Members of a Family of Drosophila Putative Odorant-Binding Proteins Are Expressed in Different Subsets of Olfactory Hairs

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Summary

A polymerase chain reaction-based method was used to generate a Drosophila melanogaster antenna1 cDNA library from which head cDNAs were subtracted. We identified five cDNAs that code for antennal proteins containing six cysteines in a conserved pattern shared with known moth antennal proteins, including pheromone-binding proteins. Another cDNA codes for a protein related to vertebrate brain proteins that bind hydrophobic ligands. In all, we describe seven antennal proteins which contain potential signal peptides, suggesting that, like pheromone-binding proteins, they may be secreted in the lumen of olfactory hairs. The expression patterns of these putative odorant-binding proteins define at least four different subsets of olfactory hairs and suggest that the Drosophila olfactory apparatus is functionally segregated.

Introduction

Olfaction, the ability to sense and to discriminate among a large number of airborne molecules, often at minute concentrations, plays a major role in the feeding and mating behaviors of most terrestrial animals. Although the morphology, organization, and complexity of olfactory organs are quite different between insects and vertebrates, several aspects of the physiology appear to be similar (Schneider, 1984; Kaissling, 1986; Snyder et al., 1988; Ronnett and Snyder, 1992). More recently, cDNAs for four proteins that appear to code for abundant, secreted proteins expressed in rat olfactory tissue have been cloned and suggested to be novel OBPs (Dear et al., 1991a, 1991b). Although odorant binding has not been demonstrated for any of them, three have sequence similarity to proteins that bind either chlorinated biphenyls or lipopolysaccharides, whereas a fourth (OBP1) shows sequence similarity to the original OBP. In moths, pheromone-binding proteins (PBPs) are expressed on the antennae of males (Vogt and Riddiford, 1981; Vogt et al., 1988, 1989). When odorant-binding protein (OBP) from rat (Peysner et al., 1988) and a frog homolog (Lee et al., 1987) were cloned, they were found to be related to a family of proteins that bind lipophilic compounds such as retinol, cholesterol, and biliverdin. As a consequence, OBPs are now thought to play the role of carrier, allowing the solubilization and concentration of hydrophobic molecules in the aqueous mucus (Snyder et al., 1988; Ronnett and Snyder, 1992). More recently, cDNAs for four proteins that appear to code for abundant, secreted proteins expressed in rat olfactory tissue have been cloned and suggested to be novel OBPs (Dear et al., 1991a, 1991b). Although odorant binding has not been demonstrated for any of them, three have sequence similarity to proteins that bind either chlorinated biphenyls or lipopolysaccharides, whereas a fourth (OBP1) shows sequence similarity to the original OBP. In moths, pheromone-binding proteins (PBPs) are expressed on the antennae of males (Vogt and Riddiford, 1981; Vogt et al., 1989, 1991a, 1991b) in specialized sensilla that are responsible for the detection of female pheromones (Steinbrecht et al., 1992). PBPs have been shown to bind directly to pheromones in vitro (Vogt et al., 1988) and are present at high concentrations in the sensilla fluid (Vogt and Riddiford, 1981). Thus, they are thought to play a role similar to that of OBPs in vertebrates despite the lack of sequence similarity (Gyorgyi et al., 1988; Raming et al., 1990; Krieger et
al., 1991; Vogt et al., 1991b). Other abundant moth antennal proteins called general odorant-binding proteins (GOBPs) are highly similar in sequence to PBPs, but equally present in both sexes (Vogt et al., 1991a, 1991b). A direct interaction of GOBPs with odorants has not been demonstrated; however, their expression in antennae and their sequence similarity with PBPs have led to the proposal that they may also be OBPs.

Drosophila has a sensitive but relatively simple olfactory system located on the third antennal segment (funiculus) (Siddiqi, 1987; Carlson, 1991). Approximately 500 sensory hairs (sensilla) can be subdivided according to three morphological types: trichoid (long, with a sharp tip), coeloconic (small, cone shaped), and basiconic (club shaped). Sensilla of the three types contain a total of only about 2500 neurons and are distributed in a stereotyped fashion on the funiculus. Almost all funicular sensilla contain multiple pores in their cuticle (Venkatesh and Singh, 1984; Itoh et al., 1991; Stocker et al., 1992), a characteristic that in other insects is always associated with olfactory function (Altner and Prillinger, 1980). Other sensilla that may have an olfactory function can also be found on the maxillary palp (Singh and Nayak, 1985; Ayer and Carlson, 1997). To identify molecules involved in Drosophila olfaction, we have developed a method for the construction of subtracted cDNA libraries from very small amounts of starting material and used it to generate a library containing antennal cDNAs from which

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**Figure 1. An Antennae-Minus-Head cDNA Library**

(A) A PCR-based method for the construction of subtraced cDNA libraries. For details, see Experimental Procedures and Results. SA, avidin.

(B) Analysis of the abundance of three specific cDNAs through successive subtractions. Southern blot analyses were performed loading equal amounts of the cDNAs indicated above the lanes. The probes used are indicated below each panel. Ant., antennae.
head cDNAs have been subtracted. Using this library, we have found 7 clones with several properties that suggest they may be Drosophila OBP's.

Results

Subtraction Cloning of Antennal cDNAs

The construction of subtractive cDNA libraries, in which ubiquitous cDNAs are removed from a collection of cDNAs from the tissue of interest, has proven useful in the identification of genes expressed in a tissue-specific manner (e.g., Wang and Brown, 1991). We have developed a polymerase chain reaction (PCR)-based method for the generation of subtracted cDNA libraries that combines two useful properties (Figure 1A). First, it is designed to use very small amounts of tissue or RNA as starting material; we have routinely used as little as 10 ng of total RNA. Second, the amplified cDNA can be used as either component in multiple rounds of subtractive hybridizations, allowing for the enrichment of cDNAs specifically present or present at higher abundance in only one of the two populations.

