRs. While simplicity could be arrived at by having fewer receptor subtypes, this does not appear to be the case. The possibility remains however, that single functional receptors may have a more primitive homo-oligomeric quaternary
structure. It remains an open question as to whether invertebrate receptors function as hetero-oligomers or homo-oligomers or both, and how different cell types regulate the receptor composition that they express.

One of the most striking features found in all of the receptor subunits that have been characterized is the retention of four hydrophobic membrane spanning domains. It would appear that this structural similarity is retained across vast species boundaries for a functional purpose, presumably to form a functional cation channel with homologous M2 domains. The analysis of other retained structural features may allow insights into functions of these receptors that must be retained throughout evolution as well. Indeed, an understanding of the utilization and alteration of retained structural and functional features should provide a unique perspective in which to consider the evolution of the presumably more complex, vertebrate nAChRs.

One approach to experimentally investigate this issue is to utilize monoclonal antibodies (mAbs) designed against specific epitopes on the vertebrate receptor to probe the structural similarity of invertebrate receptors or other functionally-related molecules. In principle, well-characterized monoclonal antibodies against the vertebrate nAChR could be utilized as probes to identify cross-reacting antigens having structural similarity to the vertebrate nAChR.
If, like the retention of four hydrophobic membrane spanning domains, structural similarity is retained for a functional purpose, then the identification and characterization of such cross-reacting antigens and the genes encoding them should provide unique insights into evolutionary relationships between the antigens that have been identified and the vertebrate nAChR. This method also may provide an alternative approach to the identification of novel nAChRs in invertebrates.

To this end, monoclonal antibodies to the well-characterized vertebrate nAChR that cross-react with Drosophila tissues were developed (Chase et al., 1986). A set of monoclonal antibodies (Table II) directed against the Torpedo californica electroplax receptor were used to identify potential cross-reacting antigens in Drosophila. Two mAbs showed prominent binding within the CNS (Figure 2). One mAb, which is directed against an epitope on the α-subunit from Torpedo, binds predominantly to axonal tracts. Another mAb, which is directed against the β-subunit from Torpedo, binds predominantly to neuropil throughout the Drosophila CNS in a pattern that is like that of markers for cholinergic function (α-Btx binding; acetylcholinesterase and choline-acetyltransferase activity). Several other mAbs directed against different epitopes on the vertebrate α-subunit cross-
react with sensory elements in the peripheral nervous system as well as areas of neuropil exhibiting cholinergic function. None of the mAbs used in the study appeared to cross-react with the α-Btx binding component in the Drosophila CNS, as none were able to immunoprecipitate α-Btx binding material from Drosophila membrane extracts. These mAbs therefore identify antigens within the Drosophila nervous system that share structural features with the vertebrate nAChR, but do not identify nAChR subunits that bind α-Btx (i.e., known α-like subunits).

Because mAbs identify structural similarities, and the vertebrate AChR is a multifunctional protein complex, a multimeric membrane-spanning, ligand-gated ion channel and hence has a number of functions which might be conserved in other receptors, ion channels or integral membrane proteins, it is possible that mAbs may not have identified AChRs but other proteins having structural and presumably functional similarity to the nicotinic AChR. To address the nature of the Drosophila antigens that were identified with one of these mAbs, mAb 27.1A.16.42 (hereafter referred to as mAb 16.42), a lambda-ZAP (Strategene) expression library in which Drosophila adult head cDNAs were inserted was screened (Slominski and Chase, unpublished results). After infection of lambda-ZAP into E. coli, fusion proteins resulting from the insertion of the cDNA adjacent to a lacZ gene were produced by inducing a
lac promoter upstream of the inserted cDNA. Proteins produced in individual phage plaques were bound on a membrane, the membrane probed with the mAb 16.42 and binding sites detected by indirect immuno-histochemistry. Three cDNAs were identified that, when expressed in *E. coli*, consistently produced fusion proteins that cross-reacted with mAb 16.42.

This study presents analyses that address whether the three cDNAs identified using mAb 16.42 derive from one or more loci encoding a known nAChR or derive from separate loci, presumably encoding either another nAChR subunit or a distinct, functionally important protein that acts in the invertebrate nervous system. Southern analyses of the cDNAs have been used to demonstrate that each of the cDNAs is unique. Partial DNA sequence analyses have been used to gain insight into the potential nature of the product of the locus identified by a particular cDNA as well as whether the cDNAs derive from known, previously sequenced genes. *In situ* hybridization of probable "sense" and "antisense" probes derived from the cDNAs with adult tissue sections has been used to relate the pattern of expression of the genes identified by the cDNAs to the pattern of mAb 16.42 cross-reactivity and verify that each cDNA may encode a product that would be found in an appropriate histological site. Finally, the identity of the chromosomal locus from which the cDNA(s) derives has been inferred from *in situ* hybridization to
*Drosophila* polytene chromosomes. This information has been used with literature searches to identify whether any of the cDNAs derive from known *Drosophila* nAChRs, as well as to assess whether extant mutants that map in the region of the identified loci might correlate with genes that have been identified by mAb 16.42. Taken together, these analyses provide a foundation on which to consider the nature of the cross-reactivity of mAb 16.42, and on which to consider whether the cDNAs identified by mAb 16.42 may encode functionally important neural proteins that warrant further study.
MATERIALS AND METHODS

Excision of cDNAs from lambda-ZAP

As it is significantly easier to analyze plasmid inserts than phage inserts, the cDNAs that were initially isolated as Lambda-ZAP (Figure 3) clones were excised from Lambda-ZAP as pBluescript phagemids, recovered as plasmids (Figure 4) and then grown for further analysis. This was accomplished using the ExAssist-SOLR system (Strategene) which is designed to allow efficient excision of the pBluescript phagemid from Lambda-ZAP vectors.

XL-1 Blue cells were streaked out on LB (Sambrook et al., 1989) agar plates containing tetracycline (12.5 μg/ml) and SOLR cells were streaked out on LB plates containing kanamycin 50 μg/ml and incubated at 32°C overnight. Colonies taken from these freshly streaked plates were then used to inoculate overnight cultures. After using the OD₆₀₀ of the cultures to estimate their density, they were diluted with 10 mM MgSO₄ to a concentration of 10¹⁰ cells/ml. To produce filamentous phagemid particles that will eventually be recovered as plasmids, 1 ml of the XL-1 Blue cells was co-infected with 10⁷ lambda-ZAP bacteriophage bearing a particular cDNA and 10⁷ ExAssist helper phage. After 15 minutes at 37°C to allow the phage to adsorb, 20 ml LB was added and the culture grown
with shaking for 3 hours at 37°C. Growth was stopped by heat shock at 65°C for 20 minutes, the cellular debris spun out and the supernatant bearing the phagemid-cDNAs as well as ExAssist helper phage was stored at 4°C. To recover the phagemid-cDNAs as plasmids, 1 μl of this supernatant was used to inoculate 20 μl of SOLR cells in 180 μl 10 mM MgSO₄, and after incubation at 37°C for 15 minutes, 100 μl was plated on LB-ampicillin (100 μg/ml) plates and incubated overnight. In the SOLR cells, the ExAssist phage are unable to replicate, contaminating Lambda-ZAP phage (from the first inoculation) are unable be adsorbed, and the phagemids are able to be recovered based on ampicillin selection. Single colonies from these plates were therefore picked and plasmids bearing cDNA inserts were recovered.

