# Molecular characterization of the eye-specific *Om(2D)* mutation in *Drosophila ananassae*

By

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# CONTENTS

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# ABSTRACT

Optic morphology (*Om*) mutations in *Drosophila ananassae* map to at least 22 loci, which are scattered throughout the genome. *Om* mutations are all semidominant, neomorphic, nonpleiotropic, and associated with the insertion of a retrotransposon, *tom*. *Om(2D)* mutants have distinctive phenotypic peculiarity among *Om* mutations. The *Om(2D)* locus has been cloned by *tom* tagging. Molecular studies on the *Om(2D)* locus of the mutants and revertants associated with chromosomal rearrangements suggested that the *tom* elements present in the locus are responsible for the mutant phenotype, and that the *Om(2D)* gene is located distal to the *tom* insertion sites in *Om(2D)* mutants.

Northern blot analysis revealed that two transcriptional units reside in the cloned region distal to the *tom* insertion sites. The present thesis concerns with molecular characterization of the two genes. One of them has been found to be a gene encoding ornithine aminotransferase (OAT) precursor, and the other gene has been shown to encode a novel protein containing histidine/proline repeats. Chapter I deals with the biochemical and molecular analyses of the OAT gene. The predicted OAT protein sequence is 433 amino acids long with a molecular mass of 47,352 Da and is highly homologous to mammalian OATs. The Drosophila OAT has a leader peptide which is commonly found in mitochondrial proteins, and is exclusively localized in the cytosolic fraction with a molecular mass of about 44 kDa. These results suggest that the Drosophila OAT may be processed and localized in the mitochondria like mammalian OATs. No significant difference of the OAT activity was found between wild-type and the mutant flies, and no aberrant eye phenotype was induced by the overexpression of the OAT gene, suggesting that the OAT gene is not responsible for the *Om(2D)* mutant phenotype.

Chapter II deals with the identification of the *Om(2D)* gene and the mechanism by which the *Om(2D)* mutant phenotype is induced. To ascertain whether the other gene would indeed be responsible for the *Om(2D)* phenotype, DNA sequence analysis of genomic and cDNA clones has been carried out. As a result, exons 4 and 5 of this gene fall within the

region deleted in the phenotypic revertant *Om(2D)63*$^{R4}$. It was thus concluded that this gene is likely to represent the *Om(2D)* gene. The *Om(2D)* RNA is not detected in wild-type eye imaginal discs, but is abundantly found in the center of the eye discs of *Om(2D)* mutants, where excessive cell death occurs. *D. melanogaster* flies transformed with the *Om(2D)* cDNA under control of the *hsp70* promoter display abnormal eye morphology when heat-shocked at the third larval instar stage. These results suggest that the *Om(2D)* gene is not normally expressed in the eye imaginal discs, but its ectopic expression induced by the *tom* element in the eye discs of third instar larvae results in defects in adult eye morphology.

Chapter III concerns with the characterization of the *Om(2D)* gene function. For this purpose, the expression pattern of the *Om(2D)* gene during fly development has been examined. The *Om(2D)* transcript was found ubiquitously in the embryonic region and intensely in the central nervous system of embryos, and in the cells of the wing disc and midgut of third instar larvae. Immunoblotting experiment using polyclonal antibodies against the *Om(2D)* protein revealed that the *Om(2D)* protein was present both in the nuclear and cytosolic fraction. These findings suggest that the *Om(2D)* gene may play an important role in development of these cells and that the *Om(2D)* protein may be involved in the process of transcriptional regulation.

# INTRODUCTION

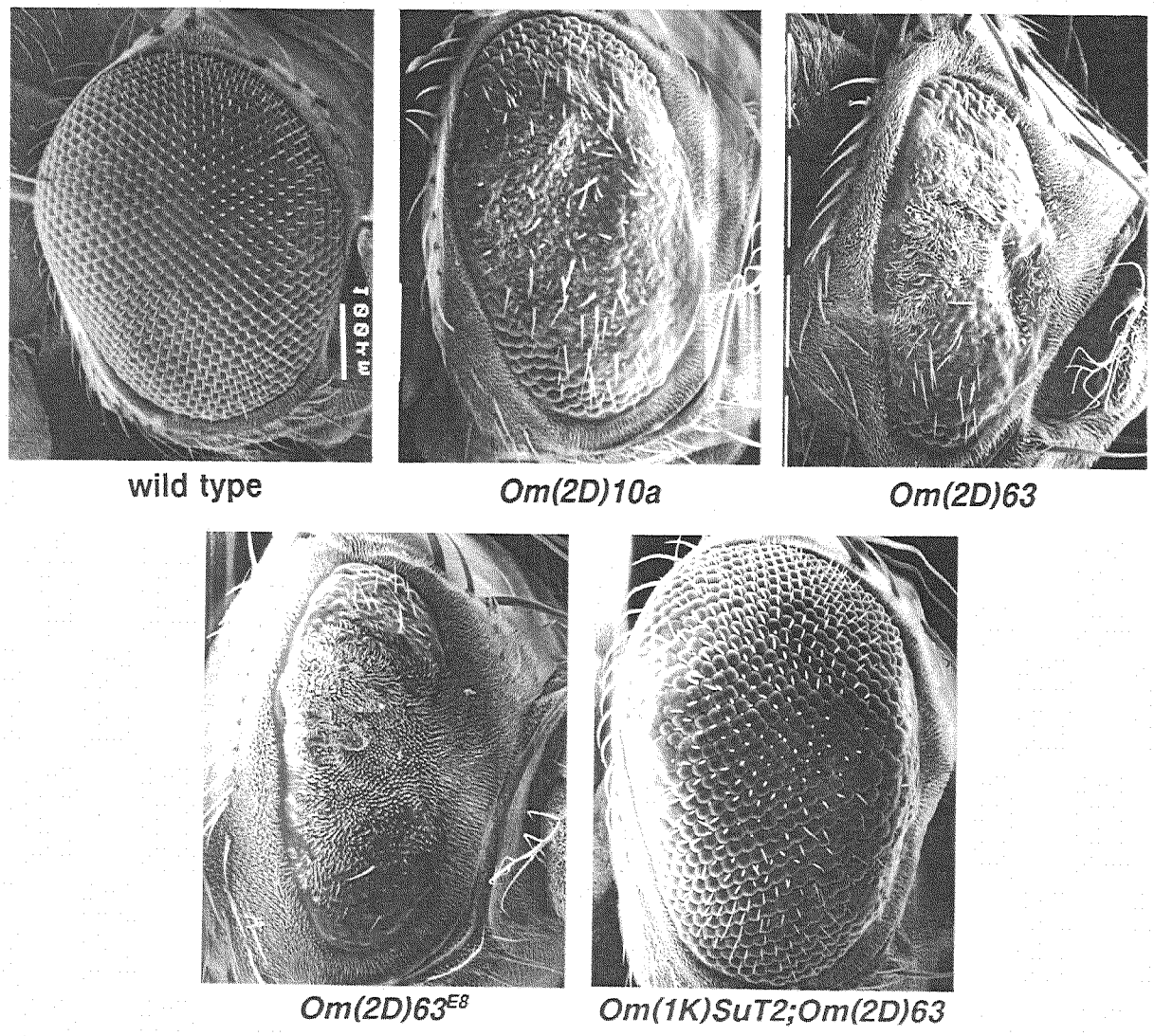



**Fig. 1A-E**. Eye phenotypes of *Om(2D)* mutants. **A**, *ca;px* is the progenitor stock of *Om* mutants and shows an $Om^+$ eye; **B**, *Om(2D)10a*; **C**, *Om(2D)63*; **D**, $Om(2D)63^{E8}$; **E**, double mutant, *Om(1K)SuT2;Om(2D)63*.

Drosophila eye is an excellent genetic system for approaching the problem of how determination and differentiation events interplay to achieve proper cellular development (Ready 1989; Rubin 1991). The adult eye consists of a hexagonal array of approximately 800 ommatidia. Each ommatidium is composed of 8 photoreceptor cells and 12 accessory cells. Despite their general similarities, the photoreceptor cells can be grouped into three functional classes (R1-R6, R7, and R8) based on morphology, axon projection pattern and spectral sensitivity. Each photoreceptor cell possesses a microvillar stack of membranes, called the rhabdomere, where the visual pigments reside. The position and the size of the rhabdomere are some of the morphological features distinguishing the three different classes of photoreceptor cells. The rhabdomeres of the photoreceptors R1 to R6 form an asymmetric trapezoid. The rhabdomere of R7 is smaller than the R1-6 rhabdomeres and occupies a central position in the distal part of the ommatidium. The R8 rhabdomere is located below R7. The cluster of eight photoreceptor cells is surrounded by pigment cells that optically insulate the unit. Four cone cells lay above the photoreceptor cells and secrete the central part of the lens.

The stereotyped arrangement of photoreceptor cells within the ommatidia is established during the last larval and pupal stage. Patterning starts at the posterior margin of the eye imaginal disc which prior to this stage consists of a single epithelial layer of unpatterned dividing cells (Ready et al. 1976). The wave of pattern formation along dorsoventral axis of the disc moves across the disc epithelium in the anterior direction accompanying a morphological indentation, called the morphogenetic furrow. In the furrow the regular spacing

of the ommatidial units is established. Individual cells spaced by approximately seven cells assume a neural fate and will become the R8 photoreceptor cells (Tomlinson and Ready 1987a). These cells are the founder cells for each ommatidial cluster. The other ommatidial cells become integrated subsequently in a fixed sequence: with the addition of R2 and R5, followed by R3 and R4, the five-cell precluster is established. Initially 1-2 additional cells, the so called mystery cells, are associated with the precluster. In contrast to the five photoreceptor precursor cells, the mystery cells do not initiate neural development and lose the association with the cluster. To the precluster the photoreceptor precursors R1, R6 and then R7 are added. Later, the integration of the cone cells and finally the pigment cells completes the ommatidial unit (Tomlinson and Ready 1987a).

Since R7 cells contain specific UV-sensitive visual pigments, they can be identified by biochemical (Zuker et al. 1987) and behavioral assays (Harris et al. 1976). This has permitted the isolation of mutations that specifically prevent the development of the R7 cell. Two genes have been identified that are required for the recruitment of the R7 cell: *bride-of-sevenless* (*boss*; Reinke and Zipursky 1988) and *sevenless* (*sev*; Tomlinson and Ready 1987b). The *sev* gene encodes a receptor tyrosine kinase localized on the R7 surface (Hafen et al. 1987; Tomlinson et al. 1987) that is thought to be activated by a *boss*-encoded ligand presented by R8 (Kramer et al. 1991). Binding of the ligand to the extracellular domain of Sev could result in the activation of the tyrosine kinase domain of Sev by which the inductive signal is transmitted into cytoplasm. Ultimately, the signal would be transduced into nucleus in which genes

necessary for the proper development of R7 reside. Recent studies revealed that this signal transduction would be achieved by a cascade containing general signal transduction components: *ras* and mitogen activating protein (MAP) kinase pathways (Hafen et al. 1993). These pathways or the homologues are shared with different signal transduction cascades not only in Drosophila, but also in other organisms (Neiman 1993).

The number and spacing of the ommatidial units are possibly defined by the specification of R8 cells, since all other cell types develop as a consequence of their direct contacts with R8 cell that have been selected earlier from the undifferentiated cells anterior to the morphogenetic furrow (Tomlinson and Ready 1987a). Several mutations have been identified to affect the R8 specification. *scabrous* (*sca*) encodes a fibrinogen-related secretory protein and its mutation gives rise to the irregular initiation of cluster formation in the morphogenetic furrow. In the wild-type eye disc, *sca* transcripts accumulate in several cells of each precluster present around the morphogenetic furrow and are found only in the R8 precursor cells in the region posterior to the morphogenetic furrow (Baker et al. 1990; Mlodzik et al. 1990). A similar phenotype is observed in *retina-aberrant-in-pattern* (*rap*) mutants (Karpilow et al. 1989). Mosaic analysis with both *sca* and *rap* indicates that the two genes are exclusively required for correct ommatidial assembly in R8 (Baker et al. 1990; Karpilow et al. 1989). Experiments with a temperature sensitive allele of *Notch*, which encodes a cell surface protein with EGF-like repeats, indicated that in the absence of functional *Notch* product in the morphogenetic furrow, too many precursor cells enter a neural pathway (Cagan and Ready 1989). In contrast,

dominant gain-of-function mutations in the gene of the *Drosophila melanogaster* EGF receptor, called *Ellipse*, result in the opposite phenotype: only very few cells enter the neural pathway (Baker and Rubin 1989; 1992). *eyes absent* (*eya*) mutants show reduction in the number of ommatidia; its severe allele shows no ommatidium at all. The mutant phenotype is caused by the increased death of the precursor cells present in front of the morphogenetic furrow (Bonini et al. 1993). A mutation in *atonal* (*ato*) gene, which encodes a transcriptional factor containing a basic-helix-loop-helix domain, leads to no expression of the *sca* gene and complete loss of ommatidia. In the wild-type eye disc, *ato* mRNA is expressed in a stripe spanning the disc on the anterior edge of the morphogenetic furrow, suggesting that *ato* is required for the earliest step of ommatidial formation (Jarman et al. 1993a,b). Although thesegeneswould play an important role in the processes of the cluster formation and R8 determination presumably occurring in the prefurrow, relatively little is known about the interaction between these genes. Furthermore, identification and characterization of other genes implicating these processes are necessary to understand the molecular mechanism of eye morphogenesis. In *D. ananassae*, a group of mutations characterized by the reduction and abnormality of the ommatidial units have been found and were named *Om* after optic morphology (Hinton 1984; 1988); hence, it has been expected that analysis of these genes may offer some clues to understanding of molecular mechanism of eye morphogenesis.

The general phenotypic characters of *Om* mutations are as follows (Tanda et al. 1993). Effects of *Om* mutations are restricted only to compound eye; no consistent abnormalities in antennae, ocelli, or bristles bordering the eye are seen

in these mutants. Most of the mutants share a reduction in eye size, particularly on the anterior side, and this is accompanied by various irregularities in ommatidial arrangement, assembly, and pigmentation. The *Om* mutants exhibit not only remarkably little pleiotropy but also relatively few variations between individuals within a stock. Although no morphological abnormality of the eye-antennal disc is observed in the late second and early third instar larvae of the *Om* mutants, from the middle of the third larval instar onward, a prominent cell death becomes evident in the center of the mutant eye discs.

*Om* mutations are semidominant and are recovered from a marker stock (*ca;px*) at a high frequency of about $2x10^{-4}$ (Hinton 1984). The mutations studied so far map to at least 22 different loci scattered throughout the genome. In Drosophila, the majority of spontaneous visible mutations are induced by the insertion of transposable elements and it has been found that several hypermutability systems results in mobilization of the transposable elements (Rubin 1983). Accordingly, Hinton (1984) predicted that *Om* mutations might be also caused by a transposable element present in the progenitor marker stock and named this putative element "*tom*" after transposon of *Om*. In addition to the *Om* mutations, alleles of *singed* (*sn*) have been found in the progenitor stock, suggesting that these mutations are caused by the same element responsible for the generation of *Om* phenotypes (Hinton, 1984). In order to identify the *tom* element responsible for the *Om* mutability system, the *sn* locus was isolated from *D. ananassae*. An insertion sequence of 7.0 kb long was found within the *sn* locus recovered from the progenitor stock. This insertion sequence was found at the cytogenetic locus where several *Om* mutations had been genetically

mapped, indicating that this sequence is evidently corresponding to the *tom* element (Shrimpton et al. 1986; Matsubayashi et al. 1992). Sequence analysis revealed that the *tom* element belongs to the *gypsy* group of LTR-containing retrotransposons, that the target sequence of the *tom* element may be TATAT, and that the entirety or a part of this sequence, ATAT, was duplicated upon the insertion of the *tom* element (Tanda et al. 1988). Since *tom* elements are present at several loci in the genome of an *Om* mutant as well as in the progenitor strain, it is unlikely that *tom* inserts only into loci that are associated with eye morphogenesis. It is therefore unlikely that the insertion site preference of *tom* may be the cause of the *Om* mutations. This does not imply, however, that *tom* has no insertion site preference at all. The fact that the *Om* mutations are all semidominant, with only a few exceptions having recessive effect, implies that *tom* seldom inserts into the coding region of the genes it mutates; rather it prefers to insert into sites where it does not have deleterious effects on the transcriptional products of the affected genes.

In order to gain insights into the role of *Om* genes in eye development, *Om(2D)* mutants which have distinctive phenotypic peculiarity among *Om* mutations were analyzed (Matsubayashi et al. 1991a, b). The *Om(2D)* locus has two alleles, *Om(2D)10a* and *Om(2D)63*. In *Om(2D)63*, ommatidia are present in two separate masses on the dorsal and ventral rims of the eye field. The pigmented tissue is seen to form a crescent in these areas. Rough cuticular tissues with scattered large bristles and short hairs are seen medially to those areas (Fig. 1). The *Om(2D)10a* stock shows a slightly weaker phenotype. Histological examination of *Om(2D)63* revealed that the ommatidial structure is

extremely disorganized. Near the deformed eye masses are cuticular tissues containing lenses of various sizes, cone cells and pigment cells. The middle of the eye field is occupied by a simple cuticular epithelium which is directly connected to the optic lobe with fatty tissues.

The *Om(2D)* locus has been cloned by *tom* tagging and chromosomal walking (Matsubayashi et al. 1991a). Two existing alleles of *Om(2D)* contain copies of the *tom* element in a 400 bp segment located in the middle of the cloned region: *Om(2D)10a* has one *tom* element and *Om(2D)63* has three copies of the *tom* elements. Southern analysis of spontaneous revertants of *Om(2D)63* revealed that a complete revertant has lost all *tom* insertions, while partial revertants have lost one or two *tom* insertions. These results suggest that the *tom* elements present in this region are indeed responsible for the *Om(2D)* phenotype, and that the cloned region may contain the *Om(2D)* gene. Moreover, revertants artificially induced by γ-ray or chemical mutagen have been subjected to analyses of the genomic structure of *Om(2D)* locus (Matsubayashi et al. 1991b). As a result, a non-lethal revertant, which is associated with the reciprocal translocation, has a break point immediately distal to the *tom* insertion sites. Other revertants, which are lethal and do not complement each other, have been shown to have some structural changes localized in a region distal to the *tom* insertion sites. These results suggest that the *Om(2D)* gene is located distal to the *tom* insertion sites in *Om(2D)* mutants.

In this thesis, I attempt to identify the *Om(2D)* gene in the cloned region. As a result, two transcriptional regions have been found distal to the *tom* insertion sites. One of them has been shown to be a gene encoding ornithine

aminotransferase (OAT) precursor, and the other gene encoding a 3.6 kb transcript is identified to be the *Om(2D)* gene. Furthermore, I will present evidence that the *Om(2D)* gene is not normally expressed in the eye imaginal disc of wild type and its ectopic expression in the disc during the third instar stage results in defects in the morphology of the adult compound eye. I also demonstrate that the *Om(2D)* gene encodes a novel protein containing histidine/proline repeats, and is ubiquitously expressed during embryogenesis as well as in wing and midgut imaginal cells at the third instar stage.

# Chapter I

# Molecular cloning and characterization of the Drosophila ornithine aminotransferase gene

# INTRODUCTION

Matsubayashi et al. (1991b) suggested that the *Om(2D)* gene may locate distal to the *tom* insertion sites in the *Om(2D)* locus. In order to identify the *Om(2D)* gene, Northern blot analysis was carried out using the cloned genomic DNA fragments as hybridization probes. As a result, at least two transcription units were localized in the distal region to the *tom* insertion sites in the cloned region. Sequence analysis of the one of these genes showed that this gene may encodes ornithine aminotransferase (OAT) precursor. The dominant nature of *Om* phenotype may result from either excessive or ectopic expression of the *Om* gene in the eye imaginal disc, so the activity of OAT in eye discs was compared between the wild type and the mutants. Moreover, I tested whether or not the isolated cDNA can bring about any mutant eye phenotype when expressed artificially in the eye imaginal disc. These experiments, however, revealed that the OAT gene is not responsible for the *Om(2D)* phenotype. Therefore, it is more likely that the remaining gene is the *Om(2D)* gene. This possibility will be dealt in the Chapter II.

