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Characterization and cDNA cloning of the pheromone-binding protein from the tobacco hornworm, *Manduca sexta*: A tissue-specific developmentally regulated protein

(olfaction/carrier protein/antenna-specific protein/Lepidoptera)

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ABSTRACT **cDNA encoding pheromone-binding protein (PBP), the major soluble protein in olfactory sensilla of male moths, has been cloned from the tobacco hornworm, *Manduca sexta*. A study of the developmental time course of PBP reveals that it is first synthesized just prior to eclosion and that the percentage of antennal mRNA encoding PBP shifts from zero to about 20% at that time. PBP is also found in sensilla from female *M. sexta* antennae. No amino acid sequence homology is observed between PBP and the vertebrate odorant-binding protein.**

For terrestrial animals to detect airborne odorants via their olfactory receptor cells the volatile molecules must be first partitioned from a gaseous to an aqueous phase. In the vertebrate nasal epithelium dendrites of the olfactory receptor cells are submerged in a hydrophilic mucus that separates and protects them from the external environment. In insects, such as moths, an analogous barrier exists between the olfactory dendrites and the atmosphere. Most of the antennal sensilla of the moths are specialized to detect odorants. In several moth species, such as the tobacco hornworm, *Manduca sexta*, only the males possess the long trichoid sensilla that are specifically sensitive to pheromone released by females (1, 2). These sensilla are hollow cuticular structures containing the receptor cell sensory dendrites (cilia) bathed in a fluid, called the sensillum lymph. Pheromone molecules are thought to diffuse through the pore tubules in the cuticular wall and across the sensillum lymph to reach receptors located in the cilia (3–5).

The pheromone-binding protein (PBP) is a 16-kDa water-soluble protein, identified in the sensilla of male *Antheraea polyphemus* by its ability to bind pheromone without metabolizing it (6). This finding and the protein's enormous 10 mM concentration in the sensillum lymph led to the suggestion that it serves to solubilize the extremely hydrophobic pheromone molecules (3–7).

In an attempt to better understand the structural and functional properties of the PBP, we undertook experiments to further characterize the protein. Here, a developmental study, the molecular cloning of the cDNA, and the complete amino acid sequence of the PBP from *M. sexta* are presented.*

MATERIALS AND METHODS

Materials. Murine leukemia virus reverse transcriptase and bacterial alkaline phosphatase were purchased from Bethesda Research Laboratories. Ribonuclease H, DNA polymerase I, Oligolabelling Kit, and ^{35}S Nucleotide Reagent Kit were obtained from Pharmacia. λgt11 vector kit, Gigapack packaging extracts, and pBluescript phagemid were purchased from Stratagene. M13mp19 DNA and all restriction enzymes came from New England Biolabs. Radiolabeled compounds were obtained from Amersham. *M. sexta* eggs were kindly provided by Michael Jackson and Jim Hobgood of the U.S. Department of Agriculture, Tobacco Research Station (Oxford, NC).

Animals. *M. sexta* were reared as described by Baumhover (8). Pupae with cuticle turned completely brown were defined as day 0. In our colony adult ecdysis most often occurs between day 14 and day 16.

Isolating Sensillar Proteins. Antennae were placed in a 1.5-ml silane-treated microcentrifuge tube that had a small hole in the cap. Pulverized dry ice was added until the tube was about ⅔ full, and the tube was vigorously swirled for 2 min. Antennal shafts were removed, and the dry ice was allowed to sublimate. Sensilla and scales were collected by rinsing the tube with diethyl ether. The ether was evaporated, and the pellet was resuspended in 2.5 mM sodium bicarbonate, pH 8.0, and sonicated for 30 sec at 4°C. Supernatants were collected after centrifugation in an Eppendorf microcentrifuge (Brinkmann) for 10 min.

Isolating Antennal and Other Tissue Proteins. Tissues were homogenized in Laemmli buffer (9) containing 2.3% (vol/vol) Triton X-100 in place of $NaDodSO_4$ for nondenaturing PAGE and in Laemmli buffer with $NaDodSO_4$ for immunoblotting studies. Supernatants were collected after centrifugation in an Eppendorf microcentrifuge for 10 min at 4°C. Proteins were separated by nondenaturing PAGE or $NaDodSO_4$/PAGE (9), followed by silver staining (10) or immunoblotting, respectively.

