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IDENTIFICATION OF *CIS*-ACTING REGULATORY SEQUENCES REQUIRED FOR TRANSCRIPTIONAL CONTROL OF THE *ard* GENE OF A NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR FROM *DROSOPHILA*

by

LEE-CHUAN YANG

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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ABSTRACT

Identification of *cis*-acting regulatory sequences required for transcriptional control of the *ard* gene of a neuronal nicotinic acetylcholine receptor from *Drosophila*

by

Lee-Chuan Yang

Adviser: Professor Thomas Schmidt-Glenewinkel

As part of a long-term goal to elucidate the cellular and molecular mechanisms controlling the expression of neuronal nAChR subunit genes in *Drosophila*, the *cis*-acting regulatory region involved in the expression of the *ard* subunit gene has been identified and characterized. DNA fragments of the 5' flanking region were fused to the *Escherichia coli lacZ* reporter gene and introduced into the *Drosophila* germ line by P-element-mediated transformation. A 325 bp fragment of the 5' flanking sequence was sufficient to direct β-galactosidase expression in the nervous tissue during development with a pattern very similar to the distribution of transcripts of the corresponding *ard* gene. The results indicate that the *ard* gene lacks a TATA box and GC-rich sequence at the transcription start site and initiates transcription at a tightly clustered site.

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This work is also in memory of Xiaoying Zhang and Xiaoping Mei.

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I. INTRODUCTION

A. Background

The problem of how development, structure and function of nervous system is specified by information from the genome and the outside world is a fundamental challenge of neurobiology. The synapses are essential structures involved in this process. They allow neurons to form intricate communication networks which are subject to plastic changes in response to a variety of stimuli. A key element of synaptic transmission and neuronal signal transduction are neurotransmitter receptors. Our current knowledge permits classification of neurotransmitter receptors into two mechanistic groups based on the signal transduction process. In one group the binding of the neurotransmitter to its receptor elicits formation of a second messenger and signal propagation through a signal transduction cascade. In the second group the neurotransmitter binds as a ligand to the receptor and activates an ion-channel in the postsynaptic membrane leading to polarization or hyperpolarization of the postsynaptic membrane.

Research in our laboratory has focussed on such a key component of the synapse - acetylcholine receptors (AChRs) which are postsynaptic neurotransmitter receptors. The AChRs can be divided into two subtypes - nicotinic and muscarinic based on their pharmacological and biochemical characteristics. The nicotinic subtype AChRs form cation channels for signal transduction while the muscarinic

subtype AChRs are guanine nucleotide-binding protein-coupled receptors composed of a single polypeptide chain and are acting through a second messenger system. The nicotinic AChR (nAChR) has been found to be a major excitatory neurotransmitter at chemical synapses of skeletal muscle, in neurons of the peripheral and central nervous system of vertebrates and in the central nervous system of invertebrates.

a. Neuromuscular nAChRs

Many molecular details of the nAChRs from the neuromuscular junction have been elucidated by studing the electric organ of *Torpedo* because it is a rich source of this receptor. It is now well established that the nAChR of the *Torpedo* electric organ is composed of four distinct subunits with a stoichiometry of $\alpha_2\beta\gamma\delta$ (Weill *et al.*, 1974; Reynolds and Karlin, 1978; Raftery *et al.*, 1980; McCarthy *et al.*, 1986). Each subunit is a glycoprotein with four hydrophobic transmembrane domains (Anderson and Blobel, 1981; Claudio *et al.*, 1983; Finer-Moore and Stroud, 1984; Lindstrom *et al.*, 1984; McCarthy *et al.*, 1986). Each α- subunit has an acetylcholine (ACh) binding site and two consecutive cysteine residues that form disulphide bridges (Karlin *et al.*, 1975; Neubig and Cohen, 1979; Sine and Taylor, 1980; Hall *et al.*, 1983). The ligand binding region has been shown to be near these cysteine residues (Kao *et al.*, 1984; Kao and Karlin, 1986). All subunits participate in the formation of the ion channel of the receptor.

Vertebrate skeletal muscle nAChRs are similar to those of *Torpedo* electric

organ, except that the γ- subunit is replaced by the ϵ- subunit, the fifth type of nAChR subunit, during development (Takai *et al.*, 1985; Mishina *et al.*, 1986; Gu and Hall, 1988). The two subtypes, $\alpha_2\beta\gamma\delta$ and $\alpha_2\beta\epsilon\delta$, differ in their gating and ion conductance properties. Prior to innervation, the $\alpha_2\beta\gamma\delta$ subtype of the nAChR is present throughout the fetal muscle cell membrane; these receptors have a low conductance and long channel opening time. Subsequently, the extra junctional nAChRs disappear and, at the synapse, the fetal form is replaced by the adult form ($\alpha_2\beta\epsilon\delta$) which has high conductance and short opening time (for review, see Schuetze and Role, 1987).

A receptor with multiple subunits raises questions about how each individual subunit assembles into a functional receptor? What mechanism control the number and distribution of acetylcholine receptors? Is the expression of the corresponding four genes to be coordinately regulated? Many studies indicate that the regulation of the number and distribution of nAChRs at the nerve-muscle synapses is very complicated. Evidence indicates that innervation plays a major role in the control of the number and distribution of nAChR expressed on the skeletal muscle fiber surface (Brehm and Henderson, 1988). In addition, muscle activity, soluble neurotrophic factors, and second messengers may also have some responsibility for the regulation (Goldman *et al.*, 1988; Goldman and Staple, 1989; Klarsfeld *et al.*, 1989; Goldman *et al.*, 1991; Martinou *et al.*,1991; Martinou and Merlie, 1991). Data suggest that these factors may regulate nACh receptor synthesis, at least in part, through

transcriptional control (Merlie *et al.*, 1984; Merlie and Sanes, 1986). In addition, forming and maintaining a high density of the mature adult form of nAChRs exclusively at synaptic sites are thought to be mediated, at least in part, by the release of chemical substances from the nerve. Nerve-activated electrical activity inhibits γ- subunit expression everywhere in the muscle, and α-, β-, and δ- subunit expression in nonsynaptic areas (Schuetze and Role, 1987). Both local synaptic control and activity-dependent control seem to be mediated, at least in part, through transcriptional regulation of the nAChR subunit genes (Changeux, 1991; Hall and Sanes, 1993). During embryogenesis when myoblasts fuse into differentiated myotubes, its expression starts at least partly because of transcriptional activation (Buonanno and Merlie, 1986). Furthermore, nuclear run-on experiments demonstrated that in differentiating muscle cells as well as after enervation, the mRNA levels are increased by transcriptional activation (Frail *et al.*, 1989; Tsay and Schmidt, 1989). Therefore, nAChR genes show dramatic changes in transcription level in response to differentiation and innervation.

Although, factors deposited by motor neurons and electrical activity were shown to be involved in control on the expression of adult-type receptor genes at the neuromuscular junctions and the inhibition of expression of these genes in extra junctional regions, the molecular mechanisms governing the stimulation and the suppression of nAChR genes expression are not understood. Further identification of the mechanisms involved will be greatly facilitated by the identification of the *cis*-

acting elements that respond to such factors. By using transfected primary cell cultures or cell lines, sequences important for tissue specific and developmental expression of the muscle nAChR genes have been identified in the chicken α and δ genes (Wang *et al.*, 1988; Klarsfeld *et al.*, 1987; Wang *et al.*, 1990), the mouse α, β, γ, δ, and ϵ genes (Prody and Merlie, 1991; Prody and Merlie, 1992; Gardner *et al.*, 1987; Baldwin and Burden, 1988; Duclert *et al.*, 1993), and in rat β, γ, δ (Berberich *et al.*, 1993; Chahine *et al.*, 1992). Promoter sequences of chicken α and mouse ϵ genes studied by using transgenic mice or by direct muscle tissue injection were also reported (Merlie and Kornhauser, 1989; Klarsfeld *et al.*, 1991; Sanes *et al.*, 1991; Duclert *et al.*, 1993; Gundersen *et al.*, 1993).

Sequence comparison of known nAChR gene promoters so far has shown few similarities. However, in all cases, the minimal regulatory region of the 5' flanking sequence contains E box elements that are defined by the nucleotides CANNTG (Lassar *et al.*, 1989). E boxes are shown to provide binding sites for helix-loop-helix proteins of the MyoD1 family (Davis *et al.*, 1987; Braun *et al.*, 1989; Edmondson and Olson, 1989; Wright *et al.*, 1989), although their functional significance for the regulation of muscle-specific gene transcription is not clear. In most of cases (α, β, and γ subunit genes), they can enhance the *in vitro* transcriptional activity (Piette *et al.*, 1990; Prody and Merlie, 1991; Jia *et al.*, 1992; Prody and Merlie, 1992), while in the ϵ promoter, E-boxes seem not to be required for the transcription activity (Numberger *et al.*, 1991).

b. Vertebrate Neuronal nAChRs

Beside their occurrence at the neuromuscular junctions, nicotinic AChRs are also found widely distributed throughout the peripheral and central nervous system of vertebrates. Pharmacological, immunological and biochemical evidence indicates similarities as well as differences between muscle and neuronal nicotinic AChRs. Like muscle nAChRs, neuronal nAChRs are cation specific but have greater permeabilities for calcium than muscle nAChRs (Séguéla *et al.*, 1993; Vernino *et al.*, 1992; for review see Sargent, 1993). Furthermore, unlike muscle nAChRs which have been found exclusively postsynaptically, nAChRs found in the central nervous system occur both pre- and post-synaptically (Lichtensteiger *et al.*, 1982; Sakurai *et al.*, 1982; Brown *et al.*, 1984).

At the molecular level, unlike muscle nAChRs, evidence indicates that the neuronal nAChRs are encoded by a family of closely related, but distinct genes (for review, see Sargent, 1993). So far, at least seven genes encoding α- subunits (α2 - α8) and three genes encoding β- subunits (β2 - β4 in rat or nα1 - nα3 in chick) have been cloned (Boulter *et al.*, 1986; Goldman *et al.*, 1987; Deneris *et al.*, 1988; Nef *et al.*, 1988; Schoepfer *et al.*, 1988; Wada *et al.*, 1988; Duvoisin *et al.*, 1989; Deneris *et al.*, 1989; Schoepfer *et al.*, 1990; Couturier *et al.*, 1990a; Boulter *et al.*, 1990; Séguéla *et al.*, 1993; Isenberg and Meyer, 1989). Human α2 - α5, α7 and β2 - β4 subunits have also been cloned (Fornasari *et al.*, 1990; Anand and Lindstrom, 1990; Chini *et al.*, 1992; Elliott *et al.*,1993; Tarroni *et al.*, 1992; Monteggia, 1995). The

definition of new subunit is based on the presence or absence of the two consecutive cysteine residues, designated α- or β- (or non α-), respectively. Chick, rat and human nAChR genes of the same subunit type are highly homologous (> 70% amino acid identity) (Decker *et al.*, 1995). All neuronal genes analyzed so far have identical structures except the α7 subunit. They contain five introns and six exons, the positions of which are conserved among most other nAChR genes. This is another difference from muscle nAChR genes which contain several additional introns (Decker *et al.*, 1995).

With in the superfamily of genes encoding neuronal nAChRs, the stoichiometry of neuronal nAChRs is still undefined. By coinjecting RNAs or cDNAs into *Xenopus* oocytes, functional neuronal nAChRs can be produced. Studies have shown that as few as one or two gene products are sufficient to produce functional AChRs in *Xenopus* oocytes. The combinations of RNAs or cDNAs encoding any one of the α2, α3, or α4 subunits and either β2 or β4 can produce functional AChRs (Boulter *et al.*, 1987; Ballivet *et al.*, 1988; Wada *et al.*. 1988; Duvoisin *et al.*, 1989; Papke *et al.*, 1989; Couturier *et al.*, 1990b; Bertrand *et al.*, 1990). The biophysical and pharmacological properties of the receptors formed with different combination of subunits are different (Luetje and Patrick, 1991). In contrast, the α7 subunit alone can form functional nAChRs when expressed in the *Xenopus* oocytes, i.e. it gives rise to homooligomeric ACh-activated channels (Couturier *et al.*, 1990a; Bertrand *et al.*, 1992; Séguéla *et al.*, 1993; Gerzanich and

Lindstrom, 1994). However, studies with subunit-specific monoclonal antibodies have indicated that native AChRs on neurons may have a more complex subunit composition (Conroy *et al.*, 1992; Vernallis *et al.*, 1991; Vernallis *et al.*, 1993). For instance, in brain, the α5 gene product is present both in α3- and in α4- based receptor subtypes, while in the ganglion it is found in an α3-based receptor subtype concentrated in the postsynaptic membrane (Conroy *et al.*, 1992). Whiting and Lindstrom proposed that the nAChRs found in the peripheral and central nervous system are assembled from only two different subunits, an α and a β subunit, with a stoichiometry most likely to be $\alpha_{2\text{-}3}\beta_{2\text{-}3}$ (Whiting and Lindstrom, 1986; Whiting and Lindstrom, 1987; Schoepfer *et al.*, 1988). Two other independent studies also demonstrated that neuronal nAChR have a pentameric stoichiometry, like their muscle siblings (Cooper *et al.*, 1991; Anand *et al.*, 1991). Therefore, the number of AChR genes normally coexpressed by individual neurons remains unclear.

The distribution pattern of nAChRs in brain areas has been studied in many experiments by using ligands (Clarke *et al.*, 1985; London *et al.*, 1988) and antibodies (Swanson *et al.*, 1987). Data have shown that the distribution of ACh and nicotine binding sites which represent functional nAChRs were similar to each other but different from that of α-bungarotoxin (α-Btx). Further immuno-cytochemical studies have shown the mAbs staining pattern being similar to the pattern found by using nicotine and ACh. Moreover, as demonstrated by *in situ* hybridization and Northern blot analysis, different neuronal nAChR subunit genes are expressed in distinct areas

of the developing and adult nervous system. In the CNS, α3, α4 and β2 mRNAs are widely distributed, the other subunit mRNAs are more restricted, yet there are many instances of overlapping expression (Wada *et al.*, 1989; Hill *et al.*, 1993; Keyser *et al.*, 1993). Other studies have shown that subunit mRNAs which are rare in the CNS are relatively abundant in several peripheral ganglia (Boyd *et al.*, 1988; Boyd *et al.*, 1991; Couturier *et al.*, 1990b; Listerud *et al.*, 1991; Corriveau and Berg, 1993). For instance, α3, α5, β2, and β4 genes are expressed in the CNS and these genes are also co-expressed with α7 in chicken ciliary ganglion neurons.

Summaries of all available data indicate that the neuronal nAChRs can roughly be divided into two classes, one of which is α-Btx sensitive and while the other is not. Both classes are different not only in the binding property to α-Btx, but also in the composition of type and number of subunits as well as in the distribution patterns. It is possible that the diversity exhibited in the neuronal nAChR family results from the differential expression of these subunits which leads to incorporation of different subunits into mature receptors. The expression of each nAChR subunit is determined (at least in part) by the transcription control of each subunit gene. Transcriptional regulation is, therefore, an important determinant of receptor subtype in a neuronal population. In addition, as mentioned earlier, many factors play roles in the regulation of the number and distribution of muscle nAChRs. It is interesting to know if those factors also contribute to the regulation of neuronal nAChRs. Neuronal and muscle nAChR gene expression may be regulated differently with respect to cell

contact, neural factors, and electrical activity. In order to understand the transcriptional regulation of neuronal nAChRs by these factors, it will be necessary to identify DNA element that control the expression of members of this family and identify factors required for the expression of these genes. Therefore, analysis the transcription control elements of each subunit becomes very interesting and necessary.

When compared with the muscle nAChR, even less is known about the transcriptional regulation of any neuronal nAChR gene. The chick α3, α4, and β2 RNA levels are regulated during development either in ciliary ganglia or the visual system (Boyd *et al.*, 1988; Matter *et al.*, 1990). Similar developing changes have been observed in rat α3, α4, and β2 RNA levels (Miyai *et al.*, 1990; Senba *et al.*, 1990), indicating differential regulation of receptor subunit genes. In addition, the chicken α7 gene is regulated at the transcriptional level by specific *cis*-acting regulatory elements which control the neuronal specificity of the promoter (Matter-Sadzinski *et al.*, 1992). The level of α3 RNA in chick ciliary ganglia decreases following both denervation and axotomy (Boyd *et al.*, 1988). The decrease following denervation is in contrast to the increase seen in the α subunit RNA level in muscle following denervation (Goldman *et al.*, 1988). Just like the muscle nAChRs, all promoter analyses were done in cell lines only. Neuronal and muscle nAChR gene expression may be regulated differently with respect to cell contact, neural factors, and electrical activity.

c. Neuronal nAChRs in the insect nervous system

In the insect nervous system, AChRs of the nicotinic type occur in high concentration. ACh is the major excitatory transmitter in many CNS interneurons, projection neurons, and most sensory input pathways (Gundelfinger, 1992). By using molecular probes from vertebrate nAChRs, many insect nAChR genes have also been identified. At least six different nAChR encoding genes have been cloned from the migratory locust (Hermsen *et al.*, 1991). In *Drosophila melanogaster*, three α-like (i.e. ALS, Dα2, Dα3) and two β-like (i.e. ARD and SBD) nAChR subunit encoding genes have been identified by either using a synthetic oligonucleotide mixture deduced from conserved region of known neuronal nAChR subunits or cross-hybridization with cDNA fragment from known nAChR subunits (Hermans-Borgmeyer *et al.*, 1986; Sawruk *et al.*, 1988; Wadsworth *et al.*, 1988; Bossy *et al.*, 1988; Baumann *et al.*, 1990; Jonas *et al.*, 1990; Sawruk *et al.*, 1990a; Sawruk *et al.*, 1990b).

The distribution of *Drosophila* nAChR subunits was studied by *in situ* hybridization. The results indicated that the expression of *Drosophila* nAChR subunit genes are highly regulated during development. The transcript level are very high in late embryonic stage, in late pupae stage, and in one-day-old adult stage (Wadsworth *et al.*, 1988; Hermans-Borgmeyer *et al.*, 1989; Jonas *et al.*, 1990; Sawruk *et al.*, 1990a; Sawruk *et al.*, 1990b). This schedule is consistent with the major periods of neurogenesis in *Drosophila melanogaster*. In addition, ALS RNA level

is also very high in the larval stage which is not found for any of the other subunits.

Studies by Dudai and Amsterdam in 1977 have indicated that specific [^{125}I] α-Btx binding is distributed almost evenly over the neuropil of the *Drosophila* CNS (Dudai and Amsterdam, 1977). Similarly, evidence from Sattelle and colleagues indicated that α-Btx also blocks the action of insect nicotinic receptors (Sattelle, 1983). After the subunits of *Drosophila* nAChRs were cloned and characterized, Schloss and colleagues showed that ALS and ARD could form one receptor subtype which can bind α-Btx with high affinity (Schloss *et al.*, 1991). By contrast, Sawruk and colleagues showed that the Dα2 subunit alone can form nicotine-gated cation channels in *Xenopus* oocytes although the concentration of agonist required to activate the channel is unusually high. The nicotine activation of this homooligomeric channel cannot be blocked by nicotinic antagonists, like α-Btx (Sawruk *et al.*, 1990a). In addition, Gorczyca and colleagues discovered a *Drosophila* nAChR subtype which is insensitive to α-Btx by electrophysiological and pharmacological method (Gorczyca *et al.*, 1991). In the locust ganglion, only a single glycopolypeptide can be detected as a component of a functional acetylcholine-gated ion channel (Breer *et al.*, 1985). All of these indicated that hetermeric nAChRs as well as homo-oligomeric nAChRs may exist in insects, similar to vertebrate neuronal nAChRs.

Furthermore, a detailed examination of the association, dissociation and equilibrium binding of [^{125}I] α-Btx to *Drosophila* head membrane extracts has

revealed a heterogeneity within the toxin-binding receptors. At least two physically distinct classes of high-affinity binding sites were observed with K_D values of ~0.1 nM (class 1) and ~4 nM (class 2) (Schloß *et al.*, 1988). Class 1 represents about 25% and class 2 about 75% of all high-affinity α-Btx binding sites found in the heads of flies. In our laboratory, we have purified a functional nAChR from *Drosophila* heads by using α-cobra toxin affinity column (Wu, 1991). Biochemical analysis revealed that there are at least two subtypes of nAChR with two types of subunits in *Drosophila*. The two types of binding sites have K_D values of 0.20 nM and 3.1nM for α-Btx, respectively. Finally, indirect evidence for the existence of various receptor subtypes also results from the work of Chase and colleagues, who used a set of monoclonal antibodies directed against the *Torpedo californica* electorplax receptor to localize immunohistochemically cross-reacting antigens in the *Drosophila* CNS. Only one subset of antibodies recognized epitopes that co-localize with the major α-Btx-binding sites (e.g. in the neuropil of the optic lobes), while others stained distinct neural structures like axonal fiber tracts, mechanosensory bristle elements and photoreceptors (Chase *et al.,* 1987).