Ornithine-δ-aminotransferase (ornithine-oxo-acid aminotransferase, OAT; EC 2.6.1.13) is a mitochondrial matrix enzyme (Ip et al. 1974) and plays a key function in the conversion of ornithine to proline and glutamate through pyrroline-5-carboxylate (Shih 1981). In man, gyrate atrophy, a rare autosomal recessive disease of the choroid and retina of eye, is associated with progressive chorioretinal degeneration leading to blindness and caused by reduction in the OAT activity; in tissues and cultured cells from gyrate atrophy patients have no or low OAT activity (Valle and Simell 1986). Although deficiency of OAT in

these patients results in a 10-15-fold accumulation of ornithine in all body fluids, there is no other clinical abnormality (Valle and Simell 1986). The molecular mechanism of gyrate atrophy is still unknown.

OAT cDNAs have been isolated from several species from yeast to human (Mueckler and Pitot 1985; Ramesh et al. 1986; Degols 1987; Schmid et al. 1993; Manenti et al. unpublished). The human and rat cDNAs have 84.3% nucleotide identity in their coding regions and encode 439-amino acid proteins with 90.4% identity (Mitchell et al. 1988). The predicted molecular mass of the rat liver OAT precursor and the mature monomer are 48,332 Da and 45,749 Da, respectively (Simmaco et al. 1986). In *Saccharomyces cerevisiae*, the OAT is cytoplasmic (Jauniaux et al. 1978) and, accordingly, lacks a mitochondrial signal peptide present in amino-terminal of the mammalian OATs (Mitchell et al. 1988).

OAT is expressed in nearly all tissues, and induced in particular tissues in response to various physiological stimuli (Valle and Simell 1986). In rat liver, OAT activity is increased 100-fold and 10-fold by a high-protein diet and by administration of glucagon, respectively (Mueckler et al. 1983). In contrast, rat kidney OAT activity levels are unaffected by dietary protein or glucagon, but are induced synergistically 10-30-fold by the administration of estrogen and/or thyroid hormone (Mueckler and Pitot 1983; Mueckler et al. 1984).

In contrast to the mammalian enzymes, little is known about insect OATs. Only a few examples are the enzymes of the blowfly and silkmoth. In both cases the OAT activities are mainly found in their fat bodies (Reddy and Campbell 1969; Tsuyama et al. 1978). As a first step to examine the regulatory mechanism of insect OAT and if illegitimate expression or suppression of the

Drosophila OAT gene could cause *Om* mutation, I studied the molecular and biochemical aspects of the Drosophila OAT gene. In this chapter, I report that the Drosophila OAT gene has a high homology to mammalian OATs and encodes 47,352-Da polypeptide, that the OAT is mainly localized in the fat body, and that its activity is elevated in the third instar stage.

# MATERIALS AND METHODS

## Drosophila stocks

Flies of *D. ananassae* were raised on standard medium containing yeast, corn meal, malt and glucose at 25°C. Flies of *D. melanogaster* were raised at 23°C on the same medium except that glucose was replaced by sugar. *ca;px* strain of *D. ananassae* (Hinton 1984) and *Canton-S* and $w^1$ strain of *D. melanogaster* (Lindsley and Zimm 1992) were used in this study.

## cDNA cloning

RNA of the *ca;px* third instar larvae was isolated by the guanidine thiocyanate and cesium trifluoroacetate-ultracentrifugation method (Okayama et al. 1987). Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose column chromatography according to the method described by Maniatis et al. (1982). A cDNA library was prepared with the poly(A)$^+$ RNA as follows: 1 μg of the poly(A)$^+$ RNA was reverse-transcribed at 37°C for 1 h with 10 pmol of oligo $dT_{12\text{-}16}$ primers (Pharmacia) and 200 Units of SuperScript reverse transcriptase (BRL). Resulting cDNAs were then converted to double-stranded DNAs. After ligation of *Eco*RI-*Not*I adaptors to the flush ended cDNAs, the cDNAs were ligated to λgt10 arms and packaged using a lambda in vitro packaging kit (Amersham) according to the manufacturer's instructions.

## In situ hybridization to polytene chromosomes

The cDNA clone labeled with digoxigenin-dUTP was hybridized to

Drosophila polytene chromosomes by the method as described (Engels et al. 1986). The hybridized probes were detected using the anti-digoxigenin-alkaline phosphatase conjugate (Boehringer), followed by staining with 0.34 mg/ml nitroblue tetrazolium (NBT) and 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

## DNA sequencing

cDNA clones and genomic DNA were subcloned into the pUC19 vector for sequencing. Deletion mutants were constructed using exonuclease III (Henikoff 1984) under the conditions recommended by the enzyme manufacturer (Takara). Sequencing was performed by the dideoxy chain- termination method (Sanger et al. 1977).

## Production of antibodies

The maltose-binding protein (MBP)-OAT-β-galactosidase fusion protein was prepared using the Protein Fusion & Purification system (NEB) to raise OAT-specific antiserum. The 551 bp *Bam*HI fragment of the longest cDNA clone, pOAT16, containing a part of the OAT open reading frame was electrophoretically isolated on 4% Sea Plaque GTG Agarose (Takara) and purified with a Prep-A-Gene DNA Purification Kit (Bio-Rad). After ligation with the *Eco*RI linker (Takara), the fragment was ligated in the pMAL-c2 vector site. This construct was introduced into *E. coli* JM109 cells by electroporation using a Pulse Controller (Bio-Rad).

Transformants carrying the above-mentioned plasmid construct were

analyzed by restriction mapping of plasmid DNAs and by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of cell lysates to examine the existence of the fusion protein. Sequence analysis confirmed that the inserted fragment was in frame.

The cells containing the fusion plasmids were grown with shaking at 37°C in one liter of a rich medium (10 g tryptone/ 5 g yeast extract/ 5 g NaCl/ 2 g glucose) with 100 mg/ml ampicillin until the culture reached 0.5 OD600. Then the expression of MBP-OAT-β-galactosidase protein was induced under the control of the Ptac promoter by shaking for 2 h at 37°C in the presence of 0.3 mM isopropyl-1-thio-β-D-galactoside (IPTG). Cells were harvested by spinning at 4,000 x g for 20 min at 4°C, and resuspended in 50 ml of the column buffer (20 mM Tris-HCl, pH 7.4/ 200 mM NaCl/ 1 mM EDTA/ 10 mM 2-mercaptoethanol/ 1 mM sodium azide). The cells were then broken by incubation with 1 mg/ml lysozyme on ice for 30 min and by sonication for 2 min. The lysate was centrifuged at 9,000 x g for 30 min. The supernatant was diluted six-fold with the column buffer, and loaded at a flow rate of 1 ml per minute on an Amylose Resin (NEB) column, and the column was washed with eight column volumes of the column buffer. Then the fusion protein was eluted with 10-20 ml of the column buffer containing 10 mM maltose, and 1.5-ml fractions were collected. The protein concentration of each fraction was measured by the Bradford assay (Bradford 1976) and the fractions containing the protein were pooled. To separate the OAT-β-galactosidase portion from MBP, the fusion protein was cleaved with the protease factor Xa and electrophoresed on a 10% SDS-polyacrylamide gel. Then the gel slice containing the OAT-β-

galactosidase protein was dialyzed against 0.2 M Tris-acetate, pH 7.4/ 1.0% SDS/ 100 mM dithiothreitol. The protein was electroeluted in 50 mM Tris-acetate, pH 7.4/ 0.1% SDS/ 0.5 mM sodium thioglycolate at 100 volts, and dialyzed against 0.2 M sodium bicarbonate/ 0.02% SDS. The purity of the protein was assayed by electrophoresis and Coomassie Brilliant Blue staining.

Five female mice were immunized with 20 μg of the purified protein emulsified in complete Freund's adjuvant (Iatron) twice at an interval of two weeks. Blood was collected from each mouse two weeks after the second injection. To collect serum, the blood was incubated at 37°C for 1 h and at 4°C overnight, and centrifuged at 10,000 x g for 10 min at 4°C. The supernatant was centrifuged again. Each supernatant (serum) was dispensed into 20 μl-fractions, and stored at -20°C. Immunoreactivity of each serum was titrated with enzyme linked immuno sorbent assay using anti-mouse IgG horseradish peroxidase conjugate (Amersham) as secondary antibodies. The antibody titers of all the serum were up to 1: 5 x $10^3$ after two weeks of the second booster injection.

## Germ-line transformation

A 1.6 kb fragment isolated from the pOAT16 by cleavage at the *Eco*RI sites within the adaptor sequences was cloned in the appropriate orientation into the blunt-ended *Xba*I site of phsp70C4, which contains the *hsp70* promoter in the Carnegie 4 vector (Tanda and Corces 1991). A 2.0 kb *Sal*I fragment containing the *hsp70* promoter and the OAT cDNA of this Carnegie construct was introduced into the blunt-ended *Bam*HI site of the CaSpeR vector (Pirrotta

et al. 1985). The resulting construct was co-injected with pπ25.7wc (Karess and Rubin 1984) into a *D. melanogaster* $w^1$ stock using the method described by Rubin and Spradling (1982).

## Immunoblotting Analysis

The third instar larvae of *D. ananassae* or *D. melanogaster* were homogenized, boiled for 10 min in loading buffer (62.5 mM Tris-HCl, pH 6.8/ 2% SDS/ 5% 2-mercaptoethanol/ 10% glycerol/ 0.01% bromophenol blue), and spun at 17,000 x g for 5 min. The supernatant was resolved on a SDS-polyacrylamide gel according to standard procedures (Sambrook et al. 1989) and electrophoretically transferred to nitrocellulose membranes at 30 volt in 50 mM Tris/ 380 mM glycine/ 20% methanol overnight using Mini-PROTEAN II (Bio-Rad). Filters were blocked in 5% nonfat dry milk/ TBS (Tris-buffered saline: 25 mM Tris-HCl, pH 8.0/ 0.8% NaCl/ 0.02% KCl) for at least 30 min at room temperature and incubated in a 1: 200 dilution of the anti-OAT antiserum for 1 h at room temperature. After washing in TBS three times for 5 min each, blots were incubated for 1 h at room temperature with a 1:500 dilution of anti-mouse IgG horseradish peroxidase conjugate (Amersham). After washing three times for 5 min each, color development was performed in 0.5 mg/ml diaminobenzidine/ 0.03% $CoCl_2$/ 0.003% $H_2O_2$/ TBS.

## RNA Analysis

Total RNA was isolated from flies of various developmental stages by the hot phenol/chloroform extraction method (Jowett 1986). Poly(A)$^+$ RNA was

isolated as described above. Two micrograms each of poly(A)$^+$ RNAs were electrophoresed on a 1.2% agarose, 2.2 M formaldehyde gel, transferred by vacuum blotting (Pharmacia LKB Biotechnology Inc.) to a Hybond-N membrane (Amersham), and hybridized to $^{32}$P-labeled probes as described by Maniatis et al. (1982).

To estimate the relative amount of transcript of the OAT gene, dot blot analysis was carried out; 1 μg each of poly(A)$^+$ RNAs was denatured, mixed with one volume of 20x SSPE (Maniatis et al. 1982), and blotted on Hybond-N membranes (Amersham) with a dot blot apparatus (Bio-Rad). Hybridization, washing, and autoradiography were carried out as described by Maniatis et al. (1982). The spots of autoradiographs were measured by densitometry with an ACI IMAGE ANALYSIS SYSTEM (ACI, Japan). *D. melanogaster ras* gene (Mozer et al. 1985) probe was used to normalize the amounts of RNA loaded on membranes. The relative amounts of the OAT gene transcripts were calculated by dividing the signal intensity of the transcripts by that of the *ras* transcripts of the same preparation.

## Measurement of OAT activity

The OAT activity of eye imaginal discs was assayed by a modification of the method of Ohura et al. (1983): five larvae at the third instar stage were dissected in Drosophila Ringer's solution, and 10 eye-antennal imaginal discs were homogenized with the reaction buffer consisting of 0.1M Hepes, pH 8.0, 0.5 mM pyridoxal phosphate, 4 mM α-ketoglutarate and 2.5 mM o-aminobenzaldehyde. After preincubation at 37°C for 5 min, the reaction was

started by adding 1 μl of 10 mM [U-$^{14}$C] ornithine (0.5 mCi/mmol), and incubated at 37°C for 1 h. The reaction was stopped by adding 90 μl of 10% trichloroacetic acid, and centrifuged after mixing with 100 μl of 30% $Na_2SO_4$ and 200 μl of *n*-butanol; 180 μl of the butanol phase was reextracted with 180 μl of 30% $Na_2SO_4$, and the radioactivity of 140 μl aliquots of the butanol phase was measured with 4 ml of ACS-II scintillator (Amersham).

The OAT activity of whole body was assayed as follows: about 0.05 g of flies were homogenized with 0.3 ml of 0.1 M Tris-HCl (pH 8.0) containing 0.3 M sucrose and 0.5 mM pyridoxal phosphate. The homogenate was then sonicated for 1 min and centrifuged for 10 min. Fifty microliters of the supernatant were incubated in a total volume of 0.5ml of 0.1M Tris-HCl (pH 8.0) containing 10 mM α-ketoglutarate, 10 mM L-ornithine, 0.5 mM pyridoxal phosphate, and 2.5 mM o-aminobenzaldehyde. After incubation for 1 h, the reaction was stopped by adding 0.5 ml of trichloroacetic acid/ethanol (5:95) and centrifuged for 5 min. The OAT activity was measured by reading absorbance at 450 nm of the supernatant against a sample blank. The enzyme activity was expressed in terms of micromoles of pyrroline-5-carboxylate formed per hour at 37°C. The molar extinction coefficient of 2.71 x $10^3$ was used to estimate the amount of pyrroline-5-carboxylate (Strecker 1965). For examining effects of antiserum on the OAT activity, 50 μl aliquots of the enzyme solution prepared as above was mixed with 1-10 μl of antiserum. After leaving at 20°C for 1 h, the enzyme activity was determined. Protein was assayed by the method as described by Lowry et al. (1951).

# RESULTS

## Cloning of the OAT gene

In order to identify the transcription units present distally to the *tom* insertion sites, Northern blot analysis of poly(A)$^+$ RNA isolated from various developmental stages was carried out using the genomic DNA fragments as hybridization probes. As a result, at least two transcription units are found in this region (Fig. 2). Since one of these units, 1.6 kb is most abundantly transcribed in the third instar stage, I screened the *D. ananassae* cDNA library derived from the third instar larvae. Several independent cDNA clones were recovered which shared the same transcription unit as judged from restriction mapping and partial sequence analysis of the cDNA and genomic DNA clones. The sequence analyses also revealed that this transcription unit is composed of three exons (Fig. 3) and highly homologous to mammalian OAT genes (GenBank database). Therefore, I postulated that this gene may be a Drosophila OAT gene. To confirm that this is the Drosophila OAT gene, I raised a polyclonal anti-serum against a fusion protein which is produced from an expression vector containing the partial fragment of the cDNA clone and tested whether the antibodies inhibit the OAT activity or not. The results showed that the OAT activity was not inhibited by adding normal serum at all, but proportionally reduced by increasing amounts of the anti-serum (Fig. 4), suggesting that the anti-serum specifically recognizes OAT protein, and hence the cDNA fragment used for production of the anti-serum encodes part of OAT.

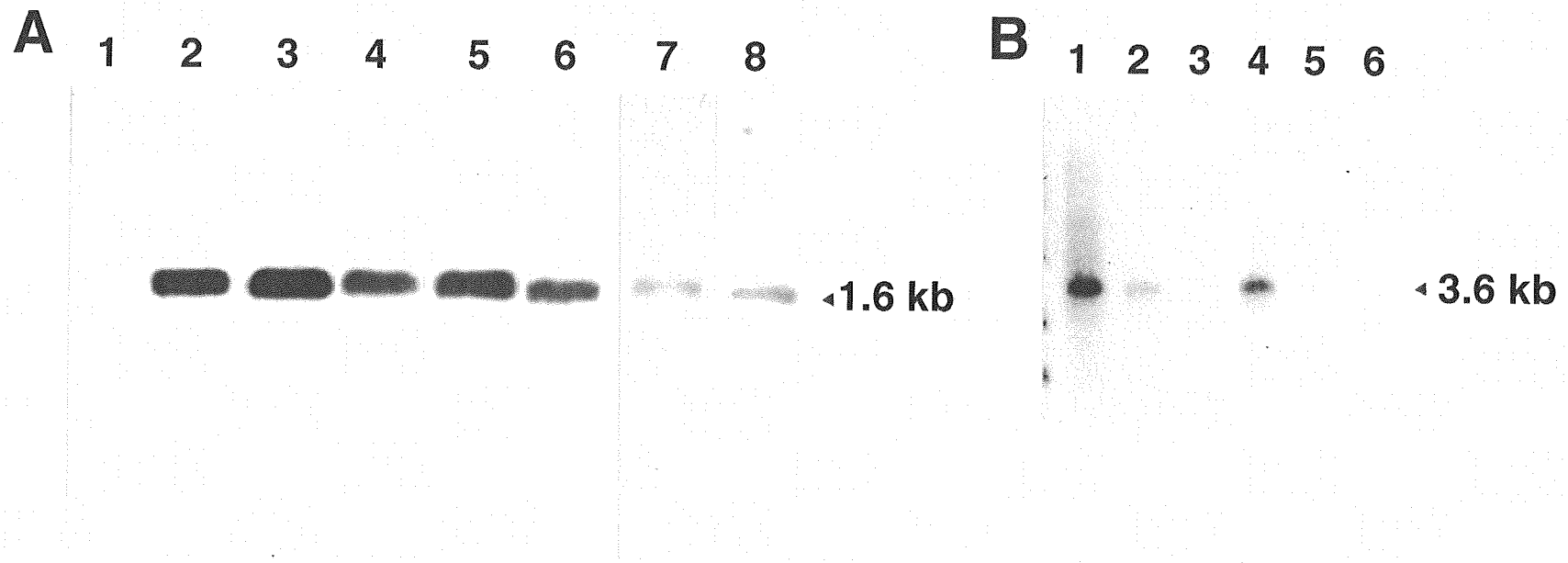



**Fig. 2A and B**. **A**, Northern blot analysis of poly(A)$^+$ RNA from various developmental stages of *D. ananassae* probed with $^{32}$P-labeled pOAT16 cDNA. The hybridized transcripts are approximately 1.6 kb long. Each lane contains 10 μg of RNA: 1, embryo; 2, first instar larvae; 3, second instar larvae; 4, early third instar larvae; 5, late third instar larvae; 6, early pupae; 7, late pupae; 8, adults. **B**, Northern blot hybridization analysis of *Om(2D)* transcript from the *ca;px* strain (lanes 1-3) and *Om(2D)63* (lanes 4-6). Poly(A)$^+$ RNA from embryos (lanes 1 and 4), late third instar larvae (lanes 2 and 5), and adults (lanes 3 and 6) were probed with λOm(2D)7. The 3.6 kb *Om(2D)* transcript is indicated by an *arrowhead*.