Antibodies to PBP. Antibodies were raised by using standard methods (11), injecting rabbits six times at 2-week intervals with PBP purified from sensillum extract by two-dimensional PAGE involving a combination of isoelectrofocusing and $NaDodSO_4$/PAGE, followed by staining with Coomassie blue (12).

Immunoblotting of Proteins. Proteins from antennae and legs were separated by $NaDodSO_4$/PAGE, transferred to a nitrocellulose membrane, and probed by using anti-PBP or preimmune serum at a 500-fold dilution (13). Antigens were visualized by the alkaline phosphatase-conjugated second-antibody method (14).

Amino Acid Sequence of the N Terminus of PBP. The N-terminal sequence of PBP was obtained by subjecting a trichloroacetic acid-precipitated, acetone-extracted sensillar preparation to protein sequence analysis by the Yale–

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Abbreviations: PBP, pheromone-binding protein; OBP, odorant-binding protein.

*The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04146).

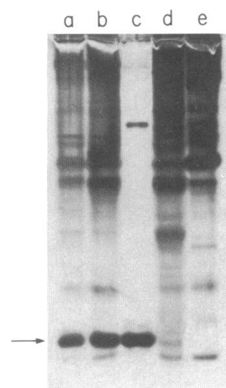

FIG. 1. PAGE analysis of proteins from different tissues of adult male *M. sexta*. Proteins were separated on a 12% polyacrylamide gel (without $NaDodSO_4$) and stained with silver. Lane a, antenna (0.5 antenna, 1.8 mg wet); lane b, antenna from which sensilla have been removed (0.5 antenna); lane c, sensilla (from 3 antennae); lane d, brain with the covering soft tissues (4 mg wet); and lane e, eye (0.8 mg wet). PBP is indicated by arrow.

Howard Hughes Medical Institute protein sequencing facility.

Construction of an Antennal mRNA-Derived cDNA Library. RNA was isolated from antennae of newly eclosed adult male *M. sexta*, followed by purification of poly(A)$^+$ RNA with oligo(dT) cellulose chromatography (15, 16). Five micrograms of poly(A)$^+$ RNA was used to synthesize oligo(dT)-primed double-stranded cDNA, which was inserted into the *Eco*RI site of λgt11 (18, 19). Approximately 150,000 recombinant bacteriophages were obtained prior to amplification of the library.

Isolation and Sequencing of cDNA Clones Encoding PBP. The amplified library was initially screened with the rabbit PBP antiserum (20). Subsequent cDNA clones were identified by probing the library with mixed, ^{32}P-end-labeled (ref. 17, p. 122) 15-residue oligonucleotides synthesized on an Applied Biosystems 380A DNA synthesizer according to the PBP N-terminal amino acid sequence from position 20 to 25, including all possible codon variations. High titer lysates were made from the positive plaque-purified clones (ref. 17, p. 293). After ultracentrifugation on cesium chloride gradients (ref. 21, pp. 80–81) phage DNA was digested with *Eco*RI and separated by PAGE. Inserts were purified and ligated into pBluescript for restriction enzyme analysis and into M13mp19 for sequencing (ref. 17, p. 391). Both strands of the cDNA clones were sequenced by the dideoxy chain termination method (22, 23). Sequences were analyzed by using the PC/GENE (IntelliGenetics, Mountain View, CA) and the GCG (24) sequence analysis software packages.

RNA Blot Analysis. Total tissue RNA (20 μg) was separated on a 1.2% agarose/formaldehyde gel, transferred to a nitrocellulose filter (ref. 17, pp. 202–203), and hybridized overnight with ^{32}P-labeled, oligonucleotide-primed cDNA (25). The blot was washed with 0.2× SSPE (ref. 17, p. 447)/0.2% $NaDodSO_4$ at 68°C. The film was exposed to the washed blot for 10 hr at −80°C. Integrated densities of the bands appearing on the exposed film were calculated by using a Visage 2000 image analyzer (Bioimage/Kodak).