B. Objectives

As outlined in the background section the nAChR plays a key role in neuronal communication. Such a structure is not static - it will require continuously active signals for its maintenance and it is expected that the receptor is influenced by a

multitude of signaling processes like cell-cell contacts, hormonal responsiveness, gradients of neurotrophic factors and neurotransmitter action during both development and physiological activity.

I anticipated that many of these signals ultimately might converge on the transcriptional machinery, in particular the protein factors which through interaction with promoters and enhancers control transcriptional activity. The objective of my thesis is to investigate the transcriptional regulation of one of the genes encoding a neuronal nAChR subunit, the *ard* gene in *Drosophila*.

The rational for the choice of the experimental system rests on the availability of germline transformation via P-element containing vectors. As discussed previously, many investigations of transcription regulation were done in transfected cell lines. Although DNA-mediated transformation of clonal cell lines provides a powerful tool of studying tissue specificity of gene regulation when cultured cells of the appropriated tissue type exist, it is obvious that this method has disadvantages for answering questions of more complex tissue-cell interactions of the type involved in neural regulation of AChR expression. *Drosophila* has molecular and genetic advantages for studying structure and function of genes not only *in vitro* but *in vivo*. Genes and their *cis*-regulatory elements can be characterized by a number of genetic and molecular approaches. Cloned DNA can be readily altered *in vitro* and then reliably integrated into the germ line via P-element vectors (Rubin and Spradling, 1982), allowing correlations of molecular information with both structural and

behavioral phenomena observed in intact animals.

Specifically, I would like to identify the *cis*-acting regulatory units required for spacial and temporal regulation of the *ard* expression. The strategy I wanted to pursue is to construct fusion genes by linking putative regulatory elements to the *lacZ* reporter gene. Expression of the fusion constructs can then be studied in the natural cellular environment. Results from these experiments will not only give insight into factors regulating the *ard* expression but also will contribute towards our understanding of neuronal plasticity and biochemical identity of cholinergic neurons.

II. MATERIALS AND METHODS

A. Materials

1. Source of materials

A *Drosophila melanogaster* (Canton S) genomic library (in EMBL3 SP6/T7 λ-vector) was purchased from Clontech Lab. Restriction enzymes, T_4 DNA ligase, T_4 DNA polymerase, and T_4 polynucleotide kinase were obtained from New England Biolab. RNA polymerase, exonuclease III and mung bean nuclease were bought from Stratagene. Calf intestine phosphorylase was obtained from Boehringer Mannheim. Nitrocellulose filters were purchased from Schleicher & Schuell. Nylon membrane (Zeta-Probe Blotting Membranes), acrylamide, bis-acrylamide, and TEMED were purchased from Bio-Rad; LM Nusieve agarose was from FMC; Sephadex G50 and a poly $(A)^+$ RNA purification kit were from Pharmacia. The T3, T7, SP6, and AChR4 primers/probes were synthesized by the Sequencing & Synthesis Facility of Hunter College. The sequences are as follows:

T3: 5'-ATTAACCCTCACTAAAG-3'

T7: 5'-AATACGACTCACTATAG-3'

SP6: 5'-TAGGTGACACTATAGAA-3'

AChR4: 5'-GGGGCGTGGCCAGCGAAATCCC-3'

2. *Bacterial strains and plasmids*

E. coli strains: XL-1 Blue and SRB were obtained from Stratagene; HB101 was a gift of Dr. Alton Meister. Plasmids used were: HZ50PL, CZ20XN, pHSS7, pHSS7S1(No.1), and pBluescript KS (+) (gifts from Dr. Leslie Pick); pBluescript II SK (+) (a gift from Dr. Shirley Raps).

3. *Drosophila strains*

The wild type strain used in this study was Oregon R. ry^{506} strain was used for germ-line transformation. The following balancer stocks obtained from Dr. Leslie Pick were used to maintain transformant lines: (1) *FM6*; ry^{506}, (2) *bAdh/CyO*; ry^{506}, and (3) *rf10/TM3*; ry^{506}. For a description of marker genes and balancer chromosomes, see Lindsley and Grell (1968). Flies were raised on a cornmeal-yeast-agar medium (Lewis, 1960) at 22° - 25°C and 65% - 75% relative humidity.

B. Isolation of a clone containing 5'-upstream sequence of the *ard* gene

1. *Screening of a genomic library*

A genomic DNA library from *Drosophila melanogaster* (Canton S), in the λ bacteriophage EMBL-3 SP6/T7, was screened by a modified procedure as described in Current Protocols in Molecular Biology (Ausubel *et al.*, 1988). A 23-mer oligonucleotide (AChR4; see Figure 1) designed based on the published *ard* sequence (Hermans-Borgmeyer *et al.*, 1986) was used as a probe. Approximately 8 x 10^4

phages were first hybridized under low-stringency condition (6X SSPE, 5X Denhardt's, 0.05% sodium pyrophosphate, 100 μg/ml sheared and denatured herring sperm DNA, and 0.5% SDS) at 60°C overnight. The filters were then washed with 3 changes of a solution of 1X SSPE and 0.05% sodium pyrophosphate at room temperature for 30 minutes and then at an increased temperature of 47°C for another 30 minutes in a prewarmed solution of 1X SSPE and 0.05% sodium pyrophosphate. After washing, autoradiography was carried out and the film was developed. The secondary screening condition was the same as the first screening. The positive clones were picked using sterile toothpicks and stored in 1ml SM (100 mM NaCl,10 mM Mg_2SO_4, 50 mM Tris (pH7.5), and 0.1% gelatin).

2. Southern blot analysis

The positive clones obtained from the genomic DNA library screen were verified by Southern blot analysis according to the procedure described in Current Protocols in Molecular Biology (Ausubel *et al.*, 1988). The DNAs were digested with different restriction enzymes, separated by 0.8% agarose gel electrophoresis, and transferred to a nylon membrane with 0.4 N NaOH as transfer buffer. The same probe (AChR4) used in the genomic library screen was end-labeled to specific activity of 1 - 3 X 10^8 cpm/μg. Hybridization was carried out under high stringency condition at 60°C overnight. The washing condition was 60°C, 1 hour in 1 X SSPE and 0.05% sodium pyrophosphate.

3. Restriction mapping

The positive DNA clone obtained from the genomic DNA library screening was digested with different restriction enzymes, single or combination. The DNA fragments were separated by agarose gel electrophoresis. By comparing the differences between single and combined digestion patterns, the locations of restriction digestion sites (HindIII, EcoRI, XbaI, BamHI and SstI) were identified. Southern blot analysis with T7, SP6 or AChR4 probes was also carried out to identified 5'-end, 3'-end and exon 2 of the *ard* gene fragments, respectively.

4. Sequence analysis

The inserts of the recombinant λ phage DNA was excised by digestion with HindIII, isolated by agarose gel electrophoresis, and purified using a Geneclean kit (BIO 101). Three Hind III fragments (i.e. 1HH (0.9 kb), 2HH (1.9 kb), and 3HH (2.5 Kb); see Figure 2) were obtained from the purification and subcloned into the Hind III site of the phagemid pBluescript KS (+). The nucleotide sequence of 1HH was determined by double-stranded DNA sequencing using the dideoxynucleotide chain termination method (Sanger, 1977) with the Sequenase II kit (U.S. Biochemicals) according to the manufacturer's instructions. T7 and T3 primers were used. Approximately 300 bases were sequenced from both ends in the first round. From these sequences new oligonucleotides were designed and used as primers for the second round. After the second round of sequencing, an overlapping sequence was

obtained and the sequence of 1HH completed.

C. Identification of the transcription start site

1. Preparation of late stage embryos

0 to 10 hours old embryos were collected on eight egg laying medium trays in each of two population cages. The embryos were then aged for 12 hours at 25°C to produce 12 to 22 hours old embryos. The aged embryos were collected on a nylon mesh (opening 140 μm) and transferred to a beaker. Dechorionation was carried out for 90 seconds in 3% sodium hypochlorite. The dechorionated embryos were rinsed several times with water, dried and quickly frozen in liquid nitrogen. The embryos can be stored at -75°C for up to several months.

2. Preparation of one-day-old fly heads

One-day-old adult flies were collected in a 50-ml Falcon tube prechilled in liquid nitrogen and stored in a Revco. Flies were mixed with equal amount of crushed dry ice and vigorously shaken in a prechilled bottle. The mixture was passed through a stack of metal sieves No. 10, 25, and 40 with opening sizes 2 mm, 710 μm, and 425 μm, respectively (Wire Cloth Company, Newark, N.J.). Separated fly heades collected on the No. 40 sieve with powdered dry ice. Isolated fly heads were then stored at -75°C in a Revco freezer overnight to allow dry ice to sublimate.

3. Preparation of total RNA

Total RNA was prepared by following the protocols described in the Rubin Lab Manual (Bowtell, 1990). 5 g of fly heads or embryos were ground into a fine powder with a mortar and pestle in liquid nitrogen. The fine powder was transferred immediately with stirring to a beaker containing 6 M guanidine isothiocyanate and 0.1 M NaOAc. The viscous lysate was sheared by passing it through an 18-G needle three times and centrifuged at 12,000 r.p.m. in an SS34 rotor at room temperature. The supernatant was transferred to a fresh centrifuge tube and centrifuged again under the same condition. The twice precleared supernatant was then carefully layered on top of 5 ml of 5.2 M CsCl and 10 mM EDTA in a polyallomer tube (14 x 89 mm). After centrifuging at 30,000 r.p.m. in an SW40.1 rotor for 16 hours at 20°C, the RNA pellet was packed on the bottom of the tube. The pellet was clear and gelatinous. It was resuspended in 200 μl of 10 mM EDTA and stored as a fine precipitate in ethanol at -75°C.

4. Preparation of poly $(A)^+$ RNA

Poly A^+ RNA was prepared according to the manufacturer's (RNA purification kit, Pharmacia) instructions. 1.25 mg of total RNA was recovered from the ethanol suspension and dissolved in 1 ml of Elution buffer (10 mM Tris-HCl, pH 7.4 and 1 mM EDTA). The RNA solution was heated to 65°C for 5 minutes and then chilled on ice. After adding 0.2 ml of Sample solution (Elution buffer containing 3.0 M

NaCl), the RNA was applied to an oligo-dT cellulose column which had been pre-equilibrated with High-salt buffer (Elution buffer containing 0.5 M NaCl). Once the entire RNA solution passed through the column under gravity, the column was centrifuged at 350 x g for 2 minutes. Using the same centrifugation routine, the column was washed twice with 0.25 ml High-salt buffer and three times with 0.25 ml Low-salt buffer (Elution buffer containing 0.1 M NaCl). The bound poly$(A)^+$ mRNA was eluted with four successive 0.25 ml aliquots of prewarmed (65°C) Elution buffer in the same manner. The poly $(A)^+$ mRNA was stored frozen at -75°C as an ethanol precipitate.

5. RNA gel electrophoresis

The RNA gel electrophoresis was performed as described in the Guide to Molecular Cloning Techniques (Ogden and Adams, 1987). The RNA sample was first denatured in a mix of 1 M glyoxal, 50% (v/v) dimethyl sulfoxide, and 10 mM sodium phosphate buffer (pH 7.0) at 50°C for 1 hour. A quarter volume of loading buffer (10 mM sodium phosphate (pH 7.0), 50% (v/v) glycerol and 0.4% bromophenol blue) was added to the glyoxylated RNA sample which had been cooled on ice and the preparation was loaded into a 1% agarose gel (in 10 mM sodium phosphate (pH 7.0)). The gel was run at 5 V/cm with constant recirculation of the buffer to maintain the pH at 7.0 until the bromophenol blue had migrated half through the gel. Following electrophoresis, the RNA was visualized by staining with

ethidium bromide (0.5 μg/ml in running buffer).

6. Primer extension analysis

The protocol from Current Protocols in Molecular Biology (Ausubel *et al.*, 1988) and part of the manufacture instruction (5'-AmpliFINDER Kit, Clontech lab, Inc.) were adapted and modified. A 25-mer oligonucleotide complementary to bp 1 to 25 of the *ard* coding sequence was synthesized and used as a primer for primer extension analysis. [γ-^{32}P]ATP labeled oligonucleotide was mixed with 9 μg of poly (A) mRNA in 30 μl of RNA hybridization buffer (4 part of formamide and 1 part of 5 x stock solution; 5 x stock solution contains 200 mM PIPES, pH 6.4, 2 M NaCl and 5 mM EDTA). After heating at 85°C for 10 minutes, the mixture was quickly moved to a 37°C water bath and incubated overnight. Following hybridization, the annealed RNA was precipitated in ethanol and redissolved in 10 μl of d-H_2O. The reverse transcription reaction was carried out by adding 20 μl of master mix (1.5 x Reverse transcriptase buffer, 50 units of RNase inhibitor, 1.5 mM dNTP mix and 12.5 units of AMV reverse transcriptase) and incubated at 37°C for 2 hours. The reaction was stopped by adding 1 μl of 0.5 M EDTA and the RNA template was removed by digesting with RNase (0.33 μg/μl) at 30°C for 30 minutes. After phenol/chloroform extraction, the cDNA was precipitated in ethanol and redissolved in 10 μl of TE buffer. The cDNA product was analyzed in a 6% polyacrylamide gel/7 M urea followed by autoradiography.

7.RACE PCR

The manufacture instructions (Marathon™ cDNA Amplification Kit, Clontech lab, Inc.) were adapted. The sequences of primers and adaptors used in this experiment are described below.

a) cDNA synthesis primer (52-mer): 5'-TTCTAGAATTCAGCGGCCGC$(T)_{30}N_1N$-3'

b) Adaptor primer (AP1, 27-mer): 5'-CCATCCTAATACGACTCACTATAGGGC-3'

c) R16-94 primer (25-mer): 5'-GCACCAAGCGCTCTTCATCTTCGGA-3'

d)cDNA adaptor:

5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3'
3'-H_2N-CCCGTCCA-PO_4 -5'

The procedure of RACE PCR is shown in Figure 3. The first stand cDNA was synthesized by mixing 10 μM cDNA synthesis primer with 1 μg of one-day-old poly $(A)^+$ mRNA and the reaction mixture (10x first-strand buffer, 10 mM dNTP mix, 1 μCi [α-^{32}P]dCTP and 100 units of MMLV reverse transcriptase) to 10 μl and incubating at 42°C for 1 hour. To synthesize the second strand cDNA, the first strand cDNA synthesis reaction solution was mixed with second-strand buffer (100 mM KCl, 10 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 0.15 mM β-NAD, 20 mM Tris (pH7.5), and 0.05 mg/ml BSA), 0.2 mM dNTP and second-strand enzyme cocktail (0.3 units/μl *E. coli* DNA polymerase I, 0.6 units/μl *E. coli* DNA ligase, and 0.0125 units/μl *E. coli* RNase H) and incubated at 16°C for 1.5 hours. 10 units of T_4 DNA polymerase

was then added to the mixture and the incubation continue for another 45 minutes. The reaction was stopped by adding an EDTA/glycogen mixture (10 mM EDTA and 0.1 mg/ml glycogen) and the mixture was extracted with phenol/chloroform. The double stranded cDNA was then precipitated in ethanol. After ligating the cDNA adaptor to both ends of the double stranded cDNA, PCR was carried out by mixing the adaptor ligated cDNA (5 µl) with AP1 primer (10 µM), *ard* sequence specific primer (R16-94;10 µM) in master mix (10x PCR buffer, 25 mM $MgCl_2$, 10 mM dNTP and 50x polymerase mix). The mixture was preheated at 94°C for 1 min. Using a DNA thermal cycler (Savant Temperature Cycler, Model TC49), 30 cycles of amplification were carried out by using a step program (94°C, 30 sec; 60°C, 30 sec; 68°C, 2 min). The PCR product was cloned into the SmaI site of pBluescript KS (+) and the sequence was determined using the Sequenase II kit (U.S. Biochemicals).

D. Germ-line transformation

1. Construction of LacZ fusion genes

The two expression vectors used in this study were P-element vectors(Rubin and Spradling, 1983), CZ20XN and HZ50PL (Hiromi and Gehring, 1987)(see Figure 4). Five constructs in the CZ20XN vector and two in the HZ50PL vector were prepared. All cloning procedures were carried out by using standard techniques (Sambrook *et al.*, 1989).

a) CZ20XN-XX & HZ50PL-XX: The XbaI/XbaI fragment (see Figure 5) was cut

from clone #4 obtained from genomic library screening and subcloned into the XbaI site of CZ20XN vector and that of HZ50PL vector, respectively. The clone with the right orientation was selected by restriction mapping.

b) CZ20XN-HX & HZ50PL-HX: The 0.8 kb HindIII/HindIII fragment (see Figure 5) was first cut from clone #4 and subcloned into pHSS7 vector (see Figure 6) to create pHSS7-HH. The XbaI-HindIII-XbaI fragment was then cut from the pHSS7-HH and cloned into CZ20XN vector and HZ50PL vector, respectively.

c) CZ20XN-1HH: The 0.9 kb HindIII/HindIII fragment (see Figure 5) obtained from clone #4 was cloned into pHSS7ΔEC to create pHSS7ΔEC-1HH. The pHSS7ΔEC is the same as pHSS7 except the EcoRI/ClaI region has been deleted. Following, the NotI fragment containing the HindIII/HindIII fragment was cut from pHSS7ΔEC-1HH and subcloned into the NotI site of CZ20XN vector.

d) CZ20XN-Mva27: The 2.7 kb MvaI fragment (see Figure 5) was cut from clone #4, blunt-ended, and cloned into the SmaI site of pBluescript KS(+). The new construct was digested with BamHI and EcoRI and the fragment containing the 2.7 kb MvaI was subcloned into pHSS7 to create pHSS7-Mva27. The 2.7 kb MvaI fragment with BamHI and EcoRI borders was obtained by partially digesting pHSS7-MvaI with NotI and cloned into CZ20XN vector.

e) CZ20XN-Mva19: The 1.9 kb MvaI fragment (see Figure 5) was cut from pHHS7-Mva27 by using NotI and subcloned into the NotI site of CZ20XN vector.

2. Construction of 5' end deletions for LacZ fusions

5'-side deletions of the 1HH fragment were generated by exo III/mung bean nuclease digestion. The protocol used was modified from a protocol obtained with an ExoIII/Mung Bean Nuclease Deletions Kit (Stratagene). The pKS1HH DNA was digested with Apa I and ClaI to generate a unique 3' overhang restriction site and a unique 5' overhang restriction site, respectively. The deletion was performed by incubating the double digested DNA with 1X exonuclease buffer, 10 mM β-mercaptoethanol and 20 U of exonuclease III (Stratagene) at room temperature. An aliquot (25 μl) was removed from the reaction at 90 seconds intervals and transferred to a tube containing 20 μl of 10 x mung bean nuclease buffer and 155 μl of H_2O. Tubes were placed in dry ice until all aliquots were removed. All samples were heated at 68°C for 15 minutes and placed on ice. The single stranded DNA was deleted by adding 15 units of mung bean nuclease (New England Biolab) and incubating at 30°C for 30 minutes. Following this step the DNAs were religated in the presence of 1 x ligation buffer and 2 units of T_4 DNA ligase (Gibco BRL) at 16°C overnight. Following transformation and sequencing, 4 clones (pKS1HH-12D, pKS1HH-22D, pKS1HH-36D and pKS1HH-37D) were chosen for further subcloning. The four chosen clones and the pKS1HH fragment were digested with KpnI and the 3' recessive end was filled-in with T_4 DNA polymerase. XbaI linkers were attached to the blunt-ended DNAs and the constructs were religated. The XbaI fragment was then cut and subcloned into the XbaI site of CZ20XN-XHM. The CZ20XN-XHM

was prepared by deleting the XbaI fragment from the CZ20XN-Mva27. Five constructs were created, i.e. CZ20XN-HHM, CZ20XN-22M, CZ20XN-12M, CZ20XN-37M and CZ20XN-36M. All five fusion constructs and CZ20XN-XHM (see Figure 7) were used for P-element mediated transformation.