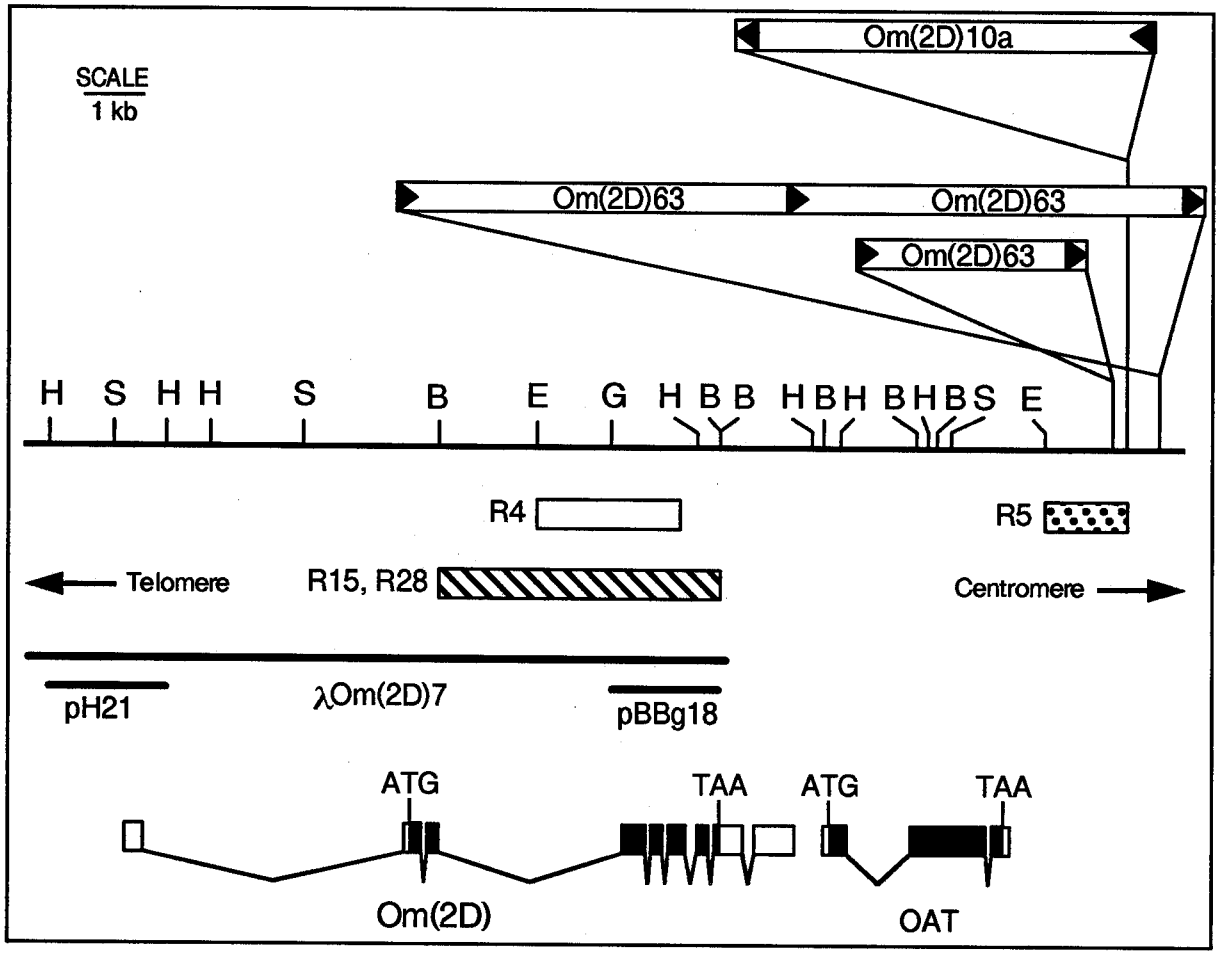



**Fig. 3.** Genomic organization of the *Om(2D)* locus. *Center line* shows restriction map of the *Om(2D)* locus in progenitor strain *ca;px*. Restriction site abbreviations are as follows: B, *Bam*HI; G, *Bgl*II; E, *Eco*RI; H, *Hind*III; and S, *Sal*I. *tom* elements found in two *Om(2D)* alleles are represented by *boxes above* the map. A *solid triangle* shows the long terminal repeat (LTR) of the *tom* element and its orientation. Two tandemly arrayed *tom* elements share one LTR. The *small box* indicates an internally deleted *tom* located adjacent to the other two elements in *Om(2D)63*. The *dotted box below* the map indicates a chromosomal breakpoint in the translocation T(2;3)48B;96A associated with $Om(2D)63^{R5}$ (Matsubayashi et al. 1991b). The *open rectangle* indicates the region deleted in $Om(2D)63^{R4}$. The *hatched box* indicates the region of structural changes in $Om(2D)63^{R15}$ and $Om(2D)63^{R28}$. *Bars below* the map indicate probes used in this study. The intron/exon structures of the *Om(2D)* and OAT gene are shown below the map. Exons are indicated by *boxes*; *solid regions* represent coding regions.

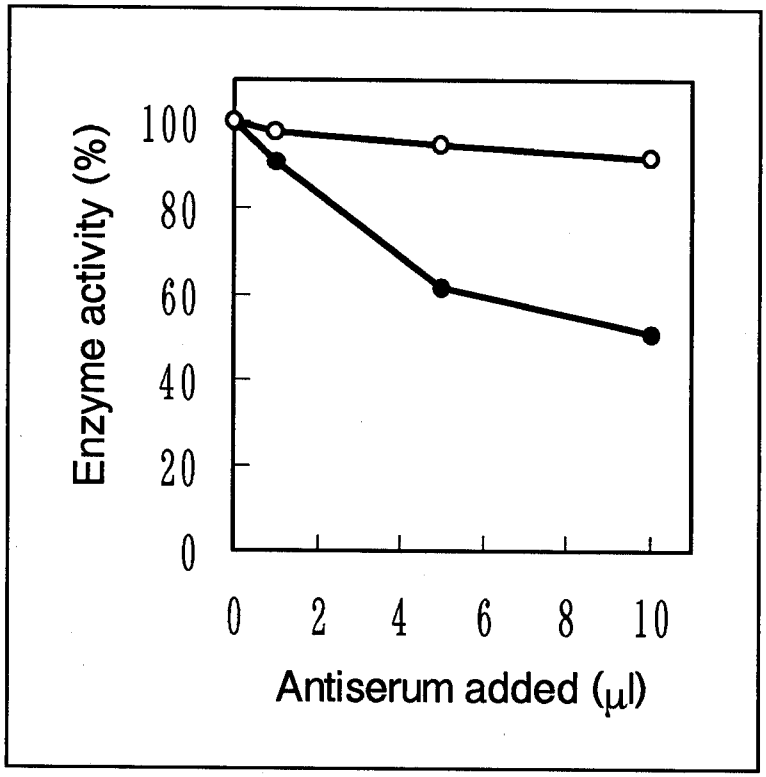



**Fig. 4.** Inhibition of OAT activity with anti-OAT antiserum. Aliquots (50 μl) of an enzyme solution prepared from the third instar larvae of *D. ananassae* were incubated with indicated amounts of mouse normal serum (*open circles*) or mouse anti-OAT antiserum (*solid circles*). The enzyme activities of the mixtures were determined as described under "MATERIALS AND METHODS".

## The OAT gene may not be the *Om(2D)* gene

To test whether or not the OAT gene is responsible for the *Om(2D)* phenotype, the following experiments were performed. The dominant nature of *Om* phenotype may result from either excessive or ectopic expression of the *Om* gene in the eye imaginal disc. Therefore, the activity of OAT in eye discs was first compared between the wild type and the mutants. As a result, no significant difference of the activity was found between wild-type and mutant flies (Fig. 5). In the second experiment, *D. melanogaster* flies were transformed with the OAT precursor cDNA under the control of the *hsp70* promoter, and heat-shocked to induce the OAT expression. However, none of the transformants showed aberrant eye phenotype in spite of the fact that their OAT activity was significantly increased by heat shock at the third instar stage (Table I). This suggests that the OAT gene is not the *Om(2D)* gene.

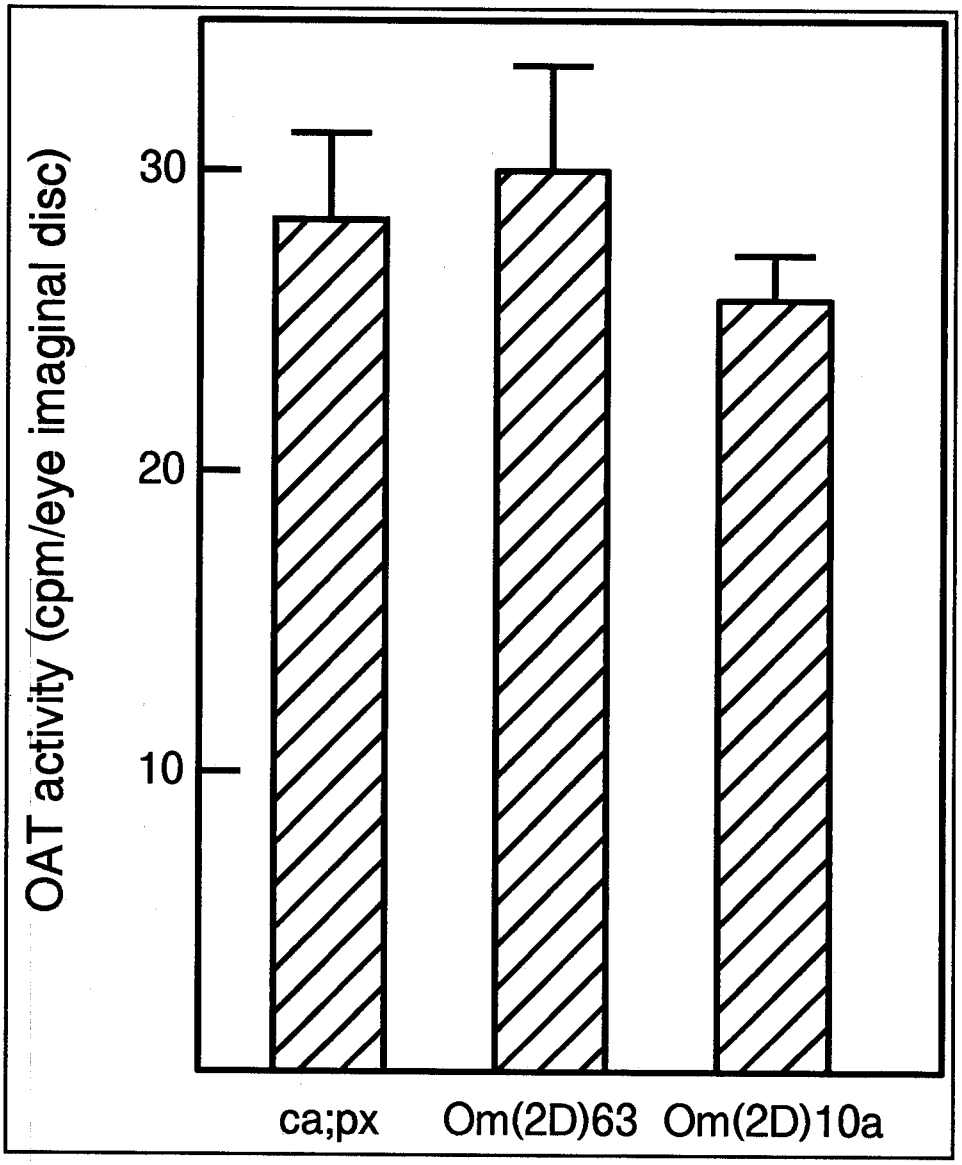



**Fig. 5.** Ornithine aminotransferase (OAT) activities of the eye-antennal imaginal discs of wild-type and mutant larvae. Enzyme activity was assayed with 10 eye-antennal imaginal discs of late third instar larvae, and is expressed in cpm of radioactivity per eye imaginal disc. The data are the averages of four or five independent experiments. *Bars* represent standard deviations.

# TABLE I

OAT activities in the third instar larvae after heat-treatment

The OAT activities in the third instar larvae of a strain transformed with the *hsp*-OAT mini gene, 6-56 and the host strain, $w^1$ were subjected to measurement of the OAT activity at normal feeding temperature, 23°C and after heat-treatment at 37°C for 2h. Indicated activities are average ±S.E. from 20 individuals in each experimental group. The activity of the transformants at 23°C increased about two-fold as much as that of the host strain, probably due to homozygosity of the chromosome inserted with the *hsp*-OAT mini gene.

| | specific activity (mmole/h/mg protein ±S.E.) | | |
|---|---|---|---|
| strain | 23°C | 37°C, 2h | Fold increase |
| $w^1$ | 0.337±0.033 | 0.403±0.040 | 1.20 |
| 6-56 | 0.846±0.053 | 3.589±0.152 | 4.24 |

## Characterization of the OAT gene

To map the OAT gene, the cDNA clone was hybridized to the polytene chromosomes of both *D. ananassae* and *D. melanogaster*. A hybridization signal was detected on the left arm of the second chromosome at 48B in *D. ananassae* and the left arm of the third chromosome at band 76C in *D. melanogaster*, respectively (Fig. 6).

The longest cDNA was 1544 bp long in accordance with the size of the transcript detected by Northern blot (Fig. 2). The complete sequence of the cDNA clone is shown in Figure 7. It contains a single open reading frame capable of encoding a protein product of 433 amino acids with a predicted molecular mass of 47,352 Da. The sequences surrounding the ATG initiation codon match to the consensus for translation start site of Drosophila genes (Cavener 1987). The cDNA sequence ends at the poly(A) stretch beginning 30 bp downstream of an AATAAA polyadenylation signal sequence. A partial sequencing of the OAT genomic region revealed that the intron-exon splice junctions are in good agreement with the previously determined splice site consensus sequences (Breathnach and Chambon 1981). A search of the NBRF-PIR protein data bank indicated that the predicted protein is homologous to ornithine aminotransferase precursors of several species. Pairwise amino acid sequence comparisons revealed that the *D. ananassae* OAT precursor shares 70.4%, 69.5% and 69.0% of amino acids with the human, rat and murine OAT precursors, respectively (Fig. 8). The *D. ananassae* OAT precursor also showed relatively high homology to that of *Plasmodium falciparum* (58%), yeast (51.6%) and mothbean (67.1%). Although the *D. ananassae* OAT precursor showed significant homology to mammalian OATs, the amino-terminal sequences

exhibited less homology. If the amino-terminal sequence of *D. ananassae* OAT is plotted on a `helical wheel' (Schiffer and Edmundson 1967), most hydrophobic residues are clustered on one side, whereas positive charged residues are located on the opposite site (Fig. 9). This amphiphilic property is one of characteristics of a mitochondrial presequence peptide (Roise et al. 1986). If the amino-terminal sequence of the OAT precursor acts as the mitochondrial presequence peptide, it is postulated that the molecular mass of the mature enzyme will be smaller than that of the protein deduced from cDNA sequence. To examine this possibility, I carried out an immunoblotting experiment using the anti-OAT polyclonal antibodies. The antibodies detected a single major band with a molecular mass of about 44 kDa from the cytosolic fraction of the third instar larvae of both *D. ananassae* and *D. melanogaster* (Fig. 10). This suggests that the OATs are processed to enter into mitochondria like mammalian OATs.

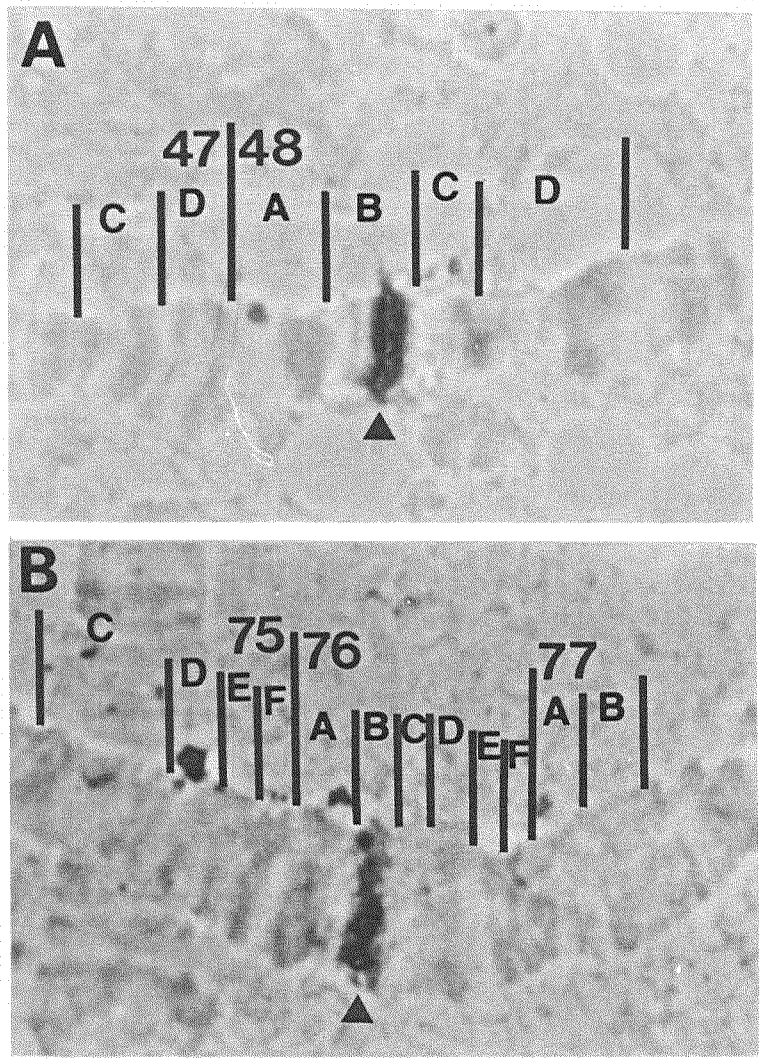



**Fig. 6A and B**. Cytological mapping of the OAT locus. The OAT cDNA labeled with digoxigenin-dUTP was hybridized to salivary gland chromosomes of *D. ananassae* (**A**) and *D. melanogaster* (**B**). A single site of hybridization was observed in each species as indicated by an *arrowhead*.

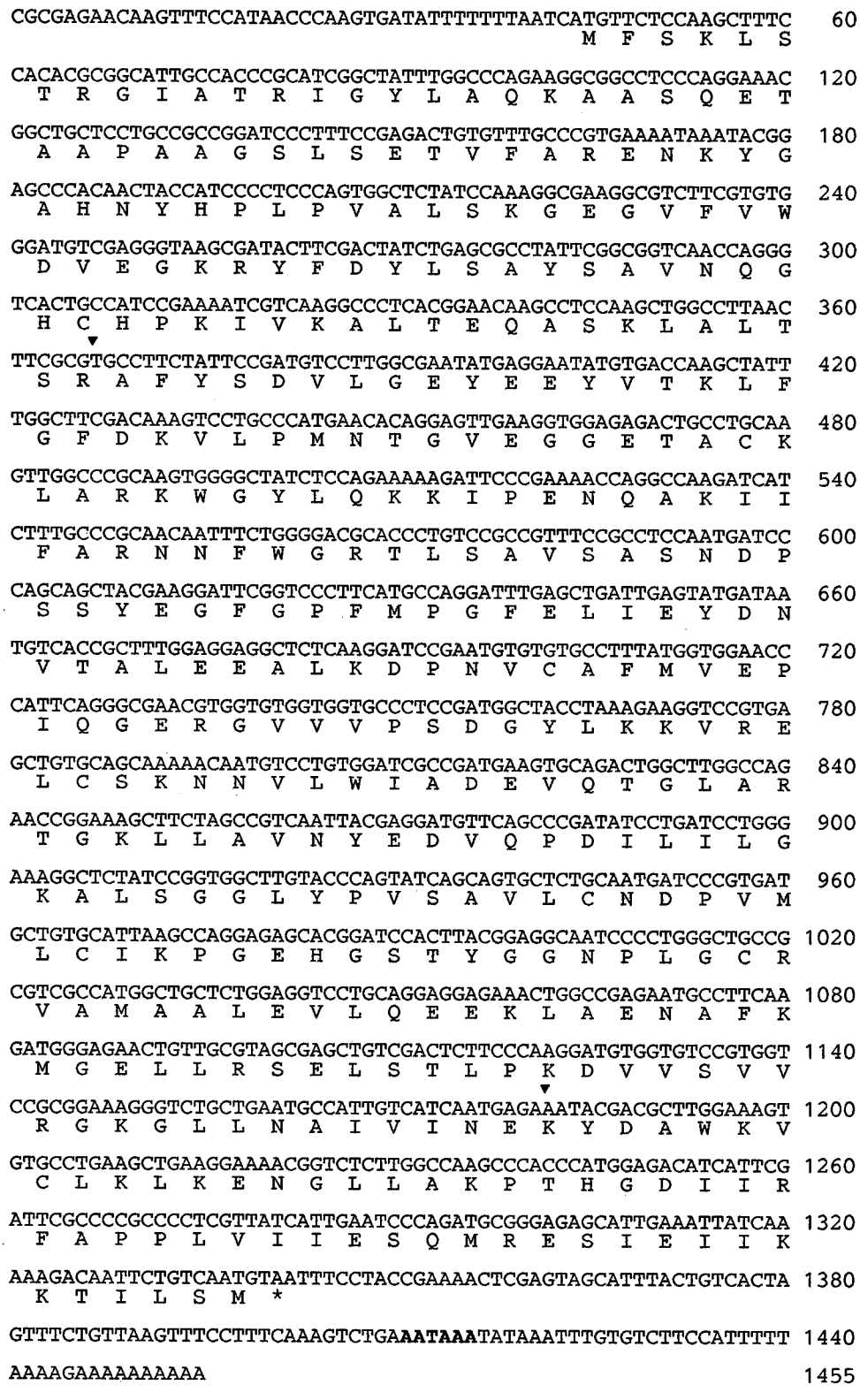



**Fig. 7.** Nucleotide sequence of the pOAT16 cDNA and deduced primary structure of the encoded ornithine aminotransferase enzyme. Splice junctions are indicated above the nucleotide sequence by *solid triangles*. Numbering of nucleotides is shown at the right margin. The polyadenylation consensus signal is shown in *bold*.