RESULTS

PBP Is Abundant and Specific to the Antenna and Its Sensilla. Fig. 1 shows proteins extracted from whole antenna, antenna without sensilla, sensilla, brain with the covering soft tissues, and eye from adult male *M. sexta*. As expected from similar, previously reported studies concerning PBP in several species of Lepidoptera, antennae and sensilla from *M. sexta* were found to contain a highly abundant protein that is not seen in nonantennal tissues (4–7). On the basis of its abundance, tissue specificity, migration profile on nondenaturing PAGE, and isoelectric point of 5.1 (data not shown), this protein was tentatively identified as *M. sexta* PBP.

The N-Terminal Amino Acid Sequence of the Protein Confirms That It Is the PBP of *M. sexta*. The protein in question is by far the most abundant protein in sensilla and is extractable in a low-salt buffer containing no detergent. Therefore, the sensillum extract with essentially no further purification was subjected to amino acid sequence analysis. The N-terminal amino acid sequence of the protein is shown in Fig. 2 along with that of bona fide PBP from *Antheraea polyphemus* (ref. 4 with correction by R. G. Vogt, personal communication). The 57% amino acid sequence identity (and 83% similarity when including conservative substitutions) affirms our presumption that the above-described *M. sexta* protein is PBP.

PBP Is Expressed During the Final Stages of Adult Development in Male and Female Moths. Fig. 3 *Left* shows proteins extracted from antennae of male animals on sequential days during adult development. The PBP can be first detected on day 14. Fig. 3 *Right* shows the developmental profile of proteins extracted from female antennae. A polypeptide with the same mobility and developmental profile as PBP from males is seen, although it is less abundant.

Antibodies Against PBP Recognize a 16-kDa Protein. PBP was isolated by two-dimensional PAGE (data not shown) and used to immunize rabbits. The specificities of the antisera obtained were tested by immunoblotting as seen in Fig. 4. As expected, the antibodies recognize a 16-kDa protein present in antennae from males and females but not in a nonantennal tissue such as male leg. The antigenic protein is most abundant in antennae of newly eclosed moths and can be seen as early as on day 13. This protein could not be detected when preimmune serum was used as probe.

Isolation and Analysis of cDNA Clones Encoding PBP. A cDNA library, derived from poly(A)$^+$ RNA from the antennae of newly eclosed male *M. sexta*, was constructed in λgt11 and amplified. Initially, 800,000 recombinant clones were screened by using rabbit PBP antiserum at a 200-fold dilution. A single clone, containing a 777-nucleotide insert (λBP6), was identified and plaque purified. Two independent lysogens, derived from the clone, were induced to produce recombinant protein, and the products were analyzed by immunoblotting. The fusion proteins were recognized by PBP antiserum and antibodies against β-galactosidase but not by preimmune serum (data not shown). Sequence analysis of the cDNA revealed a 198-nucleotide open reading frame but no part of the known N-terminal amino acid sequence. Therefore, a redundant, mixed 15-residue oligonucleotide, designed according to the N-terminal sequence of the *M. sexta* PBP (Fig. 2) from amino acid 20 to 24, was synthesized and used to rescreen the library. The oligonucleotide probe was found to hybridize to over 20% of the plaques formed by recombinant phage. Ten clones were plaque purified, and their cDNA inserts were subcloned and sequenced. Of these, nine (λBP931–939) proved to have essentially the same sequence, while one (λBP251) encoded an overlapping but, for the most part, different sequence. As shown in Fig. 5, the sequences encoded by λBP931–939 overlap those of the original clone λBP6 as well as λBP251.

FIG. 2. Comparison between the N-terminal amino acid sequence of the putative *M. sexta* PBP (MS) with that of the *Antheraea polyphemus* PBP (AP) (ref. 4 with correction by R. G. Vogt, personal communication). Both sequences were determined by analyzing the protein. Amino acid residues are denoted with the standard single-letter symbols, and common residues are enclosed in boxes.