3. Microinjection procedure

ry^{506} fly embryos were collected on apple juice agar plates (1.5% sugar in apple juice) with a yeast paste at 30 minutes intervals and brushed into a 25-ml beaker containing about 3 ml of tap water. Chorions were removed by filling up the beaker with 3% sodium hypochlorite and incubating for 1 min at room temperature. The embryo suspension was then poured through a small basket. The small basket was made by removing the bottom of a scintillation vial and drilling a hole into its cap, which was used to secure a nylon mesh (opening 140 μm). Embryos were rinsed thoroughly in a gentle stream of tap water, dried by touching with kimwipes and spread on a slide. Using a dull needle, embryos were positioned to form a line without touching one another on the edge of a double sticky tape with each embryo oriented in the same direction (posterior poles of embryos face the edge). A small strip of tape with lined-up embryos was then transferred to the edge of a coverslip while maintaining the orientation of embryos towards the edge of the coverslip. To complete the preparation for injection, embryos were desiccated in a petri dish containing Drierite (Aldrich Chem. Co. Inc.,). The time for embryo lining-up and

drying varied depending upon room temperature and humidity. Typically, 5 - 7 minutes for lining-up and 3 - 4 minutes for drying were applied for the first round. While the embryos of the first round were drying, a second round of lining-up was carried out (3 - 4 minutes) and then the second round embryos were dried for 1 -2 minutes. After desiccation, the embryos were covered with a thin layer of Halocarbon oil (Series 95; Halocarbon Products, North Augusta, SC, USA).

A glass capillary needle was connected to a needle holder which was mounted on an inverted microscope stage (Model: Bio Star; Reichert, Inc.). The needle holder was joined by a polyethylene tubing (i.d. 1.14 mm; o.d. 1.57 mm) to a 60-ml syringe. For injection, the tip of the needle was positioned within the embryo at 5% - 10% egg length (posterior pole = 0%) by moving the stage toward to it. A tiny amount of DNA solution was injected using air pressure generated by pressing the syringe and the needle was quickly withdrawn. Wrinkled embryos and overage embryos were avoided and destroyed. After microinjection, embryos were kept on the coverslip under oil in a moisture chamber and incubated for 2 days at room temperature. Eclosed larvae were transfer to vials containing cornmeal agar medium.

Needles were pulled from capillaries (o.d. 1.0 mm, i.d. 0.5 mm; World Precision Instruments, Sarasota, FL, USA) using a Vertical Pipette Puller (Model 700B; David Kopf Instruments, Pasadena, CA, USA). Sharp tips (proximal diameter of 2 mm) were generated by touching the apex of the needle with a fine tipped forceps. The glass needle was filled by suction from the syringe.

4. P-element mediated transformation

Plasmid DNA containing 5' upstream region fused to the *lacZ* reporter gene and a transposon marked with the *rosy* gene was mixed with DNA (pπ25.7wc (Karess and Rubin, 1984)) containing a helper P element capable of providing transposase and microinjected into ry^{506} mutant embryos prior to the stage of pole cell formation as described above (termed the G_0 generation; see Figures 8 & 9). Each of the newly eclosed adults of G_0 were mated to 3 ry^{506}adults of the opposite sex. The G_1 ry^+ progeny were mated singly to 3 of the opposite sex ry^{506} adults again. The selected G_2 ry^+ males were crossed to *ry* stocks containing appropriate Balancer chromosomes - *bAdh/CyO* (chromosome 2) or *rf10/TM3* (chromosome 3) to establish homozygous stocks. The self-cross of G_2 is continued until the homozygous stocks is established.

5. Frozen sections

One drop of O.C.T. compound (Lab-Tek Division, Miles) was added onto the surface of specimen holder. The holder was immersed half-way into liquid nitrogen until the O.C.T. compound began to freeze at the bottom. The holder was removed from the liquid nitrogen and placed on a flat surface. The O.C.T. continued to freeze from bottom to top. A second smaller drop of O.C.T. was added when the unfrozen area had contracted to a ring of about 0.5 cm in diameter. To ensure proper contact between the two blocks, the second drop of embedding medium was added before the first drop was completely frozen. The second drop, in which the tissue would be

embedded had a diameter of approximately 0.5 cm. Before the second drop froze, the tissue was added. Late stage pupae or adult flies were transferred to the block using a needle. Tissue was embedded at high density but not in contact with each other. The tissue was placed in the center of the drop. The block was immersed again half-way into liquid nitrogen and allowed it to freeze completely. The block was transferred into the cryostat chamber and equilibrated for approximately 30 min. After the block has been equilibrated to chamber temperature, it was mounted to the microtome and trimmed if necessary. 8 - 10 μm sections were cut and collected on subbed microscope slides. After the sections covered an area of about 18 x 36 mm of the slide, it was placed on a hot plate at about 52°C for 3 min to dry and flatten the sections onto the gelatin matrix of the slide. Now the slides were ready for fixation.

6. Detection of β-galactosidase distribution patterns in frozen sections

The frozen sections on slides were fixed with 3% formalin in PBS for 10 minutes and washed 3 times with PBS, 10 minutes each. The sections were then stained with X-gal staining solution (10 mM $NaPO_4$ (pH 7.2), 150 mM NaCl, 1 mM $MgCl_2$, 3 mM $K_4[FeII(CN)_6]$, 3 mM $K_3[FeIII(CN)_6]$, 0.3% Triton X-100, and 0.2% X-gal (in DMSO); recipe obtained from Dr. Ballinger) at 37°C overnight. The reaction was stopped by removing solution and washing with 2 changes of PBT (PBS with 0.05% Tween 20), 1 change of 70% ethanol and 2 changes of PBS sequentially. The slides were mounted in 90% glycerol, observed and photographed.

7. Detection of β-galactosidase distribution patterns in whole mount embryos

A protocol from Dr. Leslie Pick (personal communication) was adapted for X-gal staining of whole mount embryos. Aged embryos (10 - 22 hours) were collected and dechorionated in 3% sodium hypochlorite for 90 seconds. Dechorionated embryos were then fixed in freshly prepared fixation solution (heptane : 50% glutaraldehyde : PBS = 2 : 1 : 0.5) for 10 to 15 minutes at room temperature. After settling, embryos in interface were transfered onto a glass slide in small drops and allowed heptane to evaporate. Embryos were picked up on the corner of a piece of double stick tape and placed on a new glass slide with embryos facing up. Covered with a drop of Ringer's solution, embryos were devitellinized by poking them with a needle. Devitellinized embryos were collected in a microfuge tube and rinsed once with warm staining solution (10 mM $NaPO_4$, 150 mM NaCl, 1 mM $MgCl_2$, 3.05 mM $K_4[Fe(III)(CN)_6]$, 3.5 mM $K_3[Fe(II)(CN)_6]$). The embryos were then stained at 37°C overnight in pre-warmed staining solution with 0.2% X-gal (in DMSO). Stained embryos were rinsed with 70% ethanol and 100% ethanol once of each and stored in 90% glycerol/1X PBS overnight. The embryos were mounted on a slide between two small coverslips, covered with long coverslip and observed.

8. β-galactosidase activity assay

β-galactosidase activity was determined according to Kitamoto *et al.*, (1992) with slight modifications. Ten heads of each *lacZ* transformant line (1-day-old) were

homogenized in 200 µl of homogenization buffer (50 mM Tris-Cl (pH 8.0), 0.1 M NaCl, and 0.5% Triton X-100). The homogenates were centrifuged at 13,000 rpm for 15 minutes at 4°C. 25 µl of the supernatant was mixed with 50 µl of 10 mM chlorophenol red-β-D-galactopyranoside (CPRG, Boehringer Mannheim) and 450 µl of 50 mM sodium phosphate (pH 7.5) and 1 mM $MgCl_2$. Reactions were incubated at 37°C for 1 hour, and the absorbance was read at 574 nm. Samples were assayed in duplicates. The amount of protein was determined in parallel. The standard procedure of a modified Lowry assay (Peterson, 1983) was used. 25 µl of supernatant was diluted to 200 µl with d-H_2O and incubated with reagent A (1:2:1= CTC: 5% SDS: 0.8 M NaOH; CTC is a solution containing one part of 0.1% $CuSO_4$ and 0.2% potassium sodium tartrate and one part of 10 % Na_2CO_3) at room temperature for 10 minutes. After adding 100 µl of reagent B (0.33 N Folin-Ciocalteu phenol), the incubation was continued at room temperature for 30 minutes. The absorbance was read at 750 nm. β-galactosidase activity is expressed in arbitrary units as O.D. units/mg protein. Background due to endogenous *Drosophila* β-galactosidase activity and light scattering material present in the crude extracts was measured by using extracts prepared in parallel from the nontransformed strain (ry^{506}). In calculating β-galactosidase levels in transformant extracts, this background was subtracted.

E. Mobility shift assay

1. Preparation of DNA fragments

Six DNA fragments (see Figure 10) were generated using the pBluescript constructs (pKS1HH, pKS1HH-22D, pKS1HH-36D, and pKSMva2.7). All of the fragments contained at least one 5' protruding end so that they could be labeled with T4 DNA polymerase. All procedures were carried out by using standard techniques (Sambrook *et al.,* 1989).

a) F1: pKS1HH was digested with TaqI and electrophoresed using a 2% low melting agarose gel. The proper size of DNA fragment (195 bp) was excised and isolated by using β- agarase I (NewEngland Biolab) digestion.

b) F2 & F3: A 764 bp DNA fragment was released from pKS1HH-22D by digesting with KpnI and SacI. After gel purification, the fragment was digested with Sau96I. F2 (198 bp) and F3 (174 bp) fragments were purified as F1.

c) F4: The F4 fragment (157 bp) was obtained by digesting pKS1HH-36D with KpnI and XbaI. The fragment was purified as described in F1.

d)F5 (170 bp): The same as described in F4 except using XbaI and HindIII.

e)F6: The F6 fragment (142 bp) was released by using pKSMva2.7 and digesting with BamHI and HindIII. The fragment then was purified by using β agarase I digestion.

2. Preparation of embryo nuclear extracts

Embryo nuclear extracts for gel retardation assay were prepared by a modification

of the method of Han *et al.*, (1993). 10 - 22 hour embryos were grown on cornmeal agar media sprinkled with dry yeast in two mass population cages of *Drosophila melanogaster* (Oregon R). The embryos were collected on a nylon sieve (opening 100 μm) and transfered to a 500-ml beaker. The embryos then were dechorionated by filling the beaker half-full with 3% sodium hypochlorite and allowed to stand for 90 seconds. The dechorionated embryos were washed sequentially with tap water, 500 ml of 0.7% NaCl/ 0.04% Triton X-100, and 1000 ml of 0.7% NaCl and blotted dry. The procedure was continued in the cold room. The embryos were suspended in the homogenization buffer at a concentration of 3 ml buffer/g embryos and homogenized in 1500 rmp with a Teflon glass homogenizer. The homogenization buffer contained 0.35 M sucrose, 15 mM Hepes (pH7.6), 10 mM KCl, 2.5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, and the following protease inhibitors: 50 μg/ml soybean tyrosine inhibitors, 1 mM benzamidine, 1 mM DTT, 2 U/ml aprotinin, 1μg/ml antipain, 1μg/ml bacitracin. After 20 strokes, the homogenate was diluted to 100 ml with homogenization buffer, transferred to an SS-34 centrifuge tubes and centrifuged at 12,000 rpm for 10 minutes at 4°C. The whitish pellets were resuspended in same volume of homogenization buffer. The homogenization was repeated and centrifuged once more. The whitish pellets were then resuspended in lysis buffer at a concentration of 5 ml buffer/g embryos. The lysis buffer was 15 mM Hepes (pH7.6), 0.1 M KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol and the same protease inhibitors as in the homogenization buffer. The lysed suspension was

resuspended thoroughly by 10 strokes with a Teflon glass homogenizer and transferred to Ti45 centrifuge tubes. The nuclei were lysed by adding 1/10 volume of 4 M ammonium sulfate solution (pH 7.9) and rotating the tubes for 30 minutes. The lysate was centrifuged in a Ti45 rotor for 1 hour at 36,000 rpm at 4°C. The supernatant was precipitated by adding solid ammonium sulfate (0.3 g/ml) gradually during 15-minute of slow stirring and then neutralized by adding 1 N NaOH (1μl/g ammonium sulfate) and further stirring for another 15-minute. The precipitate was collected in Ti45 tubes after spinning for 25 minutes at 36,000 rpm (4°C). The pellet was resuspended in no salt HEMG buffer at the concentration of 50μl/g starting material. The no salt HEMG buffer is 25 mM Hepes, pH7.6, 0.1 mM EDTA 12.5 mM $MgCl_2$, 10% glycerol, and 1 mM DTT. The protein concentration was measured by the method of Bradford (Bradford, 1976) using a Bio-Rad protein assay solution. The sample was frozen in liquid nitrogen and stored at -70°C.

3. Gel retardation assay

The procedure was carried out essentially as described by Han *et al.*,(1993). DNA fragments were end-labeled with [α-^{32}P]dCTP and T4 DNA polymerase. 5 μg nuclear extracts was incubated with 10 fmole labeled fragment in a total volume of 25 μl reaction buffer. The reaction buffer contained 0.1 M KCl, 25 mM Hepes pH 7.9, 0.5 mM DTT, 10% glycerol, 0.5 mM EDTA and 5 μg poly(dI-dC)(dI-dC). Samples were incubated on ice for 1 hour and electrophoresed in a cold room using

a 4% native polyacrylamide gel and 0.5X TBE as a running buffer. The gel was electrophoresed at 250 volts until the bromophenol blue dye run to the bottom of the gel. The gel was then dried at 80°C and analyzed by using a phosphoimager (Molecular Dynamics).

III. RESULTS

Isolation of *ard* genomic DNA clones containing the 5'-flanking sequence

To begin with the investigation of the transcriptional regulatory elements controlling the expression of the *Drosophila* neuronal nAChR *ard* subunit gene, a *Drosophila melanogaster* genomic library was screened to isolate clones containing 5'-flanking sequence of the *ard* gene. A ^{32}P-end-labeled oligonucleotide derived from the exon2 sequence of the *ard* gene (see Figure 1) was used as a probe for screening under low stringency conditions. Seven putative positive clones (clones #1 - #7) were identified after secondary screening. Four of them (clone #1, #3, #4, and #7) showed a strong hybridization signal while the other three clones (clone #2, #5, and #6) showed ambiguous signals. To further confirm truly positive clones, Southern hybridization experiments and restriction mapping were carried out. The results revealed that two (#1 and #4) out of these seven putative positive clones were identical clones showing the same restriction patterns as part of the known *ard* gene sequence (Hermans-Borgmeyer *et al.*, 1986). The partial restriction map of the clone and part of the *ard* gene are shown in Figure 1. These two genomic clones span about 14.5 kb from approximately 8.7 kb of 5' flanking region to the middle of exon 6 of the *ard* gene. Clone #4 was used for further analysis. The other five clones were not studied further since they either do not have the same restriction patterns as described in Sawruk *et al.*, (1988) or were not positive in Southern blot analysis.

Transcription start sites

The major transcriptional regulatory elements of many eukaryotic genes are located upstream of their transcription initiation sites. We anticipated this would be the case for the *ard* gene. Therefore, as the next step in identifying regulatory elements in the 5'-flanking region of the *ard* gene responsible for its specific expression, the transcription start site(s) were analyzed by using primer extension assays and RACE-PCR. Primer extension was used to determine the length between the 5' end of the mRNA and the position of the primer. RACE-PCR was used to ascertain and define precisely the start site(s). The probe used is indicated in Figure 11, and the results of primer extension assay and the sequence of PCR product are shown in Figure 12 and Figure 13, respectively. The primer extension assay with poly $(A)^+$ RNA from one-day-old adults and late stage (10 to 22 hours) of embryos both yielded identical patterns which included three products of 331 nt, 338 nt, and 376 nt. The product of 338 nt started with an adenosine is obviously more intense than the other two products and designated as +1 of the transcription start site. The two minor products are, therefore, positioned at +8 and -38. The RACE-PCR showed only one product located at +23. Another RACE-PCR product was also observed but the size was about 54 bp shorter than the other RACE-PCR product. The results of primer extension assay and RACE-PCR suggest that the transcription initiates at multiple start sites and the major start site is located 304 bp upstream from translation start site. It appears that the *ard* subunit gene has the same transcription

start sites during development at the embryonic as well as the adult stage. It is noted that during this investigation six different primers with sequences ranging from exon1 to exon3 were also tested in the primer extension assays without giving any reaction products (see Figure 11). The reason for these failures remains unknown.

Sequence analysis and characterization of the 5'-flanking region

Once the transcription initiation sites were identified, about 750 bp of upstream sequence from the initiation sites was sequenced (see Figure 14). Examing the upstream sequence from the transcription start site, no traditional TATA and CCAAT consensus sequence were found just like other genes with multiple transcription initiation sites (Matter-Sadzinski *et al.*, 1992; Bessis *et al.*, 1993; Hu *et al.*, 1994). Although six TATA sequences are found further upstream, they are too far removed to participate in the initiation of *ard* gene expression.

Searching the upstream sequence from the transcription start sites for known regulatory sequences, eight E boxes are found within a 570 bp region. Further, eight Ets-like protein binding sites are found around the transcription start sites. Five of them are located upstream while the other three are downstream of the start sites. Five *zeste* and one GAGA binding sites are also seen around the cluster of the transcription start sites of the *ard* gene (see Figure 14). Four *zeste* binding sites are located upstream and one is down stream of the transcription start sites. It is noted that three of the four upstream potential *zeste* binding sites and the GAGA binding

site are all located within the region between -487 and -332, the minimal essential sequence for driving the expression of the *ard* gene (see below).

Strategy for identification of regulatory elements

The strategy for identification of regulatory elements was to fuse various DNA fragments from the 5'-upstream region of the *ard* gene to the *lacZ* reporter gene in a P element transformation vector. The regulatory properties of the various DNA fragments in fusion genes were then to be tested. The structures of the two P element transformation vectors used in this study - CZ20XN and - HZ50PL are illustrated in Figure 4. The HZ50PL vector contains a hybrid gene possessing a fusion of the *hsp70* basal promoter element to the *Escherichia coli lacZ* sequences. The hybrid gene was placed within a P element vector (Rubin and Spradling, 1983) containing a *rosy* gene as a phenotypic marker for transformation. The HZ50PL vector also contains a unique XbaI and a NotI restriction sites upstream of the *hsp70-lacZ* construct which served as the recipient for test fragments. The CZ20XN vector is identical to the HZ50PL vector except the *hsp70* basal promoter element has been deleted. The rationale for this two-pronged approach with both vectors rests on the assumption that test sequences inserted into HZ50PL would generate "enhancer fusions" while such constructs with CZ20XN would result in "promoter fusions".

Several transformant lines for each construct were generated and analyzed for reproducible patterns of β-galactosidase expression. Expression was examined in

one-day-old adults by using the histochemical X-gal staining and quantitative analysis of β-galactosidase activity. In some cases, late embryos and late pupae were also analysized. The list of transformant lines described in this work are presented in Tables 1, 2 and 3.

Expression specificity of the fusion constructs

A. Initial analysis

To identify transcriptional control elements regulating the transcription of the *ard* gene, transformant lines with five different restriction segments (XbaI/XbaI, HindIII/XbaI, HindIII/HindIII, MvaI-2.7 and MvaI-1.9; see Figure 5) were initially analyzed. The 0.72 kb HindIII/XbaI has the same 3'-boundary (position -333 relative to translation start site) as the 4.2 kb XbaI/XbaI while having a shorter 5' end for 3.5 kb. The 0.9 kb HindIII/HindIII fragment is the same as HindIII/XbaI fragment except the 3' end was extended 170 bp to HindIII site. The MvaI-2.7 and MvaI-1.9 fragments have the most extended 3' end to MvaI site (position -28 relative to translation start site). The 5'-boundaries of MvaI-2.7 and MvaI-1.9 differ. All fragments were cloned into either the XbaI or NotI cloning site of CZ20XN expression vector (see Materials & Methods for detailed procedures) to generate CZ20XN-XX, CZ20XN-HX, CZ20XN-HH, CZ20XN-Mva27, and CZ20XN-Mva19, respectively. In parallel, HZ50PL-XX and HZ50PL-HX were also prepared to identify any regulatory elements other than promoter. Transformant lines were

established after P element transformation was performed (see Materials and Methods for details). The expression patterns of reporter gene constructs in transformed one-day-old adult heads were examined by using X-gal staining. Table 1 and 2 show the summary of transformant lines and X-gal staining results of CZ20XN and HZ50PL constructs, respectively.

The patterns of β-galactosidase expression is determined by the extent of the specific putative regulatory DNA to drive *lacZ* expression. No X-gal staining were observed for any of the transformant lines of CZ20XN-XX and CZ20XN-HX. 5 out of 8 of transformant lines of CZ20XN-HH show similar expression patterns to distribution patterns of the *ard* transcript (Wadsworth *et al.*, 1988; Hermans-Borgmeyer *et al.*, 1989). In one-day-old adult head section X-gal staining is detected in the cortical regions of the midbrain, the cellular cortex of the optic lobe area and the cortical regions of thoracic ganglia (see Figure 15A & 16B). A more detailed view of brain area is shown in Figure 15B. A uniform X-gal staining can be seen in the cortical regions surrounding the lobular and medulla, while the cellular cortex of the lamina contains relatively low levels of X-gal staining. Only very low level or none of X-gal staining was detected in central brain ganglia. Similar expression patterns were also detected in late pupae sections (see Figure 16A). Three lines showed no significant X-gal staining, possibly indicating insertion into a nonfunctional chromosome position.