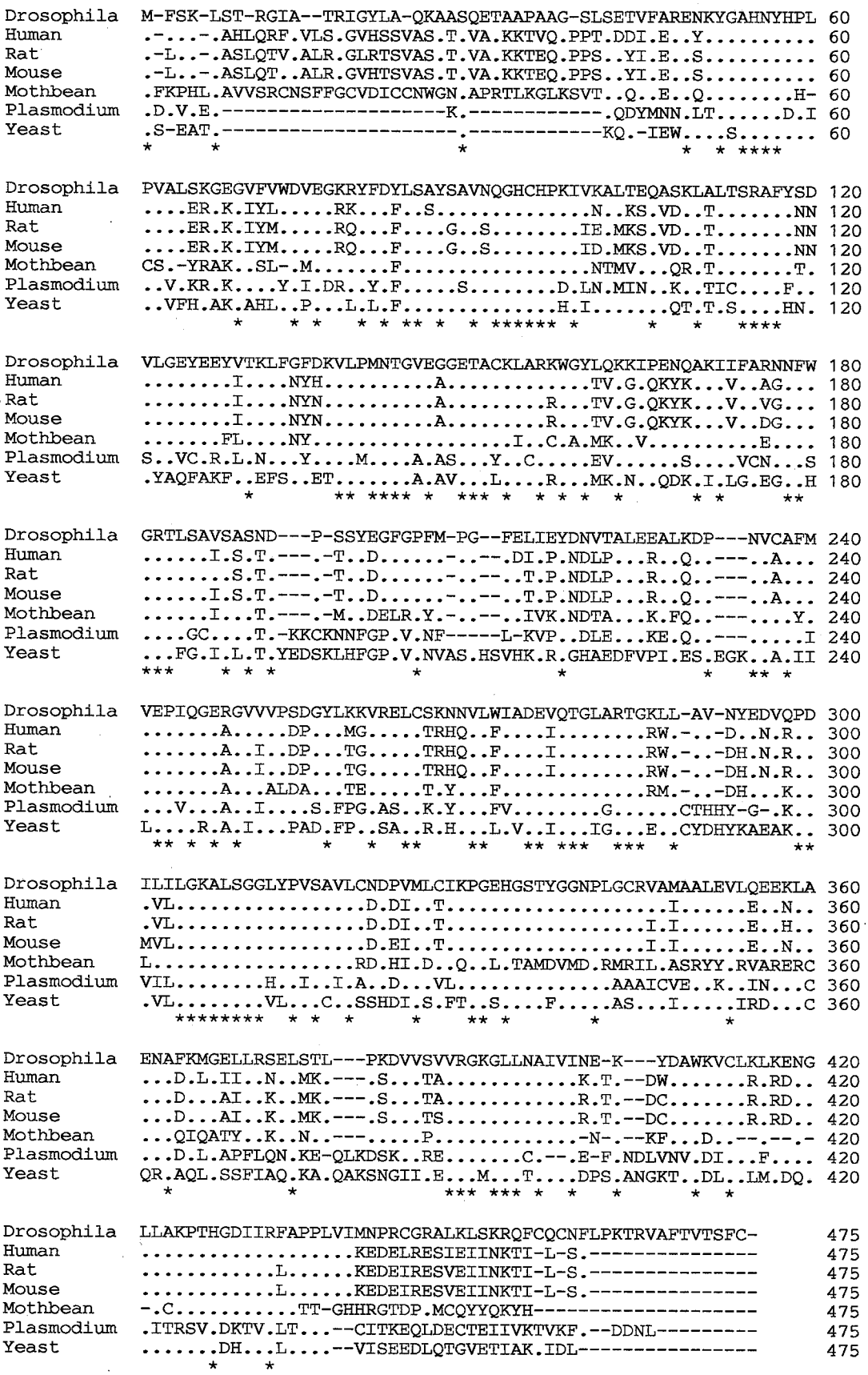



**Fig. 8.** Sequence comparisons of ornithine aminotransferases from Drosophila and other eukaryotes. Identical amino acids are shown by *dots* (.) and gaps are indicated by *hyphens* (-). Positions perfectly conserved are indicated by *asterisks* (*).

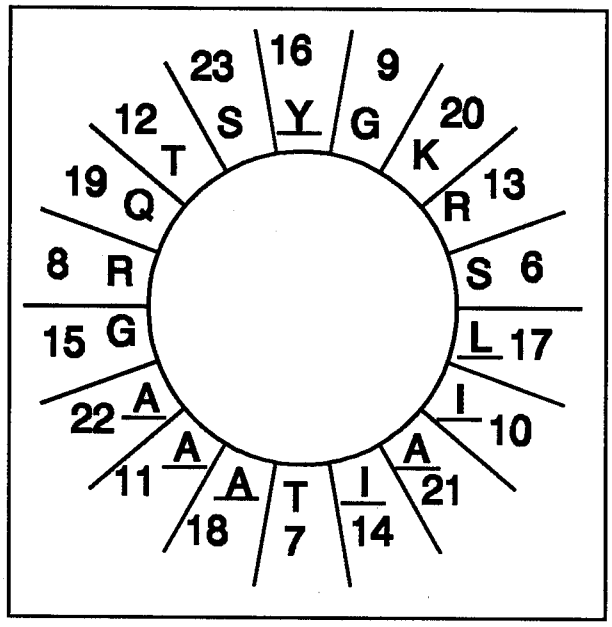



**Fig. 9.** The pre-sequence of Drosophila ornithine aminotransferase can form an amphiphilic helix. Amino-terminal residues 6-23 were plotted on a 'helical wheel' as described by Schiffer and Edmundson (1967). Hydrophobic residues are *underlined*.

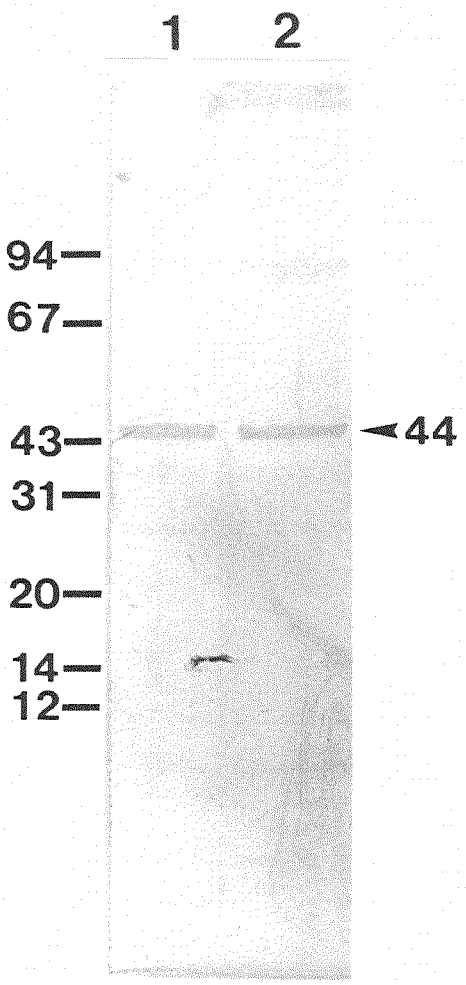



**Fig. 10**. Immunoblotting analysis of extracts from the third instar larvae detected with anti-OAT antibodies. Lane 1 contains the cell extract from *D. melanogaster* and lane 2, that from *D. ananassae*. Value indicated at the right margin is relative molecular mass (kDa) relative to those of standard marker proteins indicated at the left margin.

## Expression of the OAT

To examine the expression of OAT during fly development, I carried out quantitation of the transcript of the OAT gene by dot blot hybridization analysis of poly(A)$^+$ RNAs isolated from staged *D. ananassae* flies using the longest cDNA clone, pOAT16, as a probe (Fig. 11A). The OAT gene was poorly transcribed during embryonic stage. However, the level of the OAT expression continually increases during larval development, and the highest accumulation was observed at the late third instar stage. After that stage, the level of the expression was sharply decreased. The same developmental profile was found by the examination of OAT activities in *D. ananassae* and *D. melanogaster*; the activity was the highest at the late third instar stage, once decreased during the pupal stage, and again increased just before the eclosion (Fig. 11B, C). On the day of eclosion, the OAT activities showed no significant difference between males and females, but the activity became lower in males than in females 5 days after eclosion. Although the cause of this difference is unclear at present, it is possible that the elevated level of female OAT activity may be related to oogenesis.

To examine the tissue distribution of the OAT activity, I measured the OAT activity in *D. melanogaster* larval tissues: head (brain, eye-antennal discs, and salivary glands), gut (entire gut and Malpighian tubule), fat body, and muscle (muscle, wing and leg discs, and cuticle). As a result, the OAT activity was detected in all of the larval tissues, but about 68% of the total OAT activity was present in the fat body (Table II). Moreover, the specific activity of the fat body was about 5-15 fold as much as that of other tissues.

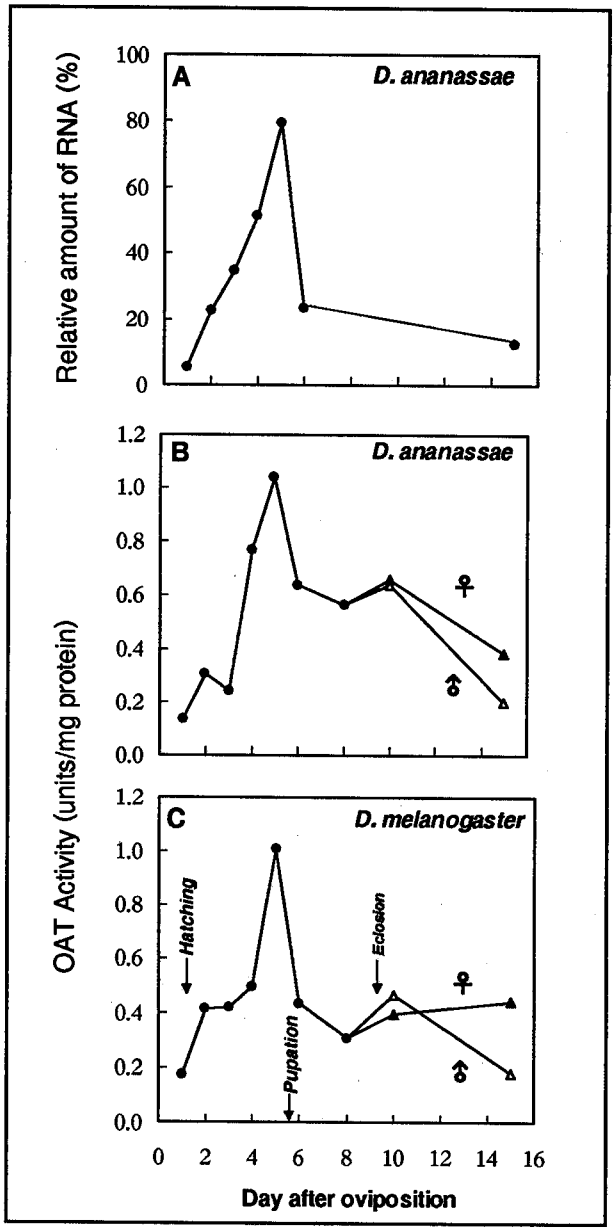



**Fig. 11A-C.** Ontogeny of OAT expression. **A**, Developmental profile in the OAT RNA level of *D. ananassae*. Dot blots of RNAs from various developmental stages were hybridized with $^{32}$P-labeled pOAT16 and autoradiographed. The signal intensity in arbitrary units of the transcript on autoradiograph was measured by densitometry and normalized with respect to that of the *ras* transcript. **B** and **C**, Developmental profiles in the OAT activity of *D. ananassae* and *D. melanogaster*, respectively. The activity of adults was measured in both female (*solid triangles*) and male (*open triangles*), separately.

# TABLE II

Tissue distribution of OAT activity in the third instar larvae of *Drosophila melanogaster*

| Tissue | Activity | Relative activity | Specific activity |
|---|---|---|---|
| | units/fly ± S. E. (n=20) | % | units/mg protein ± S. E. |
| Whole body | 162.3±13.19 | 100.0 | 1.01±0.082 |
| Fat body | 111.0±11.30 | 68.4 | 5.77±0.293 |
| Gut + Malpighian tubule | 15.0±2.39 | 9.2 | 1.07±0.086 |
| Brain + eye-antennal discs + salivary glands | 3.8±0.89 | 2.3 | 0.39±0.046 |
| Muscle + wing and leg discs + cuticle | 30.8±0.92 | 18.9 | 0.95±0.107 |

# DISCUSSION

The *D. ananassae* OAT was found to be highly homologous to mammalian OATs. Human OAT, which is localized in mitochondrial matrix, is synthesized as a precursor protein with an amino-terminal leader peptide that is cleaved incident with mitochondrial entry (Inana et al. 1986). The rat OAT precursor has a predicted molecular mass of 48,332 Da and is cleaved between Ala-25 and Thr-26, resulting in the mature monomer with a molecular mass of 45,749 Da (Simmaco et al. 1986). On the other hand, the yeast OAT is a cytosolic enzyme, so it lacks an equivalent for the mitochondrial presequence peptide (Degols 1987). The immunoblotting experiment showed that the Drosophila mature OAT enzyme has a molecular mass of about 44 kDa, whereas the molecular mass predicted from cDNA sequence is 47,352 Da, suggesting that the Drosophila OAT is also processed to be a mature enzyme. Although leader peptides of mitochondrial enzymes have no obvious homology, they possess several common features. They are generally highly basic, contain no acidic residues and have a significant high frequency of alanine, leucine, serine and arginine (von Heijne et al. 1989). The most distinctive structural feature of mitochondrial presequence peptides is amphiphilic properties (Roise et al. 1986). The amino-terminal sequence of the *D. ananassae* OAT shares this feature, suggesting that this sequence acts as a signal peptide for the mitochondrial localization of the OAT. Thus, the Drosophila enzyme may also be mitochondrial. It has been known that the blowfly OAT is exclusively localized in the mitochondria (Tsuyama et al. 1978).

The OAT activities fluctuate during development in the same way in both

*D. ananassae* and *D. melanogaster* and reach the highest level at the late third instar stage. This is also in good agreement with that of blowfly OAT which showed the highest activity in the 5-day old larvae just before the pupation and decreased thereafter (Tsuyama et al. 1978). A similar fluctuation pattern was found with *D. melanogaster* NADP-isocitrate dehydrogenase (Fox 1971), which is a member of citrate cycle enzymes present in mitochondria. $\alpha$-ketoglutarate produced by this enzyme can be converted into glutamate by OAT. Therefore, it is possible that these enzymes are functionally linked, their activities being controlled by the same regulatory system.

A mammalian OAT is thought to be a house keeping enzyme (Valle and Simell 1986), but its activity is regulated independently in each tissue in response to particular signals. For example, the rat OAT activity is present in all tissues examined so far and is high in kidney, liver, and small intestine (Herzfeld and Knox 1968). A high protein diet and glucagon administration to rats induce liver OAT activity (Mueckler et al. 1983). On the other hand, administration of estrogen or thyroid hormone to rats induce kidney OAT activity (Mueckler and Pitot 1983; Mueckler et al. 1984). In Drosophila, the OAT activity was found in every tissue of third instar larva, suggesting that Drosophila OAT also functions in housekeeping. However, the increased activity in the fat body implies the existence of the mechanism of the tissue specific activation.

In blowfly, OAT activity is found in many tissues, but about 50% of the total activity is localized in the fat body (Tsuyama et al. 1978). In the case of the Drosophila OAT, 68% of the total activity was concentrated in fat body and the specific activity is 5-15 fold as much as that of other tissues. Insect fat body

plays many important roles in metabolism including the intermediary metabolism of amino acids (Keeley 1985). The fat body also supplies many metabolic substances to hemolymph, which shows very higher amino acid levels as compared with mammalian blood (Chen 1985). Therefore, it is reasonable to assume that the OAT activity is maintained at a high level in insect fat body.

The question as to why a deficiency of OAT and subsequent ornithine accumulation in body fluid lead to the ocular tissue specific degeneration of human gyrate atrophy patients has not been answered yet. If OAT mutants are recovered from *D. melanogaster*, they would certainly be a valuable system for elucidating the molecular mechanism of the gyrate atrophy. Unfortunately, no such mutation has been found in *D. melanogaster*, although several mutations including lethal ones were reported to map near the OAT locus, 76C (Lindsley and Zimm 1992).

# Chapter II

# Retrotransposon-induced ectopic expression of the *Om(2D)* gene causes the eye-specific *Om(2D)* phenotype in *Drosophila ananassae*

# INTRODUCTION

At an early stage in our studies on *Om* mutations, we presumed that all the *Om* genes might be involved directly in eye morphogenesis, and hence might be useful for scrutinizing the molecular mechanism of eye morphogenesis. Tanda and Corces (1991) reported that the *Om(1D)* gene encodes a homeoprotein, the expression of which is seven-fold higher in the mutant eye imaginal discs than in wild type, and suggested that the eye-specific mutant phenotype of *Om* may be a consequence of the tissue-specific induction of *Om* gene expression by regulatory sequences present in the *tom* element. They also reported that the *Om(1D)* gene is expressed in clusters of differentiating photoreceptors in the eye imaginal disc; a null allele shows a rough eye phenotype with disorganized ommatidia (Tanda et al. 1993). On the basis of these findings they concluded that the *Om(1D)* gene is necessary for normal eye differentiation. Furthermore, the *D. melanogaster BarH1* gene, which is homologous to the *Om(1D)* gene is functionally required for the differentiation of R1-R6 photoreceptor precursors and primary pigment cells (Kojima et al. 1991; Higashijima et al. 1992a). Furthermore, the *Om(1A)* gene is homologous to the *cut* homeoprotein gene (Awasaki et al. 1994), which is expressed in cone cells and interommatidial bristle precursor cells of the eye imaginal disc in addition to cells of other tissues (Blochlinger et al. 1993).

In this chapter I present evidence that, unlike these *Om* genes, the *Om(2D)* gene is not normally expressed in the eye imaginal disc, but that ectopic expression in the disc during the third instar stage results in defects in the morphology of the adult compound eye. It is therefore conceivable that

some other *Om* genes may, likewise, not be involved in normal eye morphogenesis.

# MATERIALS AND METHODS

## Fly stocks

Flies were raised at 25°C on standard medium containing yeast, corn meal, malt, and glucose. Mutant alleles of *Om(2D)* used in this study are described in Matsubayashi et al. (1991a and b). *Om(1K)SuT2*, a dominant suppressor of *Om*, was kindly provided by Hiroshi Matsubayashi.

## General molecular biology procedures

Basic techniques not described in detail here are according to Sambrook et al. (1989).

## RNA isolation and Northern blot analysis

Total RNA was isolated as described in Okayama et al. (1987). Poly(A)$^+$ RNA was isolated using the RNA purification kit (Pharmacia) according to the manufacturer's instructions. Poly(A)$^+$ RNAs (3μg/lane) were separated on 1.0% formaldehyde/agarose gels and transferred to Hybond-N (Amersham). Filters were probed with $^{32}$P-labeled λOm(2D)7 recombinant phages (H. Matsubayashi personal communication).

## cDNA cloning

A cDNA library was prepared from the poly(A)$^+$ RNA of *Om(2D)63* embryos as described in Chapter I. The cDNA library was screened using the pBBg18 genomic DNA clone as a probe (Fig. 3).

## Primer extension

Primer extension was performed essentially as in Sambrook et al. (1989). An oligonucleotide complementary to nucleotides 16-36 of the *Om(2D)* cDNA sequence (Fig. 13) was used as a primer. Ten micrograms of poly(A)$^+$ RNA purified from the *ca;px* embryos was hybridized with the primer labeled at the 5' end with [$\gamma$-$^{32}$P]dATP, and extended with reverse transcriptase. A gel marker was generated by sequencing the pH21 genomic DNA clone (Fig. 3) with the oligonucleotide used for primer extension as a primer. Primer-extended products were electrophoresed on a sequencing gel along with the marker. Radioactivity of the dried gel was recorded as a digital image by Bio imaging analyzer BAS200 (Fuji Photo Film Co., Ltd.). The recorded image was then printed out by BAS-Pictrography (Fuji Photo Film Co., Ltd.).