**Preparation of plasmid DNA.**

Single colonies from the LB-ampicillin plates described above were inoculated into a 1-liter flask containing 100 ml LB and 100 μg/ml ampicillin, and grown overnight with shaking at 200-300 RPM at 37°C. The cells were transferred into a 250 ml bottle and pelleted in a chilled GSA rotor by spinning at 5,000 RPM for 5 minutes. The cells were resuspended in 1.2 ml of 0.01% glucose, 25 mM Tris-HCl, pH 8.0, 1 mM EDTA, transferred to a 35 ml snap cap tube and lysed by adding approximately 0.5 mg lysozyme and incubating on ice for 15
minutes. Bacterial chromosomal proteins and DNA and membranous material was precipitated by adding 4.8 ml of 0.2 N NaOH, 1% SDS, placing the tube on ice for 10 minutes, adding 4 ml of 25% potassium acetate, 20% glacial acetic acid and incubating the tube an additional 10 minutes on ice. After pelleting this material by spinning the tube at 10,000 RPM in chilled SS-34 rotor for 30 minutes, RNA was degraded by treating the supernatant with RNAse A (60 µl of 1 mg/ml) at 37°C for 30 minutes and remaining proteins extracted with phenol-chloroform. To obtain plasmid DNA, 0.6 volume of isopropanol was added and the DNA pelleted in a 10 minute, 10,000 RPM spin in a SS-34 rotor. After dissolving the DNA in 0.4 ml TE (10 mM Tris-HCl, pH 7.8, 1 mM EDTA), it was transferred to a microfuge tube and selectively re-precipitated on ice for 1 hour after adding 85 µl 5 M NaCl and 0.5 ml 13% PEG 8000. The pellet was recovered by spinning the tube 10 minutes in a microfuge, centrifuged at 12,000xg, washed in 70% ethanol, dried and resuspended in 400 µl TE. The yield and purity of the DNA were determined from measurements of the OD<sub>260</sub> and OD<sub>280</sub>.

**Developing A Restriction Map of the cDNAs**

To ascertain which of a set of available restriction enzymes cut within the cDNAs, each was individually treated with EcoRI, XhoI, HindIII, BamHI, SacI, SpeI, PstI, SalI, KpnI
and PvuII. Appropriate double digests were performed to elucidate the locations of restriction enzyme cleavage sites when an enzyme cut within the cDNA more than once. The products of the restriction digests were separated on an 18 cm long, 2% agarose, 1X TPE (Sambrook et al., 1989) gel run at 40 volts overnight, visualized by adding 0.4 μg/ml ethidium bromide to the gel and photographed under short-wave UV light using Polaroid Type 667 film.

Southern blot analysis

Restriction Fragment Separation and Southern Transfer. To address whether the cDNAs shared homology, Southern analyses were performed. Three sets of identical restriction digestions were set up with each group containing digests of the three cDNAs. 1 μg of each cDNA (coded 4-1, 8-1 and 10-1) was digested in triplicate with the restriction enzymes PvuII and EcoRI. After restriction digestes were complete, samples were loaded in three groups, each containing a 4-1, 8-1 and 10-1 digest, on a 2% agarose gel and restriction fragments separated and visualized as described above. The fragments were denatured by treating the gel twice in 0.5 M NaOH and 1.5 M NaCl for 30 minutes each and then the gel neutralized by immersing it twice in 1.5 M NaCl and 1 M Tris, pH 7.4 for 30 minutes each. The fragments were transferred to MagnaNT (Micron Separations, Inc.) nitrocellulose membrane by using
the Possiblot pressure blotter (Stratagene) using 10X SSPE as blotting buffer following the manufacturers recommendations. After transfer, the location of the lanes of each digest was marked on the membrane, so that each set of digests (including 4-1, 8-1, and 10-1) could be separated later. Fragments were then covalently cross-linked to the membrane with ultraviolet light (254 nm) for thirty seconds at 12,000 microjoules/cm² in a Stratalinker (Stratagene) followed by baking under vacuum at 80°C for 1 hour.

**Preparation of DNA probes.** To generate ^32^P-labeled cDNA probes for use in Southern analysis, cDNAs inserted into pBluescript were excised from the plasmid vector using appropriate restriction enzymes, purified by gel electrophoresis and fragment isolation and uniformly labeled using a random-priming labeling method. Three μg of each cDNA was digested with enzymes that would release the cDNA insert (4-1 with EcoRI + XhoI, 8-1 and 10-1 with EcoRI) and restriction fragments separated on a 1X TPE 2% agarose gel containing ethidium bromide (0.4 μg/ml). A positively charged nitrocellulose NA-45 (Schleicher and Schull) membrane was cut 1 mm wider and deeper than the lane in which the fragments had been separated and, to facilitate reversible binding of DNA fragments, treated sequentially in 10 mM EDTA, pH 8.0 (5 minutes), 0.5 N NaOH, (5 minutes) and 6 changes of sterile
nanopure water (30 seconds each). The membrane was placed in a position directly in front of the band to be isolated and the DNA run onto the membrane by electrophoresis for 1.5 hours at 50 volts. After removing the membrane from the gel, adhering agarose fragments were removed by rinsing it in a low salt buffer (0.15 M NaCl, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA) and the DNA eluted by treating it, in a 1.5 ml eppendorf tube, with two 200 μl aliquots of a high salt buffer (50 mM arginine, 1 M NaCl) at 65°C for 30 minutes each. The DNA was precipitated at -70°C for 30 minutes after adding 10 μg of carrier tRNA, 80 μl 10 M ammonium acetate, and 800 μl 100% ethanol and recovered after spinning at 12,000 g in a microfuge. The resulting pellet was rinsed in 70% ethanol, dried, and resuspended in 20 μl of TE.

Probe-labeling and hybridization. The probes (4-1, 8-1, and 10-1) were labeled using °P-dATP and a random-priming method (Ambion, DECAprime II DNA labeling kit). The three identical Southern blots bearing digests of each cDNA were wetted with 2X SSPE, and placed in separate hybridization chambers containing 10 ml of pre-hybridization solution (50% formamide, 5X SSPE, 0.1 %SDS, 1X Denhardt’s Reagent [0.02% ficoll-400, 0.02% polyvinylpyrolidone and 0.02% bovine serum albumin, fraction V] and 100 μg/ml denatured, sheared fish sperm DNA). The chambers were placed in a hybridization oven and slowly rotated for 1 hour at 42°C. A different heat-
denatured $^{32}$P-labeled cDNA probe was then added to each of the three hybridization chambers, and hybridization allowed to proceed for 16 hours at 42°C with slow rotation. Excess probe was then removed and the membranes were washed in 0.1% SDS, 0.1X SSPE at 50°C, with three changes of solution after successive 20 minutes intervals. The membranes were wrapped in Saran Wrap and exposed to Kodak XRP-5 X-ray film for varying lengths of time, with 30 minutes of exposure providing good images. The resulting autoradiograms were used to identify cross-reacting DNA fragments.

**DNA Sequencing**

The three cDNAs, cloned into pBluescript were sent to the University of Nebraska - Lincoln DNA sequencing facility for automated DNA sequencing. Sequence was obtained by using the T3 and T7 primers flanking the cDNA inserts. Analysis of the DNA sequences was performed using the GCG DNA sequence analysis program at the University of Nebraska Medical Center (Omaha).

**In situ hybridization to polytene chromosomes**

Chromosomal *in situ* hybridizations were performed essentially as described by Laverty (1991). Briefly, late-third instar wild-type (CS) animals were harvested, salivary-glands dissected and fixed in a lactic acid/acetic acid
fixative and squashed on clean glass slides. Uniformly labeled biotinylated cDNA probes were prepared using a nick-translation method and biotin-11-dUTP (ENZO Biochemicals), hybridized to the squashed chromosomes and detected using a DETEK-1 HRP kit (ENZO Biochemicals) using 3'-3'-diaminobenzidine (GIBCO-BRL) as a substrate. The chromosomes were lightly counterstained using Geimsa stain in 10 mM sodium phosphate buffer (pH 6.8), dried and mounted using Permount (Fisher). The chromosomes were viewed under phase-contrast optics at using a 10X ocular and 40X and 100X (Oil immersion) objectives on a Zeiss standard microscope. Photographs were taken using Kodak Kodachrome ASA 100 film with a 80A filter to provide color correction due to tungsten illumination.