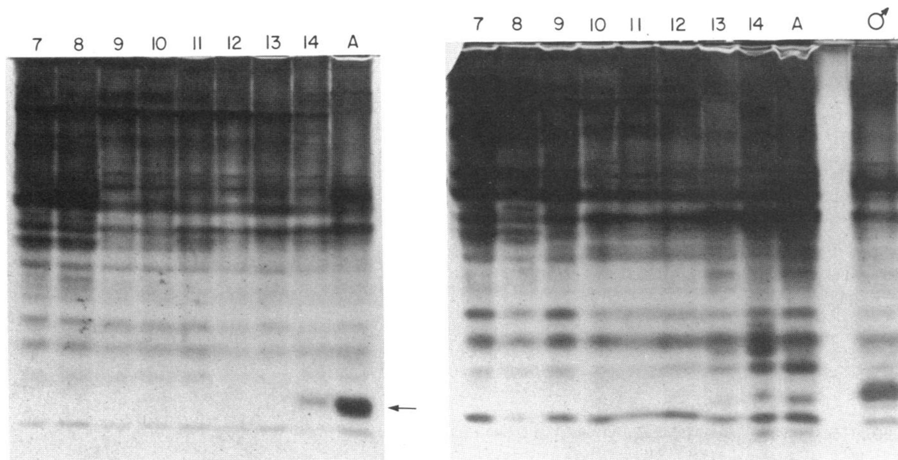

FIG. 3. Appearance of the PBP during adult development. PAGE analysis of male (*Left*) and female (*Right*) *M. sexta* antennal proteins at different developmental stages (7 to 14, day 7 to day 14; A, adult animal on the day of eclosion). Proteins were separated on 12% polyacrylamide gels (without $NaDodSO_4$) and stained with silver. In *Left* proteins extracted from 0.5 male antenna per day were loaded in each lane. In *Right* proteins from 2 female antennae per day were loaded in each of the first 9 lanes and proteins from 0.5 of an adult male antenna were loaded in the last lane (♂). PBP is indicated by the arrows.

On the basis of the overlapping sequences of the cDNA inserts, the nucleotide sequence for the cDNA encoding PBP and the predicted amino acid sequence for PBP could be determined (Fig. 6). This cDNA consists of 1363 nucleotides and contains an open reading frame of 504 nucleotides. By matching the known N-terminal sequence (Fig. 2) with the deduced amino acid sequence we were able to define the first amino acid of the mature (secreted) protein. The predicted N-terminal amino acid sequence of the mature protein precisely matches the one derived from protein sequencing. The hydrophobic region toward the N terminus of the deduced amino acid sequence [(−26)–(−1)] is characteristic to the signal sequence of many secreted proteins (26).

The predicted mature polypeptide consists of 142 amino acid residues with a calculated molecular mass of 15,768 Da and a theoretical isoelectric point (pI) of pH 4.65. These data correlate well with those observed in our laboratory (16 kDa; pI = 5.1). PBP from *A. polyphemus* analyzed by isoelectrofocusing on ultrathin-layer PAGE was shown to have a pI of pH 4.7 (7).

Temporal Expression of PBP mRNA Is Similar to That of the Protein. By combining cDNAs PB6 and PB931 to probe an RNA blot, the size, developmental expression, and tissue specificity of mRNA encoding PBP were determined. As shown in Fig. 7, the probes hybridize to an RNA ≈1400 nucleotides in length. This mRNA can first be clearly seen on day 13, and it is tissue specific and present in antennae of both

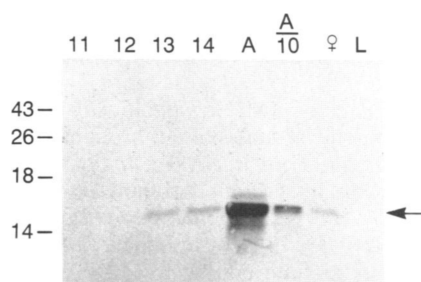

FIG. 4. Immunoblot analysis of the specificity of the rabbit anti-PBP polyclonal antiserum. Proteins from male and female *M. sexta* antennae and male leg were separated on $NaDodSO_4$/15% PAGE, transferred to nitrocellulose, and probed with the polyclonal antiserum (at 1:500 dilution) and alkaline phosphatase-conjugated second antibody. Lanes 11 to 14, day 11 to day 14 male pupal antenna (0.5 antenna per lane); lane A, antenna from newly eclosed adult male (0.5 antenna); lane A/10, antenna from newly eclosed adult male (0.05 antenna); lane ♀, antenna from newly eclosed adult female (0.5 antenna); lane L, leg from newly eclosed adult male (0.5 leg, 1.8 mg wet). PBP is indicated by the arrow. Positions of prestained molecular mass standards (Bethesda Research Laboratories) are shown on the left and indicated in kDa.

sexes but predominantly in males. The PBP mRNA is 7-fold more abundant in antennae from males collected on day 14 compared to antennae from females of the same age.