## Labeling of probe for in situ hybridization

A modified method of Kramer and Zipursky (1992) was used to label DNA fragments for whole mount in situ hybridization. The *Om(2D)* cDNA (500 ng) was digested with *Alu*I and *Cla*I to cut into pieces less than 400 bases and suspended in 1 µl of buffer 5 (0.5 M Tris, pH 7.2/ 0.1 M $MgCl_2$/ 1 mM dithioerythritol/ 2 mg/ml BSA/ 3 mg/ml hexanucleotide random primer, Boehringer Mannheim), 4 µl of 25 mg/ml hexanucleotide random primer (Pharmacia) and 5 µl $H_2O$. The DNA in the mixture was denatured by boiling for 10 min, quickly chilled on ice, and added to 1 µl of buffer 5, 2 µl of buffer 6 (1 mM each dATP, dCTP, dGTP, 0.65 mM dTTP/ 0.35 mM digoxigenin-11-dUTP, Boehringer Mannheim), 1 µl of Klenow fragment (2 U/µl, Takara) and

6 µl of $H_2O$. The mixture was incubated at 16°C overnight, and then incubated at 37°C for 4 h. The labeling reaction was stopped by adding 4 µl of 0.5 M EDTA. To remove free nucleotides, labeled DNAs were heated for 15 min at 80°C and passed through a Sephadex G-50 (Pharmacia) spun column with 50 µg of yeast tRNA and 2 ml of 0.1% Tween 20/ PBS (phosphate buffered saline: 137 mM NaCl/ 3 mM KCl/ 10 mM $Na_2PO_4$/ 2 mM $KH_2PO_4$). Another 2 ml aliquot of 0.1% Tween 20/ PBS was spun in a G-50 spun column and added to the former. The probes thus prepared were diluted to a concentration of 2 ng/ml, and stored with an equal volume of 100% deionized formamide at -20°C. The probes were used at a final concentration of 10 ng/ 100 µl for embryos or 40 ng/ 100 µl for imaginal discs.

## In situ hybridization to embryo and imaginal discs

In situ hybridization was performed with a modified method of Tautz and Pfeifle (1989). Embryos were collected on grape juice plates at 25°C (Wieschaus and Nusslein-Volhard 1986), washed with water, dechorionated in 50% commercial bleach for 3 min, and washed with 0.05% triton X-100. They were fixed in 4% paraformaldehyde (pH 7-7.5)/ PBS for 15-20 min on ice and then fixed in 4% paraformaldehyde/ 0.6% Triton X-100/ PBS for 15 min at room temperature. Embryos were washed in PBT five times for 5 min each in 1:1 PBT-hybridization buffer (50% deionized formamide/ 5 x SSC/ 200 µg/ml yeast tRNA/ 100 µg/ml sonicated, boiled herring sperm DNA/ 0.1% Tween 20) for 10 min, and in the hybridization buffer for 10 min, and prehybridized in the hybridization buffer for at least 1 h at 48°C.

The heat-denatured probes were then added and incubated for 24 h at 48°C. After hybridization, excess probes were removed, and the embryos were washed in the hybridization buffer for 20 min at 48°C in 1: 1 PBT-hybridization buffer for 20 min at 48°C, and in PBT for 10-12 h at 48°C with five changes. Then the embryos were incubated in a 1: 2,000 dilution of anti-digoxigenin alkaline-phosphatase-conjugated antibodies (Boehringer Mannheim) which had been preabsorbed for 1 h at 4°C. After incubation for 1 h at 4°C, the embryos were washed in PBT four times for 2 h each, soaked in buffer 3 (100 mM Tris-HCl, pH 9.5/ 100 mM NaCl/ 50 mM $MgCl_2$) for 15-20 min, and stained with 3.5 μl BCIP (50 mg/ml)/ 4.5 μl NBT (75 mg/ml) per 1 ml of buffer 3. Third larval imaginal discs were dissected in PBS, and then treated with the same procedure as for embryos. After staining, the samples were mounted in 80% glycerol.

## Immunocytochemistry

Eye imaginal discs were dissected in Ringer's solution and fixed for 45 min in 3% paraformaldehyde in PBS on ice. The eye imaginal discs were washed for at least 15 min in 0.05% Triton X-100 in PBS (PBT), and incubated in 10% sheep serum in PBT (PBTS) for 15-30 min, followed by overnight treatment with monoclonal antibody (MAb) 22C10 (Fujita et al. 1982) at a 1:200 dilution in PBTS, washing for 30 min in PBT and incubation for 2-4 h with horseradish peroxidase conjugated anti-mouse IgG (Promega) at 1:200 in PBTS. After washing for 15 min with several changes of PBT, the antibodies were visualized by incubation in 0.5 mg/ml diaminobenzidine, 0.01% $NiCl_2$, 0.01%

$CoCl_2$, and 0.03% $H_2O_2$ in PBS. The discs were then washed in PBT, dehydrated though a graded series of ethanols, cleared in methylbenzoate, and mounted in Bioleite (Ohken Shoji Co.).

## Acridine orange staining

Eye imaginal discs were stained with acridine orange according to the protocol of Spreij (1971) in order to visualize dying cells. After dissection in Ringer's solution, discs were incubated for 5 min in $1.6\times10^{-6}$M acridine orange in Ringer's solution, rinsed, and examined with fluorescence filters.

## Germ-line transformation

A 3.5 kb fragment isolated from the *Om(2D)* cDNA clone R35 by cleavage at the *Eco*RI sites within the adaptor sequences was cloned in the appropriate orientation into the blunt-ended *Xba*I site of phsp70C4, which contains the *hsp70* promoter in the Carnegie 4 vector (Tanda and Corces, 1991). A 3.9 kb *Sal*I fragment containing the *hsp70* promoter and the *Om(2D)* cDNA of this Carnegie construct was introduced into the blunt-ended *Bam*HI site of the CaSpeR vector (Pirrotta et al. 1985). The resulting construct was co-injected with pπ25.7wc (Karess and Rubin, 1984) into a *D. melanogaster* $w^1$ stock using the method described by Rubin and Spradling (1982).

## Electron microscopy

Flies stored in 25% ethanol were dehydrated to 100% ethanol, passed through a graded series of hexamethyldisilazane (HMDS) in ethanol up to 100%

HMDS, and vacuum-dried. They were then coated with gold and examined with a scanning electron microscope (JEOL JSM-5400LV).

# RESULTS

## Identification of the *Om(2D)* gene

Localization of the *Om(2D)* coding region was first studied by genomic Southern blot analysis with *Om(2D)* mutants and revertants, induced by γ-ray mutagenesis and associated with chromosomal rearrangements (Matsubayashi et al. 1991b).

The *Om(2D)63*$^{R5}$ revertant, which is associated with a reciprocal translocation, T(2;3)48B;96A, had a breakpoint immediately distal to the *tom* insertion sites in the *Om(2D)* region, whereas the *Om(2D)63*$^{R4}$, *Om(2D)63*$^{R15}$ and *Om(2D)63*$^{R28}$ revertants had rearrangements within a nearby 5 kb *Bam*HI segment (Fig. 3). To characterize the *Om(2D)63*$^{R4}$ rearrangement in detail, Southern analysis was performed with *Om(2D)63*$^{R4}$*/NG2* heterozygotes. The result showed that the *Om(2D)63*$^{R4}$ chromosome has a deletion in between the *Sph*I and *Eco*RI sites (Fig. 3). On the basis of these findings, I concluded that the *Om(2D)* coding region resides distal to the *tom* insertion site, and that part of the coding region may be included within the deleted region.

Detailed characterization of the *Om(2D)* coding region was performed by analyzing poly(A)$^+$ RNA by Northern blots hybridization and also by characterizing cDNAs. Northern blot analysis showed that a single 3.6 kb transcript in poly(A)$^+$ RNAs from various stages of *D. ananassae* development hybridizes with λOm(2D)7; the transcript is more abundant in the embryo than in other developmental stages (Fig. 2B). A cDNA library was then constructed from poly(A)$^+$ RNA of the *Om(2D)63* embryos and screened with the pBBg18

genomic clone as probe. The structure of the 3.6 kb transcript, shown in Figure 3, was deduced by restriction mapping and limited sequencing of the longest isolated cDNA clone, R35, and of the corresponding genomic regions. The transcription unit encompasses 11 kb, consisting of nine exons. Exons 4 and 5 fall within the region deleted in the *Om(2D)63*$^{R4}$ revertant. It was thus concluded that the 3.6 kb transcription unit is likely to represent the *Om(2D)* gene.

## Sequence of the *Om(2D)* gene product

Primer extension was carried out to map the *Om(2D)* transcription start site (Fig. 12). A 5' end-labeled oligonucleotide complementary to nucleotides 16-36 of the cDNA R35 sequence (Fig. 13) was annealed to embryonic poly(A)$^+$ RNA of *Om(2D)63* and extended with reverse transcriptase. As a result, a single extension product of 200 nucleotides long was detected, suggesting that the C in the third position of the heptanucleotide sequence TTCAGTT is the start site (Fig. 13). This sequence has a significant degree of homology to the Drosophila initiation site consensus for RNA polymerase II-mediated transcription of RNAs (Hultmark et al. 1986). The cDNA R35 sequence ends at the poly A stretch of 34 bp that begins 20 bp downstream of an AATAAA polyadenylation signal sequence. The sequence from the transcription start site to the end of the cDNA R35 thus amounts to 3,574 bp including the poly A tract and is in accordance with the size of the transcript detected by Northern blots (Fig. 2B).

The complete sequence of the cDNA clone R35 (Fig. 13) contains a single

large open reading frame capable of encoding a protein product of 671 amino acids with a predicted molecular mass of 71,412 Da. Since sequences surrounding the second ATG initiation codon show a perfect match to the consensus for translation start sequences of Drosophila genes (Caverner, 1987), the translation of the *Om(2D)* gene probably starts at this codon. If this holds true, the predicted molecular mass of the protein product would be 66,307 Da.

Comparison of the predicted *Om(2D)* amino acid sequence with the GenBank protein database revealed no significant overall homology to any other proteins so far reported, apart from a short segment of alternating histidine and proline residues similar to the PRD repeat motif (Frigerio et al. 1986). This motif has been found in a number of Drosophila transcriptional regulatory proteins and could suggest that the *Om(2D)* gene may be involved in transcriptional regulation. However, the functional role of this motif is uncertain at present, although a recent report suggests that it might mediate protein-protein interaction in a pH-dependent fashion (Janknecht et al. 1991).

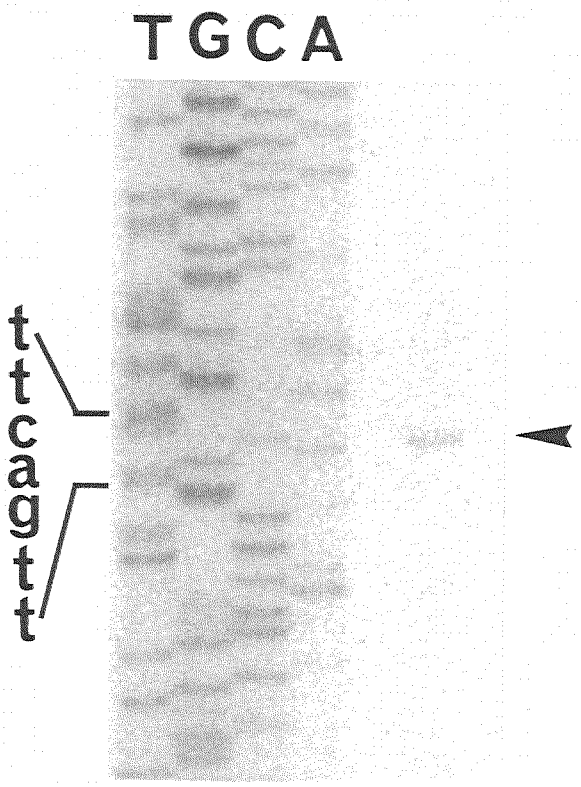



**Fig. 12**. Primer extension analysis. The primer extension product of poly(A)$^+$ RNA (10 μg) from *ca;px* embryo is indicated by an *arrowhead*. Sequence around a putative transcription start site is shown on the left-margin. The pH21 genomic clone (Fig. 3) was sequenced and used as a size marker.

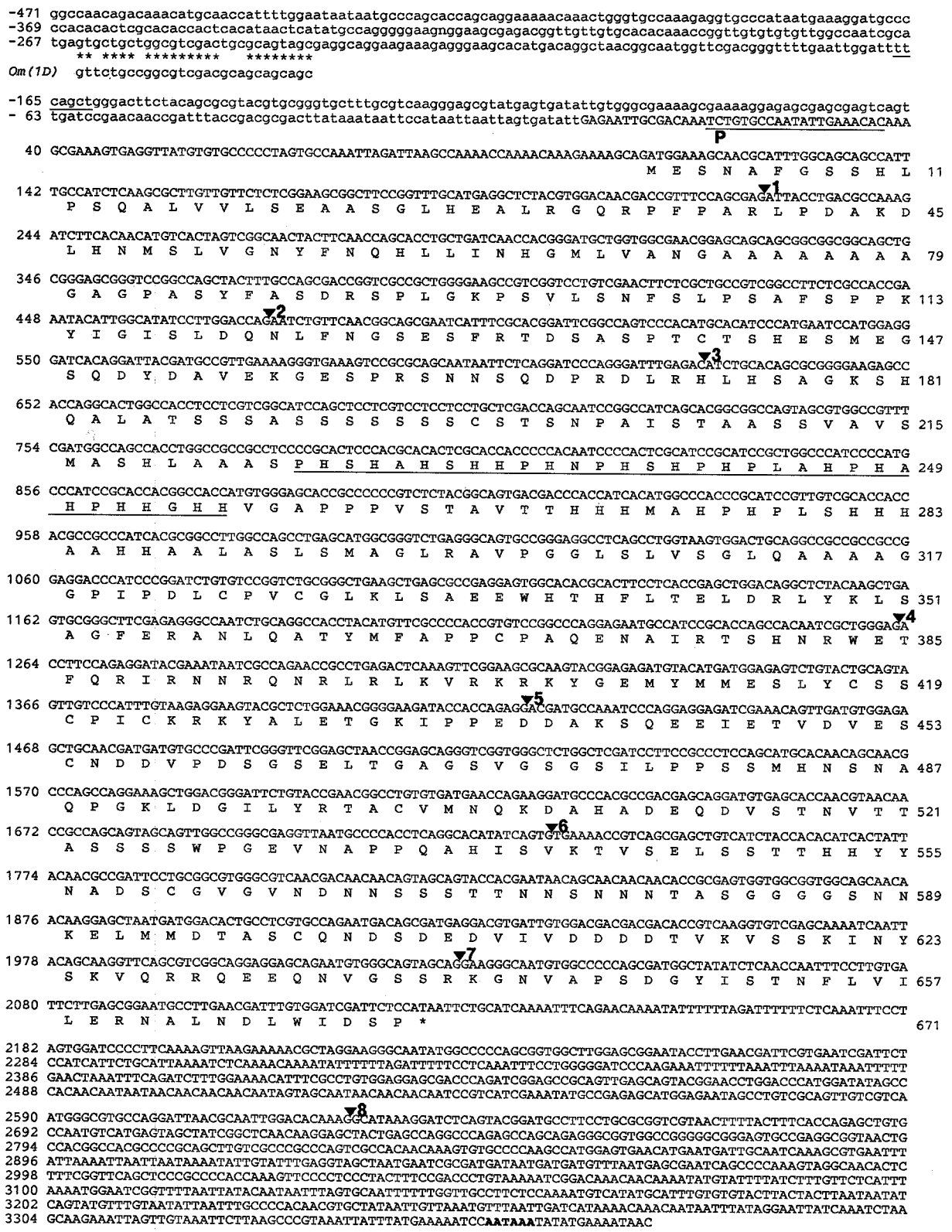


**Figure 13.** (*See following page for legend.*)

**Fig. 13.** Sequence of the *Om(2D)* cDNA clone R35 and the predicted *Om(2D)* protein product. *Numbers* at the *left* represent nucleotides starting from the 5' end of cDNA clone R35 and at the *right* indicate amino acid positions in the predicted *Om(2D)* protein sequence. Sequences derived from genomic DNA are shown in *lowercase letters*. *Numbered triangles* (1-8) indicate intron/exon junctions within the cDNA sequence. The sequence found 207 bp upstream of the transcription start site of the *Om(1D)* gene (Tanda and Corces 1991), which is homologous to part of the *Om(2D)* gene (*asterisks*), is shown in line 5. The *underlined lowercase* nucleotide sequence is homologous to the Drosophila initiation site consensus. The primer used in the primer extension experiment is indicated by 'P'. The PRD repeat motif in the *Om(2D)* protein is *underlined*; the *asterisk* marks the termination codon; a polyadenylation signal (AATAAA) is shown in *bold*. The nucleotide sequence data reported in this study will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with the following accession numbers D17581 and D26553.

## *Om(2D)* transcripts in developing embryos

Northern blot analysis showed that the *Om(2D)* transcript is abundantly present in wild-type and mutant embryos (Fig. 2B). To localize the sites of the *Om(2D)* gene expression in normal developing embryos, in situ hybridization experiments were performed using the *Om(2D)* cDNA as a probe. The *Om(2D)* transcript detected throughout the entire embryo even at the blastoderm stage (Fig. 14A) is probably of maternal origin, since zygotic transcription begins ~1.5-2 h after egg laying (Edgar and Schubiger 1986). Ubiquitous distribution of the transcript was continuously observed after the gastrulation stage throughout the period of germ-band extension and retraction (Fig. 14B-F), with a slightly higher level of the transcript in the mesodermal layer (Fig. 14B). No obvious difference in spatial or temporal expression patterns of the *Om(2D)* gene were observed between wild type and mutants, except that a considerable accumulation of the *Om(2D)* RNA was found along the cephalic furrow in the mutant (data not shown).

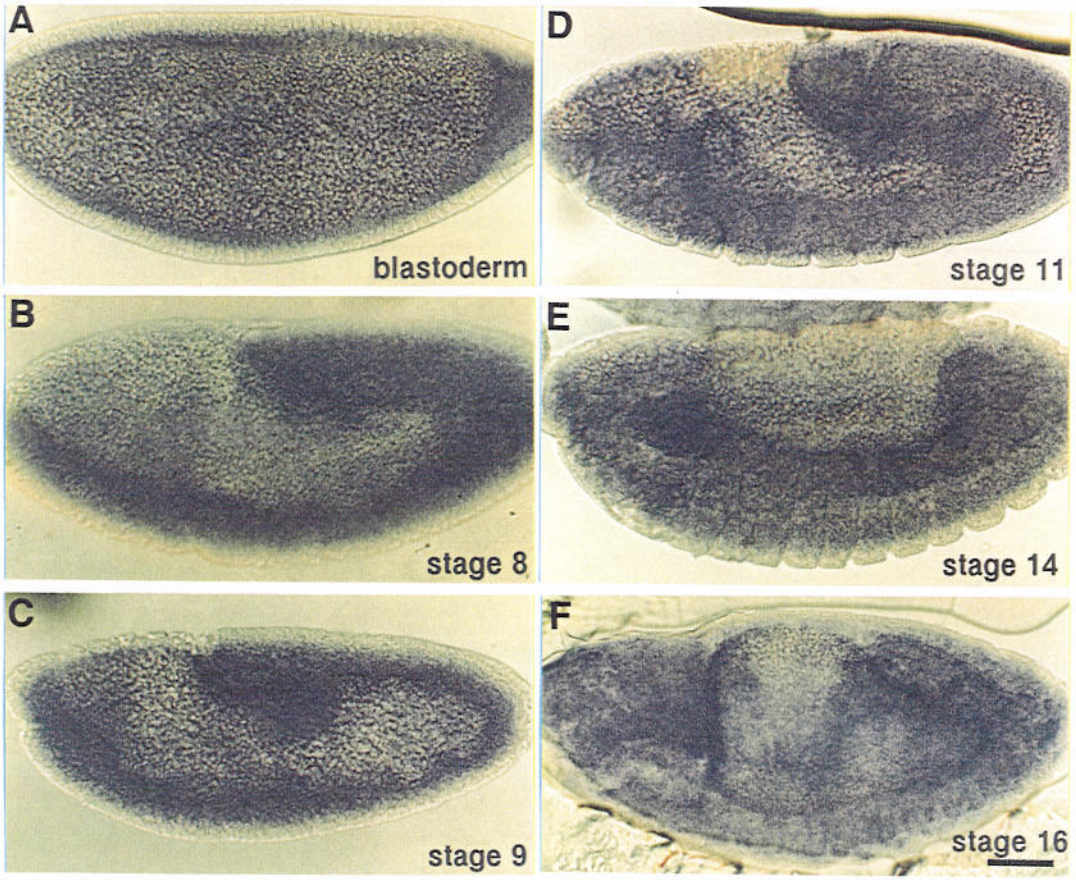



**Fig. 14A-F**. Localization of *Om(2D)* transcripts in developing embryos. Wholemount preparations of staged embryos were hybridized in situ with an *Om(2D)* cDNA probe labeled with digoxigenin. The anterior is to the left and the dorsal surface is at the top. Embryonic stages are as follows; **A**, Late cellular blastoderm; **B**, Stage 8; **C**, Stage 9; **D**, Stage 11; **E**, Stage 14; **F**, Stage 16 (Campos-Ortega and Hartenstein 1985).