The method we used for cDNA synthesis is based on the one previously described by Belyavsky and colleagues (Belyavsky et al., 1989) with modifications designed to cope with two major difficulties. First, the efficiency of the PCR is very dependent on the size of a DNA molecule, such that long cDNAs tend to be out-competed by smaller ones (Lisitsyn et al., 1993). To overcome this limitation, our method leads to the specific amplification of cDNAs representing only the 400-600 nucleotides at the 3' end of mRNAs, regardless of their size. RNA is partially base hydrolyzed to an average size of 500 nucleotides. After reverse transcription with an oligo(dT) primer, cDNAs of 400-600 nucleotides are purified on a polyacrylamide gel. Second, the earlier method calls for the G tailing of the first strand cDNA, then allowing PCR amplification of the cDNAs with both an oligo(dT) primer (complementary to the natural poly(A) tail) and an oligo(dC) primer (complementary to the G tail). However, we have found that conditions compatible with annealing of the oligo(dT) primer tend to allow nonspecific priming by the oligo(dC) primer as seen by the amplification of nontailed products (data not shown). This is probably due to large differences in the melting temperatures of the two homopolymers and results in a lack of reproducibility in the sequence representation in the amplified cDNA population. To solve that problem, we designed an oligo(dT) primer with a string of deoxycytidines at its 5' end. This allows the amplification of the G-tailed first strand cDNA with a single oligo(dC) primer under stringent hybridization conditions, during which nonspecific priming is minimized. The reproducible representation in the amplified cDNA of sequences from the 3' ends of a variety of large, as well as small, mRNAs was confirmed by hybridizing Southern blots with specific probes. We found less than a 2-fold variation between duplicate samples that were processed in parallel (data not shown).

We used this method to amplify cDNAs made from total RNA extracted from a dozen hand-dissected antennae (10-20 ng) and from an equivalent amount of RNA extracted from heads without antennae. The amplified cDNAs were then used in two symmetrical subtractive hybridizations. One of the cDNAs (tracer) was radioactively labeled and hybridized to a 10- to 20-fold excess of the other cDNA (driver), which contained covalently linked biotin residues. The driver and hybrid cDNAs were then removed by avidin chromatography. The resulting subtracted cDNAs are designated antennae-minus-heads (antennal cDNA tracer, head cDNA driver) or heads-minus-antennae (head cDNA tracer, antennal cDNA driver). Since we expected the first round of hybridization to subtract preferentially abundant sequences (Wang and Brown, 1991), we proceeded to a second and third round using antennae-minus-heads as tracer and an equal mixture of heads and heads-minus-antennae as driver. The cDNAs from one and two additional rounds of subtraction are designated (antennae-minus-heads)² and (antennae-minus-heads)³ cDNAs, respectively. After each round of hybridization, the efficiency of the subtraction was estimated from the percentage of radioactively labeled tracer that did not bind to the avidin beads. Whereas the first hybridization was greater than 90% effective in removing the tracer, hybridization to the driver decreased to 85% for the second round and to less than 50% for the third round. This suggested that most of the antennal sequences present after two rounds of subtractive hybridization were absent or rare in head cDNA.

To confirm this interpretation, we measured by Southern blotting the representation of several well-characterized mRNAs in the various cDNAs (Figure 1B). The level of the abundant and ubiquitous ribosomal protein 49 message (O'Connell and Rosbash, 1984), equal in antennal and head cDNA, decreased by a factor of 25 after two rounds of subtraction. The message for the much less abundant, neuronal specific protein embryonic lethal abnormal visual system (elav) (Campos et al., 1987) increased 2- to 3-fold in the first round-subtracted cDNAs. The opsin message, very abundant in the head and absent from antennae (Zuker et al., 1985), increased 2- to 3-fold in the heads-minus-antennae cDNA (data not shown). We find that abundant sequences such as opsin (which is absent from antennae) and several abundant, antennal-specific cDNAs (data not shown) are only slightly enriched in heads-minus-antennae and antennae-minus-heads, respectively, even though they are absent from the corresponding driver. This could be due to the fact that abundant sequences tend to form networks which are more likely to be lost through nonspecific
Figure 2. Five Drosophila Members of a PBP-Related Family of Proteins

(A) Multiple sequence alignment of the Drosophila and moth members of the PBP-related family of antenna1 proteins. The PILEUP program was used, except for the final alignment of PBPRP-4 (near cysteine residue 1), which was done manually. For PBPRP-4, the 22 amino acids between positions 40 (H) and 41 (V) are written on the next line. Two known moth sequences, Antheraea polyphemus PBP and Antheraea pernyi GOBPI, are left out of this comparison because they are too similar to A. pernyi PBP and M. sexta COBPI, respectively. Cysteines are boxed. Residues in common between three or more members of the family are shaded; when ~o choices are possible, only one is shown. Putative signal peptides were defined as hydrophobic regions in a Kyte-Doolittle profile (Kyte and Doolittle, 1982) and obey criteria for eukaryotic signal peptide sequences (van Heijne, 1985); they are underlined. Sequences are from (Vogt et al., 1991b) or references therein. PBPApe, A. pernyi PBP; PBPMs, M. sexta PBP; GOBP1Ms, M. sexta GOBPI; GOBP2Ms, M. sexta GOBP2.

(B) Plot of the overall sequence similarity in a multiple sequence alignment such as the one shown in (A), except that PBPRP-4 was left out. The PLOTSIMILARITY program was used. The position of the cysteines in the multiple sequence alignment is indicated, as well as the minimal and maximal distances between any two successive cysteines.