Predicted Hydrophobicity. The predicted hydrophobicity of the mature PBP has been analyzed by the method of Kyte and Doolittle (27). There are two major hydrophobic domains along the sequence, one being residues 49–56, the other comprising the last 11 C-terminal residues. In addition, there are four other, less strongly hydrophobic regions (residues 6–13, 35–37, 88–94, and 106–116) in the protein.

PBP Is Not a Member of the Binding Protein Family. The nucleic acid sequence and the predicted amino acid sequence of the PBP cDNA were compared to those in the GenBank and EMBL sequence data bases on January 21, 1988, and no significant homologies were noted. Also, we observed less than 13% amino acid identity between the PBP and the members of the binding protein family that includes the human retinol-binding protein, the bovine β-lactoglobulin, the rat odorant-binding protein (OBP), a frog olfactory tissue specific secretory protein (BG), the *M. sexta* insecticyanin, and other hydrophobic ligand-binding proteins (reviewed in refs. 28–34). The two stretches of amino acid residues that are conserved among the binding proteins listed above were not detected in the PBP amino acid sequence (34).

DISCUSSION

PBP is by far the most abundant soluble protein in antennal sensilla from adult male *M. sexta*. Its mRNA is also abundant, as over 20% of the cDNA inserts from a recombinant phage library representing mRNA from adult male antennae encode PBP. All three methods used to examine the developmental expression of PBP show that the synthesis of both mRNA and protein occurs around the time of eclosion, although the precise onset of PBP synthesis is difficult to determine. By silver staining and PAGE analysis the PBP appears on day 14, typically 1 day prior to eclosion, while by immunoblot analysis the protein is first detected on day 13.

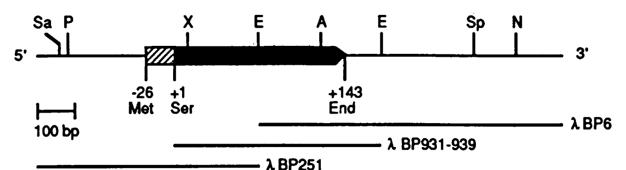

FIG. 5. Physical map of PBP cDNA and the positions of the isolated overlapping cDNA clones. The coding region of the mature peptide is solid, the predicted precursor region is hatched. The initiating methionine (Met), the first amino acid of the mature peptide (Ser), and the end of the peptide sequence (End) are indicated below the map; bp, base pairs. Restriction sites are shown above the map: A, *Apa* I; E, *Eco*RI; N, *Nhe* I; P, *Pst* I; Sa, *Sal* I; Sp, *Spe* I; and X, *Xba* I.