**mRNA Localization Using In Situ Hybridization**

The protocols for mRNA localization using in situ hybridization were adapted from a number of protocols; including those described in literature provided by Boehringer Mannheim.

**Animal Preparation and Sectioning.** Wild-type Drosophila adult male and female animals raised at 25°C were collected within several hours of eclosion. To assess whether mRNA expression varied as a function of age or sex, the animals were divided into groups according to sex and then aged for either one or seven days prior to fixation. The animals were
fixed using a modification of a procedure provided by Paul Ingham (Cambridge, UK). The fixative used was freshly prepared Carnoy's fixative for insects (6 parts isopropanol, 3 parts chloroform, 1 part formic acid). In order to allow penetration of the fixative, the mouthparts, wings and legs were removed. After one hour of fixation, the animals were transferred through a graded ethanol series (20 minutes each in 50%, 70%, 95%, 2x100%), cleared in xylene and transferred to embedding molds containing molten paraffin (60°C). After paraffin embedding overnight, the specimens were oriented and the blocks allowed to cool. Serial, 10 µm thick sections were cut on a microtome and placed on several drops of DEPC treated water on poly-L-lysine coated slides. The sections were allowed to expand for a few minutes at 44°C on a slide warmer, excess water was drained and the sections were allowed to dry on the slide warmer overnight.

**Preparation of digoxigenin-labeled DNA probes.** Probes were prepared by linearizing the plasmid DNA through cleavage at either the T3 or T7 end of the cDNA insert (Figure 4), and then using Taq DNA polymerase and the T7 or T3 primer, respectively, to generate digoxigenin-labeled sense or antisense single-stranded DNA probes. To linearize the "dual promotor vector DNA" containing the cDNA, two restriction digestions were set up: 2 µg of each cDNA (4-1, 8-1, or 10-1) was cut with either Hind III (which cleaves at the T7 end of
cDNA insert, allowing T3 primed replication) or with Sac I (which cleaves at the T3 end of cDNA, allowing T7 primed replication) in a total volume was 40 µl at 37°C for 1.5 hours. After heat inactivating the restriction enzymes at 65°C for 15 minutes, the DNAs were used in a replication reaction using Taq DNA polymerase, the appropriate (T3 or T7) primer, a digoxigenin-labeled substrate and thermal cycling. The replication reaction (a "unidirectional polymerase chain reaction") was carried out in 0.5 ml polypropylene tubes in a Coy thermal cycler, and contained 2.5 µl 10X buffer mix (Boehringer Mannheim; 0.5 M KCl, 0.1 M Tris-HCl, pH 8.3, 15 mM MgCl₂, 0.01% gelatin), 5 µl 5X dNTP mix (Boehringer Mannheim; 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM Digoxigenin-11-dUTP), 1.5 µl 25 mM MgCl₂, 7 µl primer (T3 or T7, Promega; diluted in sterile water to 21 ng/µl), 8 µl template DNA (diluted to 50 ng/µl) and 1 µl H₂O. After 40 µl sterile mineral oil was overlain on the reaction mixture, it was centrifuged briefly and then boiled for 5 minutes to denature the plasmid DNA template. 2.0 µl Taq DNA Polymerase (1.25 U) was added and the replication reaction was carried out by cycling the reaction mixture in the Coy thermal cycler as follows: 95°C for 45 seconds (denaturation), 55°C for 30 seconds (primer annealing), 72°C for 1 minutes (elongation of replicating DNA strand). After the thermal cycling run, 75 µl of distilled water was added and the sample spun briefly in a
microfuge. 90 μl of the reaction mixture was removed from beneath the oil, and the DNA probe precipitated at -70°C overnight after adding 10 μl carrier tRNA, NaCl to 0.1 M and 3 volumes of 100% ethanol. The resulting pellet was washed with 70% ethanol, dried and resuspended by adding 30 μl 20X SSC (DEPC treated), 30 μl 0.1 M Tris-HCl, pH 7.6, and 7.5 μl salmon sperm DNA (10 mg/ml). After the labeled DNA dissolved, the solution was boiled (using a temperature block) for 60 minutes to shear the probe to a size (50-100 bases) appropriate for use in the in situ hybridization procedure. After boiling, the probe was chilled on ice and 150 μl formamide, 7.5 μl sodium pyrophosphate solution (200mg/ml) and 75 μl Denhardt’s solution was added. The probe was then stored at -20°C until use.

Pretreatments of sectioned material. The sections were dewaxed and hydrated by immersing the slides in the following wash series: 2x15 minutes in xylene, 2x15 minutes in 100% ethanol, 10 minutes in 95% ethanol, 10 minutes in 70% ethanol, 10 minutes in 50% ethanol and 2x10 minutes in DEPC water. The slides were then treated with 0.2 N HCl for 20 minutes at room temperature and immersed in 0.3% Triton X-100 in 1X PBS (10X PBS contains, per liter, 80 gm NaCl, 2 gm KCl, 14.4 gm Na₂HPO₄, 2.4 gm KH₂PO₄, pH adjusted to 7.4 with 0.1 N HCl) to wet the slides. To minimize non-specific binding of the probe and allow the probe access to the tissue, the sections were
treated with 10 μg/ml of Proteinase K in 0.1 M Tris-HCl, pH 8, 50 mM EDTA for 30 minutes at 37°C. To stop this protease reaction, the sections were rinsed in 0.2% glycine and post-fixed for 5 minutes in freshly prepared 4% paraformaldehyde (pH 7.4) and then placed in 1X PBS. To help minimize non-specific binding, the sections were acetylated by treatment in 0.25% acetic anhydride containing 0.1 M triethanolamine for 10 minutes and then placed in 1X PBS.

_Hybridization and Immunological Detection._ After prehybridization in 50% formamide and 50% 2X SSC (Sambrook et al., 1989) for 2 hours at 37°C, 50 μl of probe was overlaid onto each slide and the slides covered with a parafilm coverslip (note that a siliconized coverslip could also be used, and might have been better to use). Hybridization was allowed to proceed overnight (16 hours) at 42°C in a sealed humid box. After the parafilm coverslip was removed by immersing the slide in 4X SSC, the sections were washed as follows: twice in 2X SSC for 15 minutes at room temperature, once in 0.1X SSC for 20 minutes at 42°C, and once in 0.1X SSC for 10 minutes at room temperature. The slides were then rinsed in PBT (1X PBS, 0.1% Tween 20) and the sections overlaid with 100 μl of 1:500 (in PBT) diluted alkaline-phosphatase conjugated anti-digoxigenin antibody (Boehinger Mannheim) for 2 hours at room temperature. Excess primary antibody was removed by washing the slides in PBT 4 times,
15 minutes each. The slides were then washed three times for five minutes each in a solution containing 100 mM NaCl; 50 mM MgCl₂; 100 mM Tris, pH 9.5; 1 mM levamisole (to inhibit endogenous lysosomal alkaline phosphatases); 0.1% Tween 20 and then overlaid with a 1 ml of the above solution to which Nitro-Blue tetrazolium and X-phosphate, substrates for alkaline phosphatase, had been added according to manufacturer’s instructions (Boehringer Mannheim Genius Kit). The detection reaction was allowed to proceed overnight at room temperature and stopped by placing the slides in PBT. The sections were mounted in an aqueous mounting media (Kirkegaard & Perry Laboratories mounting media for fluorescent microscopy, Catalog #71-00-16), and viewed under bright field optics using 10X ocular and 10X, 16X and 40X objectives on a Zeiss Standard microscope. Photomicrographs were taken using Kodak Kodachrome ASA 100 film using a 80A filter to correct for color changes with tungsten filament illumination.
RESULTS