Interactions with the driver. In contrast, sequences corresponding to the IP3 receptor, which has been shown to be encoded by a relatively rare mRNA 5 times more abundant in antennae than heads (Hasan and Roshbash, 1992), were further enriched by a factor of 25 after two subtractions (Figure 1B). In summary, subtractive hybridizations seem to be having two different and sometimes contrary effects on the relative abundance of individual cDNAs. First, rare sequences are favored at the expense of abundant ones. Second, sequences present in the tracer but not in the driver are enriched after each round of hybridization, as exemplified by the case of the IP3 receptor.

Five Antennae Proteins Have Sequences Similar to Those of Moth PBPs

After cloning the (antennae-minus-heads)cDNA into a plasmid vector, we picked 8 insert-containing clones at random and analyzed their expression by Northern blotting using RNA extracted from bodies (without heads, wings, or legs), heads (without antennae), and appendages (legs, antennae, and wings; data not shown). We found that 5 of the 8 clones show either specific or enriched expression in appendages. Partial sequence data gave clues to the identity of 2 of the clones, a2 and a5 (see below). In the case of a2, although no obvious match could be found with known sequences in computerized searches, we were struck by the fact that it codes for a small protein (148 amino acids) containing a putative signal peptide and six cysteine residues, as do several moth antennal proteins, PBPs and GOBPs (Raming et al., 1990; Vogt et al., 1991b). The spacing of the cysteine residues in A2 is also very similar to the spacing found in the moth proteins: (X41-Cys-X2-A-Cys-X3-Cys-X3-Cys-X42, in which X stands for any p amino acids) for the PBPs and GOBPs, and (X41-Cys-X2-A-Cys-X3-Cys-X2-Cys-X3) for A2. In a multiple sequence alignment, other similarities can be found between A2 and the moth PBPs and GOBPs (see below). This led us to conclude that A2 is structurally related to this family of moth antennal proteins implicated in olfaction. Although A2 is likely one of the Drosophila counterparts of moth GOBPs (Vogt et al., 1991b), the spacing of the cysteine residues in A2 is also very similar to the spacing found in the moth proteins: (X41-Cys-X2-A-Cys-X2-Cys-X3-Cys-X3-Cys-X42, in which X stands for any p amino acids) for the PBPs and GOBPs, and (X41-Cys-X2-A-Cys-X2-Cys-X3-Cys-X3-Cys-X3) for A2. In a multiple sequence alignment, other similarities can be found between A2 and the moth PBPs and GOBPs (see below). This led us to conclude that A2 is structurally related to this family of moth antennal proteins implicated in olfaction. Although A2 is likely one of the Drosophila counterparts of moth GOBPs (Vogt et al., 1991b), we prefer the name PBPRPs for PBP-related proteins because it makes no assumption as to their properties. a2 has thus been renamed pbprp-1.

PBPRP-1 is much less similar to any of the moth proteins than they are to each other (Figure 2A). In particular, the introduction of several small deletions and insertions is required to optimize the alignment to the moth proteins that are perfectly collinear between the cysteines (Vogt et al., 1991b). The fact that the moth Manduca sexta has at least three related proteins (GOB1, GOB2, and PB; Vogt et al., 1991a, 1991b), coupled with the fact that pbprp-1 is only expressed in a fraction of olfactory hairs (see below), suggested the existence of other Drosophila pbprp clones. However, the low level of sequence similarity of PBPRP-1 to its moth counterparts and the absence
A Family of Drosophila Olfactory Hair Proteins

of any cross-hybridizing band on low stringency Southern blots (data not shown) suggested that strategies based on sequence similarity might not succeed in finding other members of this family in Drosophila. As an alternative, we reasoned that as the moth genes and pbprp-7 are both expressed at relatively high levels in antennae and since pbprp-7 was discovered after sampling only 8 random clones, proteins of this family might comprise a large proportion of the clones in our subtractive library. Sixty-two clones were picked at random, and their inserts were analyzed on Southern blots using probes made from antenna1 and head cDNAs. Using this assay, 17 clones were found to have a higher level of expression in antennae than in heads. Cross-hybridization showed that these clones correspond to 11 different sequences, and partial sequence data indicated that 2 clones (pbprp-2 and pbprp-3) code for members of the PBPRP family. Partial sequencing of 33 clones, which were insufficiently abundant to be detected in the differential hybridization, showed that 2 more (pbprp-4 and pbprp-5) are part of the same family, bringing the total number to 5.

In addition to the sequence similarity with moth PBPs and GOBPs, all five members of the family have putative signal sequences at their amino termini, suggesting that, like their moth relatives (Vogt et al., 1991a; Steinbrecht et al., 1992), they are secreted in the lumen of olfactory hairs (Figure 2A). A multiple sequence alignment shows that there is much more sequence divergence among the Drosophila members of this family of proteins than is the case among their moth counterparts. In particular, although all the moth proteins show identical spacing between the cysteines, each of the Drosophila PBPRPs has its own unique pattern except for PBPRP-2 and PBPRP-5. The higher degree of similarity existing between PBPRP-2 and PBPRP-5 on one hand and PBPRP-1 and PBPRP-3 on the other may correspond to subfamilies of OBPs. A graphic representation of the similarity of all the proteins in the multiple sequence alignment (Figure 2B) shows that, in addition to the hydrophobic leader peptide, three regions of the proteins, near cysteine 1, between cysteines 2 and 3, and near cysteines 4, 5, and 6, are more conserved and may thus take part in a structurally important motif.

The low level of sequence conservation among Drosophila pbprp clones was confirmed by the fact that low stringency genomic Southern blots with any of the pbprp clones gave no hint of cross-hybridizing genes (data not shown), making it impossible to estimate directly the total number of pbprp clones in Drosophila.