Most of CZ20XN-M27 and CZ20XN-M19 transformant lines (6 isolines total

each) also showed the same expression patterns as the *ard* transcript. Like the transformant lines of CZ20XN-HH, some lines showed no X-gal staining. The staining pattern observed for all positively expressing lines was qualitatively similar. No X-gal staining were detected in control transformant lines of CZ20XN. These results indicate that the essential *cis*-acting regulatory elements responsible for spatial *ard* gene expression are located within the 0.9 kb HindIII/HindIII region.

In addition, the β-galactosidase expression patterns of HZ50PL-XX and HZ50PL-HX were also tested in one-day-old adult heads. No X-gal staining were detected in control transformant lines of HZ50PL (see Table 2). Most transformant lines of HZ50PL-XX constructs and HZ50PL-HX constructs show no X-gal staining either. X-gal staining can only be detected in 4 out of 12 isolines of HZ50PL-XX and 3 out of 15 isolines of HZ50PL-HX. These ratios are much lower than that of CZ20XN constructs. In addition, the expression patterns of both HZ50PL constructs are different from each other as well as to those in CZ20XN constructs. For the HZ50PL-XX, the X-gal staining was detected mainly in the optic lobe area and the cortical region of the mid-brain (see Figure 21A & 21B). Unlike the expression patterns seen in the CZ20XN constructs, the X-gal staining was seen in the neuropil regions all over the optic lobe while the lamina has the most intense staining. The X-gal distribution patterns in HZ50PL-HX are seen in the cell body area of lamina and cortical regions of Modula and lobular. In addition, some axons in medulla and lobular were also lightly stained (see Figure 22A & 22B).

B. Analysis of 5'-deletions of the 0.9 kb HindIII/HindIII fragment

Since the 0.9 kb HindIII/HindIII fragment contains the essential regulatory elements, it is interesting to define more precisely the properties and molecular limits of the *cis*-regulatory elements within the 0.9 kb HindIII/HindIII region. In order to study this further, a series of 5'-deletions were constructed by using exonuclease III/mung bean nuclease and fused to the bacterial reporter gene *lacZ* in CZ20XN vector. Six new constructs were created: CZ20XN-HHX, CZ20XN-22M, CZ20XN-12M, CZ20XN-37M, CZ20XN-36M and CZ20XN-XHM. At least two transformed lines were established for each construct. Again, each transformed line was examined for its resulting β-galactosidase expression patterns in one-day-old adults. Table 3 presents a summary of the distribution patterns for each construct included in this study. All of the transformed lines of each construct showed positive X-gal staining except CZ20XN-XHM. The expression patterns of one-day-old adults are the same as that of the *ard* transcript and that of CZ20XN-HH transformant lines. In addition, the X-gal staining of the late stage of embryo and late pupa of CZ20XN-36M were also tested. The distributions of expression patterns are identical to those of the *ard* transcript. All transformant lines showed essentially the same pattern relative to each other. Much lower or no X-gal staining was detectable in CZ20XN-XHM.

Quantitative analysis of expression

Levels of expression from the transformed *lacZ* fusion genes were determined quantitatively with a simple liquid β-galactosidase assay system that uses CPRG as a substrate. CPRG produces a water-soluble product and is preferable to ONPG because it is reported to be ten times more sensitive (Simon and Lis, 1987). For crude fly extracts, CPRG has the additional advantage over ONPG that the product absorbs at a wavelength not significantly interfered with by fly pigments. In CPRG assay of crude fly extracts, the rate of color development was constant over at least four hours of reaction time at 37°C and the relationship between extract concentration and CPRG cleavage was linear over a 100-fold range of extract concentration (Simon and Lis, 1987).

The results of β-galactosidase activity assay were shown in Figure 23. The transformant line of seven 5'-deletion constructs and CZ20XN were studied. The β-galactosidase activity is expressed in arbitrary units as O.D. units/mg protein. Background due to endogenous fly β-galactosidase activity and light scattering material present in the crude extracts was measured by using extracts prepared in parallel from the nontransformed strain (ry^{506}). This background was subtracted numerically from β-galactosidase levels in transformant extracts. As expected, the transformant lines with the expression vector alone (i.e. CZ20XN) has only background level of β-galactosidase activity. All of the transformant lines with various 5' flanking regions showed various levels of β-galactosidase activity. The

β-galactosidase activities directed by HHM, 22M, 12M, 37M and 36M were in a range between 1.5 and 2.5 O.D. units/mg protein. The β-galactosidase activity directed by HH seems a little lower (< 1 O.D. unit/mg protein) than in any of the other positive lines. The β-galactosidase activity dropped dramatically to almost background level in the transformant lines of CZ20XN-XHM. These results correspond to the results of X-gal staining.

Gel mobility shift assay

To identify the essential region required for interacting with trans-regulators, gel mobility shift assays were carried out. The 1.03 kb of HHM fragment was cut into six sub-fragments (F1 - F6) (see Figure 10) by digesting different constructs from previous experiments with appropriate restriction enzymes (for details see Materials and Methods). ^{32}P-labeled DNA fragments were incubated with 10 - 22 hr *Drosophila* embryo nuclear extracts in the presence of poly(dI-dC)(dI-dC). The DNA-protein complexes were separated from the free probes by a 4% native polyacrylamide gel. The results indicate that among six fragment probes tested by mobility shift assays, almost all of them interacted with proteins in the 10-22 hours embryo nuclear extract.

The competition result with F4 is shown in Figure 24A. There are four F4-protein interaction products (bands a,b,c and d). Only one of them (band b) is specific and can be competed partially by F2 but not by any other fragment probes.

Band a, c and d are competed by non-specific DNA (SK; a 102 bp plasmid fragment from pBluescript II SK(+)) and therefore considered non-specific products. As shown in Figure 24B, there are four F5-protein interaction products but only one (band a) is specific. The specific binding can be competed by F4 and partially by F1, F2 and F6 but not by F3 and SK. The competitors were in a 50-fold molar excess of the probes. The specific interaction between F3 and proteins from late stage embryonic nuclear extract was also observed when competed by unlabeled probe (see Figure 25A). But the F3-protein binding (band b) can only be partially competed by F1 and F2. It is interested to note that the F3-protein binding was not competed by F3' which is the same as F3 except having a shorted 5' end with 54 bp. In addition, competition with F6 is also observed (see Figure 25B). The interaction between F6 and proteins from late stage embryonic nuclear extract seems more complex. There are four F6-protein interaction products (band a, b, c and d). In self-competition, band d is completely competed out by unlabeled F6, while the competitions of band a, b, and c are not quite complete. Within these four products, bands b and d are considered specific and bands a and c are non-specific based on the competition by SK. Band b is competed by F1 and partially by F2. Band d is competed by F1 and partially by F2, F3 and F4.

IV. DISCUSSION

Isolation and characterization of the gene coding for the *Drosophila* nAChR *ard* subunit gene has been described previously (Wadsworth *et al.*, 1988; Sawruk *et al.*, 1988). The temporal and spatial distributions of the *ard* transcript was detected through development. During mid-embryogenesis, during metamorphosis, and in newly enclosed adults, the *ard* transcript was readily detectable while the *ard* transcript levels dropped significantly during early embryogenesis, in larvae, and early stage of pupae. The expression pattern of the *ard* gene was detectable in the developing central nervous system but was not detected in any other tissues. In agreement with the temporal distribution, the *ard* transcript were either undetectable or at a low level in the central nervous system during early embryogenesis, in larvae, and early pupae stage. The main aim of our study was to understand the temporal and spatial regulation of the *ard* subunit gene and eventually that of the nAChRs in central nervous system.

The primer extension analysis and RACE-PCR suggested multiple transcription start of the *ard* gene. The major start site is located 304 bp upstream from the translation start site. Two minor start sites are positioned +8 and -38 relative to the major start site. Using transfection assays in various cell lines, the multiple transcription start sites were also observed in the promoter regions of other vertebrate nAChR genes, such as the chicken nAChR $\alpha 2$, $\alpha 7$, and $\beta 4$ subunit genes (Matter-

Sadzinski *et al.*, 1992; Bessis *et al.*, 1993; Hu *et al.*, 1994). Common start sites were identified in both late embryonic stage and one-day-old adult, indicating a conservation of transcription initiation during development in embryonic as well as one-day-old adult stages.

By using P-element mediated transformation, our experimental results indicated one region of promoter activity within 325 bp around the transcription start sites. The HindIII/HindIII (0.9 kb) but neither HindIII/XbaI (0.72 kb) nor XbaI/XbaI (4.2 kb) drive the expression of *lacZ* fusion gene. This supports the identification of the transcription start sites since the start sites are located at the 3' end of HindIII/HindIII fragment which is beyond the XbaI site. Our 5' deletion experiments suggest that the distal region of 0.57 kb flanking sequence (region between -1056 and -487 relative to the translation start site) does not contribute significantly to control the expression of the *ard* gene. Only when the 5' deletion was extended further to -332 relative to the translation start site which is very close to the transcription start sites was a drastic reduction in transcriptional activity observed. Therefore, the sequence between - 487 and -332 is suspected to harbor at least one essential element for driving the expression of the *ard* gene. The results of β-galactosidase activity assay also support this observation. In addition, comparing the β-galactosidase activity between transformant lines with different 5' deletion constructs, it is noted that the activity directed by the construct with HH fragment seems to be lower than in any other positive lines. The similar phenomena was observed in X-gal staining. The X-gal

staining of transformant lines of CZ20XN-HH was less sensitive than that of CZ20XN-36M. The reason of this difference needs further investigation.

While this investigation was in progress, a similar result was reported in one-day-old adult flies by Hess and colleagues (Hess *et al.*, 1994). The cluster of start sites we identified within the 5'-flanking sequences correspond to that reported by Hess *et al.*, (1994) although the number of start sites reported by these investigators are different from that we identified. By using primer extension assay and RACE, Hess and colleagues identified four and six transcription start sites, respectively. Our major start site agrees to the most 5'-end minor start site of the primer extension products reported by this group. In addition, our RACE-PCR product agrees exactly to the 3'-end minor start site of primer extension products identified by this group (see Figure 14). The discrepancy between these results needs further investigation.

Moreover, Hess and colleagues (Hess *et al.*, 1994) identified a 900 bp DNA fragment spanning ~760 bp upstream and ~140 bp downstream of the transcription start sites containing the essential information for specific expression of the *ard* gene which is in agreement with the data presented in this thesis. However, in this investigation, we have reduced the essential sequence for the *ard* gene specific expression to 325 bp around the transcription start sites. Our data as discussed above show that this 325 bp DNA fragment is sufficient to drive correct expression during the development including late embryonic, late pupae and one-day-old adult stages and the expression patterns are similar to those of the endogenous *ard* transcript.

Comparing our competition assay results of gel retardation analysis with embryonic nuclear extract, there was no obvious competition between tested fragments (i.e. F3, F4, F5 and F6) but it seems that they were all competed by F1 and F2 with one exception (there was no competition between F1 and F4). Together with the 325 bp essential sequence required for correct expression of the *ard* gene, it indicates that the transcriptional regulation of the *ard* gene is complex and needs further investigation.

RNA polymerase II transcription initiation usually begins about 30 bp downstream of a TATA box (Breathnach and Chambon, 1981). Analysis of the nucleotide sequence upstream of the cluster of the transcription start sites reveals the absence of TATA and CCAAT boxes. Although six TATA box sequences are found further upstream, they are located too far away to be associated with control of *ard* expression. In the absence of TATA and CCAAT boxes, initiation is heterogeneous.

There are two classes of promoters that lack obvious TATA boxes, one is GC-rich and the other is not. The GC-rich promoters are found primarily in housekeeping genes that are constitutively active, contain several transcriptional initiation sites spread over a fairly large region, and usually contain several binding sites (i.e. 5'-CCCCTCCC-3') for the transcription factor Sp1(Dynan and Tjian , 1983; Sehgal *et al.*, 1988). The other class which does not contain GC-rich sequence are regulated during development and initiate transcription at only one or a few tightly clustered sites. In addition, this type of promoters contains multiple copies of the binding sites

for the transcription factors, ZESTE and GAGA (Biggin and Tjian, 1988; Yao and White, 1994). The promoter structures of several developmentally important *Drosophila* genes, such as *engrailed* (*en*), *Ultrabithorax* (*Ubx*), *Antennapedia* (*Antp*) and *E74* have been reported to fall in this class (Soeller *et al.*, 1988; Biggin and Tjian, 1988; Perkins *et al.*, 198; Thummel, 1989).

Inspection of the sequence around the *ard* transcription start site, together with the data discussed above, indicated that the *ard* promoter belongs to the second class. Within the sequence between HindIII (-1056) and the translation start site, there are no GC-rich sequence and no potential Sp1 binding sites. In contrast, five *zeste* and one GAGA binding sites are seen around the cluster of the transcription start sites of the *ard* gene (see Figure 14). Four *zeste* binding sites are located upstream and one is downstream of the transcription start sites. It is noted that three of the four upstream potential *zeste* binding sites and the GAGA binding site are all located within the region between -487 and -332, the minimal essential sequence for driving the expression of the *ard* gene.

Searching for more possible sequence motifs of known *cis*-regulatory elements, eight potential Ets-like protein binding sites in both orientations are found within 1.05 kb fragment which including the cluster of putative transcription start sites (see Figure 14). The Ets family members have a primary role in the formation of the initiation complex on minimal core promoters lacking the TATA sequence (Wasylyk *et al.*, 1993) and are classical activators that stimulate transcription from a distance. The Ets

proteins have been found to bind specifically to these sequences contain an invariant core motif C/A GGA A/T in the middle of 10 bp of DNA. Ets1 directly contacts all 10 bp and interacts with GGA in the major groove and with flanking sequences in the minor groove. The flanking sequences are variable and there is growing evidence that they help determine which Ets protein will bind. The *ets* motif may be located up- or down-stream from the transcription start site. Indeed, three out of eight potential Ets-like binding sites are located downstream of the transcription start sites.

The E boxes with consensus sequences CANNTG has the binding sites for the transcription factors containing helix-loop-helix (HLH) domain. E boxes have been reported to exist in all 5' flanking regions of characterized muscle nAChR subunit genes within their putative promoter regions, although they are different in their number and distance to the transcriptional start site. At least two functional E box elements, separated only by a short stretch of nucleotides, are present in the promoter regions of the chicken, mouse and rat γ subunit gene and in the α subunit enhancer of chicken and mouse. In contrast, the mouse β, the rat and mouse ϵ and rat δ minimal promoter regions contain only one CANNTG consensus sequence. In addition, a number of transcription factors bearing a basic helix-loop-helix (bHLH) motif have been described that are required for regulation of the neural programme. In *Drosophila*, the proneural genes containing the HLH motif have also been found to be involved during neurogenesis (for review see Campos-Ortega, 1991). They are important for development of the central and peripheral nervous system in the

embryo. Such proteins are thought to form two amphipathic helices separated by a loop that mediates dimer formation. This would enable the formation of homodimers or heterodimers with specific DNA-binding and transcription regulatory properties. Expression of these genes is thought to provide the cells with neural potential. Homologues of these genes have been found in a number of vertebrate species in which they are expressed in developing nerve cells. Analyzing the upstream sequence from the transcription start site, eight E boxes are found within 570 bp region. One of them is within the sequence from -487 to -332. Whether this single E box is involved in the expression regulation or additional E boxes are required need further investigation.

The 1.05 kb *ard* upstream region was further compared to the 5' flanking regions of other genes encoding proteins involved in neurotransmission in the *Drosophila* nervous system, such as acetylcholinesterase (*ace*) (Fournier *et al.*, 1989) and dopadecarboxylase (*ddc*) (Scholnick *et al.*, 1986; Johnson and Hirsh, 1990). No significant sequence similarity has been found between the *ard* regulatory region and the promoter regions of those genes.

So far, five different subunits of nAChR genes in *Drosophila melanogaster* have been identified, i.e. ALS, Dα2, Dα3, ARD and SBD. Two of the subunits (i.e. ALS and ARD) have been suggested by using immunoprecipitation experiments to be components of the same receptor complex (Schloss *et al.*, 1991; Schloss *et al.*, 1992). In addition, ALS, Dα2, and ARD have been shown to share a widespread similar

distribution in the central nervous system of *Drosophila* and their expression may be overlapping (Schuster *et al.*, 1993; Jonas *et al.*, 1994). These subunit genes must be regulated in a highly coordinate way. Therefore, it is interesting to compare the promoter regions between these subunits genes. To date, only the 5'-flanking regions of *ard* and *Dα2* have been reported (Hess *et al.*, 1994; Jonas *et al.*, 1994). Just like the *ard* gene, the *Dα2* gene does not have TATA box-like sequence. The only common sequence between *ard* and *Dα2* in the 5' upstream regions is located between -998 and -984 relative to the translation start site of the *ard* gene and between -676 and -662 relative to the transcription start site of the *Dα2* gene. This region has 14 out of 15 identical sequence and includes an E-box. It is noted that, in the *Dα2* gene, six GAGAG sequence motifs are found within the first 350 bp upstream of the transcription start site.

In summary, by using P-element-mediated transformation, we were able to identify the *cis*-acting regulatory elements which confer neuronal specificity on the *Drosophila* nAChR *ard* subunit gene. During development (including late embryonic, late pupae and one-day-old adult stages), the 0.3 kb upstream region of the *ard* gene directed reporter gene expression to the particular area of central nervous system of fly head. The expression pattern is similar to that reported for the *ard* transcript. Our results suggest that the 325 bp DNA fragment around the transcription start site contains at least one *cis*-acting regulatory element and is sufficient for confer spatial and temporal transcriptional specificity. However, it cannot be excluded that

additional *cis*-acting elements in the *ard* subunit 5' flanking DNA contribute to the expression pattern of the *ard* subunit gene. In addition, some possible sequence motifs of known *cis*-regulatory elements have been identified within the 1.05 kb fragment while the functional significance of all potential *cis*-elements remains to be established.

Figure 1. The partial restriction map of the positive genomic clones #1 & #4 (bottom) and part of the corresponding *ard* gene (top)

The *ard* gene shown above was reported by Hermans-Borgmeyer *et al.*, 1986. The exons are shown by partially filled boxes. The transmembrane domains are shown as filled boxes. Numbers initial with 'i' are introns. "SP", signal peptide.

The 14.5 kb positive genomic clones contain 8.7 kb of 5'-flanking region and 5.8 kb of the *ard* sequence. The clones originated from the EMBL3 T7/SP6 vector. The T7 and SP6 promoter sequences are shown by horizontal arrows, respectively. The oligonucleotide probe (AChR4) used in genomic library screening is indicated by vertical arrows.

B, BamHI; E, EcoRI; H, HindIII; N, NotI; S, SacI; X, XbaI.