Characterization of cDNAs

To facilitate analysis of the three cDNAs identified with mAb 16.42, they were excised from lambda-ZAP and rescued as double-stranded plasmids able to express ampicillin resistance (pBluescript II SK). Restriction map analysis with ten restriction enzymes has shown that the three cDNAs are not identical (Figure 5). If they derive from one gene, they may either represent cDNA copies of alternatively-processed mRNAs (either by alternative RNA splicing, alternative transcription start sites, alternative poly-adenylation sites, or a combination of these) or different partial length cDNAs. Alternatively, they may derive from more than one gene. When digests of the three cDNAs were separated on an agarose gel, and replica Southern blots probed with each of the cDNA inserts, only fragments corresponding to the probe cDNA show hybridization (Figure 6). For example, probe 8-1 only hybridizes to cDNA 8-1, not 4-1 or 10-1. The hybridization signal in the 4-1 and 8-1 lanes of the blot probed with the 10-1 cDNA arises from contaminating vector sequences in the probe. The hybridization signal in the 8-1 and 10-1 lanes in the blot probed with the 4-1 cDNA is an artifact due to the processing of the blot itself, as it does not align with any
bands seen in the gel. Therefore, at the level of Southern analysis, the three cDNAs do not share significant homology, and thus are unlikely to derive from one gene. It is still possible that they derive from one locus, but do not share any exons.

In situ hybridization to adult fly tissue section

Given the result described above, one general concern is whether mAb 16.42 indeed recognizes three different gene products, or whether one or more of the cDNAs that was identified is a false positive. One test of the hypothesis that the three cDNAs are not false positives is that the loci they derive from should be expressed in a pattern consistent with the pattern of cross-reactivity of mAb 16.42.

Gene products expressed in adult Drosophila neuropil should be transcribed in the nuclei of the adult CNS cell bodies, namely in the cellular cortex surrounding the neuropil. Transcripts may also be found in the neuropil itself, if the transcripts are transported away from the cell bodies prior to being translated. Therefore, if the cDNAs indeed encode antigen recognized by mAb 16.42, they should be transcribed in many, if not all, regions of the CNS cortex, in other words, they should be transcribed in a pattern consistent with mAb 16.42 cross-reactivity.

To experimentally address this hypothesis, single-
stranded digoxigenin-labeled DNA probes were generated for each cDNA by making use of the T7 and T3 primers that flank the cDNA insert in pBluescript and unidirectional-PCR. The two different primers were used to create an anti-sense strand and a sense strand probe, of which the later should serve as a control. As transcription is directional (5' to 3') for each pair of probes deriving from one cDNA, one should hybridize to mRNA (i.e., it is an anti-sense probe) while the other should not hybridize (i.e., it is a sense probe). While it is not yet certain which strand is sense and which is anti-sense, preliminary sequence data (see below) suggest that the T3-primed probes are anti-sense probes, while the T7-primed probes are sense probes.

In a section through the brain hybridized with a T3-primed probe (antisense) for cDNA 4-1, there is intense signal over the cell bodies of the adult Drosophila CNS (Figure 7a), while in a similar section hybridized with a T7-primed probe (sense) for cDNA 4-1, there is only faint staining in the same cortical areas (Figure 7b). In other sections hybridized with T3-primed and T7-primed probes for both cDNA 8-1 and 10-1, the result are similar (Figures 8 and 9). The intensity of the signal for the three putative anti-sense probes are much more intense than the signals for the three putative sense probes. Therefore, the three cDNAs identified by mAb 16.42 appear to derive from loci transcribed in CNS cell bodies and mAb 16.42
may indeed bind to three distinct antigens expressed in neuropil.

There did not appear to be any qualitative or quantitative difference in the level of signals seen in the putative anti-sense probes among adults of different ages or sex-types. As can be noticed from comparison of Figures 7, 8 and 9, there may be some quantitative difference in the level of expression of the transcripts detected with the three different probes. Clarification of this issue will require further analysis however.

**In situ hybridization to chromosomes**

To analyze the loci identified by the cDNAs further, it is essential to know where these genes are located on chromosomes. This information allows inferences as to whether they may have been characterized genetically and in particular, facilitates an understanding of whether the cDNAs derive from loci that are known to encode nAChRs. Therefore, *in situ* hybridization to chromosomes was used to identify the cytological position of these loci.

When *in situ* hybridization to chromosomes was performed with probes from cDNAs 4-1, 8-1 and 10-1, hybridization signals were detected at positions 87A-B, 98C-F and 86D-E respectively (Table III; Figures 10, 11 and 12). To date, five genes which encode *Drosophila* nAChR subunits have been
reported. Comparison of the cytological position of the three cDNAs with that of these known fly nAChRs reveals that none of the cDNAs derive from known nAChR loci (Table III).

**Sequence analysis of cDNAs**

To address what products the loci identified by the cDNAs might encode, sequence analysis was initiated. Partial DNA sequence of the cDNAs was generated using the T3 and T7 primers flanking each cDNA insert (Figure 4). For cDNA 4-1, the 510 and 440 bases whose sequence was reliably obtained using the T7 and T3 primers, respectively, are shown in Figures 13 and 14. For cDNA 8-1, the 590 and 580 bases whose sequence was reliably obtained using the T7 and T3 primers, respectively, are shown in Figures 15 and 16. For cDNA 10-1, the 690 and 460 bases whose sequence was reliably obtained using the T7 and T3 primers, respectively, are shown in Figures 17 and 18.

While the sequencing data are incomplete, comparison of the sequences that were obtained for each cDNA with one another using the GCG DNA sequence analysis program revealed no regions of significant homology, indicating that each cDNA derives from a unique gene. Likewise, comparison of the obtained sequences to invertebrate sequences present in the Genbank (January 1994 version) database failed to reveal any identical sequences, and suggests that the loci identified by
the cDNAs are novel. Two small regions of homology with a known fly nAChR gene were found for cDNA 8-1 (Figure 15). Both of these regions are in non-coding regions of a nAChR gene, and the spatial relationship of the regions of homology in cDNA 8-1 is not like that in the nAChR gene. Therefore the sequence data are consistent with the results of the chromosomal localization of the loci identified by the cDNAs, and supports the conclusion that these cDNAs are not derived from known nAChR genes. The GCG program was also used to search the available sequence data for open reading frames, so that a possible protein product of these loci could be inferred. None of the sequences displayed a significantly long open reading frame, and thus the nature of the protein products of the loci that were identified with these cDNAs remains unknown.

In order to sequence each cDNA in its entirety, two sets of exonuclease III nested deletions were made for each cDNA (Sambrook et al, 1989). By making a set of nested deletions that sequentially delete increasingly greater portions of the cDNA starting near either the T3 or T7 ends of the cDNA, the complete sequence of both strands of each cDNA may be obtained. These deletions are currently being used to gather complete sequence data for each cDNA. With this data, more extensive analysis of these cDNAs will be possible.
This thesis has explored the basis for the cross-reactivity in *Drosophila* of a mAb that is directed to an epitope on the vertebrate nAChR. The three cDNAs that were identified using the mAb in a screen of an expression library derive from unique, novel loci that do not encode known *Drosophila* nAChRs. These loci are however expressed in a pattern consistent with the cross-reactivity of the mAb, which in turn is found in regions of cholinergic function. These data raise two intriguing questions. First, what is the structural basis for the shared cross-reactivity of the mAb with the nAChR and the products of these loci? Second, is there any functional relationship between the cross-reacting antigens and the vertebrate nAChR. More specifically, do these loci encode receptor subunits, in particular nAChR receptor subunits, or other proteins sharing a function with nAChR subunits.