Members of the Drosophila PBP-Related Family Are Expressed in Different Subsets of Sensilla

Northern blot analysis was carried out using RNAs extracted from bodies, heads, and appendages (see

Figure 3. Northern Blot Analysis of Putative OBPs

Five micrograms of total RNA from appendages, bodies, and heads was loaded in each lane as indicated. Probes are indicated under each panel. Number of counts and hybridization times were normalized for filters in same panel (except for rp49 in [B]). (A) PBP-related clones pbprp-1 to pbprp-5. (B) Two other antennal clones, a5 and a10 (pbprp-1 and rp49 are included for comparison).
A Family of Drosophila Olfactory Hair Proteins

Figure 3A. The members of this family are expressed at different levels that are consistent with the initial Southern blot screen. pbprp-2 is expressed at the highest level, mostly in appendages but also in heads. The other four cDNAs, pbprp-1, pbprp-3, pbprp-4, and pbprp-5, only show detectable expression in appendages, with pbprp-3 and pbprp-4 showing the highest and lowest levels of expression, respectively.

To delineate better the expression pattern of these 5 clones, we performed in situ hybridizations on frozen sections of adult Drosophila heads. Except for pbprp-2 (see below), and in agreement with the Northern blot data, no staining could be detected in any part of the head other than the third antennal segment (data not shown). It remains possible that one or more of these genes is expressed in small numbers of cells in other parts of the body. The staining patterns for each of the pbprp genes on the funiculus suggest expression in various subsets of sensilla (Figure 4A). We found that the fraction of sensilla in which a given mRNA is present

Figure 4. Differential Antennal Expression Patterns of Putative OBPs

(A) Horizontal sections of heads at approximately the same level were hybridized to probes made from all 5 PBP-related clones. In these sections, only the third segment of the antennae (the most distal from the head) is visible. For each of the pbprp clones, a view of the right antenna is shown. Note that the number of cells stained with each probe in these sections is not representative of the total number of stained cells in the third antennal segment. Ant, anterior; Med, medial; Post, posterior. In the lower right-hand corner, a horizontal section of a whole head (hybridized with an a10 probe) is shown for orientation. A, antenna; E, eye.

(B) Sagittal sections of heads were hybridized with a5 and a10 probes. Views of the right antenna are shown in each case. S, sacculus.

(C) Schematic frontal view of the right antenna. Area 1 corresponds approximately to expression of pbprp-1 and pbprp-3. Similarly, pbprp-5 expression is approximated by area 2. A horizontal section is pictured to illustrate the three surfaces of the antenna: Ant, anterior; Post, posterior; Med, medial. Note that this horizontal section is upside down compared with the ones shown in (A).
Figure 5. *pbprp-2* Is Expressed in Taste Organs in the Head

Sagittal sections of the head at the level of the proboscis were hybridized with a *pbprp-2* probe. (A and B) Two successive sections through the proboscis show the ventral cybarial sensory organ at the level of the sensory hairs (A) and at that of the support cells (B), which appear to be the site of *pbprp-2* expression (see text). OE, oesophagus; VCSO, ventral cybarial sense organ.

(C) Taste bristles at the tip of the proboscis. TB, taste bristles; N, neuron; SC, sheath cells.
corresponds generally to the relative expression levels detected by Northern blotting. pbprp-2, the most highly expressed gene of the family, also exhibits the most widespread expression, corresponding to regions of the antennae containing sensilla of all three types, basiconic, coeloconic, and trichoid (Figure 4A). However, pbprp-2 expression does not seem to occur in all sensilla (data not shown). In addition to its antennal expression and consistent with the Northern blot analysis, pbprp-2 mRNA is also detected in the maxillary palps (data not shown) and in cells at the bases of the taste hairs on the proboscis and internal taste organs of the head (Figure 5; see Stocker and Schorderet, 1981). In the case of the taste bristles on the proboscis, the larger size of the sensilla and a better tissue preservation allow the resolution of the two types of cells present (Figure 5C). It appears that, as has been shown for PBP (Steinbrecht et al., 1992), pbprp-2 is expressed in the sheath cells close to the surface of the cuticle and not in the more deeply located neurons.

pbprp-1 and pbprp-3 mRNAs are observed in ventrolateral regions mostly on the anterior surface of the third antennal segment (Figure 4A; Figure 4C, area 1); those regions contain almost exclusively trichoid sensilla (Venkatesh and Singh, 1984). Although the resolution of this technique does not allow us to determine whether the two genes are coexpressed in the same hairs, neither of them is expressed in all regions containing trichoid hairs. Direct counting of staining cells in successive sections suggests that about 80 cells, corresponding to close to half the number of trichoid sensilla (150) present on the funiculus (Venkatesh and Singh, 1984), express each of the two genes.

Sensilla showing pbprp-5 expression are restricted to a different area mostly on the medial and posterior surface of the antennae with little or no overlap with pbprp-1 and pbprp-3 expression patterns (Figure 4A; Figure 4C, area 2). This expression pattern correlates well with the distribution of basiconic sensilla (Venkatesh and Singh, 1984). pbprp-4 mRNA is only observed in a small number of hairs scattered over the surface of the funiculus, consistent with the fact that it has a lower level of expression than any other gene in this family. Note that a single section is shown for each probe in Figure 4A and that the number of staining sensilla may underrepresent (pbprp-1 and pbprp-3) or overrepresent (pbprp-4) the number of staining sensilla on the whole funiculus. Because of the male specific expression patterns observed for moth PBPs, expression of each Drosophila pbprp was examined by in situ hybridization on sections from male as well as female animals; no difference was observed.