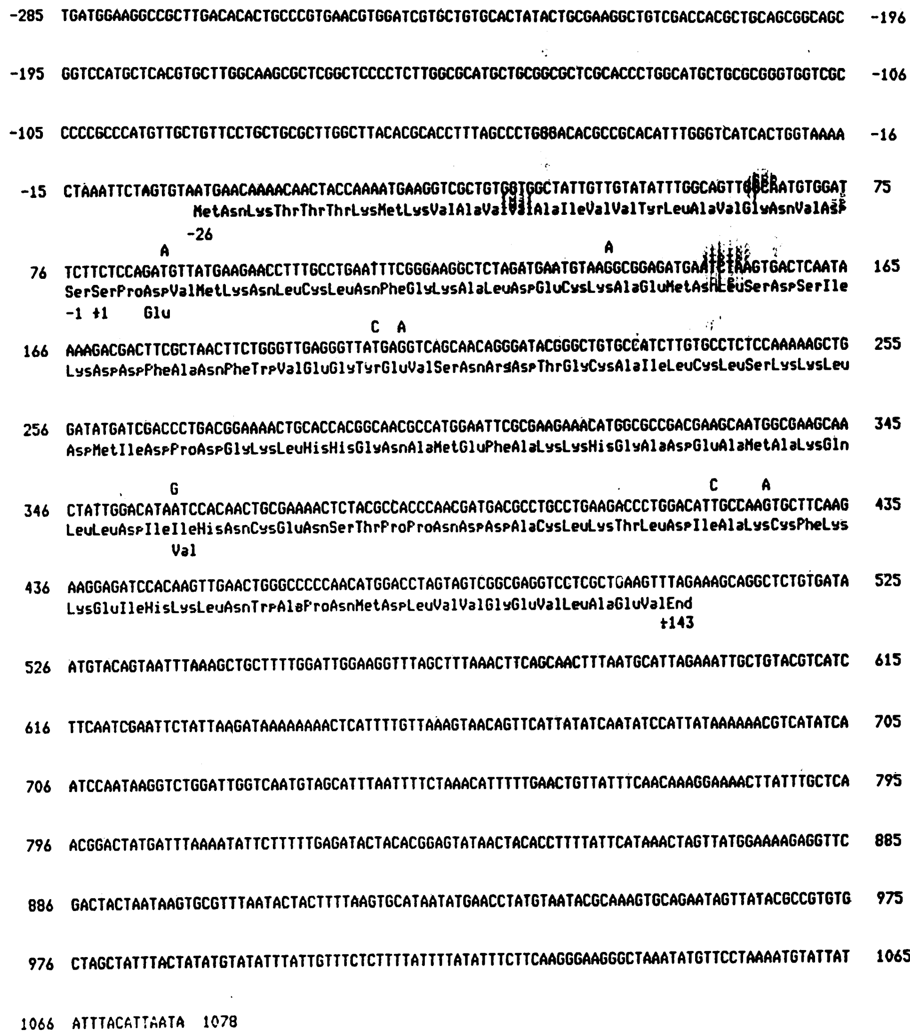

FIG. 6. Nucleotide sequence of PBP cDNA derived from sequencing the overlapping λBP clones, and the predicted amino acid sequence. Nucleotides are numbered beginning with the first base of the ATG initiator codon. Nucleotides to the 5′ side are designated by negative numbers. Amino acids are numbered from the N-terminal (serine) residue of the mature protein, while negative numbers mark the putative secretory signal sequence. Variations in the nucleotide sequence detected in cDNAs λBP932 and λBP935 and the resulting amino acid sequences are indicated above and below their respective sequences.

On Northern blots the mRNA is apparent on day 13, but with longer exposure times (3–4 days) it can be seen as early as on day 12, 3 days before eclosion (data not shown). These disparities probably reflect the natural variations in developmental rates among individual animals observed in our colony as well as the different sensitivities of the assays used in these experiments.

In view of the striking activation of the PBP gene near the time of eclosion, it is perhaps relevant that over a decade ago a similar time course was found for the first detectable electrical response of antennae to a blend of pheromone extracted from the abdominal tip of female *M. sexta* (35). This is also the time during adult development when an antenna-specific aldehyde oxidase (36) and a male olfactory neuron-specific antigen identified with a monoclonal antibody (MOSA) are produced (37, 38). The coincidence of timing between the synthesis of PBP and other proteins that are probably important for pheromone reception is consistent with the hypothesis that the function of PBP is to transport pheromone through the aqueous lymph to receptors located on olfactory cilia.

There is evidence that in the late pupal development the rapid decline in circulating 20-hydroxyecdysone, an active metabolite of the steroid hormone ecdysone, induces RNA expression in *M. sexta*. The role of ecdysteroids in this developmental phase is best characterized in the process of programmed neuronal death in the abdominal ganglia (39). The regulating effects of these hormones have also been seen in other tissues of the moth (40). Furthermore, it is known that, if an animal is decerebrated a few days after pupal ecdysis, the antennae still develop and become electrically responsive to odorant stimuli, as in normal animals (41). Thus, if the PBP is relevant for olfaction the factor that regulates its expression must originate from tissues other than brain. Ecdysone is synthesized by the prothoracic gland and then converted by peripheral tissues into various metabolites (42). A closer study of the regulatory region of the PBP gene is necessary to determine what role ecdysteroids play in the transcriptional control of the PBP gene during development. The characterization of the chromosomal gene should also yield information about elements responsible for tissue specificity of the PBP gene expression.