The specificity of mAb 16.42 is to bind the β-subunit of the vertebrate nAChR (Chase et al., 1987). The fact that the mAb was initially generated against native, Triton-X extracted *Torpedo* nAChR suggests that the epitope it recognizes is a naturally occurring one. The nature of this epitope is clearly of interest to the current study. As the mAb does not
bind to the ligand-binding subunit of the receptor, it is not directed against the site of ACh binding. Also, as this particular mAb does not bind intact mouse muscle (Chase et al., 1987) and therefore does not bind to the ectodomain of the intact receptor, it is probably not directed to an externally accessible surface of the intact receptor. Therefore, the epitope the mAb recognizes on the β-subunit of the vertebrate nAChR may be a structure found on the cytoplasmic side of the receptor or a structure within a portion of one or more of the transmembrane domains.

The nAChR subunits have four hydrophobic membrane spanning domains (M1-M4). One of these, the M2 helix, is presumed to line the pore of the ion channel. If mAb 16.42 recognizes the transmembrane region, it may interact with that part of the protein that interacts with the membrane, and so it may be identifying transmembrane proteins in Drosophila. If the mAb recognizes part of the M2 portion of the transmembrane domain, the domain lining the cation channel itself, it may recognize structures in Drosophila that participate in forming an ion channel.

In both vertebrates and invertebrates, molecular and pharmacological characterization of the nAChRs has suggested that there is considerable heterogeneity in the types of subunits of the nAChR used within the CNS (Sawruk et al.,
In addition, the nAChRs are members of the superfamily of neurotransmitter receptors that include the GABA-A and glycine receptors. Subunits of these receptors are membrane-spanning glycoproteins having four transmembrane regions. The receptors themselves have similar structural organization and are all ligand-gated ion channels. Therefore, it is possible that mAb 16.42 recognizes either novel nAChRs or other members of this superfamily of neurotransmitter receptors.

Knowledge of the cytological position of the loci that have been identified with mAb 16.42 can be used to find out whether there are any known mutations in these regions that might correspond to functions important in the CNS, and in particular, correspond to known AChRs or other neurotransmitter receptors. A survey of loci in these regions, summarized in Table IV, reveals that at least one of the loci, that identified by 10-1, contains a gene that may be of considerable interest. This is Rst, one of the genes in cytological region 86D that was identified by cDNA 10-1. Mutations, now lost, had been found that were resistant to nicotine sulfate (Lindsley et al., 1992). It would therefore be of interest to re-generate such mutations, and ascertain whether they are at the locus identified by cDNA 10-1.

In all of the cytological regions identified, a number of loci affecting neural or muscular function have been found (Lindsley and Zimm, 1992). For some of these, sequence data
has been generated previously and extensive phenotypic and molecular analyses have been performed (e.g., svp). Searches of Genbank for homology with the cDNA sequences did not identify any of these loci. Therefore, it would appear that the cDNAs identified by mAb 16.42 do not derive from such loci. Nevertheless, it is still possible that the cDNAs derive from identified loci. Not all of the loci identified in these regions have been characterized molecularly, and some are identified only as a recessive-lethal complementation group for which little additional phenotypic characterization has been performed. Thus, it will be of interest to pursue further genetic studies in parallel with molecular investigations of the loci identified by these cDNAs.

Because of high specificity of mAbs in general, one might have expected that a single mAb would be directed solely to one protein. But in this study, the results suggest that the three cDNAs identified by mAb 16.42 derive from different, unique genes. This is presumably because they share a common structural epitope. This is consistent with several other reports that have suggested that certain receptors may share functional domains with other proteins (Downward et al., 1984; Mostov et al., 1984; Russell et al., 1984; Sudhof et al., 1985).

Using well-characterized monoclonal antibodies against the vertebrate nAChR has provided a useful tool to identify
cross-reacting antigens having structural similarity to the vertebrate nAChR. Also, they will be invaluable to probe the functions of the shared antigenic determinants in Drosophila, to identify potential AChRs and functionally similar proteins, indeed, to find new and interesting genes.
Figure 1

Structural Model of Invertebrate nAChR Subunits*

S-S: conserved disulfide bridge; M1 to M4: putative membrane-spanning α-helices; CHO: N-glycosylation site conserved among all neuronal nAChR subunits. The two adjacent cysteines which are characteristic of all ligand-binding nAChR α-subunits are indicated (HS).

*After Gundelfinger and Hess (1992)
Structural Model of nAChR Subunits

mAbs directed against different epitope domains on the vertebrate nAChR specifically cross-react with different subsets of *Drosophila* neural tissue. This figure shows the patterns of cross-reactivity of two mAbs on unfixed frozen sections and visualized by indirect immunofluorescence with rhodamine-conjugated secondary antibodies.

Panels a and b show phase contrast and fluorescent images, respectively, obtained using mAb 27.43.37.

Panels c and d show phase contrast and fluorescent images, respectively, obtained using mAb 16.42.

r-retina; la-lamina; m-medulla; lo-lobula; lp-lobula plate; 1-1st optic chiasma; 2-2nd optic chiasma; c-cellular cortex

*From Chase et al. (1987)*
After screening, lambda plaques recognized by the hybridization probe are isolated and allowed to infect cells which are co-infected with filamentous helper phage. Inside the cell, trans-acting proteins from the helper phage recognize two separate domains (Initiator and Terminator) positioned within the Lambda ZAP II vector arms. Both of these signals are recognized by the helper phage gene II protein and a new DNA strand is synthesized, displacing the existing strand. The displaced strand is circularized and packaged as a filamentous phage by the helper phage proteins, then packaged and secreted from the cell. pBluescript plasmids are recovered by infecting an F’ strain and plating on ampicillin plates, giving bacterial colonies.

*From Stratagene (1993)
1. Construct DNA library
2. Isolate positive clone

3. Excise the pBluescript phagemid containing the cloned DNA insert by co-infection with helper phage
The pBluescript II SK phagemid is a 2961 basepair phagemid derived from pUC19. The SK designation indicates the ploylinker is oriented such that lacZ transcription proceeds from Sac I to Kpn I.

LacZ: (lac promoter: 816-938 bp) This portion of the lacZ gene provides α-complementation for blue/white color selection of recombinant phagemids. An inducible lac promoter upstream from the lacZ 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.

*From Stratagene (1993)
pBluescript® II SK +/- 2.96 kb

T3 primer
5’ ATTAAACCTCACTAAAG 3’

SK primer
5’ TCTAGA CTAGTGATC 3’

BstXI II promoter 5’ BstXI II promoter 3’
SaeI SacII NotI XbaI BamHI SmaI EcoRI

KS Primer
5’ GCTATGGCAGCTGGAGC 3’

M13 -20 Primer
5’ GATATCA ATGGCTATTACGCGCGCTCACTGGCCGTCGTTTTACAA 3’

T7 promoter
5’ T7 promoter 3’

GCTATGGCAGCTGGAGC 3’

T7 Primer
5’ GATATCA ATGGCTATTACGCGCGCTCACTGGCCGTCGTTTTACAA 3’

M13 -20 Primer
5’ GATATCA ATGGCTATTACGCGCGCTCACTGGCCGTCGTTTTACAA 3’
Figure 5
Restriction Map of cDNAs 4-1, 8-1 and 10-1

Each cDNA was individually treated with EcoRI, XhoI, HindIII, BamHI, SacI, SpeI, PstI, SalI, KpnI and PvuII. The letters represent sites cut by corresponding restriction enzymes. Appropriate double digests were performed to elucidate the locations of restriction enzyme cleavage sites when an enzyme cut within the cDNA more than once. The maps of the three cDNA are different.
Restriction Map of cDNA's Isolated With mAb 16.42

**cDNA 4-1, 2.2 kb**

(X) P PT X T (E)

**cDNA 8-1, 2.6 kb**

(E) S P T TP P X TP (E)

**cDNA 10-1, 2.7 kb**

(E) P P T (E)

0.5 kb

E - Eco RI
P - Pvu II
X - Xho I
T - Pst I
S - Sal I
Figure 6
Southern Blot Analysis of cDNAs

Panel A shows the cDNA digested with EcoRI and PvuII. Panel B shows the digest blotted and probed with the indicated cDNA. The cDNAs do not share homology. For example, probe 8-1 only hybridized to cDNA 8-1, not 4-1 or 10-1.