Two Other Antennal Proteins Are Expressed in Different Subsets of Olfactory Hairs and Have Putative Signal Peptides at Their Amino Termini

Since members of the multigene family are expressed in different sensilla, we considered the possibility that this might also be true for antennal-specific cDNAs in our subtracted library which are not related to PBP. Four additional clones of this type were analyzed by in situ hybridization. The 2 most abundant ones, A5 and A10, are shown in Figure 6. A5 and A10 Have Candidate Signal Peptides at Their Amino-Terminal Ends (A) A5 is similar to a family of proteins from yeast, nematode, rat, and bovine. The sequences of the five members of this family were aligned using the PILEUP program in the GCG70 sequence package. Shaded residues are those in common between two or more members of the family. The putative signal peptides of A5 and the O. volvulus antigen were defined as hydrophobic regions in a Kyte-Doolittle profile (Kyte and Doolittle, 1982), obey criteria for eukaryotic signal peptide sequences (von Heijne, 1985), and are underlined. To maximize the sequence similarity, a T was deleted at position 453 of the published sequence (Lobes et al., 1990), changing the frame after Tyr-134 (at position 171 of the alignment shown). B. p21, bovine brain p21 protein (Schoen-}

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and a10 (Figure 3B), have detectable and distinctive expression patterns (Figure 4B).

The expression of a5 is restricted to cells at the bases of a few scattered sensilla on the posterior surface of the antenna (Figure 4B). The distribution of these a5-positive cells suggests that they may be part of coeloconic sensilla. This expression is confirmed by the lack of effect of the lz mutation, which selectively removes basiconic sensilla (Stocker and Gendre, 1989), on the a5 staining pattern (data not shown). The expression of a10 shows the most spatially restricted pattern of the seven we have characterized (Figure 4B). The a10 probe hybridizes primarily to cells within the largest cavity of the sacculus. The sacculus is a sac-like indentation of the posterior surface of the antennae, and its largest cavity has been shown to contain several types of coeloconic sensilla (Venkatesh and Singh, 1984; ltoh et al., 1991). In addition, a10 is expressed in a few cells that lie outside of the sacculus on the anterior and posterior surfaces of the antennae, in sensilla that are probably also coeloconic (Venkatesh and Singh, 1984).

As mentioned above, no similarity exists between the sequences of A5 and A10 and those of the PBP-related family of genes described here. However, the presence in their open reading frames of candidate signal peptides (Figures 6A and 6B) (von Heijne, 1982, 1985) and the absence of any potential transmembrane domain suggest that they too may be secreted in the lumen of the sensilla. In addition, we found significant similarities between A5 and sequences in the databases that may give clues to its function.

The A5 Protein Is Highly Similar Both to Vertebrate Brain Proteins That Bind Hydrophobic Ligands and to Yeast and Nematode Proteins

The open reading frame present in the a5 clone is highly similar to members of a previously described group of proteins (Figure 6A), including two vertebrate brain proteins and one yeast protein (TFS1), the latter implicated in the ras/cdc25 signal transduction pathway (Tripp et al., 1989; Robinson and Tatchell, 1991). Both vertebrate proteins have been shown to bind with some specificity to hydrophobic ligands. One is a rat 23 kd protein that binds specifically to the correct stereoisomer of morphine, levorphanol (Grandy et al., 1990). The other, a bovine brain 21 kd protein, binds to phospholipids with a preference for phosphatidyl-ethanolamine (Schoenfield et al., 1987). In addition, A5 also has sequence similarity to a major antigen expressed by the nematode Onchocerca volvulus, the etiologic agent of onchocerciasis in humans (Lobos et al., 1990). A multiple alignment shows that the O. volvulus protein is also part of the same group of proteins, although in this case optimal alignment requires the introduction of an arbitrary frameshift in the published sequence (see Figure 6A, legend). Although neither the rat brain 23 kd protein nor the yeast TFS1 seems to have a signal peptide (as determined by their nucleotide sequence), both the A5 and O. volvulus open reading frames start with stretches of highly hydrophobic amino acids. In the case of the O. volvulus antigen, the secreted nature of this protein has been directly demonstrated by immunocytochemistry (Lobos et al., 1990).

To explore the possibility that any of the cDNAs described above correspond to known mutations in olfaction-related genes, we mapped their locations on the cytological genetic map of larval polytene salivary gland chromosomes. pbprp-1, a10, pbprp-3, and pbprp-4 map on chromosome III at positions 69D, 73F, 63C/D, and 64D, respectively. a5 and pbprp-5 map to 22A/B and 28B, respectively, on chromosome II. pbprp-2 is at 19D on the X-chromosome. No relevant mutation has been mapped to any of these locations.

Discussion

Drosophila melanogaster olfaction has been studied from the genetic (Ayyub et al., 1990; Carlson, 1991) and behavioral (Tompkins et al., 1980; Venard and Jallon, 1980; Gailey et al., 1986; Ferveur et al., 1989) points of view; however, there have been relatively few detailed molecular studies. Cloning and characterization of previously characterized olfactory mutants are ongoing (Hasan, 1990; Carlson, 1991), and recently, the Droso phila IP3 receptor gene has been cloned and shown to have enhanced expression in the antennae (Hasan and Rosbash, 1992; Yoshikawa et al., 1992). The enhancer trap method allows for the generation of mutants in genes screened for their expression in antennae, and this approach is being actively pursued to identify new olfactory genes (Riesgo-Escovar et al., 1992; Vijayraghavan et al., 1992).