## *Om(2D)* transcripts in the eye imaginal disc

The dominant nature of the *Om* phenotype may result from either excessive or ectopic expression of the *Om* gene in the eye imaginal disc, brought about by the *tom* insertion. To distinguish between the two possibilities, in situ hybridization experiments were performed with eye imaginal discs from the late (crawling) third instar larvae, using the *Om(2D)* cDNA R35 as a probe. No *Om(2D)* RNA was detected in wild-type eye discs (Fig. 15A), whereas it was abundantly found in the region just anterior to the morphogenetic furrow in the *Om(2D)63* mutant, the amount decreasing toward the dorsal edge of the disc (Fig. 15B). A considerable amount of the RNA was also found in the region posterior to the morphogenetic furrow (Fig. 15B). In the extreme derivative, $Om(2D)63^{E8}$ (Fig. 1D) the RNA accumulates in the region posterior to the morphogenetic furrow to a much higher level than is seen in *Om(2D)63*, concomitantly with a large increase in the region anterior to the morphogenetic furrow (Fig. 15C). The degree of *Om(2D)* expression thus seems to be correlated with the extent of the mutant phenotype (Fig. 1). A corollary of these findings is that the *Om(2D)* phenotype may result from ectopic expression of the *Om(2D)* gene in the eye disc, even though the *Om(2D)* gene function may not be a prerequisite for normal eye development.

Second-site dominant suppressor mutations (*OmSu*) that reverse *Om* mutant phenotypes to the wild type have been isolated by Hinton (1984, 1988). In order to examine the effects of a suppressor mutation on the *Om(2D)* gene expression, the *Om(1K)SuT2* gene was introduced into the *Om(2D)63* mutant, and the eye discs of the resulting *Om(1K)SuT2;Om(2D)63* double homozygote,

which shows a nearly normal eye phenotype (Fig. 1E), were subjected to in situ hybridization with the cDNA as a probe. The result showed that the RNA is only detectable in a narrow region anterior to the morphogenetic furrow and is no longer found in the posterior region of the disc (Fig. 15D). Such a dramatic reduction in expression of the gene may be responsible for the reversion of the eye phenotype, suggesting that the suppressor mutation may act on the *Om(2D)* expression at the transcriptional level.

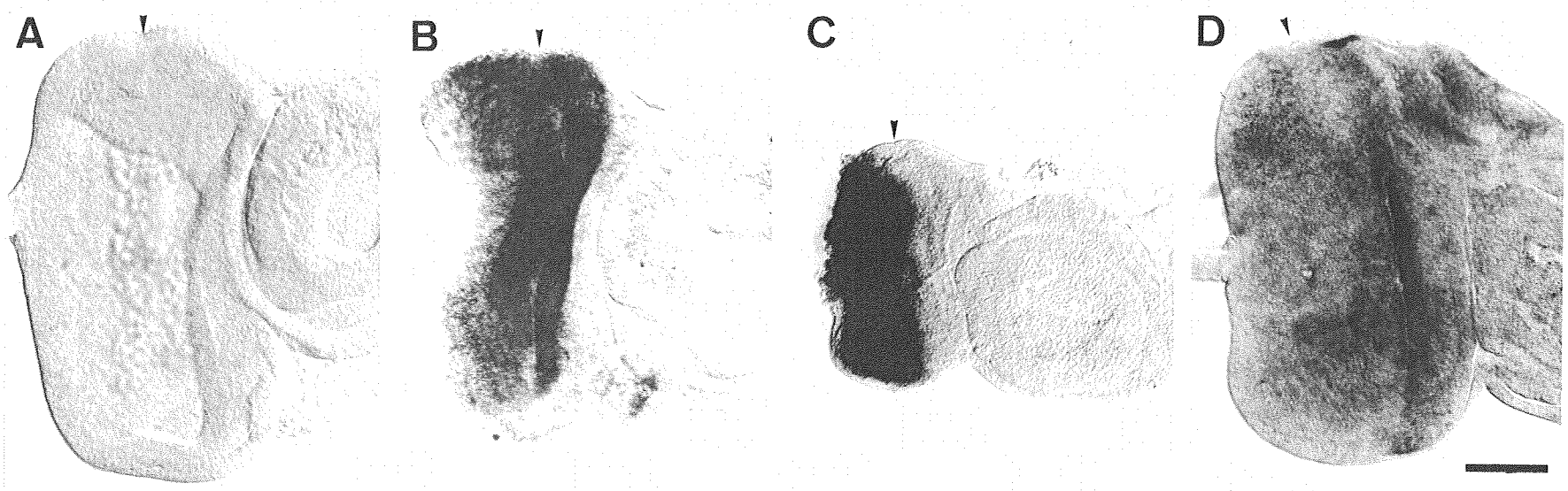



**Fig. 15A-D**. Localization of *Om(2D)* transcripts in the eye-antennal imaginal disc from late third instar larvae. Wholemount preparations were hybridized in situ with the *Om(2D)* cDNA probe labeled with digoxigenin. *Arrowheads* mark the morphogenetic furrow. **A**, *ca;px*; **B**, *Om(2D)63*; **C**, *Om(2D)63*$^{E8}$; **D**, *Om(1K)SuT2;Om(2D)63*. The anterior is tc the right. *Bar*, 50 mm.

## Neural differentiation and cell death in the eye imaginal discs of *Om(2D)* mutants

To examine the effect of the *Om(2D)* gene expression in the mutant eye discs on eye development, the eye imaginal discs of late third instar larvae were stained with the neuron specific monoclonal antibody 22C10. In eye discs of wild-type, *Om(2D)63* and *Om(2D)10a* strains, differentiating photoreceptor neurons formed a regular array of cell clusters behind the morphogenetic furrow, but their numbers were apparently reduced in the mutants (Fig. 16A-C). In the extreme derivative, $Om(2D)63^{E8}$, the clusters disappeared from the center of the eye disc and those remaining in the periphery were irregularly arranged (Fig. 16D). Such abnormalities may result from cell death; a considerable increase in the number of dead cells occurred in the mutant eye imaginal discs, particularly, in $Om(2D)63^{E8}$ (Fig. 16E-H). These findings suggest that the ectopic expression of the *Om(2D)* gene in the eye imaginal discs may cause cell death leading to malformation of the adult compound eye.

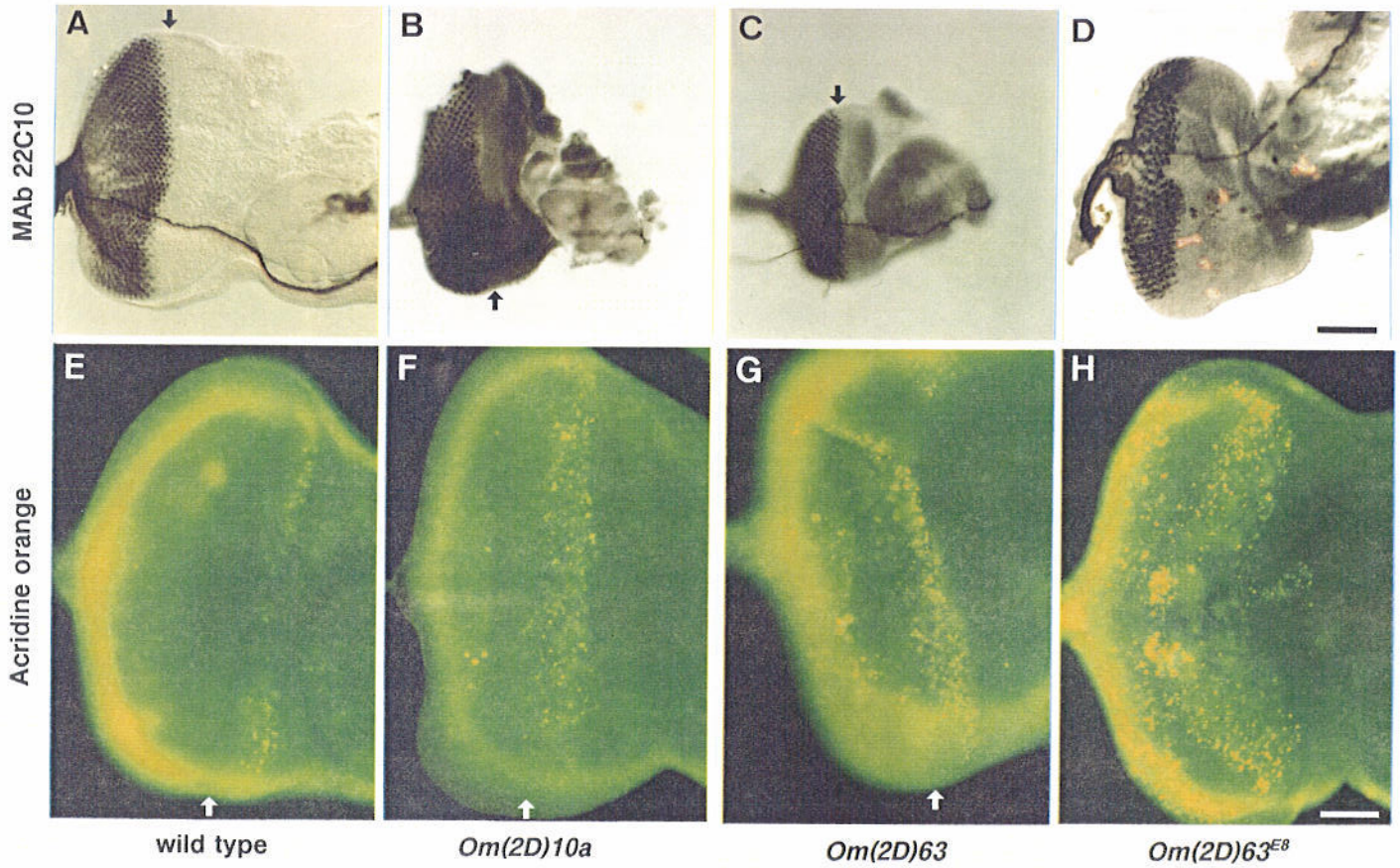



**Fig. 16A-H**. Neural differentiation and cell death in the eye-antennal imaginal disc of late third instar larvae. Wholemount preparations were stained with monoclonal antibody 22C10 (*upper row*) and with acridine orange (*lower row*). *Arrows* mark the morphogenetic furrow. **A**, **E** *ca;px*; **B**, **F** *Om(2D)63*; **C**, **G** *Om(2D)10a*; **D**, **H** *Om(2D)63$^{E8}$*. The anterior is to the right. *Bars*, 50 mm.

## Artificially induced ectopic expression of the *Om(2D)* gene

To test whether artificially induced ectopic expression of the *Om(2D)* gene could cause a phenotype similar to those of *Om(2D)* alleles, a cDNA fragment containing the entire *Om(2D)* protein-coding region was fused to the *D. melanogaster hsp70* promoter, cloned into the CaSpeR P-element transformation vector, and injected into preblastoderm embryos of the *D. melanogaster* $w^1$ stock. A total of 12 independent transformant lines were established which showed no phenotypic abnormalities when raised at 25°C.

Heat treatment was first performed with early (feeding) third instar larvae of all the transformant lines (three 1 h exposures to 37°C at 2 h intervals of 25°C). While $w^1$ mutants carrying no insert showed wild-type eye morphology (Fig. 17A), all the transformants bearing an insertion of the *hsp-Om(2D)* minigene exhibited abnormal adult eye morphology when subjected to the heat treatment; about 50% reduction in the number of ommatidia was observed, along with irregular arrays of deformed ommatidia (Fig. 17B). In the next experiment, embryos and second and third instar larvae and pupae of two transformant lines were subjected to heat pulsing at 37°C for 1 or 2 h. The results showed that the heat pulsing had no effect on embryos or second instar larvae and pupae, but effectively induced abnormal eye morphology similar to that described above when applied to third instar larvae; the effect was most severe with late third instar larvae (Fig. 17C).

None of the heat-pulsed transformants mimicked the *Om(2D)* phenotype (Fig. 17D). It is conjectured, therefore, that the *Om(2D)* gene must be continuously expressed at a certain rate during a certain period of ommatidial

development to mimic the *Om(2D)* phenotype, and that such a characteristic mode of gene expression would be difficult to reproduce experimentally in vivo, and may only be possible using the *tom* element. In general, tissues other than the eye imaginal discs were not affected by heat treatments. A few of the several hundred heat-shocked transformants examined had one or two tarsal segments instead of five, or had bent tarsal segments; a possible explanation is that the leg discs might be somewhat sensitive to high levels of *Om(2D)* protein during a short developmental period.

## Effects of *tom* on an adjacent gene

The *tom*-induced mutation affects almost exclusively the morphology of the optic system, which implies that the *tom* element may have a tissue-specific enhancer sequence. This possibility was examined in the following experiment to determine whether the putative enhancer could influence adjacent genes other than the *Om* genes.

Near the *tom* insertion sites of *Om(2D)* alleles there are at least four independent coding regions (data not shown), and the gene immediately downstream of the *Om(2D)* gene has been identified as the OAT precursor gene (see Chapter I). Effects of the *tom* element on the OAT gene were studied by assaying OAT activities in the eye imaginal discs of wild-type and mutant larvae. However, no significant difference was found between the activities of the wild type and mutants (Fig. 5). It seems that the *tom* element is capable of acting on *Om* genes, but not on other nearby genes. This is in accordance with the finding that mutations recovered with the *Om* mutability system map to only 22 loci, although the *tom* elements are found at many more loci in the genome.

It is likely that promoters of the *Om* genes may share a homologous sequence, which interacts with an enhancer of the *tom* element. A search for such sequences in the *Om(1D)* and *Om(2D)* genes showed that the 28-bp sequences located 207 bp and 99 bp upstream of the transcriptional start sites, respectively, are 78.6% homologous to each other (Fig. 13). Whether or not these sequences are actually involved in the interaction between the *tom* element and the *Om* genes remains to be determined.

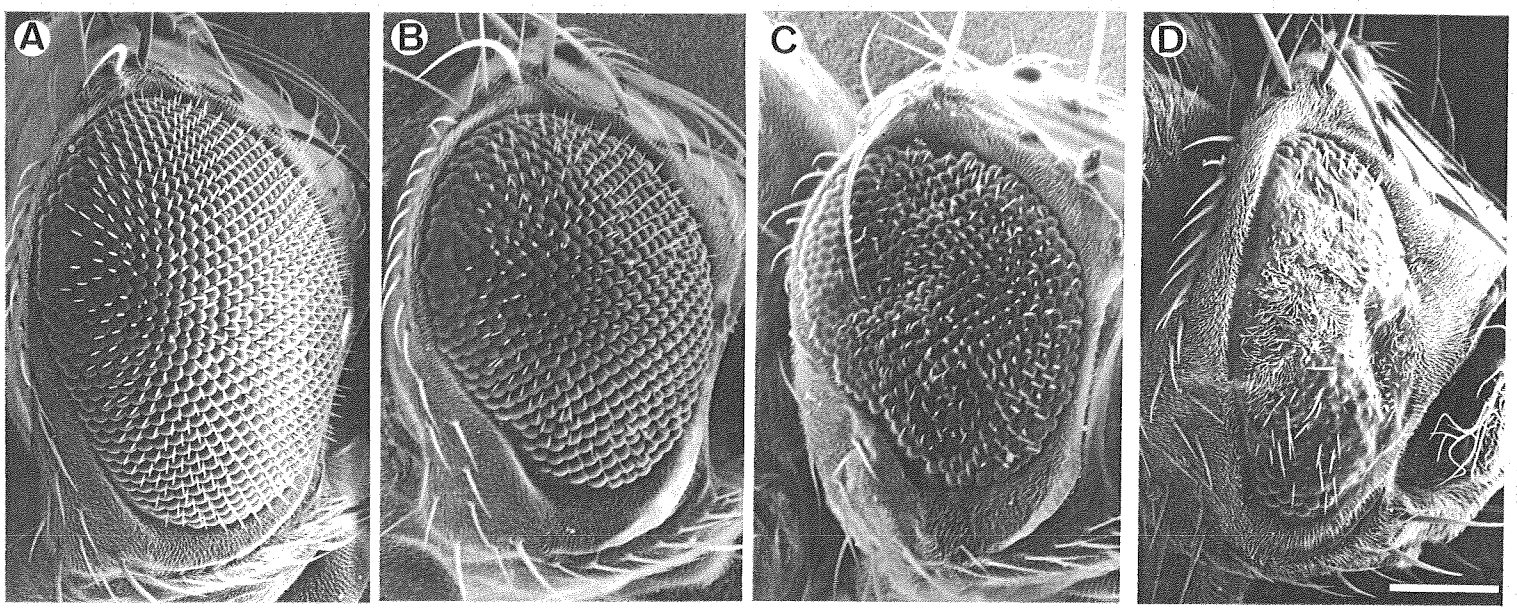



**Fig. 17A-D**. Effect of artificially induced ectopic expression of the *Om(2D,* gene on adult eye morphology. Scanning electron micrographs of the eye of heat-shocked flies. Animals were heat shocked at the third instar stage. **A**, $w^1$ mutant was heat shocked at the early third instar stage. **B**, Transformant with insertion of the *hsp-Om(2D)* minigene was heat shocked at the early third instar stage. **C**, Transformant was heat shocked at the late third instar stage. **D**, Phenotype of *Om(2D)63* in *D. ananassae*. The anterior to the right.

# DISCUSSION

Based on their findings with the *Om(1D)* mutants, Tanda and Corces (1991) proposed that a tissue-specific transcriptional enhancer, present in the *tom* element, stimulates expression of the *Om(1D)* gene when the *tom* element inserts in close proximity to the *Om(1D)* gene. Moreover, I have shown here that the *Om(2D)* transcript was found to be abundantly expressed in the center of mutant but not in wild-type eye discs. Further support of this hypothesis is provided by our recent findings that the *Om(1A)* product is ectopically expressed in the eye imaginal discs of the *Om(1A)* mutants (Awasaki et al. 1994). In this context, it is worth mentioning that many retroviruses have tissue-specific enhancers in their long terminal repeats (LTR). For instance, the LTR of mouse type C retrovirus possesses several enhancer elements which determine the viral pathogenicity (Golemis et al. 1990). In murine T lymphomas, the *c-myc* oncogene is activated by a murine leukemia virus LTR enhancer present in its vicinity (Corcoran et al. 1984). Furthermore, *int* genes (*1*, *2* and *3*) are expressed ectopically in mammary glands under the control of a tissue-specific enhancer present in the LTR when mouse mammary tumor provirus is inserted near the genes (for review see Nusse 1988). It is therefore reasonable to assume that the *tom* LTR may also have a tissue-specific enhancer. The possibility that such an enhancer is present in the *tom* LTR is now being studied directly, using transformants carrying *tom* LTR ligated to the *hsp* promoter plus a reporter gene.

In *Om(2D)* mutant larvae, expression of the *Om(2D)* gene was activated in the eye disc by the *tom* element, whereas the adjacent OAT gene is not activated by *tom*, suggesting that the transcriptional regulatory sequences present

in *tom* may be able to distinguish *Om* genes from other genes. Such selective interactions between transcription elements have repeatedly been observed with enhancers and promoters. For instance, defective P elements can activate glucose-6-phosphate dehydrogenase (G6PD) promoter but not the normal actin 5C promoter of *D. melanogaster*. However, the actin 5C promoter can be activated by the P elements if the 20 bp sequence around the G6PD transcription start site is present in front of the promoter (Ito et al. 1993). It has also been reported that when the synthetic polymerase II promoter is linked to a reporter gene with different combinations of upstream promoter elements and enhancer elements, the strength of the promoter varies depending on the combination of the elements; for example, combination of a CACCC box and an AP1 enhancer does not stimulate the transcription of the reporter gene, whereas the combination of a GC box and AP1 stimulates transcription efficiently (Wang and Gralla 1991). This indicates a specific interaction between a sequence in the upstream promoter elements and enhancers. It is thus feasible that *Om* genes may share a regulatory sequence in common, which proteins that bind the enhancer of *tom* may act upon. The homologous sequences found in the upstream region of the *Om(1D)* and *Om(2D)* genes might be involved in such a selective interaction.

One of the complete revertants, $Om(2D)63^{R4}$, was associated with a deletion within the *Om(2D)* coding region, and hence this may be regarded as a null mutant for *Om(2D)*. Animals homozygous for $Om(2D)63^{R4}$ die at the first instar stage, but no abnormalities were found either in the cuticular pattern of dead first instar larvae or in the central nervous system of embryos

(Matsubayashi et al. 1991b). In situ hybridization experiments revealed that the *Om(2D)* gene is expressed ubiquitously during embryogenesis, suggesting that the *Om(2D)* gene may not be involved in differentiation of specific cell types, but might play a role in fundamental pathways in all cells.