The hybridization signal in the 8-1 and 10-1 lanes in the blot probed with the 4-1 cDNA is an artifact due to the processing of the blot itself, as it does not align with any bands seen in the gel.
Southern Analysis of cDNAs

Panel A: Plasmid DNA digested with Eco RI & Pvu II.

Panel B: Southern blot probed with the indicated cDNA.
Figure 7

Spatial Expression of the Locus Identified by cDNA 4-1

This figure shows *in situ* hybridization to tissue sections of adult *Drosophila*.

Panels A shows the pattern of hybridization of digoxigenin-labeled, T3 primed, single-stranded (anti-sense) probe from cDNA 4-1. There is intense signal over the cell bodies of the CNS. This staining pattern is consistent with the locus being transcribed in the cell bodies of the optic ganglia and their protein products being transported into axonal and synaptic regions.

Panels B serves as a control obtained with the T7 primed, (sense) probe from cDNA 4-1. There is only faint staining over the cell bodies.
Figure 8

Spatial Expression of the Locus Identified by cDNA 8-1

This figure shows *in situ* hybridization to tissue sections of adult *Drosophila*.

Panels A shows the pattern of hybridization of digoxigenin-labeled, T3 primed, single-stranded (anti-sense) probe from cDNA 8-1. There is intense signal over the cell bodies of the CNS. This staining pattern is consistent with the locus being transcribed in the cell bodies of the optic ganglia and its protein products being transported into axonal and synaptic regions.

Panels B is a control obtained with the T7 primed, (sense) probe from cDNA 8-1. There is much less staining over the cell bodies.
Figure 9

Spatial Expression of the Locus Identified by cDNA 10-1

This figure shows in situ hybridization to tissue sections of adult Drosophila.

Panels A shows the pattern of hybridization of digoxigenin-labeled, T3 primed, single-stranded (anti-sense) probe from cDNA 10-1. There is intense signal over the cell bodies of the CNS. This staining pattern is consistent with the locus being transcribed in the cell bodies of the optic ganglia and its protein products being transported into axonal and synaptic regions.

Panels B is control obtained with the T7 primed, (sense) probe from cDNA 10-1. There is almost no signal detected over the cell bodies.
Chromosomal Location of the Locus Identified by cDNA 4-1

Chromosome squashes were prepared from third-instar larval salivary glands and hybridized with a biotinylated probe prepared from the cDNA 4-1. The hybridization site was at position 87A-B (indicated by the arrow)
Figure 11

Chromosomal Location of the Locus Identified by cDNA 8-1

Chromosome squashes were prepared from third-instar larval salivary glands and hybridized with a biotinylated probe prepared from the cDNA 8-1. Arrow indicates site of hybridization at 98C-E.
Figure 12
Chromosomal Location of the Locus Identified by cDNA 10-1

Chromosome squashes were prepared from third-instar larval salivary glands and hybridized with a biotinylated probe prepared from the cDNA 10-1. Arrow indicates site of hybridization at 86D–E.
Figure 13
Partial (T7-end) DNA sequence of cDNA 4-1

The three cDNAs, cloned into pBluescript were sent to the University of Nebraska - Lincoln DNA sequencing facility for automated DNA sequencing.

This figure shows partial sequence was obtained by using T7 primers flanking the cDNA 4-1 insert.
Partial (T7-end) DNA Sequence of CDNA 4-1

1  AAAGAAACAC TACCACGTGG GTGGATATCT TGTGGATGGA AATCGACATG
51  ATATCGAGCA CTGCTACTAT CATGGCACC TGAAAGGATTA TCCCCGAGCC
101  AGTGCAGCAT TCCATACCTG CAACGGTGTA AGTGGCGTCA TTCACATCGG
151  TAACGAGACC TTTGTTATCC ATCCCTTCTA CGCGGAGAT CTGTCCAAAC
201  ATCCACATGT CATCTTGGAG GCTCGCACAA AGGCAGAACAA AGGTTGCCCC
251  AACTCAGGCA ATCTGGACTC ATGGCGTCTA TCCCCGCGCA CCAAGCACTT
301  GTCCGCCGGT GTTGCTGGCG TCGTGGAGGA GATCCCTCCAG CGGGGAGTTC
351  CGCGCAACAA GCAGGATGTG CGCGAGGCCA CCAAATACAT TGAGACGCCC
401  ATCATCGTTG ACAAGGCAAT GTTCGATAAG CGCAATGCCA GCGCTCGTGC
451  CGAGGTCACTC CAGGATGCCA TCCAGTGGC CAACATAGCC GATCTGTACT
501  TTCCGCACGTG AAAACGCGGC GTCTCGCTTG TCTACATTGA GACCTGGCGC
551  AAAACCGGGC GTTATCGATG CAGCAAGATA TCAGCAAGGA ATATCAACTT
601  TATGACTACA CTCAAGGAAA CGTCCCAATCG ACGAGATACA CACGCTCTGC
651  CTCCAACACG CCGTGCGCTC AGCTGCACTG CACCGAAGACTGTTG
The three cDNAs, cloned into pBluescript were sent to the University of Nebraska - Lincoln DNA sequencing facility for automated DNA sequencing.

This figure shows partial sequence was obtained by using T3 primers flanking the cDNA 4-1 insert.
Partial (T3-end) DNA Sequence of cDNA 4-1

1 AATTCCTTTT TCTTTTTTTT TTTTTTTGGA AGTTTTAAAA AATATATTTT
51 TATGTTACTT TTTNNNNNNNC TTGTTCTTTT TGTGTTGTA ATTTGTATTT
101 GTATTTAAAG CTGGCTTTTT CATTACTTCT TTTCAACTAA CCTTAGACAC
151 AGGGAGAGT TTCTATGTAC TACAACGTAC CTGTAGCACA ATGCCATCAA
201 GGTGAATGTT ATAAAAACGA ACCCTACAAC TGACATTAGA ACAACGACTA
251 GGAACACTGT ATTTGAGCCG TGGTAGCTCT CATATGGGGT TTCTTTCTTC
301 TCCATGTTGTA TGTTGTTTTTC CGGCGTGGGC AGTGCTTCCG TTGTCAGTGC
351 TGGTGAGGC AGCAGACAG AACTGCAGTC GTTGCGGCCC CATCCCATGT
401 CGCAGAAAACA GCGATTGTAT TGGTGAGAAC GCATGTCTGA GCACTCTAGA
451 CCCTGTGAAT TGGCGGGGAT TTGGTCGTAT CCACGTGGGG AAAGAGACTG
501 ACGACGTCTG GTGGAGGAAA TTAGATATCA CGGACCGAGT TCCGTCCTTA
551 ACCAGGCCTG CTCCGGATAC CTGGTGGGAC ACTTGCCGAC TGGCGGCTTG
601 CATCAATTCC TGGCTTTTCT CGAATGAGGT TCGAGTAGA CTGATCGTG
651 ATCGTGCATG CTGCGGTCCC A
The three cDNAs, cloned into pBluescript were sent to the University of Nebraska - Lincoln DNA sequencing facility for automated DNA sequencing.