As an alternative method to identify genes involved in olfaction in Drosophila, we have generated a subtracted cDNA library enriched for clones expressed at higher levels in antennae than in the rest of the head. In a preliminary characterization of this library, we find that approximately half of the clones (7 of 12; data not shown) may be expressed specifically or preferentially in antennae. These results validate the method described here and suggest that it should be useful in other situations in which the amount of available tissue is a limiting factor.

PBPRPs from Drosophila and Moths Contain Six Cysteines in a Conserved Distribution

We have described a group of Drosophila proteins related in sequence to moth PBPs and COBPs. The greater degree of sequence variation displayed by the Drosophila proteins than by their moth counterparts allows for a better analysis of the conserved features of this family than was previously possible (Raming et al., 1990; Vogt et al., 1991b). In particular, although the moth proteins are almost perfectly collinear, the alignment of the Drosophila members requires the inclusion of several small deletions or inser-
tions. The most striking feature shared by all these proteins, already noted in the case of the moth proteins (Kaming et al., 1990; Vogt et al., 1991b), is the presence of six cysteines. Although the intercysteine spacing is generally conserved among all members of this family, the intervals between cysteines 1 and 2, 3 and 4, and 4 and 5 show a limited amount of variation (27-37, 33-43, and 9-11 residues, respectively), whereas those between cysteines 2-3 and 5-6 are strictly conserved (4 and 9 residues, respectively; Figure 2A). This suggests that some domains of the proteins may be more critical than others to a common secondary structure. Indeed, when the overall similarity of the nine proteins (Drosophila and moths combined) is graphically displayed, three domains appear to be more highly conserved (near cysteine 1, between cysteines 2 and 3, and between cysteines 4, 5, and 6; Figure 2B). This general pattern of conservation in the vicinity of the cysteine residues suggests that all the proteins in this group have a similar structure, in which the cysteines play a critical role. A good candidate for such a role would be the establishment of intramolecular disulfide bonds (Raming et al., 1990), although the apparent requirement for a strict spacing between cysteine residues is more reminiscent of metal-binding domains (O’Halloran, 1993). Interestingly, three vertebrate OBPs (rat OBP, OBPα, and frog BC) and a fourth protein secreted by salivary glands and possibly involved in taste are part of a group of carrier proteins that share a general structure, unrelated to that of PBPRPs, in which intramolecular disulfide bonds play a critical role (Lee et al., 1987; Pevsner et al., 1988; Dear et al., 1991b; Schmale et al., 1990).

We have found 5 pbprp clones through random sampling of a small number of clones (62) in our subtracted antennal library. Three of these clones were found only once and are present at frequencies of approximately one in a thousand (data not shown) in this library, suggesting that we have analyzed only a small fraction of all such cDNAs. Extrapolating from our results leads to the estimate that there should be at least 15 such clones in our library (p < 0.05; data not shown).

The fact that only three PBPRPs have so far been found in any given moth may be due to such studies having been initiated through the biochemical purification of proteins, which would greatly bias the search toward the most abundant molecules. This bias may also explain the relatively small amount of sequence variation observed in the moth proteins compared with Drosophila PBPRPs.

**Varied Expression Patterns of the Drosophila PBPRPs Are Consistent with a Function in Olfaction**

The moth PBPs have been implicated in olfaction by their specific expression in pheromone-sensing hairs and by the fact that they bind pheromone. We have now shown that the Drosophila pbprp clones are expressed in hairs which vary in morphology, location, and even primary sensory function, but are all consistent with the chemosensory detection of volatile molecules. Except for pbprp-2, expression of all the pbprp clones appears to be limited to cells on the surface of the third antennal segment, which is almost exclusively devoted to olfaction, although it may also contain some contact chemoreceptors (Stockier et al., 1992). pbprp-2 is also expressed in regions of the second antennal segment, on the maxillary palps, and in taste hairs in the head, both on the proboscis and in the internal taste organs. Expression in the maxillary palps is observed at the base of sensilla, which may correspond to olfactory hairs present in this second antenna1 segment (data not shown; Ayer and Carlson, 1992). Although the function of the pbprp-2-expressing cells on the second antennal segment is uncertain, blowfly taste organs homologous to the ones showing pbprp-2 expression have been shown to be sensitive to hydrophobic molecules in two ways. First, behavioral studies have shown that, after ablation of all known olfactory organs, the presence of even low concentrations of hydrophobic molecules in a mixture whose taste is otherwise attractive to the fly will either prevent proboscis extension or promote regurgitation (Dethier, 1955). Second, electrophysiological recordings have shown that concentrated vapors of hydrophobic substances will provoke specific responses by some taste hair neurons (Dethier, 1972). The sensitivity of hairs primarily involved in taste to hydrophobic volatile molecules has been suggested to be important in preventing insects from ingesting food contaminated with toxic substances. In summary, the expression of the Drosophila PBPRPs, although more widespread and diversified than that of the moth PBPs, is confined to chemosensory organs and is consistent with a function in olfaction or closely related chemosensory processes.