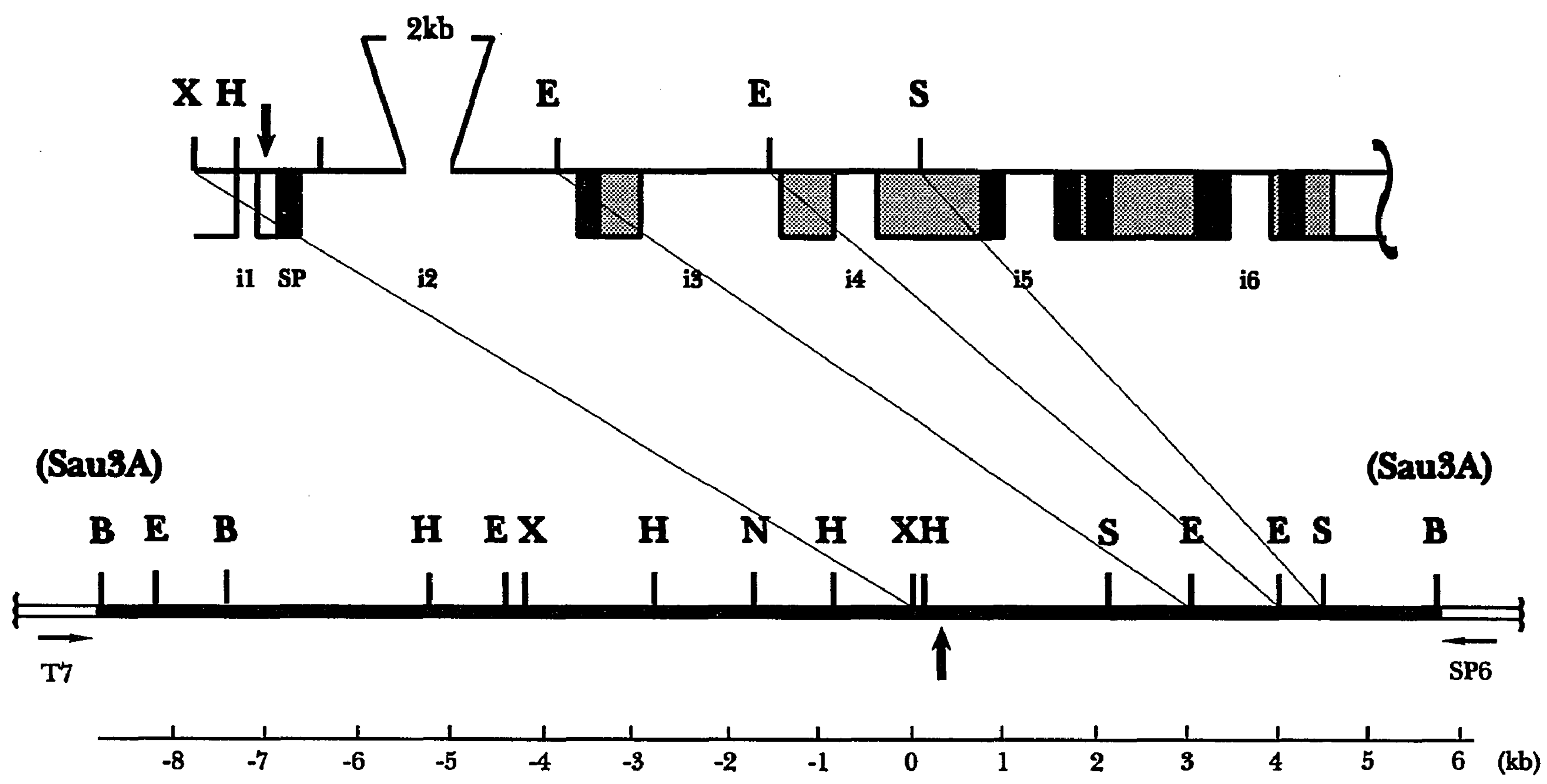

2kb
X H
E
E
S
i1 SP
i2
i3
i4
i5
i6
(Sau3A)
(Sau3A)
B E B
H E X
H
N
H
XH
S
E
E S
B
T7
SP6
-8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 (kb)

Figure 2. Three HindIII fragments

The fragments were subcloned into the HindIII site of the pBluescript KS (+). The nucleotide sequence of 1HH was determined by double-stranded DNA sequencing using the dideoxynucleotide chain termination method.

The closed arrow indicates the translation start site. The open arrow points to the transcription start area.

B, BamHI; E, EcoRI; H, HindIII; N, NotI; S, SacI; X, XbaI.

(2.5 kb) 3HH

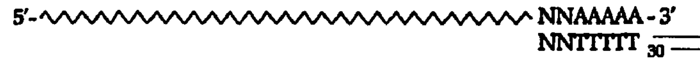
(1.9 kb) 2HH

(0.9 kb) 1HH

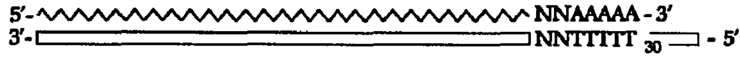
Figure 3. The Overview of RACE-PCR

The cDNA synthesis primer (5'-TTCTAGAATTCAGCGGCCGC$(T)_{30}$NN-3') was first annealed to poly $(A)^+$ RNA. After the first and second strand of the cDNA were synthesized, the adaptor (partial filled boxes) was ligated to both ends of the double stranded cDNA. 30 cycles of amplification (94° C, 30 sec; 60°C, 30 sec; 68° C, 2 min) were then carried out with preheating at 94°C for 1 min. The *ard* primer (5'-GCACCAAGCGCTCTTCATCTTCGGA-3') is shown as a closed arrow. The AP1 primer (5'-CCATCCTAATACGACTCACTATAGGGC-3') is indicated as an open arrow. (Adapted from "Marathon™ cDNA Amplification Kit", Clontech Lab, Inc.)

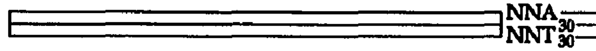
poly A^+ RNA



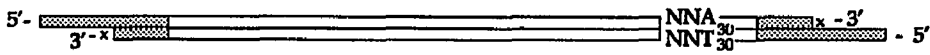
first strand synthesis



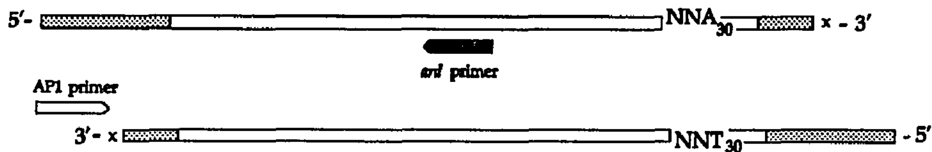
second-strand synthesis

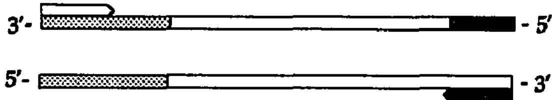


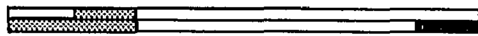
adaptor ligation



PCR







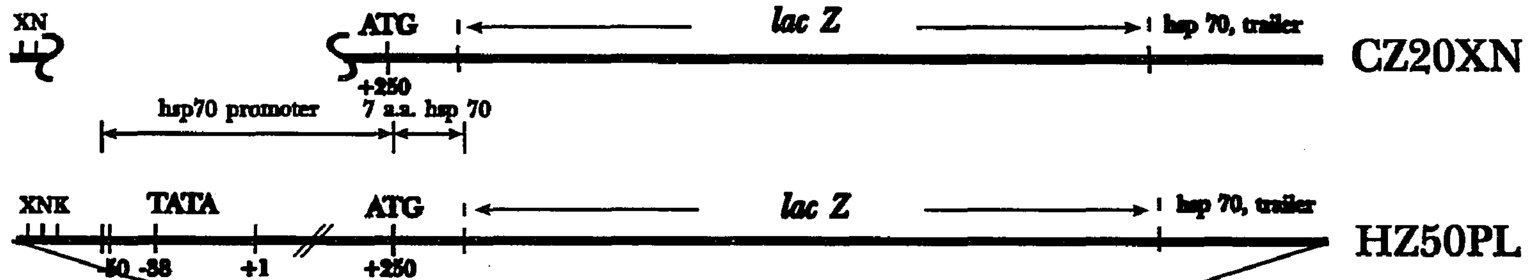
PCR products

30 cycles

Figure 4. The expression vectors CZ20XN and HZ50PL*

CZ20XN contains a *ry+* and a *lacZ* reporter gene. A polycloning site with XbaI and NotI is inserted between *ry+* and *lacZ.* HZ50PL contains an additional *hsp70* promoter 5' flanking to *lacZ.* *ry+*, *rosy+* (a phenotypic marker for transformation) H, HindIII; E, EcoRI; K, KpnI; N, NotI; X, XbaI.

* Dr. Leslie Pick personal communication.


XN
ATG
lac Z
hsp 70, trailer
CZ20XN
+250
hsp70 promoter
7 a.a. hsp 70
XNK
TATA
ATG
lac Z
hsp 70, trailer
HZ50PL
-50 -38
+1
+250

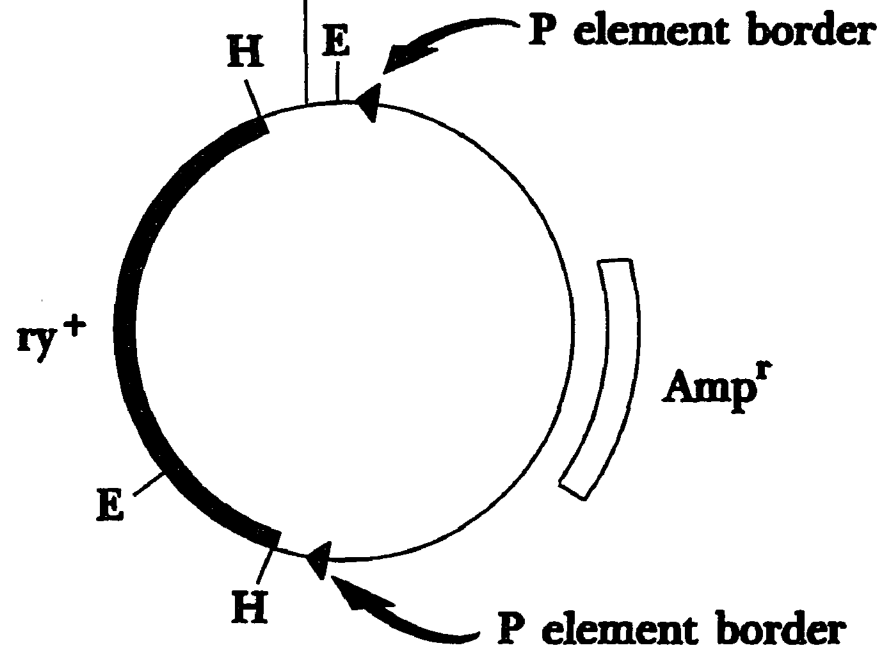
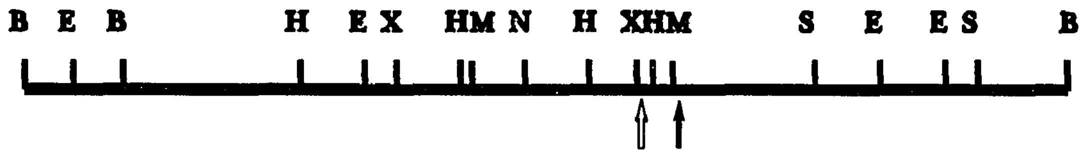

H
E
P element border
ry+
Ampr
E
H
P element border

Figure 5. The first set of restriction fragments tested for their *cis*-regulatory activity in this study

The drawing indicates different portions of 5' flanking sequence fused to the *lacZ* gene.

"open arrow", transcription start sites.

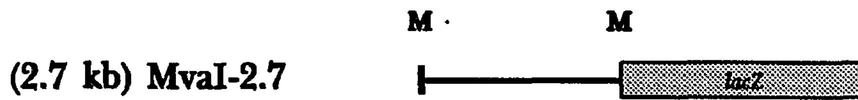
"closed arrow", translation start site.


B E B H E X HM N H XHM S E E S B

X X
(4.2 kb) XbaI/XbaI
lacZ

H X
(0.72 kb) HindIII/XbaI
lacZ

H H
(0.9 kb) HindIII/HindIII
lacZ


M M
(2.7 kb) MvaI-2.7
lacZ

N M
(1.9 kb) MvaI-1.9
lacZ

Figure 6. pHSS7*: the shuttle vector used in this study

* Dr. Leslie Pick personal communication.

Not I
EcoR I
Cla I
Hind III
Xba I
Bgl II
Pst I
BamH I
Not I
Sma I

Pst I
Bgl II
Filled in HindIII site
Not I

Km^r
185 bp M13 origin Fragment
ori

pHSS7

100 bp

Not I EcoR I Cla I Hind III Xba I Bgl II

GCGGCCGCGGAATTCATGTTTGACAGCTTATCATCGATAAGATTCTAGAGATCT

Pst I

TCCATACCTACCAGTTCTCCGCCTGCAGCAATGGCAACAACGTTGCCC

BamH I Not I Sma I

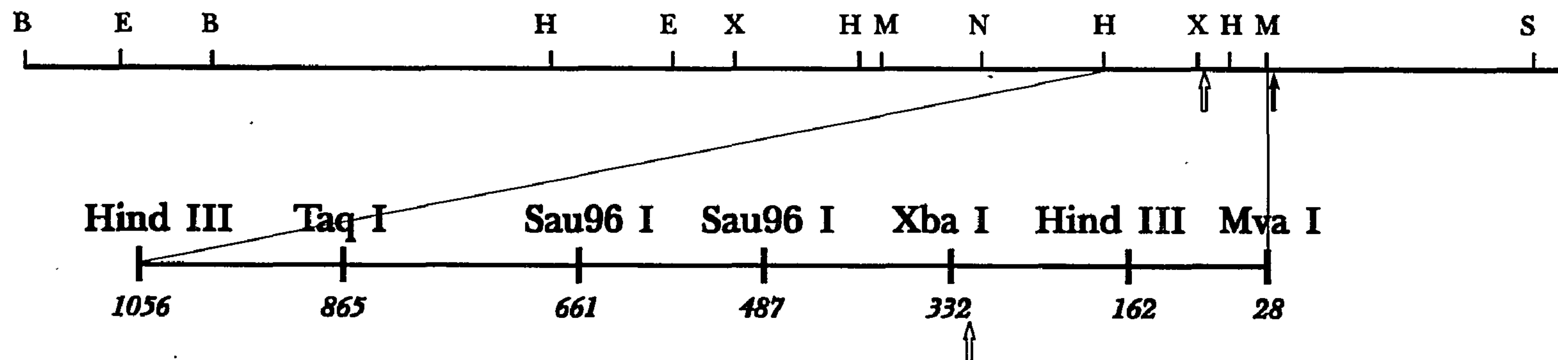
GGATCCGCGGCCGCCCGGG

Figure 7. The deletion fragments tested for *cis*-regulatory activity in this study

The drawing indicates different parts of the 1HH fragment with the same 3' end (MvaI, 28 nt related to translation start site) fused to the *lacZ* gene. The top portion shows the corresponding location of 1HH fragment to the *ard* gene.

"open arrow", transcription start site

"closed arrow", translation start site


B E B H E X H M N H X H M S
Hind III Taq I Sau96 I Sau96 I Xba I Hind III Mva I
1056 865 661 487 332 162 28

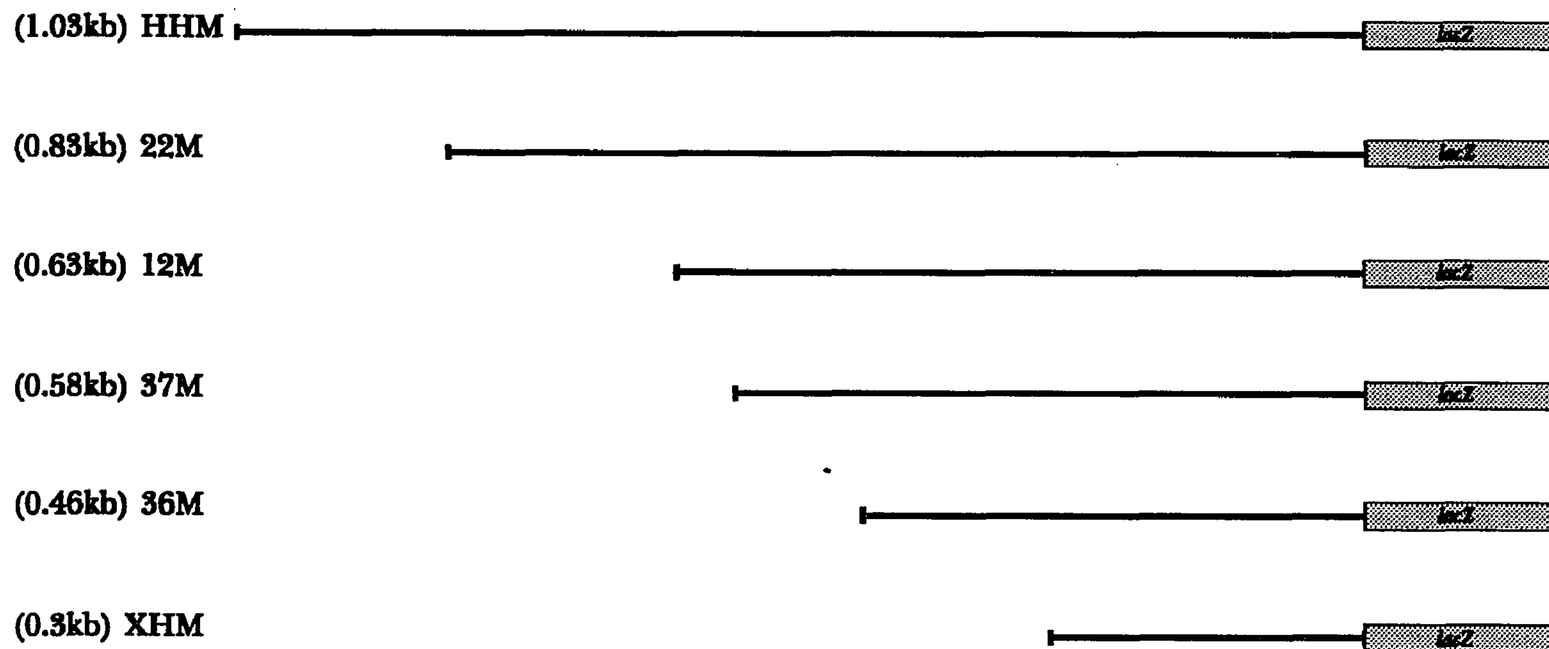

(1.03kb) HHM
lacZ
(0.83kb) 22M
lacZ
(0.63kb) 12M
lacZ
(0.58kb) 37M
lacZ
(0.46kb) 36M
lacZ
(0.3kb) XHM
lacZ

Figure 8. The principle of P-element mediated transformation

The plasmid DNA, pP[(ry+)A], containing fused 5' upstream region to the *lacZ* gene and a transposon marked with the *rosy* gene was co-injected with a helper plasmid, pπ25.7wc, containing a P element into *Drosophila* embryos. The expressed transposase recognized the intact P element borders in pP[(ry+)A] but not the defect P element borders in pπ25.7wc. Therefore, only the portion within the intact P element borders in pP[(ry+)A] was expressed and inserted into chromosomes. (Adapted from "Spradling, 1986").

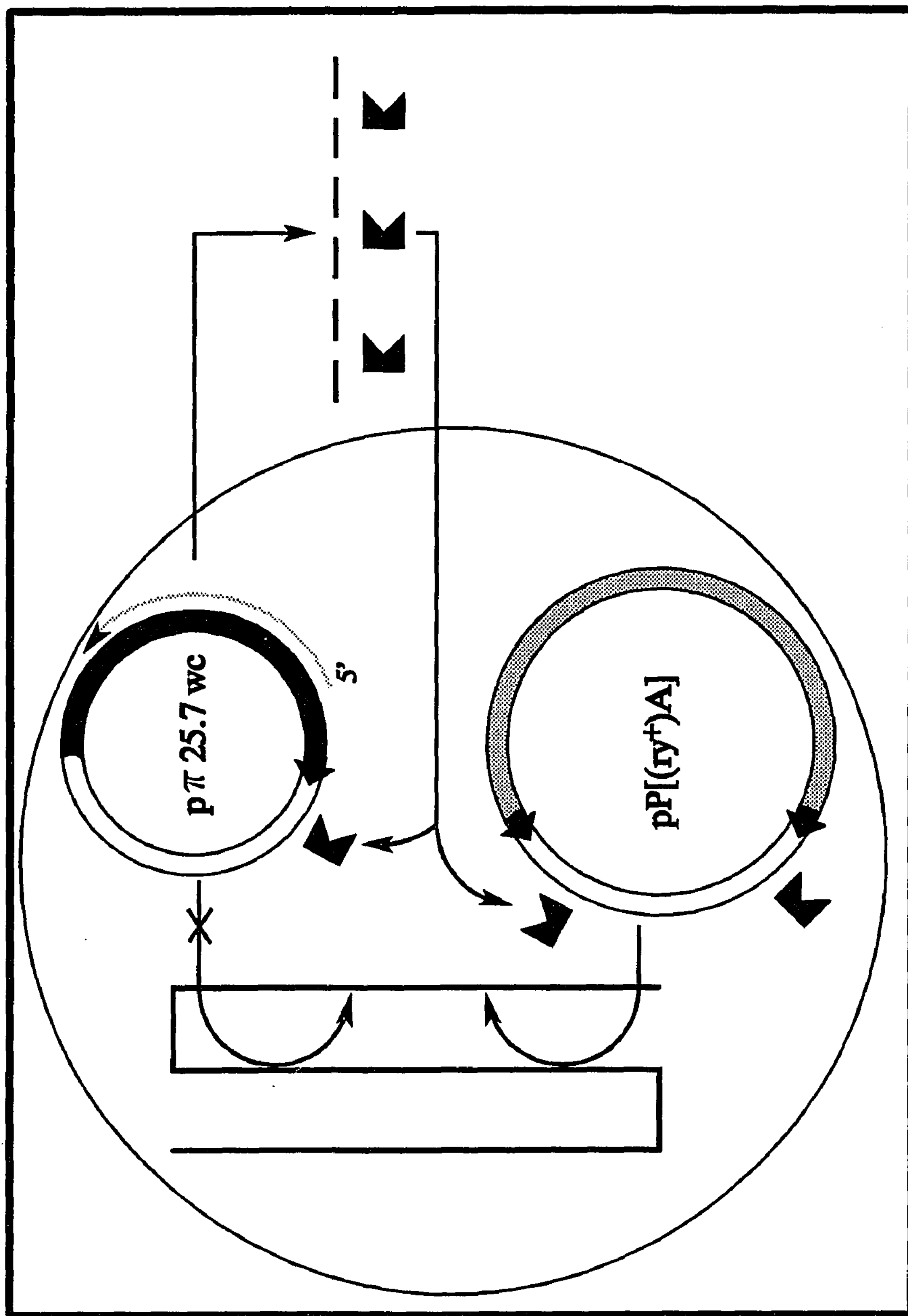

pπ 25.7 wc
5'
pP[(ry+)A]

Figure 9. Overview of balancer crosses

The pP[(ry+)A] was co-injected with pπ25.7wc into *ry*^{506} embryos (G_0). The adults of G_0 were back crossed with 3 *ry*^{506} flies of the opposite sex. The selected *ry+* of G_1 were back crossed again with 3 *ry*^{506} flies of the opposite sex to establish isolines. The G2 *ry+* males were crossed to balancer strains - *bAdh/Cyo* (chromosome 2) and *rf10/TM3* (chromosome 3) to establish homozygous stocks. (Modified from "Spradling, 1986").