This figure shows partial sequence was obtained by using T7 primers flanking the cDNA 8-1 insert. Underlined sequences show significant homology with a known *Drosophila* nAChR gene, but both of these regions are in non-coding regions of a nAChR gene.
Partial (T7-end) DNA Sequence of cDNA 8-1

1  GAAAAGTATC TCAATCCGCA AAAGCTCAAG CAGCAGTCC TTAGGAGCGA
51  GAAGCTGCGA TCCATTCTGG AGCACTATGC CAAGGAGTCG GGCACGCCGC
101  TCAAGCAGAT GGAGGGTCAG GCCCGGGCGA TACATTGATGA AATCCGCCCTC
151  GATCAGGAAATA TGGCCATCAT CCGGTGGGCA GGCATTGCAAA TTACGGCGAT
201  CGGCAAGCGG ATCTGGCAGAT GATTCATATGT ACAATTCCAGC AGCATGGCGCA
251  ATGTGCGCAA GGATATGGGC CAGTCCCCCA TTCTCTATCT GCCAAGTCAT
301  CGCAGCTACA TGGACTTCAT CCTGATGTCC TACATCTGCT TATACTACGA
351  CATCGAGATA CCAGGAATAG CAGCCGGCAT GGATTTCCAC TCCATGTTCG
401  GAATGGGCAC CAGTGGGAG AAAAAAGAGGAG CGTTCTTTCA ATCGGCCGACG
451  TTCTCCCAACG ATGAGCGTATA CTGGGATATT TTCCGGGAGT ATATGCGGCG
501  CCTGGGTGCA AAATACGACAT GGCGGTTGAG TTTCTGATTT AGGGCACGGG
551  TCCAGGATTT AAGGATCTCG CTTCCCAAAA TTGACTACTC TCGATGGCTT
601  GGTGACCATAT TTACAGGGCG AGTGCCGGATG TATGATTTGT GCCAGTGAGT
Figure 16

Partial (T3-end) DNA sequence of cDNA 8-1

The three cDNAs, cloned into pBluescript were sent to the University of Nebraska - Lincoln DNA sequencing facility for automated DNA sequencing.

This figure shows partial sequence was obtained by using T3 primers flanking the cDNA 8-1 insert.
Partial (T3-end) DNA Sequence of cDNA 8-1

1 ATTCCTATCA GTGTTAGAC TATAAGATTTC TCTATGCACT CGCATACACT
51 AACACTCAAG GATGGGTGCA TTTGCATAGT CACAAAATTTG TAGTTCAATC
101 TTATTCTAGC ATAGGTCATT TGTGTTCCCA ACAAATGGGTG GTGCTATGTT
151 ACATTATCAA AAACCTTTAA ATGCAGGGTG GAAAATAGGGG AATGTTAATT
201 ATGCCAATAT AAATAAGGATA CTATTAGTAT CGACCCATCC TAAACCAGACA
251 CACCGTTAAC CTACGTAATG CCGTGGGTGG ATACGCTGTTT CCACTGGAGC
301 AGCTGCCACA GCGGGCACAQ CACCAGCTTG GTGCAAAGTA TGCAGATGCT
351 GCGACAATTC TTGAGCAGGG CGACAACAAA CTGCATGACC TGCATGGGCA
401 ATGACATGTG ATTTGACTA TACTGATTGC GACCGACTG AGATATCGGTA
451 CTCGCATTTG TGTCCCTTCC CTTNNNNNCTT CCTTGCAAGCG CAATGTCGTT
501 TTGCAGTTAT ACATCTGCTA GTTAGCCTTT GCCTCTGGTT GGCAGACAGCG
551 GTATTTGTTGA AATGCCATNG TGGCCAGTCTT GACCCCCAGT TTTACATTT
601 GGCACGAATA CCCAAGTGGT GAGCCATAAT TGATCGCATC CTTCGGTGCG
651 TGGTCGCCCT CTGCTTGCCCT TTGGC
The three cDNAs, cloned into pBluescript were sent to the University of Nebraska - Lincoln DNA sequencing facility for automated DNA sequencing.

This figure shows partial sequence was obtained by using T7 primers flanking the cDNA 10-1 insert.
Partial (T7-end) DNA Sequence of cDNA 10-1

1    AATTCGAGCC AACGATAAAG AGTTCAATGC CCAGTTCAAA TATCATGTAA
51   GTAAATACAA TATTAACGGT TCATCTAGAA TGCACACTAA ATAAAGTTTT
101  TCTATTTGGT TAACTATTAT TTATAATCCG CATTAATTTC CAGAACAACCT
151  ACATCAAGAC CTCCAAGTAT TCGCTGTCTA CGTTTCTGCC ATTCGAATCTG
201  CTGGAAGCAAT TTCAGCGGCT GGGCAAACCTC TATTTCTTCN NCCTGCTGGT
251  CCTCCAGCTG ATACCCGGCA TATCCTCACT CACCCCAGTG ACCACAGCAA
301  TTCCCCCTGAT AGGAGTACTT ACTCTGACGG CTGTAAGGAA TGGTATGAT
351  GATATTGTGA GTACAAAAATA CTACGCTGTA ACTTCATAAT TGCCAATCTG
401  TATTTTCCCT TCAGCAACGT CATATTTCTG CACTCGCAGG TAACACAATCG
451  CAAGTCGAAA ACGCTGCAGA ATGGCAAGTT GGTGGAAGCC AAGTGGTCGG
501  AGGTACAGGT GGGCGATGTC ATTCGGCTGA CAACGACAGT TTCGTGGCCG
551  CCACACCTGT TGCTGTCCAG ATCCGAGCCA AATCGTCTGT GTTTCATCGA
601  GACAGCCGAA CTGATGGGGA GACGATCTCA AGGCAAGCAG CTCTGACCGA
651  ACCATCGAGC TCGGCTGAGC CCATGACTGC TATGGACTTC AATGCGAGAT
701  ATTTGCACGC GCCAACAACC TGCTGAACAA TTGATGGCAT
Figure 18
Partial (T3-end) DNA sequence of cDNA 10-1

The three cDNAs, cloned into pBluescript were sent to the University of Nebraska - Lincoln DNA sequencing facility for automated DNA sequencing.

This figure shows partial sequence was obtained by using T3 primers flanking the cDNA 10-1 insert.
Partial (T3-end) DNA Sequence of cDNA 10-1

1  AATTCACGAC TCTGCTGTGG GTATATTGTG TGTATCAGAA AAAAGGGTTG
51  TGTACATCAT ACTTTCAAAA TAATAAATAC TTTTATCGTG TTAAAGCCAA
101  AAAAATTCCG GTCGGGTGAAA AGCGGCATAC ATACATGGCG AAAACACATA
151  ACAAGACAT AACAAGATAC TAATTGATAA TAGTGAATGT GAATTTAAGG
201  ACATGCAGCA TGCAATACGTG CACTCTGTGA GGCGACGAAC TCCAATTGGT
251  GGCGAATGCC TTGGATCGAT GGTGGTGGTG CTTGGTGGTG CTAAGTGTGG
301  ATGTGGATTG TGGCTCTCTG CCGAGGTGGT GCTCTAGCTG GGAGATGACA
351  ACACCCAGCG TGGCGTTGAT GGCGCTTTTT TAAACCGGAT CTGACTAAT
401  CGCTTNNNTT GTGACTTGGAA TGGTGCAATGT TGTGTGTTTC TGTCTGTGTC
451  ATGTGCATGCA AAATCTTTAC TTTGTCAGCA ACTGCTAGTG CGGACAATTG
501  CGCTGCATAC ATCGGCTCCA CTCCTCNNNN NNNCGACTAA TTTCGCTTCC
551  TCATTTGCTCA CGCTGGCATA AGTCTCCACT TCCATCAACT GGTGCAGAAC
601  GACACGGTTTC GATCTGCATC TACTGGGCCG GAGTATGCAC ATGCTTGCTC
651  GCNNNGTTCT ACTGGATCCA CGTGCGCA
Table I