Although PBP is thought to specifically solubilize and transport pheromone, it may have a broader role involving odorants in general. Female *M. sexta* do not respond to pheromone but can detect nonpheromonal odorants such as *trans*-2-hexenal just as well as males can (35). Nonetheless, PBP and its mRNA are present in female antennae, and the same developmental time course for PBP is seen in both sexes (Fig. 3). While the amount of PBP is less in female antennae than in ones from males, the total sensillar volume for female antennae, which lack the large male specific pheromone-sensitive trichoid sensilla, is also less than for male ones (43). Though we are aware that our approach is qualitative, we surmise that the concentrations of PBP in sensillum lymph are similar in the two sexes.

The question remains as to whether the binding of different odorants is specific. Sequencing of several clones revealed few variations in the nucleic acid sequences, and these variations either did not affect the amino acid sequence or created only conservative substitutions (Fig. 6). This indicates that sequence differences in PBP do not provide for

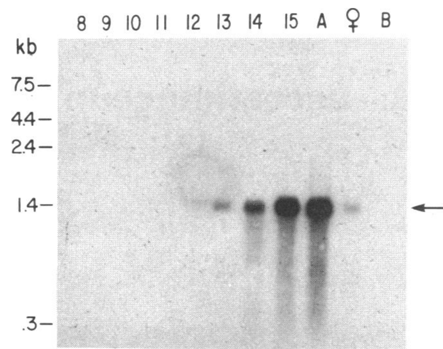

FIG. 7. Blot hybridization analysis of total RNA prepared from male *M. sexta* antennae at different adult developmental stages, female antennae, and a male body from which the antennae had been removed (lanes 8 to 15, antennae from day 8 to day 15 male pupae; lane A, antennae from newly eclosed adult male moths; lane ♀, antennae from day 14 female pupae; lane B, body without antennae of a day 14 male pupa). Each lane was loaded with 20 μg of total RNA. After transfer the filter was probed with ^{32}P-radiolabeled oligonucleotide-primed cDNA from clone λPB6 and λPB931. The mRNA hybridizing to the probes is indicated by the arrow. The markers to the left correspond to an RNA size ladder from Bethesda Research Laboratories; kb, kilobases.

odorant discrimination. While it is possible that other, more variant PBP clones exist, we believe that the PBP described here has a general, nonspecific role in binding and solubilizing both pheromones and nonpheromonal odorants.

With the help of a Kyte–Doolittle analysis (27) two major hydrophobic domains of the PBP could be identified, one being from the 49th to the 56th amino acid residues, the other the last 11 C-terminal residues. These domains may play roles in forming an odorant-binding pocket. Further structural analysis, such as x-ray crystallography, will be necessary to precisely define how the protein is shaped to bind ligand.

Finally, is PBP related to OBP, which is found in the olfactory mucus of vertebrates (44–46)? Both proteins are thought to be involved in facilitating the translocation of hydrophobic odorants from the air to the olfactory cilia. While both PBP and OBP can bind odorants and are small acidic proteins, we believe that they are evolutionarily not related. OBP belongs to a family of binding proteins (BP) on the basis of shared amino acid sequences (30, 32). Several of these proteins are known to be carriers of small organic molecules. According to our analysis there are no similarities between the amino acid sequences of the PBP and those of any of the BP family members. On the other hand, insecticyanin, a prominent serum protein in *M. sexta* that binds biliverdin, is a member of the BP family (29). Thus, the lack of similarities between PBP and the BP family of proteins is unlikely to be due to evolutionary drift from a common progenitor during the course of divergence of insects and vertebrates. If the similar suggestions concerning the roles of PBP and OBP turn out to be correct, these proteins would appear to represent interesting examples of convergent evolution.

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