<u>Generation</u> P[(ry$^+$)A] + pΠ25.7wc

microinject

G 0 ry^{506} ♂ or ♀ ⊗ ry^{506} ♂♂ or ♀♀

select ry$^+$

G 1 P[(ry$^+$)A];ry^{506} ♂ or ♀ ⊗ ry^{506} ♂♂ or ♀♀

select ry$^+$

G 2 Isolines

balancer crosses

G 3 Balanced Stocks

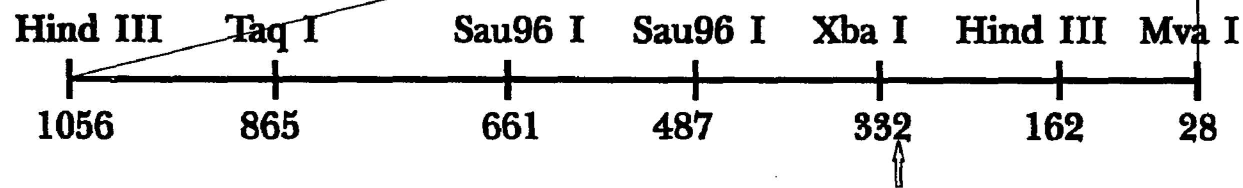
G 4 Homozygous Stocks

Figure 10. The restriction fragments used in the gel retardation assay

The top of the figure shows the corresponding location of the 5' flanking portion used in gel retardation assay. The bottom of the figure shows the six fragments (F1 - F6) used in gel retardation assay.

Translation and transcription start sites are marked with closed and open arrows respectively.

B
E
B
H
E
X
H
M
N
H
X
H
M
S


Hind III
Taq I
Sau96 I
Sau96 I
Xba I
Hind III
Mva I
1056
865
661
487
332
162
28

F1
F3
F5

F2
F4
F6

Figure 11. The seven oligonucleotides used in the primer extension assay

DNA sequence of the region of the XbaI site at -333 related to the translation start site and the end of exon3 (reported by Sawruk *et al.*, 1988).

The exons are indicated by upper case letters and the introns are indicated by lower case letters. The long intron2 is shown partially (i.e. about 2.6 kb is omitted).

"partial closed box", the only primer gave primer extension products.

"open boxes", the primers failed the primer extension assays.

TCTAGAAAGT CAGAATCCCA TTGATTTTTA TCCAGATTCC CGAAGTAGAG TTCTTAATCC GCACCCAAAC TGACGGCTCC AGTTCAAGAC

GGAAAGTTCG GAAAGAAAAT TCCCTGAAAA ATCAGTGAAA ATTAAGAAAA TATTCATAAA GCACTAGCTA AAATTAAGAA AAGCTTGAGA

TAAGgtgaag tgtgctgttt ttaagaggtt ttgtctggat taaaggcctc atttattgtg tctacagTTT TGGGGCGTGG GCCAGCGAAA

TCCCAATGCG AAAAAATATA AATTAGCCAT GTCCCTGGAG TAAGTGAAAA GTGTGGAAAA ATCATGGAGT CTTCCTGCAA ATCCTGGCTG

TTGTGCAGCA TCCTGGTGCT TGTGGCCTTT TCGTTGGgta cgtattttgg gctacgaaga agaagactgc cacgcccctt tcgggcaagg

gccgttatgt aatcgtaaag gaaagaaatg caaatgaagg cctaaaaact ctgttaac....................=2.6 KB ....

....gaattc ctatatgtgt gtgtacactc ttccgttgtt tttcgttcgc ttccgtttcc gttgtgtttt ttagTCAGTG CATCGGAAGA

TGAAGAGCGC TTGGTGGGTG ACCTCTTTCG AGGCTACAAT AAACTCATAC GAGCCGTACA GAATATGACA CAAAAAGTTG GAGTAAGATT

TGGTTTGGCG TTCGTACAGC TAATCAATGT C.........

Figure 12. Primer extension analysis

Three products were observed in primer extension assays with poly A^+ mRNA from both late embryonic stage (Em) and one-day-old adult (Ad). 338 nt is more intense than the other two products and designated as +1 of the transcription start site. The two minor products are positioned at +8 and -38. The right portion of the figure shows a marker sequence.

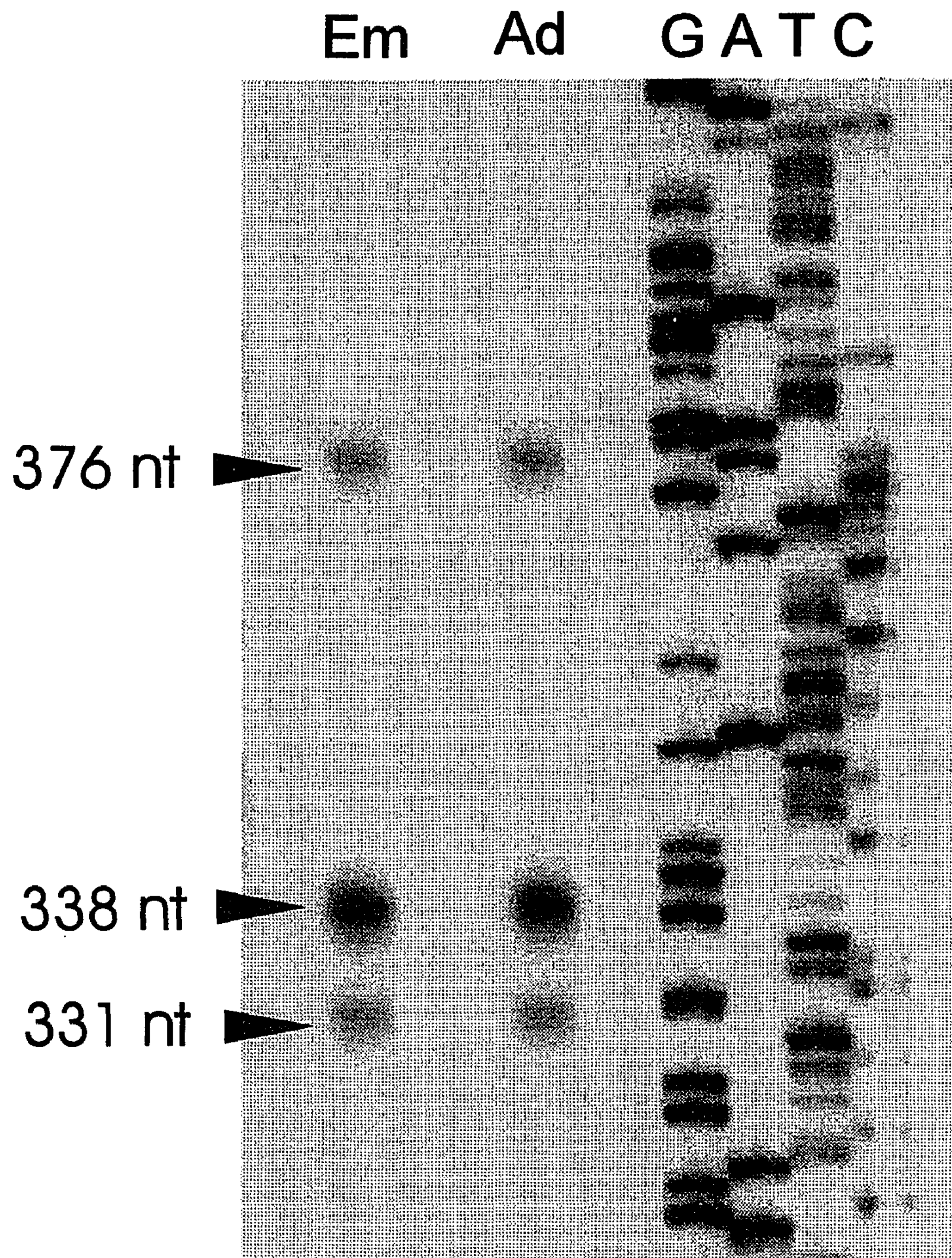

Em
Ad
G A T C
376 nt
338 nt
331 nt

Figure 13. The result of RACE-PCR

The figure shows the partial sequence of the RACE-PCR clone combining the sequences of pBluescript KS(+), adaptor and the *ard* gene. The transcription start site is indicated by a bold letter T.

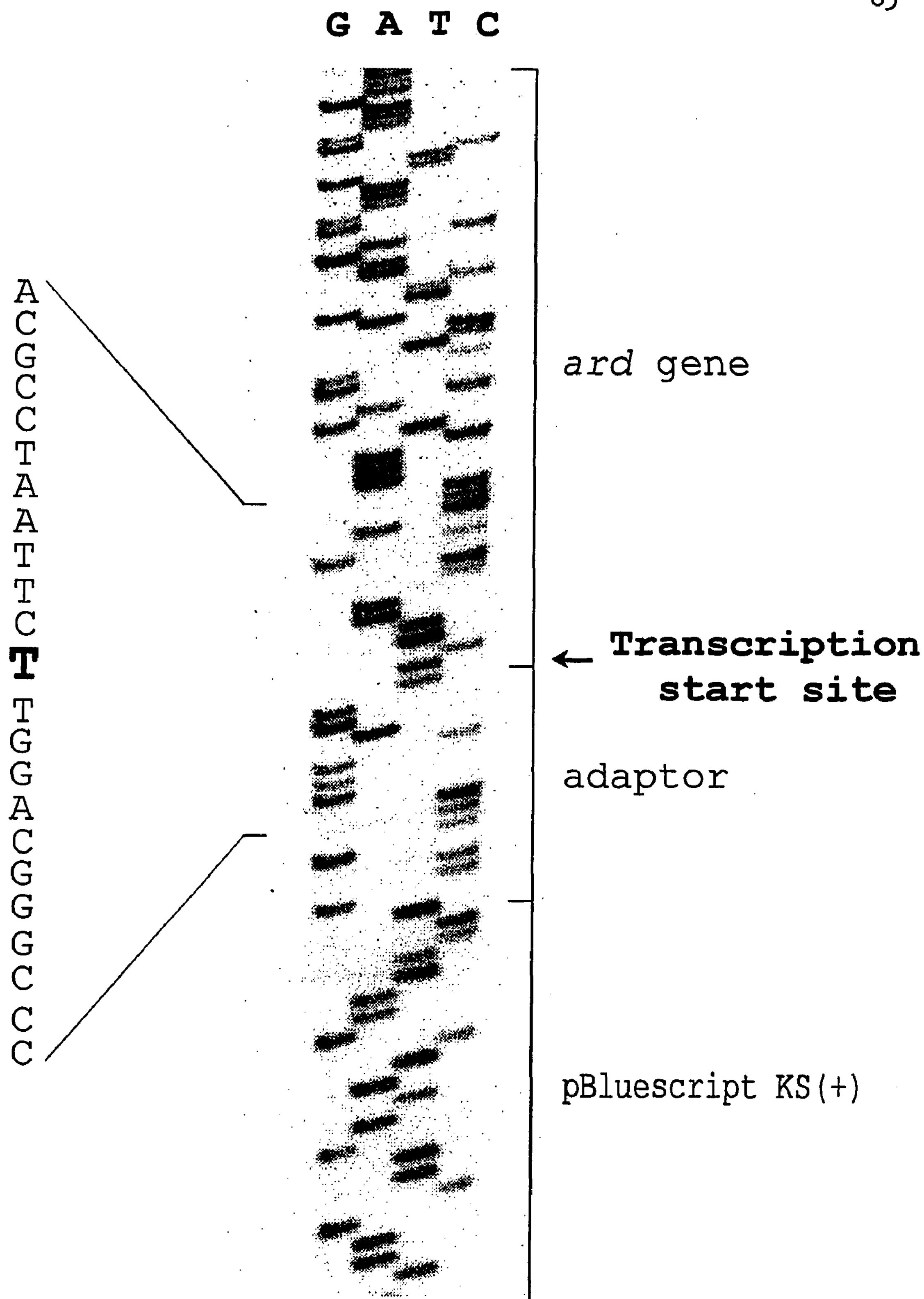

G A T C
A
C
G
C
C
T
A
A
T
T
C
T
T
G
G
A
C
G
G
G
C
C
C
ard gene
Transcription start site
adaptor
pBluescript KS(+)

Figure 14. DNA sequence of the region between the HindIII site at -1057 and the translation start site

(i) The cluster of the transcription start sites is shown by a heavily filled box. The primer extension products are indicated by open downward arrows above the sequence. The bigger one is designated arbitrarily as +1 of the transcription start site. The product of RACE-PCR is indicated by a close downward arrow above the sequence. The corresponding primer extension products reported by Hess *et al.*, (1994) are also shown (arrow heads underneath the sequence).

(ii) E-boxes are lightly filled boxes. Ets-like protein binding sites are indicated by horizontal arrows underneath. GAGA binding site is shown by an open box. *zeste* binding sites are indicated by horizontal arrows above the sequence.

(iii) The fragments used in the gel retardation assay are indicated by horizontal broken arrows underneath with corresponding fragment numbers.

1057 AGCTTACAAG CCCAAAGCCA TTTGGCAATA TAATTCAAA TGTAATTAAA GGTTAATATA AGTGCTGACA GCTGAATGAG AATTGGGAAT

F1

967 TTGACCAAAA GGTCAAACTG AAGCCATATT ATACAAGAGT TTAAAAATAA TGCACTTGCA TTTTTAGAAA TCTGGAACAA ATATCGTGAA

877 ATGTGGGTAC TCGAAAGCAT TGCCGTTGTT GGGAAACATT TATTTAAATC ATTTACGCGC CATGAGGAAC TGCGTTTTCC TAATGATAAC

F1 F2

787 CATAAGCGAT TGGACGCTGT GAAAAAATAT ATAAAAAAAA CTATGAAATA ACAAATTAAA TTCCACTCCC ACCTGACCTT TACGTCGTTA

697 GTTTTTTTTT TTTTTTGTTA TTCGCTTAAT TTATAGGCCC AAGTCTTATG TCATAACTAA ACATTTTTAT AACAAAATAA AACGAGATTG

F2 F3

607 CCGGATAGCG GAAGCATTCT TCCACTAACT TTTCACAATC ACTGTGGTGG AAACTTTTTG TGGGCGCCGG CAACTCTGGA GGCCAAGCAC

517 ATGTAAACAA ATATTATCAA CAGCTGATAG GGCCAACTGC TATTGTTTAC AAACTAAAGA TTATCAAAAG GGTTGGTTTT GGAAAAAACA

F3 F4

427 CCTTTAGAAA TGAGAGAGCT TTCCATTTTC ATCTCAGTTA TCCGAATTTT TCGCGCCTGC GCTCAGCTCT TTTTGTCAGA TTTGGCTCGT

337 GCGCTCTAGA AAGTCAGAAT CCCATTGATT TTTATCCAGA TTCGCGAAGT AGAGTTCTTA ATCCGCACCC AAACTGACGG CTCCAGTTCA

F4 F5

247 AGACGGAAAG TTCGGAAAGA AAATTCCCTG AAAAATCAGT GAAAATTAAG AAAATATTCA TAAAGCACTA GCTAAAATTA AGAAAAGCTT

F5 F6

157 GAGATAAGGT GAAGTGTGCT GTTTTTAAGA GGTTTTGTCT GGATTAAAGG CCTCATTTAT TGTGTCTACA GTTTTGGGGC GTGGGCCAGC

67 GAAATCCCAA TGCGAAAAAA TATAAATTAG CCATGTCCCT GGAGTAAGTG AAAAGTGTGG AAAAATC**ATG**

F6

Figure 15. Distribution of β-galactosidase activity in CZ20XN-HH transformed isolines

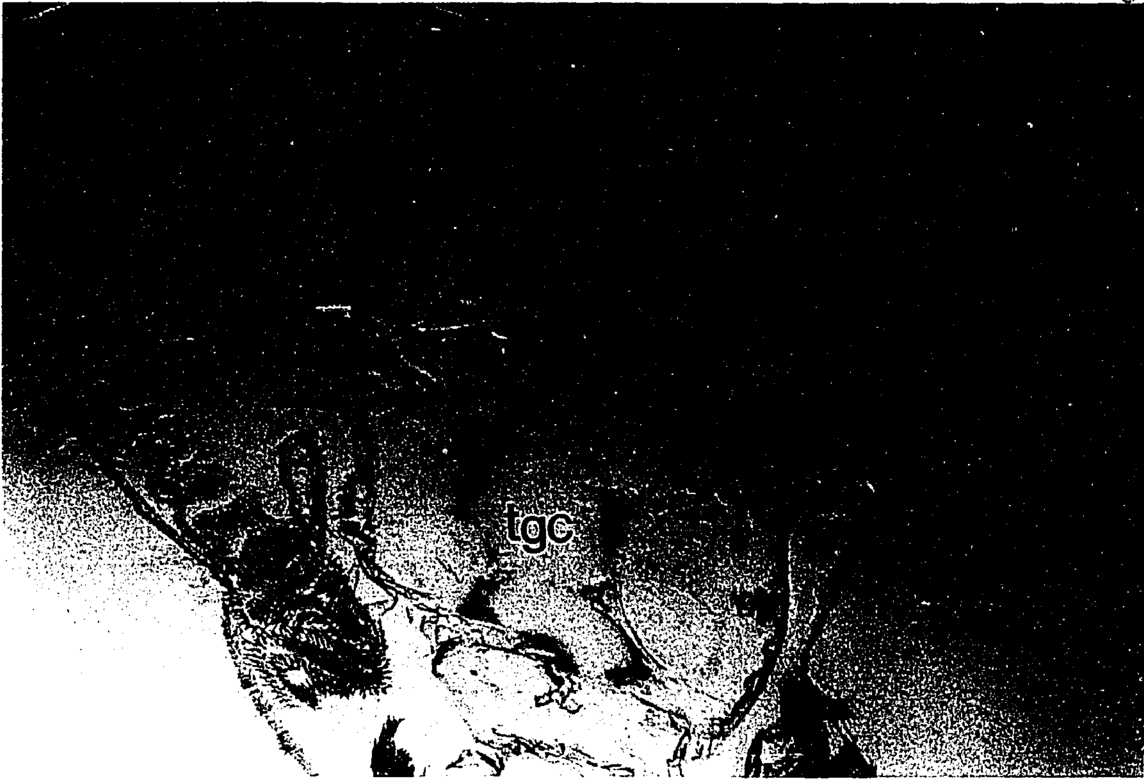
The X-gal staining patterns in horizontal section of one-day-old adult brain (A) and an enlarged view of right side optic lobe (B) are shown. b, brain; o, optic lobe; r, retina; l, lamina; m, medulla; lo, lobular; lp, lobular plate; cb, central brain.

b

B
cb

Figure 16. Distribution of β-galactosidase activity in CZ20XN-HH transformed isolines

The X-gal staining patterns in late stage of pupae (A) and the adult thoracic ganglionic center (B) are shown. b, brain; o, optic lobe; r, retina; tgc, thoracic ganglionic center.