**Spatial Segregation of the Olfactory Apparatus Is Revealed by the Expression Patterns of Putative OBPs**

It has been pointed out that, unlike the situation in vertebrates in which all olfactory neurons bathe in a common aqueous medium (mucus), the segregation of insect olfactory neurons in sensilla affords the possibility of differentiation of the sensillar fluid as exemplified by the restriction of PBPs to male-specific sensilla (Vogt et al., 1991a; Steinbrecht et al., 1992). Our data provide the first evidence that sensilla present in animals of both sexes also differ in the composition of their sensillar proteins. First, the presence of putative signal peptides and the overall sequence similarity suggest that, like PBPs, PBPRPs are secreted in the sensillar fluid. Since they also have potential putative signal peptides, this may also be the case for A5 and A10. Second, our analysis of the spatial expression patterns of each of these proteins reveals a surprising diversity in expression patterns, both for the five PBPRPs and for the other two putative sensillar proteins. Although pbprp-2 expression is widespread and
probably covers sensilla of all three morphological types, it seems not to be expressed in some sensilla (data not shown). At least four different, more restricted and partially overlapping expression patterns are displayed by the other 6 clones. The expression patterns of pbprp-1 and pbprp-3 on the one hand and pbprp-5 on the other are confined to regions containing trichoid or basiconic sensilla, respectively (Figure 4C, areas 1 and 2). In both cases, however, only part of the area containing sensilla of the corresponding type is stained. pbprp-4 and a5 are each expressed in small numbers of cells scattered over a large fraction of the antennal surface. Although their expression patterns may overlap with each other and with those of pbprp-1, pbprp-3, and pbprp-5, they are also expressed in regions from which the previous three genes are excluded and, for as in particular, containing mostly coeloconic sensilla. a10 expression is mainly confined to the sacculus. Since we have only sampled a small number of clones in our library, it seems likely that many other sensilla proteins exist which display other expression patterns. Finally, the variety in sequence and the similarity to proteins (PBP other olfactory-specific protein, strongly suggesting that these sensilla proteins bind to different odorants and that their presence in a particular sensillary fluid may be related to the odorant specificity of that hair (Vogt et al., 1991a). The spatially segregated expression of putative OBPs may therefore correspond to a functional segregation of the peripheral olfactory apparatus.

A correlation between the restricted expression patterns of putative Drosophila OBPs and a functional segregation is consistent with previous morphological and physiological studies. In insects as well as vertebrates, the first synaptic relay of olfactory information occurs in anatomically separate structures in the antennal lobe, the glomeruli. In both cases, specific odorants activate one or a small number of glomeruli, suggesting the existence of a topographic map of odors in the brain (Lancet et al., 1982; Rodrigues, 1980; Hansson et al., 1992; Shepherd, 1992). In addition, a relationship between specific glomeruli in the antennal lobe and groups of sensilla of similar morphology and location on the antenna has been suggested by retrograde labeling studies that correlate well with our findings (Stocker et al., 1983). In particular, retrograde filling of trichoids in an area showing pbprp-1 and pbprp-3 expression has shown that all neurons project to the same two glomeruli (VA1 and DA1; Stocker et al., 1983). Similarly, all neurons from basiconic sensilla in an area expressing pbprp-5 have projections on the V, VM1, and DM1 glomeruli. Finally, single hair recordings (Siddiqi, 1987), as well as electroantennograms from different regions of the antennae (Ayer and Carlson, 1992), have shown a relationship between position on the antennal surface and odorant specificity.

The organization of the Drosophila antennae can be compared with that of the vertebrate olfactory epithelium, recently revealed by the analysis of the expression patterns of individual odorant receptors (Nef et al., 1992; Ngai et al., 1993a, 1993b; Ressler et al., 1993; Vassar et al., 1993). In all cases, only one or a small number of olfactory receptors is expressed in each olfactory neuron. In catfish, neurons expressing any given receptor appear to be randomly distributed throughout the olfactory epithelium (Ngai et al., 1993a). In rats and mice, the expression of each receptor is limited to cells restricted to one of three broad bilaterally symmetrical regions, within which it also appears random (Nef et al., 1992; Ressler et al., 1993; Vassar et al., 1993). This segregation is probably maintained in the projection to the olfactory bulb and may correspond to an initial sorting of the olfactory information. The Drosophila olfactory apparatus appears to be simpler than that of vertebrates in the number of smells which can be discriminated (Sidiki, 1987), in the number of olfactory neurons (Carlson, 1991), and in the number of glomeruli (Stocker et al., 1983). Therefore, in the absence of cloned odorant receptors from Drosophila (or any other invertebrate), our finding that at least four different subsets of olfactory hairs can be defined on the basis of their putative OBPs may indicate a greater importance of the organization of the peripheral olfactory system in insects than in vertebrates.

In vertebrates, one olfactory receptor has been shown to respond to odorants in the absence of any other olfactory-specific protein, strongly suggesting that OBPs are not absolutely required (Raming et al., 1993). It is likely that a similar situation is found in insects (Stengl et al., 1992). However, the existence in both cases of a variety of demonstrated or potential OBPs in the aqueous medium bathing olfactory neurons suggests that these proteins play an important role in olfaction. OBPs could increase the solubility of hydrophobic odorants in the aqueous environment bathing olfactory neurons and thus result in an increased sensitivity (Snyder et al., 1988; Vogt et al., 1991a). In an extension of this model, the OBP-odorant complex would be recognized as a whole by the membrane-bound receptor. Alternatively, binding to an OBP could be one of several processes involved in the odorant inactivation necessary for the proper temporal resolution of olfaction (Kaisling, 1986; Stengl et al., 1992). Further studies using reverse genetics and site-directed mutagenesis should allow testing of the role these putative Drosophila OBPs play in olfaction.