The amino acid sequence of the *Om(2D)* protein, as deduced from the nucleotide sequence of the cDNA, is novel and is characterized by the presence of the $(HX)_n$ PRD repeat motif (Frigerio et al. 1986). The PRD repeat is not only present in a number of Drosophila homeodomain proteins, including *Om(1D)* (Tanda and Corces, 1991), but it is also conserved in a number of homologous vertebrate homeodomain proteins (Janknecht et al. 1991). Moreover, this motif has been found in several types of Drosophila transcription factors, including E74, an Ets oncoprotein-related protein (Janknecht et al. 1989); *odd-skipped*, a zinc-finger protein (Coulter et al. 1990); and *daughterless*, a basic helix-loop-helix protein (Cronmiller et al. 1988). Although the functional role of this motif is unknown at present, it is reasonable to suppose that *Om(2D)* protein may be involved in transcriptional regulation.

In the eye imaginal disc of *Om(2D)*, the site of active expression of the *Om(2D)* gene seems to correspond to that of abnormal cell death, suggesting that the ectopic expression of the *Om(2D)* gene in the eye imaginal disc may result in cell death. However, the gene had no effect on imaginal discs other than the eye disc when expressed artificially by heat shock at the third instar stage, suggesting that an eye-disc specific factor causes cell death upon interaction with the *Om(2D)* protein. The existence of such a putative tissue-specific factor has also been suggested by the previous finding that two modifiers of *Om(2D)*, which are themselves recessive eye mutants, act as *Om(2D)* specific-dominant

enhancers (Matsubayashi et al. 1991b). It is thus feasible that products of those enhancers may interact with the product of the *Om(2D)* gene.

An early stage in our studies on *Om* mutations, we presumed that all the *Om* genes might be involved directly in eye morphogenesis. The *Om(1D)* gene encodes a homeoprotein and is expressed in clusters of differentiating photoreceptors in the eye imaginal disc, a null allele showing a rough eye phenotype with disorganized ommatidia (Tanda and Corces 1991, Tanda et al. 1993). The *Om(1A)* gene is homologous to the *cut* homeoprotein gene (Awasaki et al. 1994), which is expressed in cone cells and interommatidial bristle precursor cells of the eye imaginal disc (Blochlinger et al. 1993). In contrast, the *Om(2D)* gene appears not to be a prerequisite for normal eye development, because in situ hybridization experiments failed to show *Om(2D)* RNA in wild-type eye discs. It may consequently be noted that the only common characteristic of *Om* genes may be nothing other than the fact that their aberrant expression causes defects in the compound eye morphology, irrespective of their normal function.

The present study has demonstrated that the *Om(2D)* gene is not normally transcribed in the imaginal eye disc, but is transcribed actively in embryonic cells, and that its ectopic expression at the third instar stage causes malformation of the adult compound eye, but not of other tissues. Ubiquitous expression of the *Om(2D)* gene in embryos implies that it plays a role in a fundamental pathway, but this is in apparent contradiction to the observation that the gene has no effect on imaginal discs other than the eye disc when ectopically expressed. This paradox may again be explained by postulating the involvement of an eye disc-specific regulatory factor.

# Chapter III

# Characterization of the *Om(2D)* gene of *Drosophila ananassae*

# INTRODUCTION

One of the complete revertants, *Om(2D)63*$^{R4}$, was associated with a deletion within the *Om(2D)* coding region, and hence this may be regarded as a null mutant of *Om(2D)*. Animals homozygous for *Om(2D)3*$^{R4}$ die at the first instar stage, but no abnormalities were found in the cuticular pattern of dead first instar larvae as well as in the central nervous system of embryos (Matsubayashi et al. 1991b). In situ hybridization experiments revealed that the *Om(2D)* gene was expressed ubiquitously during embryogenesis, suggesting that the *Om(2D)* gene may not be involved in differentiation of specific cell types, but might play a role in fundamental pathways of all cells. The *Om(2D)* gene may not be prerequisite for normal eye development, because in situ hybridization experiments failed to show *Om(2D)* RNA in wild-type eye discs. On the contrary, the gene had no effect on imaginal discs other than the eye disc when expressed artificially by heat shock at the third instar larval stage, suggesting the presence of an eye-disc specific factor which causes cell death upon interacting with the *Om(2D)* protein.

The amino acid sequence of the *Om(2D)* protein, as deduced from the nucleotide sequence of the cDNA, is novel and characterized by the presence of $(HX)_n$ PRD repeat motif (Frigerio et al. 1986). The PRD repeat is not only present in a number of Drosophila homeodomain proteins including *Om(1D)* (Tanda and Corces, 1991), but it is also conserved in a number of homologous vertebrate homeodomain proteins as well (Janknecht et al. 1991). Moreover, this motif has been found in several types of Drosophila transcription factors, including E74, an Ets-oncoprotein related protein (Janknecht et al. 1989); *odd-*

*skipped*, a zinc-finger protein (Coulter et al. 1990); and *daughterless*, a basic helix-loop-helix protein (Cronmiller et al. 1988). Although the functional role of this motif is unknown at present, it is reasonable to suppose that the *Om(2D)* protein may be involved in transcriptional regulation.

In this chapter, I have examined the expression pattern and cellular localization of the *Om(2D)* gene product in the embryo and the third instar larvae, using the antibody raised against the *Om(2D)* protein as well as a *Om(2D)* cDNA fragment, in a hope to obtain information on the physiological role of the *Om(2D)* gene.

# MATERIALS AND METHODS

## Purification of the *Om(2D)* protein

Purification of the *Om(2D)* protein was carried out according to the method described in Chapter I. The *Pvu*I-*Stu*I fragment containing a part of the *Om(2D)* open reading frame was ligated in frame with the gene encoding the maltose binding protein (MBP) in the pMAL-c2 vector (New England Biolabs). The *E. coli* cells (JM109) containing the fusion plasmid were stimulated to express the fusion protein by adding IPTG to the medium. The fusion protein was purified by an amylose resin affinity column chromatography following the manufacturer's instruction. The purified fusion protein was used to raise polyclonal anti-serum in mice as described in Chapter I.

## Immunoblotting analysis

Samples were homogenized in 62.5 mM Tris-HCl (pH6.8) containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue, boiled for 10 min, and centrifuged. The supernatant was resolved on a SDS-PAGE and transferred to nitrocellulose membrane by the method described by Harlow and Lane (1988). The blotted membrane was incubated with anti-*Om(2D)* antiserum at a 1:200 dilution and subsequently with goat anti-mouse IgG alkaline phosphatase conjugate. The membrane was then stained with NBT and BCIP.

## Antibody staining

The embryos were collected, dechorionated in 50% commercial bleach for 3 min, washed with PBS/ 0.05% Triton X-100, and fixed in 10:9:1 of *n*-

heptane:PEM (0.1 M PIPES/ 2 mM EGTA/ 1 mM $MgSO_4$, pH 6.95):37% formaldehyde with shaking for 15-30 min at room temperature. After the aqueous layer was removed, one volume of 100% methanol was added for devitellinization. Then the devitellinized embryos were treated with 100% methanol for 10 min, PBT twice for 10 min each, and blocking solution (3% bovine serum albumin/ PBT) for 10 min. The samples were incubated in a 1:200 dilution of the anti-*Om(2D)* antiserum in the blocking solution either at room temperature for 4 h or at 4°C overnight. After washing in the blocking solution three times for 10 min each, the samples were incubated in a 1:500 dilution of anti-mouse IgG horseradish peroxidase conjugate (pre-absorbed against homogenized embryos for 1 h at 4°C) for 2 h at room temperature. The samples were washed in PBS three times for 10 min each and stained in PBS containing 0.5 mg/ml DAB/ 0.03% $CoCl_2$/ 0.003% $H_2O_2$. The staining reaction was stopped by addition of sodium azide to 2%.

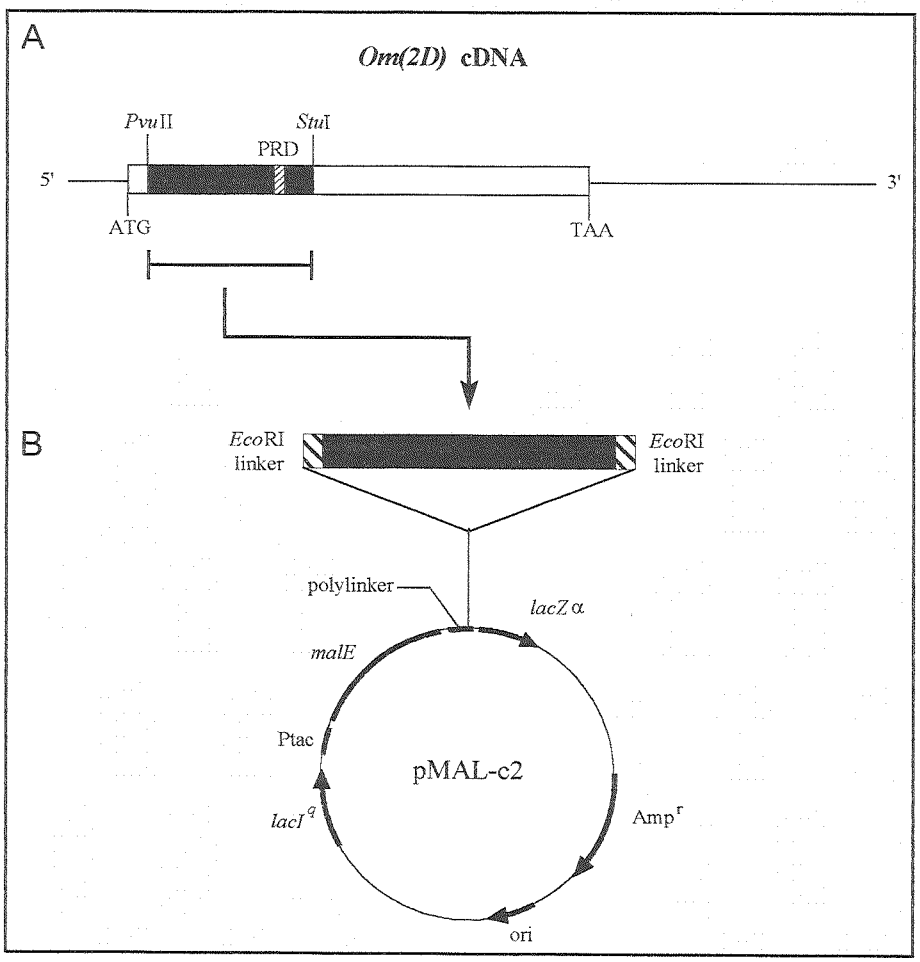
Imaginal discs of third instar larvae were dissected in PBS on ice, fixed in 4% paraformaldehyde in PEM for 30 min at room temperature, washed in PBT for 15 min, and blocked with the blocking solution for 15 min. Immunostaining of imaginal discs were carried out with the same procedure used for embryos. Preimmune mouse serum was used as negative control for the anti-*Om(2D)* antiserum. Neither embryos nor imaginal discs used in this study were stained with the preimmune mouse serum. The samples were washed in 2% sodium azide/ PBS for several times, mounted in Aquatex (Merck) and examined using Nomarski optics (Nikon).

# RESULTS

## Preparation of antisera against the *Om(2D)* protein

Comparison of the predicted *Om(2D)* amino acid sequence with the GeneBank protein database showed no significant homologies, as a whole, to any other proteins so far reported, besides a short segment of alternating histidine and proline residues like the PRD repeat motif (Frigerio et al. 1986). The functional role of this motif is uncertain, but it has been found in a number of Drosophila transcriptional regulatory proteins. Therefore, possibility exists that *Om(2D)* protein may be involved in transcriptional regulation. In order to test this possibility, I attempted to examine the localization of *Om(2D)* gene transcripts in various developmental stages of flies. To do this, I have first prepared antibodies against the *Om(2D)* protein.

A schematic structure of the *Om(2D)* cDNA and its open reading frame are shown in Figure 18A. A 679 bp *Pvu*I-*Stu*I fragment containing the PRD repeats was subcloned into an expression vector pMAL-c2 (Fig. 18B) which enables the translation of the MBP-*Om(2D)*-β-galactosidase fusion protein. With this clone were transformed *E. coli* JM109 cells, and the *Om(2D)* fusion protein was induced by IPTG (Fig. 18C). The fusion protein was cleaved to MBP and *Om(2D)*-β-galactosidase, purified, and injected to mice with adjuvant. Each serum obtained from the mice was titrated as described in MATERIALS AND METHODS. The immunological specificity of the antibodies was tested by immunoblotting using partial purified fusion proteins as antigen. As a result, all lots of antisera recognized the *Om(2D)* fusion protein (Fig. 19A).



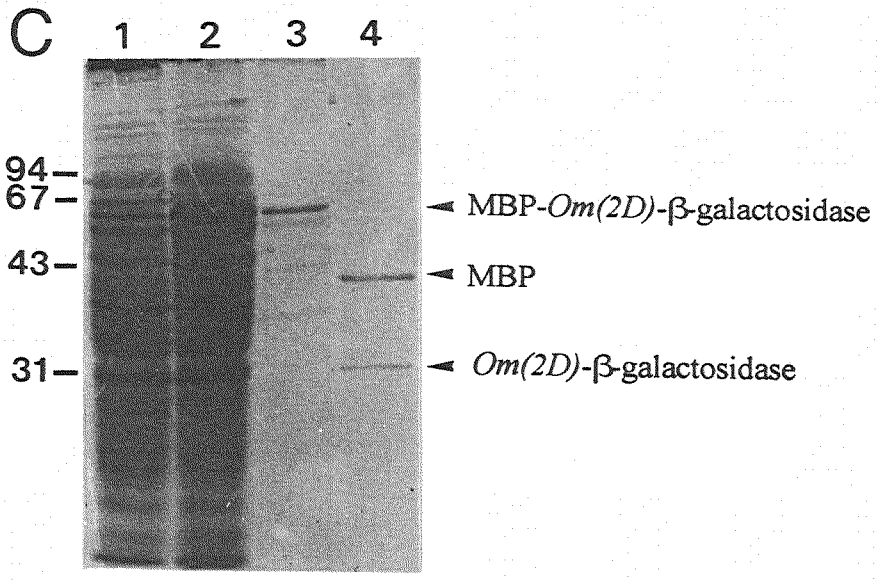




**Fig.18A-C** (*See following page for legend.*)

**Fig. 18A-C.** Structures of the *Om(2D)* cDNA and plasmid pMALc2-*Om(2D)*. **A**, Structure of the *Om(2D)* cDNA. Open reading frame (ORF) is indicated by a *white bar*, and the fragment cloned into the vector is indicated by a *black bar* in ORF. The PRD repeat is indicated by *hatched areas*. **B**, Structures of plasmid pMALc2-*Om(2D)*. A *Pvu*I-*Stu*I fragment of *Om(2D)* was cloned into an expression vector for raising MBP-*Om(2D)*-β-galactosidase fusion protein. An *Eco*RI site on the pMALc2 indicates the cloning site where a *Pvu*I-*Stu*I fragment flanked by *Eco*RI linkers was cloned into. *Arrows* indicate the direction of transcription. *malE* encodes MBP, while *lacZ α* encodes β-galactosidase. The Ptac promoter is for transcription of *malE*, *lacZa* and the gene inserted between them. $lacI^q$ encodes the Lac repressor which turns off transcription from Ptac unless IPTG is added. The origin of replication is represented by *ori*. The ampicillin resistance gene is shown as $Amp^r$. **C**, Expression and purification of the *Om(2D)* fusion product. SDS-PAGE were stained with Coomassie Brilliant Blue. Bars indicate molecular size markers. Lane 1: Uninduced cells. Lane 2: Induced cells. Lane 3: Purified protein eluted from an amylose column with maltose. Lane 4: Purified protein after factor Xa cleavage. *Arrowheads* indicate the *Om(2D)*-β-galactosidase fusion protein.

## Molecular mass of the *Om(2D)* protein in vivo

The complete sequence of the *Om(2D)* cDNA clone contains a single large open reading frame capable of encoding a protein with a predicted molecular mass of 71,412 Da. To examine the actual molecular mass of the *Om(2D)* protein, embryonic extract was resolved by SDS-PAGE, and then the *Om(2D)* protein was detected using the anti-*Om(2D)* antiserum by immunoblotting analysis. As a result, 64, 56, and 49 kDa of *Om(2D)* products were detected (Fig. 19B, lane 1). The strongest signal of 64 kDa corresponds to the predicted size of the *Om(2D)* protein, provided that it is translated from the third ATG initiation codon of the *Om(2D)* ORF. This suggests that the *Om(2D)* protein may be processed in several ways in vivo.

## Subcellular localizations of the *Om(2D)* protein

To examine subcellular localizations of the *Om(2D)* protein, homogenates of third instar larvae were separated into nuclear and cytosol, and each fraction was probed using the *Om(2D)* antibodies by immunoblotting. As a result, the *Om(2D)* protein was detected in the nuclear fraction (Fig. 19B, lane 2) as well as in the cytoplasm (Fig. 19B, lane 3). This result supports the idea that the *Om(2D)* protein may be involved in the process of transcriptional regulation.

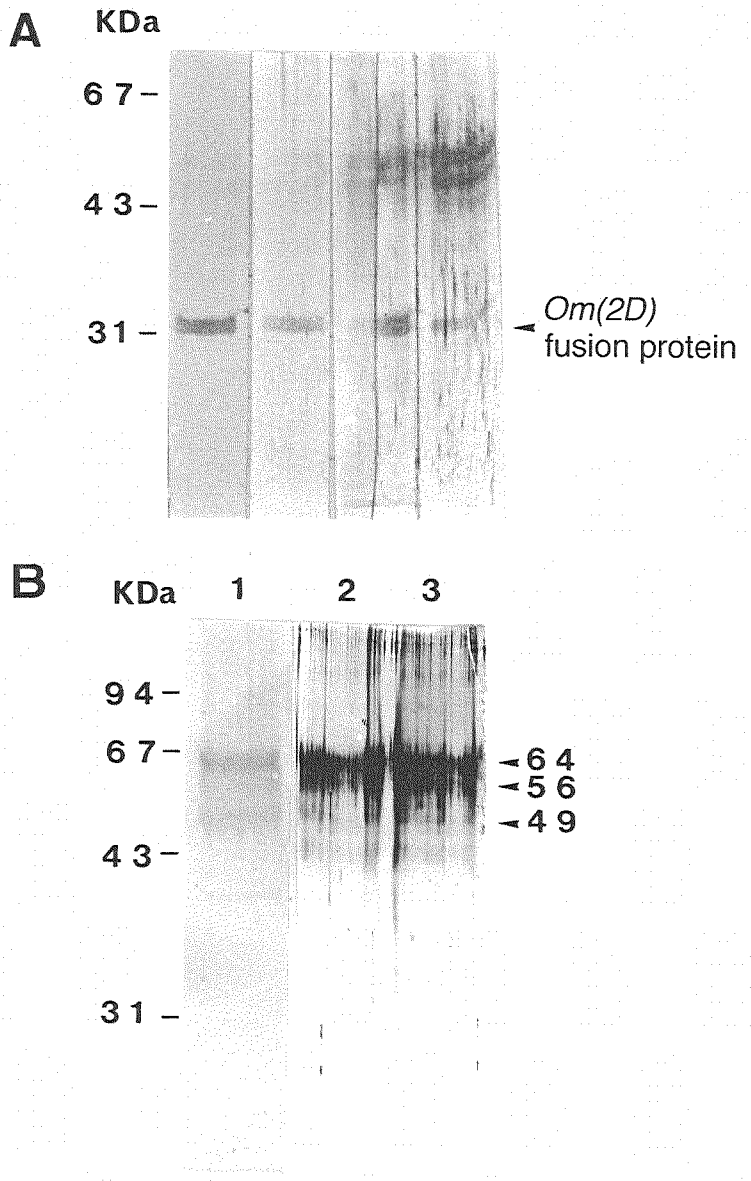



**Fig. 19**. Immunoblotting analysis. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. The *Om(2D)* protein was detected with mouse anti-*Om(2D)* antiserum. **A**, The protein used for immunization was detected by all lots of antiserum on five pieces of nitrocellulose membrane. **B**, Immunostaining of fly extracts with *Om(2D)* protein. Lane 1, Extract of *ca;px* embryos. Extract of *ca;px* third instar larvae was separated into nucleic (lane 2) and cytosol fractions (lane 3). *Bars* indicate molecular size markers. *Arrowheads* indicate locations of the signals.