**Known *Drosophila* nAChR Loci***

<table>
<thead>
<tr>
<th>Locus</th>
<th>Putative function</th>
<th>Chromosomal localization</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>α-Like Subunits</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-like subunit-1 (ALS)</td>
<td>ligand binding</td>
<td>96A</td>
<td>Bossy et al. 1988</td>
</tr>
<tr>
<td>α-like subunit-2 (Da2/SAD)</td>
<td>ligand binding</td>
<td>96A</td>
<td>Sawruk et al. 1990</td>
</tr>
<tr>
<td>α-like subunit-3 (Da3)</td>
<td>ligand binding</td>
<td>7E</td>
<td>Mülhardt et al. (unpublished)</td>
</tr>
<tr>
<td><strong>Non-α like Subunits</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nAChR from <em>Drosophila</em> (ARD)</td>
<td>structural</td>
<td>64B</td>
<td>Hermans-Borgmeyer et al. 1986</td>
</tr>
<tr>
<td>Second β-subunit (SBD)</td>
<td>structural</td>
<td>96A</td>
<td>Sawruk et al. 1990</td>
</tr>
</tbody>
</table>

*After Gundelfinger & Hess (1992).*
### Table II

Characterization of Cross-Reacting mAbs Against The Vertebrate nAChR*

<table>
<thead>
<tr>
<th>mAb</th>
<th>IgG subtype</th>
<th>Subunit specificity*</th>
<th>Binding to ectodomain†</th>
<th>Binding to MIR‡</th>
<th>Immunoprecipitation of <em>Drosophila</em>[^125]I-α-BTX binding component§</th>
<th>Cross-competition for Torpedo AChR</th>
<th>Cross-reaction with mouse muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>27.34.52</td>
<td>IgG1</td>
<td>α</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>27.35.74</td>
<td>+</td>
</tr>
<tr>
<td>27.35.74</td>
<td>IgG1</td>
<td>α</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>27.34.52</td>
<td>+</td>
</tr>
<tr>
<td>23.55.13</td>
<td>IgG3</td>
<td>α</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>27.43.37</td>
<td>IgG1</td>
<td>α</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>27.1A.16.42</td>
<td>IgG1</td>
<td>β</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Determined by Western blots of affinity purified *Torpedo* AChR subunits separated by SDS-polyacrylamide gel electrophoresis.

† Determined by ability to bind to intact mouse muscle cells.

‡ The MIR, or Main Immunogenic Region, is a highly conserved extracellular region on the α-subunit which has been shown to be highly immunogenic.

§ None of the mAbs have been able to immunoprecipitate a [125I]α-BTX binding component from *Drosophila* head membrane extracts.

# From Chase et al. (1987)
Table III

Position of Known *Drosophila* nAChR Genes Compared To cDNAs

<table>
<thead>
<tr>
<th>Known <em>Drosophila</em> nAChR Loci*</th>
<th>Chromosomal Location</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>α-like Subunits</strong></td>
<td></td>
</tr>
<tr>
<td>α-like subunit-1 (ALS)</td>
<td>96A</td>
</tr>
<tr>
<td>α-like subunit-2 (Da2/SAD)</td>
<td>96A</td>
</tr>
<tr>
<td>α-like subunit-3 (Da3)</td>
<td>7E</td>
</tr>
</tbody>
</table>

| Non-α-like Subunits           |                     |
| nAChR from *Drosophila* (ARD) | 64B                 |
| Second β-subunit (SBD)        | 96A                 |

<table>
<thead>
<tr>
<th>Loci Identified Using mAb 16.42</th>
<th>Chromosomal Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA 4-1</td>
<td>87A-B</td>
</tr>
<tr>
<td>cDNA 8-1</td>
<td>98C-E</td>
</tr>
<tr>
<td>cDNA 10-1</td>
<td>86D-E</td>
</tr>
</tbody>
</table>

Table IV
Previously Characterized Genes In The Cytological Regions Of Loci Identified With mAb 16.42*

<table>
<thead>
<tr>
<th>Loci</th>
<th>Gene</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>sad (shadow)</td>
<td>embryonic lethal with no differentiation of cuticle</td>
<td></td>
</tr>
<tr>
<td>man (mandarin)</td>
<td>eye color bright orange</td>
<td></td>
</tr>
<tr>
<td>mudl (mudlike)</td>
<td>eye color grayish brown</td>
<td></td>
</tr>
<tr>
<td>DipC</td>
<td>structural gene for dipeptidase C</td>
<td></td>
</tr>
<tr>
<td>Mfcp (myofibrillar contractile protein)</td>
<td>structural genes for several contractile polypeptides of approximately 22,500 daltons = Msp (Muscle specific proteins)</td>
<td></td>
</tr>
<tr>
<td>mus309</td>
<td>mutagen-sensitive mutation</td>
<td></td>
</tr>
<tr>
<td>svp (seven-up)</td>
<td>recessive-lethal mutation whose gene product is expressed in subsets of neuroblasts and the eye</td>
<td></td>
</tr>
<tr>
<td></td>
<td>plus additional 19 recessive-lethal complementation groups</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Loci</th>
<th>Gene</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>87A-B (4-1)</td>
<td>Hrbl</td>
<td>hnRNA binding protein</td>
</tr>
<tr>
<td></td>
<td>Doa (Darkener of apricot)</td>
<td>causes a copia insertion in the white locus to produce more pigment than normal; homozygous lethal</td>
</tr>
<tr>
<td></td>
<td>Glu</td>
<td>possible structural gene for membrane-bound beta-glucuronidase</td>
</tr>
<tr>
<td></td>
<td>Pkc3</td>
<td>gene encoding an isoform of protein kinase</td>
</tr>
<tr>
<td>98E-F (8-1)</td>
<td>Sevc (Serendipity cognate)</td>
<td>structural gene for a protein of 868 amino acids with eight TFIIIA-like fingers.</td>
</tr>
<tr>
<td></td>
<td>yem (yema)</td>
<td>cluster of maternal effect genes active in oogenesis</td>
</tr>
<tr>
<td></td>
<td>Dr (Drop)</td>
<td>mutation affecting eye facet number</td>
</tr>
<tr>
<td></td>
<td>LapA, LapD</td>
<td>Leucine aminopeptidase A and D structural gene</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Loci</th>
<th>Gene</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>86D-E (10-1)</td>
<td>odh</td>
<td>structural gene for Octanol dehydrogenase</td>
</tr>
<tr>
<td></td>
<td>ants</td>
<td>antenna modified</td>
</tr>
<tr>
<td></td>
<td>Rst(3)ns</td>
<td>Resistance to nicotine sulfate (lost mutation)</td>
</tr>
<tr>
<td></td>
<td>cu</td>
<td>curled wings</td>
</tr>
<tr>
<td></td>
<td>Er (Erect)</td>
<td>bristle mutation</td>
</tr>
<tr>
<td></td>
<td>plus additional 6 recessive-lethal complementation groups</td>
<td></td>
</tr>
</tbody>
</table>

*Based on available data from Lindsley & Zimm (1992)
REFERENCES


Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger and Waterfield, M. D.


Stratagene (1993). Cloning System Catalog, La Jolla, CA.