Experimental Procedures

Construction of a Subtracted (Antennae-Minus-Heads) cDNA Library using PCR

RNA was extracted using standard protocols (Maniatis et al., 1982) from ten manually dissected antennae and a few heads (without antennae). The amount of RNA was estimated using quantitative PCR with 449 primers (Hasan and Rosbash, 1992) and approximately 10 ng was used in each case. Corresponding
to either ten antennae or one-twentieth of a head. Each RNA was partially base hydrolyzed in the presence of 40 mM NaHCO₃, 60 mM Na₂CO₃ (pH 10.2) for 10 min at 60°C. Hydrolysis was stopped by the addition of an one-tenth volume of 3 M sodium acetate (pH 6.5), 5% acetic acid, and the RNA was ethanol precipitated. Reverse transcription was carried out with AMV reverse transcriptase (Life Sciences) in the presence of 20 pmol of a poly(T) oligonucleotide ([C₅₋₇]AAGCTT[CG]₃) for 10 min at room temperature and 45 min at 37°C. The sample was denatured in formamide sample buffer and loaded on a 5% polyacrylamide, 7 M urea gel. The cDNA fractions corresponding to 400-600 nucleotides were eluted from the gel. Size-fractionated cDNA was G tailed with 4 units of terminal transferase (Pharmacia) in the presence of 10 mM GTP for 30 min at 37°C. PCR was carried out in 100 μl in the presence of 3.75% dimethyl sulfoxide and 20 pmol of a poly(C) oligonucleotide (ATCAGGTCACCTCCA[C]₇) to avoid mispriming, the mixtures were kept on ice until the addition of Taq polymerase and were then transferred immediately to a thermal cycle that had been preheated to 80°C. The PCR program (for a GeneAmp 5600 using thin-walled 200 μl tubes) was 1 min at 95°C; 30 at 95°C, 30 at 60°C, 1 min at 72°C for 3 cycles; followed by 30 s at 95°C, 1.5 min at 72°C for 27 cycles; and 4 min at 72°C. Special precautions were taken to avoid contamination of the samples; whenever possible, manipulations were carried out in a laminar flow hood, and controls without RNA or cDNA were incorporated at the appropriate steps to detect any contaminant. In addition, the optimal number of cycles was determined in preliminary experiments to keep the amplification in its exponential phase. To generate driver cDNA, ten PCR reactions of 100 μl each were performed under conditions identical to the cDNAs described above with XATCAGTCAGCTCTCA[G]₃ as the biotinylated residue; the oligonucleotide was purchased from Midland and gel purified to eliminate contamination with nonbiotinylated oligo as primer. For generation of the tracer, a 100 μl reaction was performed in the presence of 10 μCi of [α³²P]dCTP. A total of 1-2 μg of tracer DNA and 10-20 μg of driver DNA were generated, mixed, ethanol precipitated, and purified on a Sefi-15L Ultracruz-Prime inc. column. After ethanol precipitation, the mixture was resuspended in 10 μl of water containing 10 μg of each of the two primers ([CG]₅₋₇AAGCTT[CG]₃) and ATCAGGTCACCTCCA[C]₇. The presence of a molar excess of these oligonucleotides prevents hybridization of the cDNAs through terminal sequences. Subtractive hybridization was performed essentially as described (Straus and Ausubel, 1990). The hybridization mixture was incubated at 100°C for 1 min, frozen, resuspended in 2.75 μl of H₂O, 1 μl of 10× EE (100 mM N-[2-hydroxyethyl]piperazine-N-3-propanesulfonic acid [pH 8.25], 10 mM EDTA), and 1.25 μl of 4 M NaCl, covered with mineral oil to prevent evaporation, and incubated at 95°C for 5 min and then at 65°C for 20 hr. The hybridization was stopped by dilution in 100 μl of EEN (1× EE, NaCl 0.5 M) and 100 μl of a 5% suspension of avidin-conjugated beads (Baxter), which had been previously equilibrated in EEN. After a 30 min incubation at room temperature, the mixture was loaded onto a Millipore ultrafree MC unit (0.22 μm), and unhybridized tracer was recovered in the flowthrough after a short spin in a microfuge. The filter was rinsed once with 200 μl of EEN. The estimate of the fraction of tracer left after hybridization was obtained by comparing radioactivity in the 400-600 nucleotide region of a 5% acrylamide urea gel in aliquots of tracer and flowthrough. The subtracted cDNA was gel purified on a preparative 5% acrylamide gel and ethanol precipitated before additional amplification by PCR and subsequent use in a second and third round of subtraction. In the second and third rounds of subtraction, the driver contained equal amounts of head cDNA and of head-minus-antennae subtracted cDNA to improve subtraction of rare sequences present in both heads and antennae (Wang and Brown, 1991).**

Cloning and Sequencing

The subtracted cDNA was digested with HindIII and Sall and cloned into pGEM4 (Promega), and relevant clones were sequenced using the Sequenase Kit (US Biochemicals). The S end of each of the three cDNAs described in this work was cloned using RACE (Frohman et al., 1988).** Briefly, specific primers were designed for each mRNA and were 5' end labeled with γ³²P ATP and polynucleotide kinase. Primer extension was carried out with poly(A) RNA from appendages, heads, or bodies. In each case, a unique appendage-specific extension product was observed of a size consistent with the one calculated from the size of the mRNA and the position of the primer on the S' partial clone. The cDNA was then gel purified and G tailed as described above and amplified by PCR using the specific primer and the (C₇)₅ primer. In each case, a PCR fragment of the appropriate length was obtained, cloned, and sequenced. Once the full-length sequence was known, two primers flanking the coding sequence were synthesized and used for PCR on appendage cDNA. This PCR fragment was directly sequenced using the fmol DNA Sequencing System (Promega) to correct any errors introduced during Taq amplification of the initial PCR product. Direct sequencing of the PCR product avoids sequence errors that can occur in the analysis of single clones.

Sequence Analysis

Sequence analysis was performed using the CCG70 package from Genetics Computer Group, Inc. Database searches were performed at NCBI using the BLAST network service (Altshul et al., 1990).

Northern and Southern Blot Analyses and In Situ Hybridizations

Frozen Drosophila adults were fractionated into bodies, heads, and appendages as described (Oliver and Philips, 1970). Northern and Southern blot analyses were performed using standard protocols (Maniatis et al., 1982). In situ hybridizations to adult head sections and to larval polytene chromosomes were as described (Hasan and Rosbash, 1992).

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References