## Expression of the *Om(2D)* gene in developing embryos

To localize the *Om(2D)* protein in developing embryos, immunocytochemical staining was carried out using the anti-*Om(2D)* antisera (Fig. 20). Throughout embryogenesis, the expression pattern of the *Om(2D)* protein was similar to that of the *Om(2D)* RNA detected by the in situ hybridization experiment (Fig. 14). However, at the late embryonic stage, the *Om(2D)* protein was abundantly found in the head region and developing central nervous system (Fig. 20F).

*Om(2D)63*$^{R4}$ is a revertant allele from *Om(2D)63* and lacks a few exons of the *Om(2D)* gene (Fig. 3). The homozygote of *Om(2D)63*$^{R4}$ allele is first larval lethal (Matsubayashi et al. 1991b). Some dissimilarity in the *Om(2D)* gene expression is expected between wild type and homozygotes of the recessive lethal allele, so that their embryos were examined in the expression pattern. However, no difference was found between wild type and the mutant (data not shown). It is thus assumed that the chromosomal deletion in the *Om(2D)63*$^{R4}$ does not affect the expression of the gene, but brings about its structural abnormality.

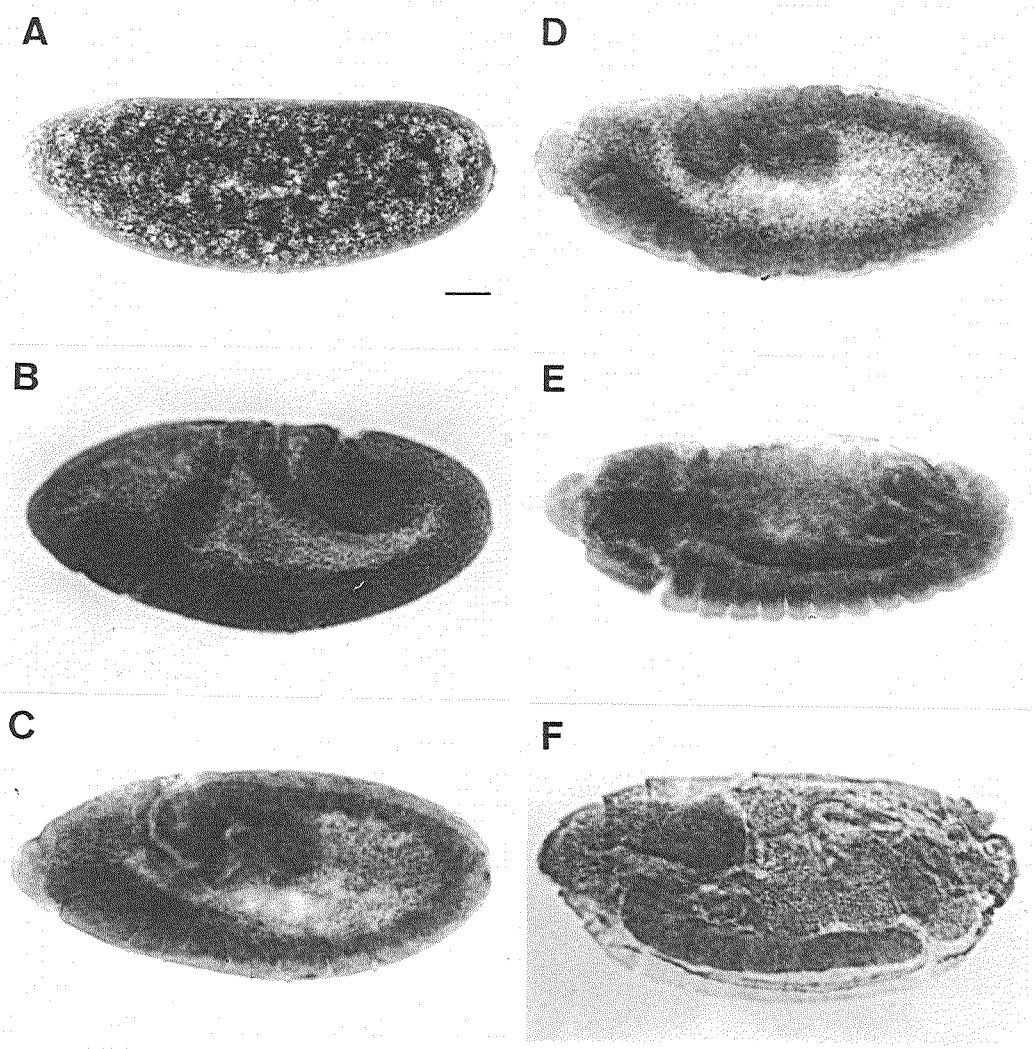



**Fig. 20**. Detection of the *Om(2D)* protein in whole mounts of *ca;px* embryos. Anti-mouse IgG horseradish peroxidase was used for the secondary antibody. The developmental stages were according to Campos-Ortega and Hartenstein (1985). Anterior is *left* and dorsal is *up*. *Bar* is 50 μm. Wholemount preparations were photographed using Nomarski optics. **A**, Stage 4; **B**, Stage 7; **C**, Stage 10; **D**, Stage 11; **E**, Stage 12; **F**, Stage 16.

## Expression of the *Om(2D)* gene in imaginal discs other than eye-antennal discs

The expression of *Om(2D)* transcript was examined in imaginal discs other than eye-antennal discs of third instar larvae of wild type and the mutant. As a result, the *Om(2D)* gene was expressed in the wing discs, haltere discs (Fig. 21A, B) and midgut (Fig. 21C) of both wild type and the mutant. The wing discs showed a characteristic staining pattern in the shape of a hook in its lateral region which is destined to develop the front wing hinge composed of the humeral plate, the proximal costa and the sensilla campaniformia (Bryant 1975). A characteristic staining was also found in the haltere discs in the primordium of the ventral part of the haltere pedicel (Ouweneel and Van der Meer 1973). Staining was also observed in many spots scattered throughout the midgut. Such a staining pattern reminds of the midgut imaginal islands. The results suggest that the *Om(2D)* gene may normally act in differentiation and development of those cells.

Immunocytochemical experiments were performed using the anti-*Om(2D)* antisera in those tissues. Beyond expectation, however, all the antisera could not detect the *Om(2D)* protein in those tissues. Although, the reason for this failure is unknown at present, it is likely that the *Om(2D)* protein is not translated in those tissues, or it is unable to react with the anti-*Om(2D)* antisera owing to its peculiar subcellular localization or to its tissue specific variation in the conformation or structure.

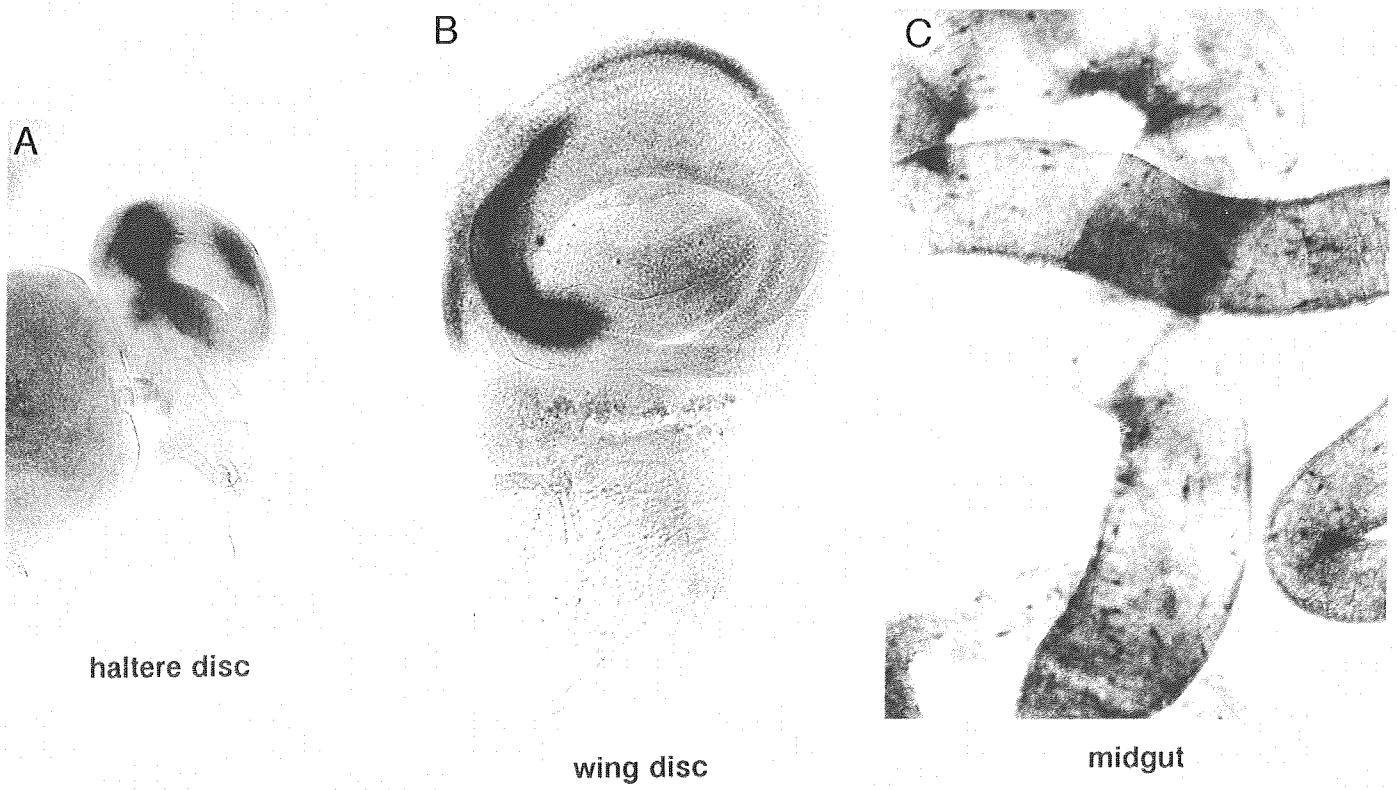



**Fig. 21**. Wholemount in situ hybridization in late third larval haltere (**A**), wing (**B**) imaginal discs, and a midgut (**C**) using *Om(2D)* cDNA probe labeled with digoxigenin.

# DISCUSSION

The amino acid sequence of the *Om(2D)* protein, as deduced from the nucleotide sequence of the cDNA, is novel and characterized by the presence of a $(HX)_n$ PRD repeat motif (Frigerio et al. 1986). The PRD repeat is not only present in a number of Drosophila homeodomain proteins including *Om(1D)* (Tanda and Corces, 1991), but it is also conserved in a number of homologous vertebrate homeodomain proteins as well (Janknecht et al. 1991). Immunoblotting analysis of the embryonic extract showed that the *Om(2D)* protein can be detected in the nuclear fraction as well as in the cytosol with the *Om(2D)* antibodies. Therefore, it is reasonable to suppose that the *Om(2D)* protein may be involved in transcriptional regulation, although further examinations are needed for confirmation of this possibility.

At the third instar stage, in situ hybridization experiments revealed that the *Om(2D)* gene is expressed in the specific cells of the wing and haltere imaginal discs and midgut. The expression pattern is not different in wild type and the mutants, suggesting that the expression of the *Om(2D)* gene in these tissues is brought about by a normal regulatory machinery of the *Om(2D)* gene itself, but not by the *tom* element inserted in the *Om(2D)* locus. These findings suggest that the *Om(2D)* gene may play an important role in development of these cells. However, the immunocytochemical experiment failed to detect the *Om(2D)* protein in these tissues. This might be explained if the characteristics of the *Om(2D)* protein in these cell types are different from that in the eye discs and embryos, so that anti-*Om(2D)* antibodies can not easily react with the epitopes of the *Om(2D)* protein. Alternatively, the *Om(2D)* transcripts might not be

translated in these tissues. To distinguish between these possibilities, further examinations should be done. The wing disc cells which express the *Om(2D)* gene can develop the front wing hinge composed of the humeral plate, the proximal costa and the sensilla campaniformia (Bryant 1975). The haltere disc cells which express the *Om(2D)* gene can develop the ventral part of the haltere pedicel (Ouweneel and Van der Meer 1973). These regions of the two discs may be topographically identical. Although many genes have been reported to act during the wing morphogenesis (Bate and Arias 1993), none of them so far studied show the spatially restricted expression as the *Om(2D)* gene did. The cells which express the *Om(2D)* gene in the midgut may correspond to the midgut imaginal islands. Genes which are specifically expressed in the midgut imaginal islands have never been reported before. Therefore, further investigations of the *Om(2D)* gene function provide a useful system with which to analyze the molecular mechanism that regulates the development of those cell types.

# GENERAL DISCUSSION

Cytological locations of the *tom* element had been determined in *Om* mutants and their progenitor stocks *ca;px* by in situ hybridization experiments to polytene chromosomes using a cloned *tom* element as a probe (Matsubayashi et al. 1992; Shrimpton et al. 1986). In each *Om* mutant so far examined, a hybridization signal is always found at the site corresponding to the genetical locus of the *Om* mutation, suggesting that all *Om* mutations are caused by the insertion of the *tom* element. Furthermore, despite the fact that almost all *Om* mutants originated from the *ca;px* stocks carrying *tom* elements at six cytological sites, *Om* mutants now vary in the number of the *tom* element from 2 to 16, and have several new sites of *tom* insertion other than *Om* loci. Additionally, the number and location of the *tom* element had been changed even between various *ca;px* stocks maintained separately (unpublished data). These not only suggest that the *tom* element may move at a higher frequency than that expected from *Om* emergence, but also indicate that there are many silent insertions of *tom* elements without any visible phenotype. A question then arises as to what determines whether a new *tom* insertion leads to a dominant eye phenotype.

Sequence analysis of *tom* insertion sites suggests that the target sequence of *tom* insertion may be the pentanucleotide T/AATAT (Tanda et al. 1988; Matsubayashi et al. 1991a). However, it is not likely that *tom* insertions occur randomly at the pentanucleotide in the genome. In the *Om* mutation system, where the *tom* element may transpose actively, very few recessive visible mutations have been found on the X chromosome, suggesting that the *tom* element might prefer to insert into regions where no coding regions nor regulatory sequences are present. Furthermore, such non-randomness of *tom*

insertion is also suggested by the following observations: in two *Om(2D)* alleles, all the sites of the *tom* insertions in the *Om(2D)* locus are within a 0.4 kb segment. Likewise, five of the six inserts in the *Om(1D)* locus are present in two clusters, each about 500 bp long and 3 kb apart (Tanda et al. 1989). Moreover, *Om(2D)63*, *Om(1D)9*, and *Om(1A)15E* possess tandem arrays of the *tom* element in their loci, and most *tom* elements so far examined have been found in the 3' region of the *Om* genes (Awasaki et al. 1994; Matsubayashi et al. 1991a; Tanda et al. 1991). These results suggest that the insertion of the *tom* element may be influenced by not only its target sequence preference, but also by some feature of the chromatin structure around the insertion sites. Alternatively, the *tom* element might have to insert into restricted regions of *Om* loci for exerting its mutagenic effect on *Om* genes. The non-randomness or hot spots of transposon insertion have been reported for *copia* element in the *white* gene (Rubin et al. 1982), and for *gypsy* element in the *scute* gene (Campuzano et al. 1985).

I have shown here that ectopic expression of the *Om(2D)* gene induced by the insertion of the *tom* elements was restricted in the region anterior to the morphogenetic furrow of mutant eye discs. That the ectopic expression of *Om* genes is confined only to eye discs was also noted in other *Om* mutants (Tanda and Corces 1991; Awasaki et al. 1994). These observations strongly suggest that a cis-acting regulatory sequence, present in the *tom* element, stimulates expression of *Om* genes when the *tom* element inserts in close proximity to the *Om* genes. Hence, it is predicted that a factor interacting with the regulatory sequence is specifically present anterior to the furrow. If it holds true, this factor would play an important role in determination and differentiation of

ommatidia. The development of the ommatidia may be accomplished by many genes, such as *ato*, *eya*, *rap*, *sca*, *Notch*, *Dl*, and *DER* (Jarman et al. 1993a,b; Bonini et al. 1993; for review see Basler and Hafen 1991), but the interaction between these genes and also the molecular mechanism of the ommatidial development are still unknown. Therefore, the identification of the eye-disc specific factor interacting with the *tom* sequence is worth further consideration.

The putative regulatory sequence present in the *tom* element may be regarded as a tissue specific enhancer based on several criteria. In the *Om(2D)* locus, the *tom* element found in *Om(2D)10a* is in the same direction as that of the *Om(2D)* transcription, but in the case of *Om(2D)63* it is reversed. In addition, the distance between the insertion site of the *tom* element and the transcription start site of the *Om* gene varies from 66 kb in *Om(1A)81* to 10 kb in *Om(1E)59a* (Awasaki et al. 1994; Juni et al. unpublished). Furthermore, the *tom* elements are present in the 5' region of the *Om(1E)* gene in *Om(1E)53*, in the intervening region of the *Om(1A)* gene in *Om(1A)42*, and in the 3' region in the other *Om* mutants. These features are all consistent with an assumption that the *tom* regulatory sequence may be an enhancer. However, there was a gene which had not been activated at all by the enhancer of *tom*; the OAT gene is not stimulated in the *Om(2D)* mutant-eye discs, even though the *tom* element resides in close proximity to the gene. Although the reason why the OAT gene expression could not been affected by the enhancer sequence in the *tom* element is unknown, it is possible that the OAT gene may be governed by a specific regulatory system different from that of *Om* genes, because the OAT gene belongs to housekeeping genes, so the enhancer might be ineffective in stimulating the OAT transcription in eye discs. Alternatively, only *Om* genes

may possess some sequences interacting with the enhancer, and the OAT gene may not. In addition to the OAT gene, a number of genes not affected by the *tom* enhancer may be present in the genome. For example, the *tom* enhancer could not stimulate genes which are inactivated by some gene modification such as methylation and intercalation of heterochromatin and are suppressed by some negative regulator such as a silencer. Consequently, not all genes located near the *tom* element are activated in the eye disc by the *tom* enhancer, and only genes stimulated by the enhancer would be competent to become as an *Om* gene.

When *D. melanogaster* flies transformed with the OAT cDNA under the control of the *hsp70* promoter were treated by heat shock at the third instar stage, elevation of the eye-disc OAT activity did not result in an aberrant eye phenotype. This indicates that there would be many genes which do not affect eye morphogenesis even though they are overexpressed in eye disc by the *tom* enhancer. Conversely, overexpression of the *Om(2D)* gene in the mutant eye disc results in the eye malformation, although its normal function is not required for eye morphogenesis. The *D. melanogaster* gene, *Dfd*, which is normally required for development of the maxillary palp and the rostral membrane, causes deletion of lower half of the eye when ectopically expressed in the eye disc under the control of the *hsp70* promoter (Chadwick et al. 1990). If this gene can be expressed in the eye disc under the influence of the *tom* element, its ectopic expression would lead to an eye malformation such as an *Om* phenotype. Therefore, only genes whose products can influence the eye morphogenesis may be recognized as *Om* genes, irrespectively of their normal function in fly development. Incidentally, the locus of *Dfd* homologue in *D. ananassae* appears

not to be any of *Om* loci (M. Matsuda, personal communication), so it is possible that its chromosomal locus in *D. ananassae* is a region where the *tom* element is difficult to insert or that this gene is insensitive to the tissue-specific effect of the *tom* enhancer.

The *Om(2D)* gene had no effect on imaginal discs other than the eye disc when expressed artificially by heat shock at the third instar stage. Additionally, the *Dfd* gene affects only eye morphology even though it is ectopically expressed in the whole body under the control of *hsp70* promoter (Chadwick et al. 1990). These results suggest that some factors resulting in eye malformation upon interaction with the gene products may be exclusively present in the eye-disc cells. Furthermore, given that the factors were specifically present in the eye disc, they would fundamentally be involved in eye morphogenesis.

While we started the analysis of *Om(2D)* mutation expecting that the *Om(2D)* gene itself may be the eye-disc specific factor involved in eye morphogenesis, the *Om(2D)* gene may not be normally involved in eye morphogenesis because it was not transcribed at all in the wild-type eye disc. However, it is suggested that the factors interacting with the *tom* enhancer or to the *Om* gene products are specifically present in the developing eye imaginal disc, and that these factors would play an important role in cell determination and differentiation during eye development. Therefore, identification of these factors should provide valuable information on the molecular mechanism underlying eye morphogenesis.

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