tgc

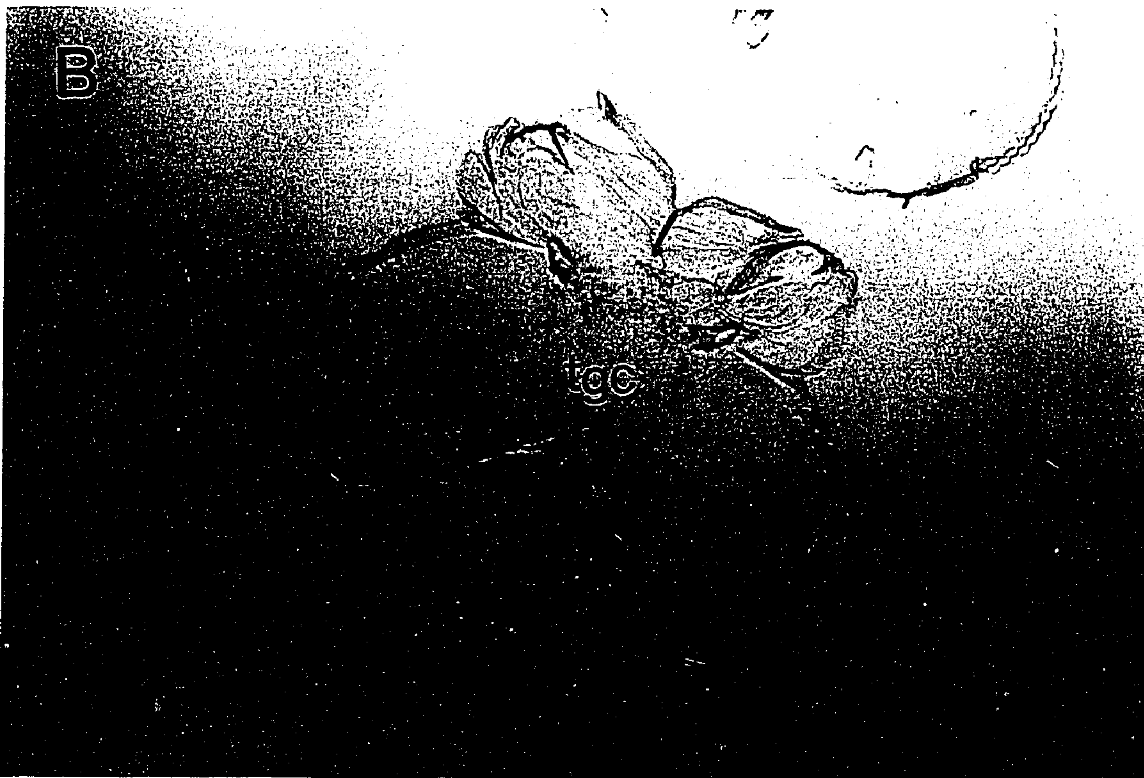
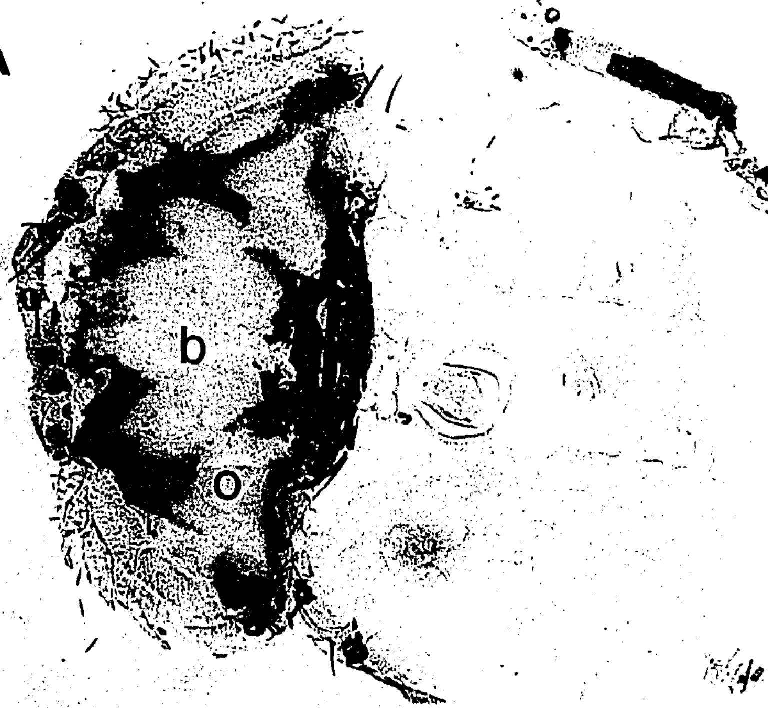

B
tgc

Figure 17. Distribution of β-galactosidase activity in CZ20XN-36M transformed isolines

The X-gal staining patterns in horizontal section of one-day-old adult brain (A) and an enlarged view of right side optic lobe (B) are shown. b, brain; o, optic lobe; r, retina; l, lamina; m, medulla; lo, lobular; lp, lobular plate; cb, central brain.


A
b
o

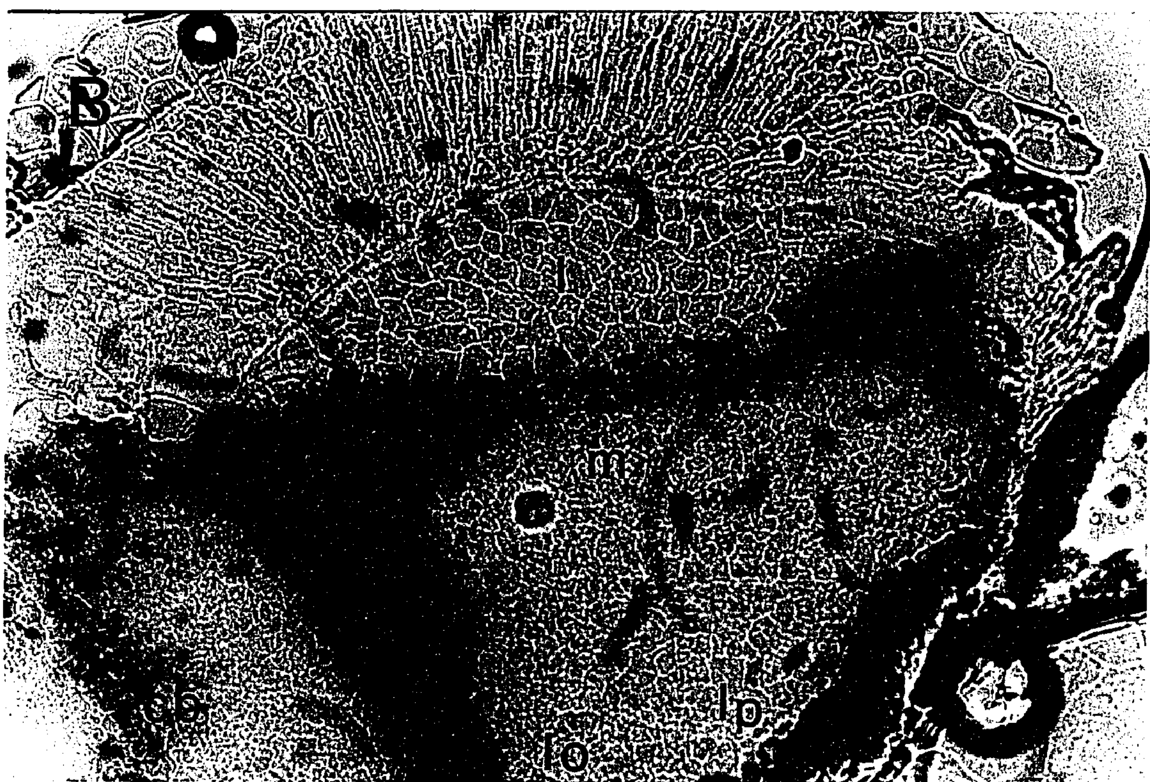
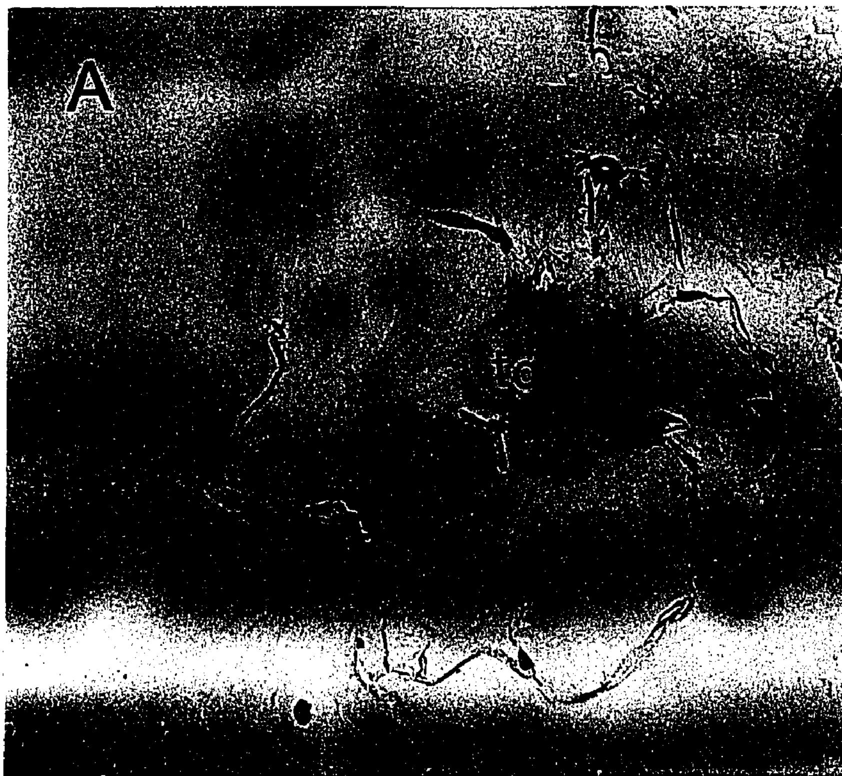

B
r
m
lp

Figure 18. Distribution of β-galactosidase activity in CZ20XN-36M transformed isolines

The X-gal staining patterns in horizontal section of one-day-old adult thoracic ganglionic center (A) and an enlarged view of thoracic ganglionic center (B) are shown. tg, thoracic ganglionic.


A
tg

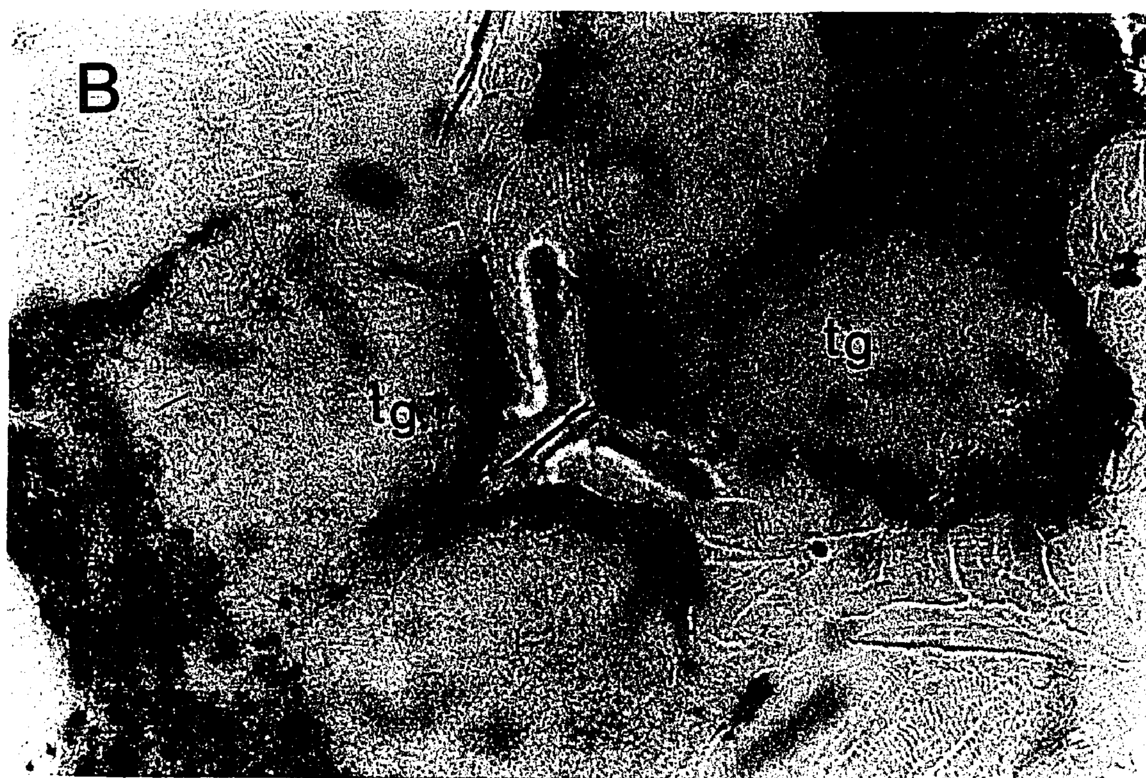

B
tg
tg

Figure 19. Distribution of β-galactosidase activity in CZ20XN-36M transformed isolines

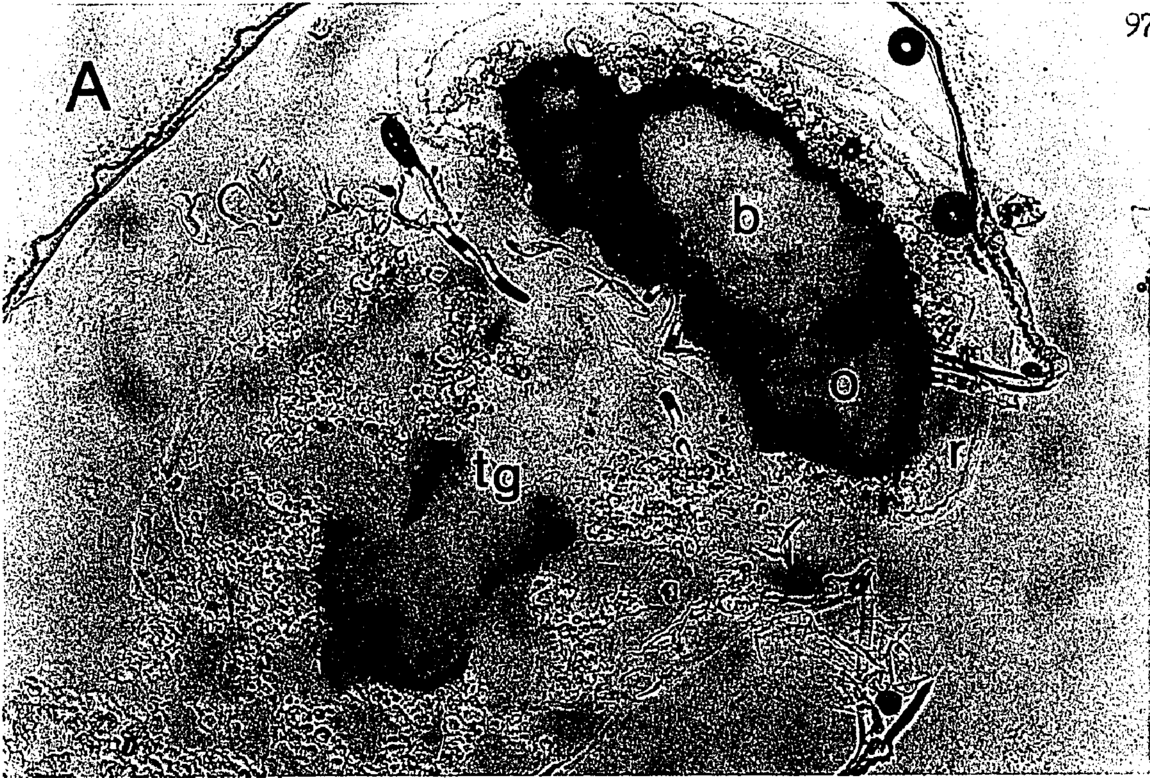
The X-gal staining patterns in whole mount preparations of late embryos (A) and an enlarged view (B) are shown. vns, ventral nervous system.

A

B

Figure 20. Distribution of β-galactosidase activity in CZ20XN-36M transformed isolines

The X-gal staining patterns in horizontal section of late stage pupae (A) and an enlarged view of part of brain (B) are shown. b, brain; o, optic lobe; tg, thoracic ganglion; cb, central brain; m, medulla; lo, lobular; lp, lobular plate.


A
b
o
r
tg

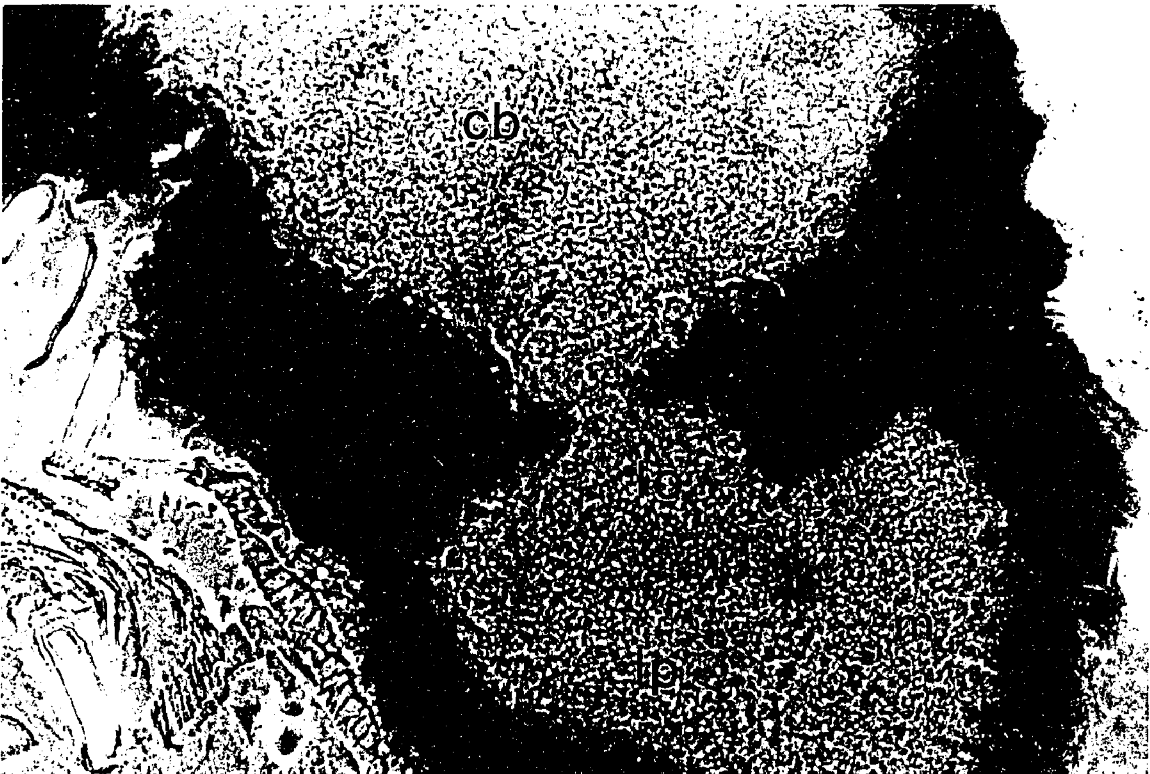
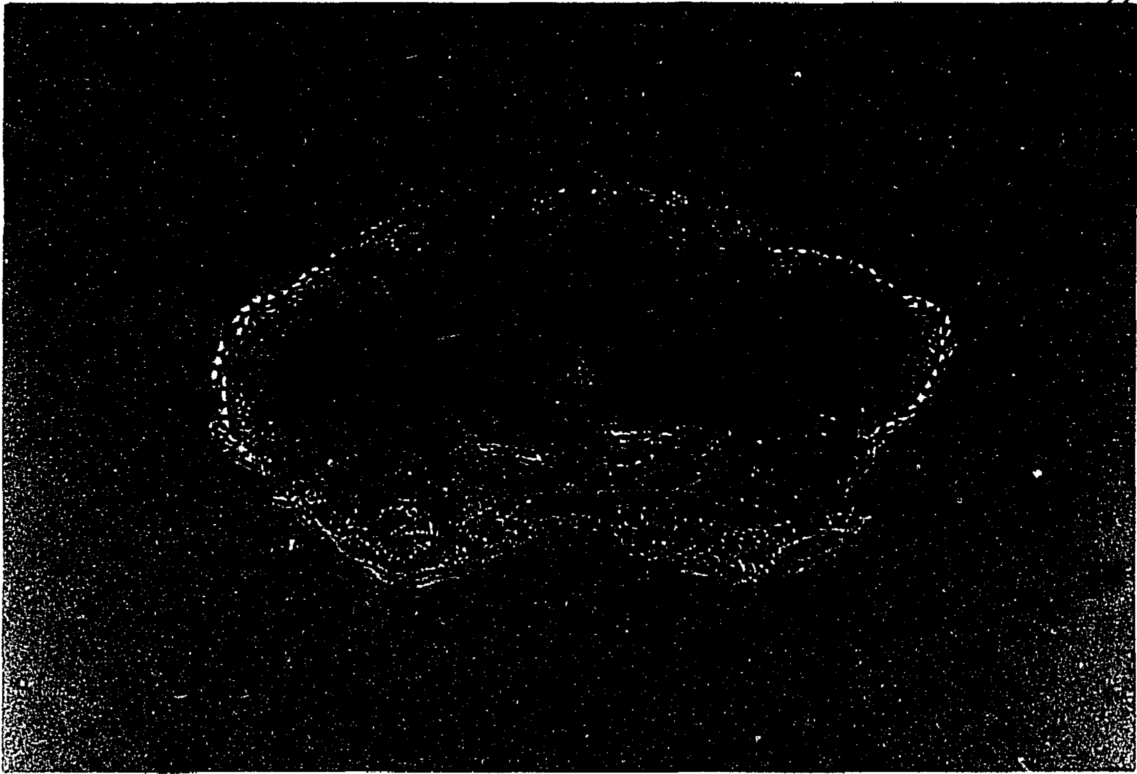

cb
lp

Figure 21. Distribution of β-galactosidase activity in a HZ50PL-XX transformed isoline

The X-gal staining patterns in frozen section of one-day-old adult brain (A) and an enlarged view of right side optic lobe (B) are shown. b, brain; o, optic lobe; r, retina; cb, central brain.



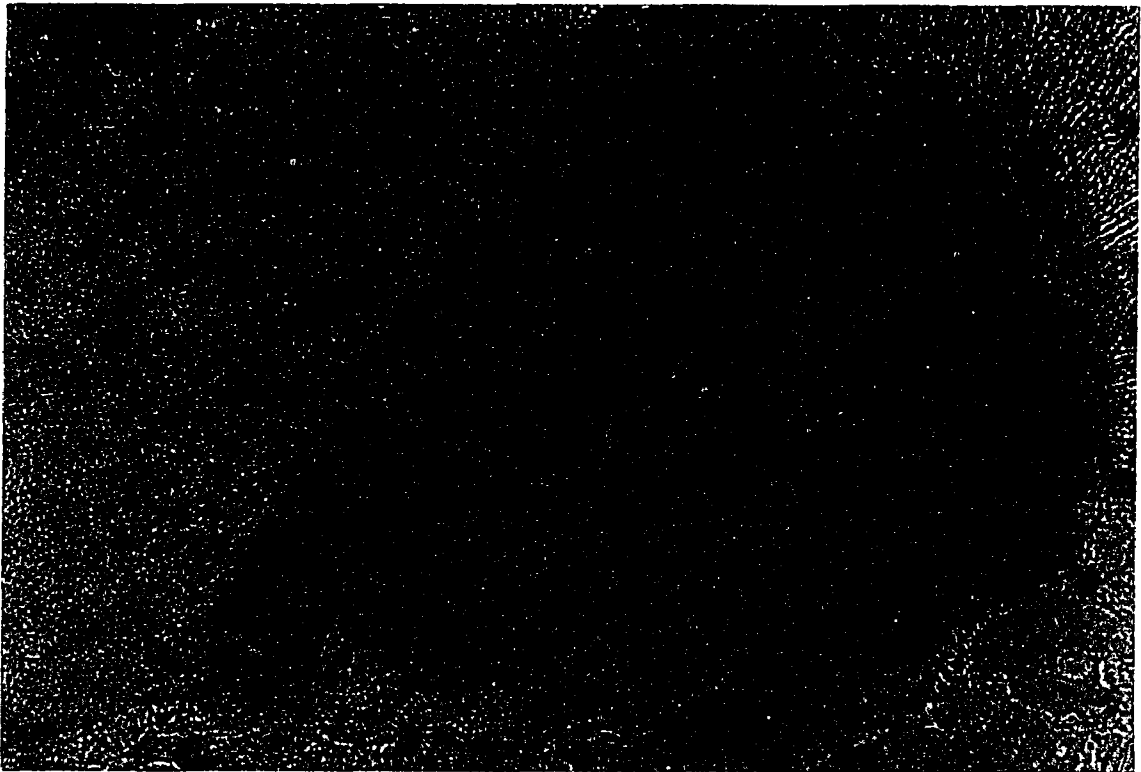
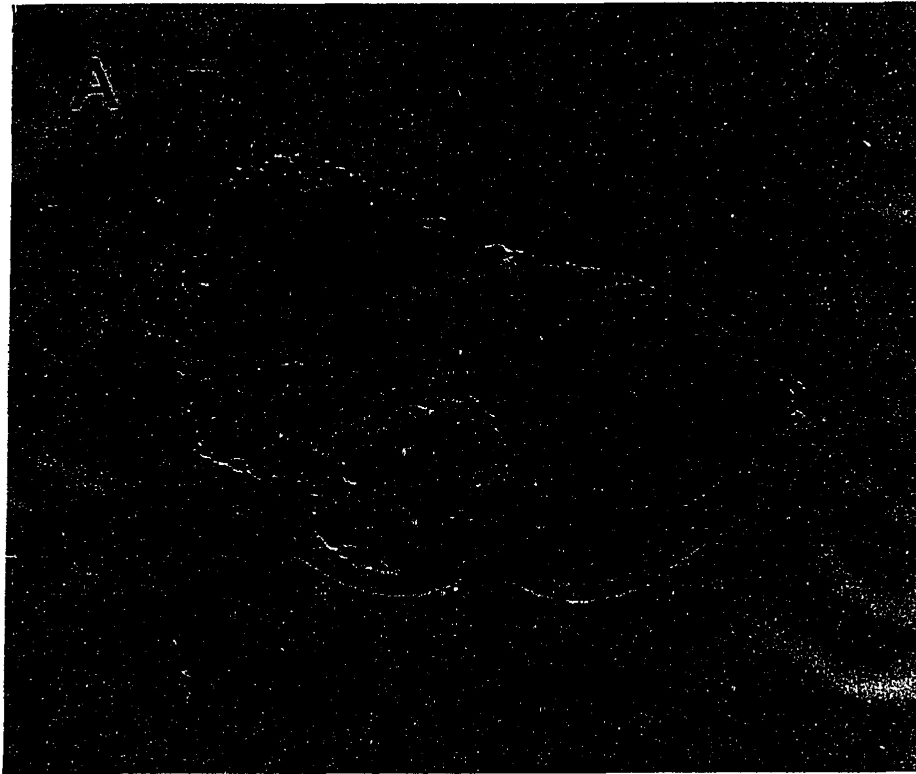


Figure 22. Distribution of β-galactosidase activity in a HZ50PL-HX transformed isoline

The X-gal staining patterns in frozen section of one-day-old adult brain (A) and an enlarged view of left side of optic lobe (B) are shown. b, brain; o, optic lobe; r, retina; l, lamina; m, medulla; lo, lobular; lp, lobular plate; cb, central brain.

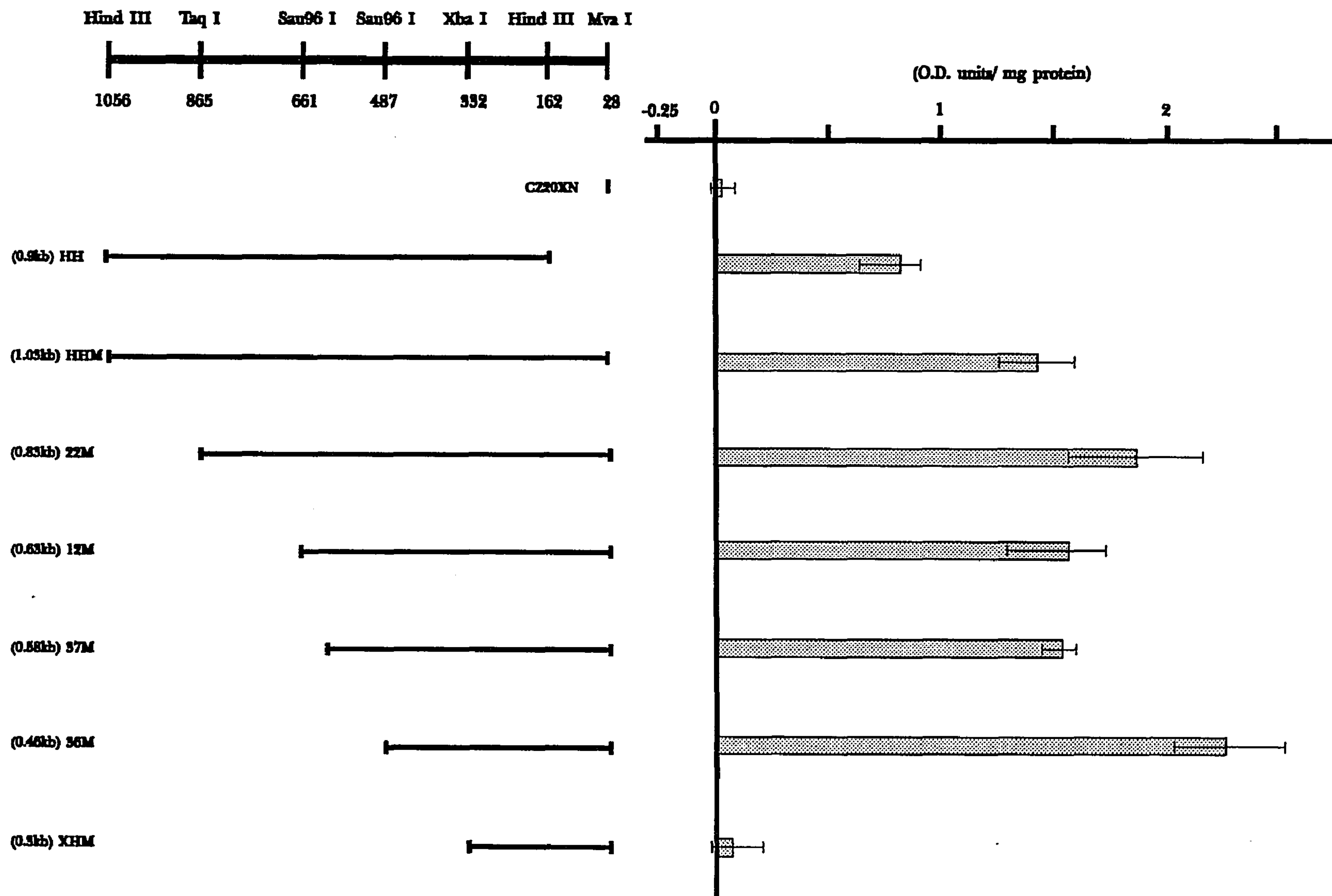

A

B
r
c
cb

Figure 23. Schematic representation of β-galactosidase activity

The left part of the figure indicates the deletion fusion constructs used in this assay. The right part of the figure shows the results of β-galactosidase activity assay. The activity is expressed in arbitrary units as O.D. units/mg protein. The bars indicate the average of all available isolines* of each construct. The background was measured by using extracts prepared in parallel from the nontransformed strain (ry^{506}) subtracted numerically from β-galactosidase levels in transformant extracts.

*: For each isoline, only balanced transformant flies were tested.



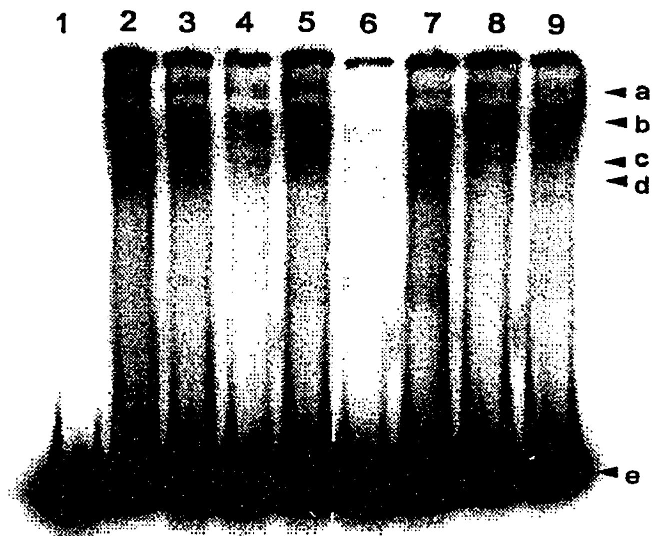
Hind III
Taq I
Sau96 I
Sau96 I
Xba I
Hind III
Mva I
1056
865
661
487
332
162
28
(O.D. units/ mg protein)
-0.25
0
1
2
CZ20XN
(0.9kb) HH
(1.03kb) HHM
(0.83kb) 22M
(0.63kb) 12M
(0.58kb) 37M
(0.46kb) 36M
(0.3kb) XHM

Figure 24. Autoradiographs of polyacrylamide gels of gel retardation assays using fragments F4 and F5 and 10 - 22 hour *Drosophila* nuclear extracts

(A) The competition assay of F4 with other fragments (F1 - F3, F5 and F6) is shown. Lane 1 is a control lane with free labeled F4 fragment only. Lane 2 shows the interaction of F4 and proteins from late embryo nuclear extracts. Lane 3 to Lane 9 show the competition of all six fragments and a non-specific competitor (SK; a 102 bp plasmid fragment from pBluescript SK (+)) to F4. Bands a, c, and d are non-specific products. Band b is a specific product. Band e is free labeled F4.

(B) The competition assay of F5 with other fragments (F1-F4 and F6) is shown. Lane 1 is a control lane with free labeled F5 fragment only. Lane 2 shown the interaction of F5 and proteins from late embryos nuclear extracts. Lane 3 to Lane 9 show the competition of all six fragments and SK to F5. Bands b, c, and d are non-specific products. Band a is a specific product. Band e is free labeled F5.

A

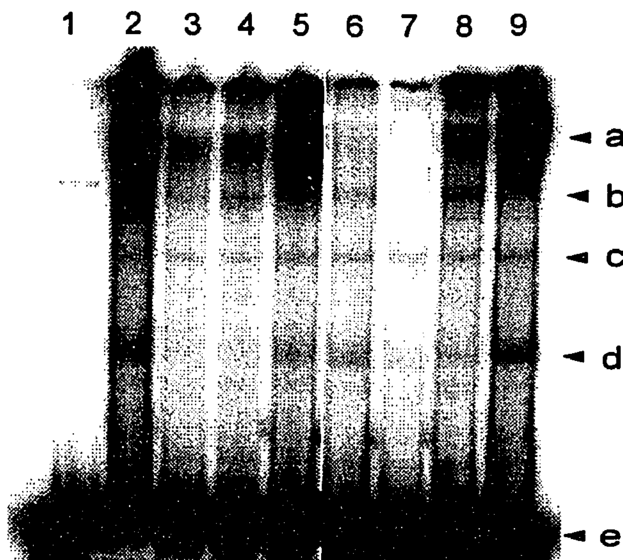


labeled DNA F4 →

competitor DNA - - F1 F2 F3 F4 F5 F6 SK

embryonic extract - + + + + + + + +

B



labeled DNA F5 →

competitor DNA - - F1 F2 F3 F4 F5 F6 SK

embryonic extract - + + + + + + + +

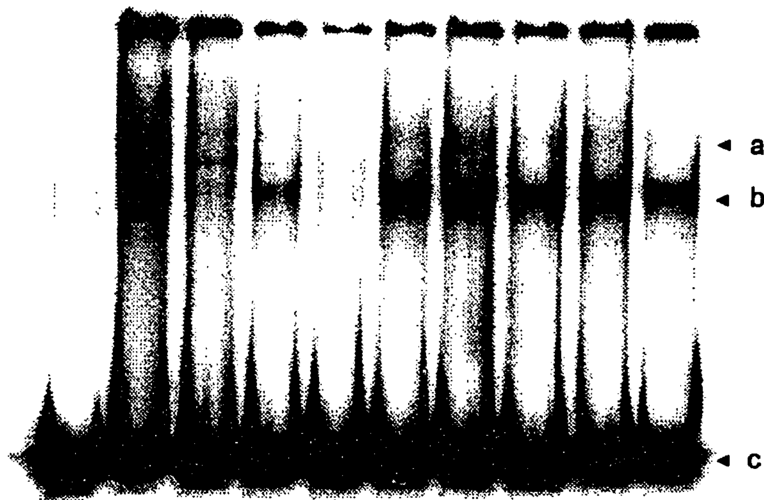
Figure 25. Autoradiographs of polyacrylamide gels of gel retardation assays using F3 and F6 fragments and 10 - 22 hour *Drosophila* nuclear extracts

(A) The competition assay of F3 with other fragments (F1, F3- F6) is shown. Lane 1 is a control lane with free labeled F3 fragment only. Lane 2 shows the interaction of F3 and proteins from late embryo nuclear extracts. Lane 3 to Lane 10 show the competition of all six fragments and SK with F3. F3' is the same fragment as F3 minus 54 bp of the 5' end. Band a is non-specific. Band b is specific. Band c is free labeled F3.

(B) The competition assay of F6 with other fragments (F1 - F5) is shown. Lane a is a control lane with free labeled F6 fragment only. Lane 2 shows the interaction of F6 and proteins from late embryo nuclear extracts. Lane 3 to Lane 9 show the competition of all six fragments and SK to F6. Bands a and c are non-specific. Bands b and d are specific. Band e is free labeled F6.

A

1 2 3 4 5 6 7 8 9 10



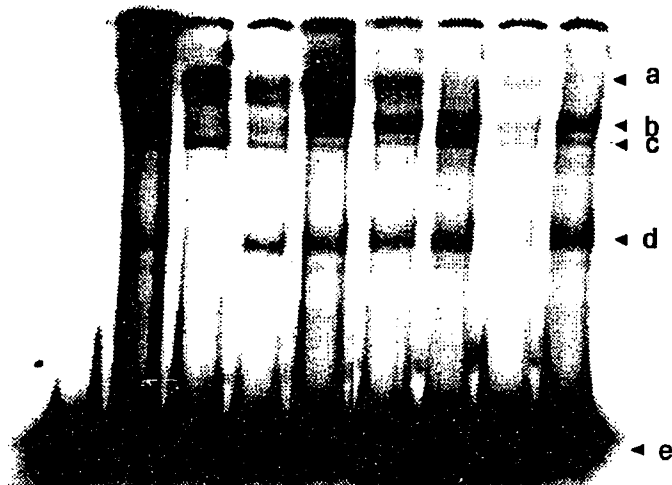
labeled DNA F3 →

competitor DNA - - F1 F2 F3 F3' F4 F5 F6 SK

embryonic extract - + + + + + + + + +

B

1 2 3 4 5 6 7 8 9



labeled DNA F6 →

competitor DNA - - F1 F2 F3 F4 F5 F6 SK

embryonic extract - + + + + + + + +

Table 1. Summary of X-gal Staining Data of CZ20XN constructs I

"-", no staining detectable

"+", positive staining in CNS.

"*", there are eleven isolines totally and all are X-gal staining negative. Only five isolines are shown here.

Constructs		Chromosome location	X-gal staining (one-day-old adults)
CZ20XN	1.	II	-
	2.	III	-
	3.	III	-
CZ20XN-**XX***	1.	III	-
	2.	III	-
	3.	II	-
	4.	III	-
	5.	II	-
CZ20XN-**HX**	1.	III	-
	2.	III	-
	3.	III	-
	4.	II	-
	5.	X	-
	6.	III	-
CZ20XN-**HH**	1.	II	+
	2.	III	+
	3.	III	-
	4.	III	-
	5.	II	+
	6.	III	+
	7.	III	+
	8.	X	-
CZ20XN-**Mva27**	1.	III	+
	2.	III	+
	3.	II	-
	4.	III	-
	5.	II	+
	6.	II	+
CZ20XN-**Mva19**	1.	III	+
	2.	II	-
	3.	II	+
	4.	III	+
	5.	III	-
	6.	III	-

Table 2. Summary of X-gal Staining Data of HZ50PL constructs

"-", no staining detectable

"+", positive staining in CNS with different expression patterns from CZ20XN constructs.

Constructs	Transformant lines	X-gal staining (one-day-old adults)
HZ50PL	1 - 5	-
HZ50PL-XX	1 - 4	+
	5 - 12	-
HZ50PL-HX	1 - 3	+
	4 - 15	-

Table 3. Summary of X-gal Staining Data of CZ20XN Constructs II - Deletions

"-", no staining detectable.

"+", positive staining in CNS.

Constructs	Chromosome location		X-gal staining one-day-old adults
CZ20XN-**HHM**	1.	III	+
	2.	III	+
CZ20XN-**22M**	1.	II	+
	2.	III	+
CZ20XN-**12M**	1.	III	+
	2.	II	+
	3.	X	+
	4.	III	-
	5.	III	+
	6.	II	-
	7.	III	+
	8.	III	+
	9.	II	+
	10.	III	+
CZ20XN-**37M**	1.	III	+
	2.	III	+
	3.	II	+
CZ20XN-**36M**	1.	III	+
	2.	III	+
	3.	II	+
	4.	III	+
	5.	III	+
CZ20XN-**XHM**	1.	II	-
	2.	III	-
	3.	III	-
	4.	III	-

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