Chem. Senses 27: 453–459, 2002

A Cluster of Candidate Odorant Receptors from the Malaria Vector Mosquito, *Anopheles gambiae*

A.N. Fox[1], R.J. Pitts[1] and L.J. Zwiebel

Department of Biological Sciences, Program in Developmental Biology and Center for Molecular Neuroscience, Vanderbilt University, Nashville, TN 37235 USA

[1]*These authors contributed equally to this work*

Correspondence to be sent to: L.J. Zwiebel, Department of Biological Sciences, Program in Developmental Biology and Center for Molecular Neuroscience, Vanderbilt University, Nashville, TN 37235 USA. e-mail: l.zwiebel@vanderbilt.edu

Abstract

Olfaction is critical to the host preference selection behavior of many disease-transmitting insects, including the mosquito *Anopheles gambiae sensu stricto* (hereafter *A. gambiae*), one of the major vectors for human malaria. In order to more fully understand the molecular biology of olfaction in this insect, we have previously identified several members member of a family of candidate odorant receptor proteins from *A. gambiae* (AgORs). Here we report the cloning and characterization of an additional AgOR gene, denoted as AgOr5, which shows significant similarity to putative odorant receptors in *A. gambiae* and *Drosophila melanogaster* and which is selectively expressed in olfactory organs. AgOr5 is tightly clustered within the *A. gambiae* genome to two other highly homologous candidate odorant receptors, suggesting that these genes are derived from a common ancestor. Analysis of the developmental expression within members of this AgOR gene cluster reveals considerable variation between these AgORs as compared to candidate odorant receptors from *D. melanogaster*.

Introduction

Chemoreception in general and olfaction in particular represent critical sensory inputs into many behaviors, including host preference selection, among insect agricultural pests and disease vectors (Takken, 1991; Hildebrand and Shepherd, 1997). These economically and medically important insects include several species of mosquitoes that transmit malaria, dengue, West Nile encephalitis, and yellow fever. While all of these diseases pose significant threats to human health, malaria, which is transmitted by several species of Anopheline mosquitoes, remains of particular concern, as it is responsible for millions of deaths per year in Africa alone (Collins and Paskewitz, 1995). The overall ability of these mosquitoes to transmit malaria (vectorial capacity) is, in part, determined by host preference selection. Therefore a molecular analysis of mosquito olfaction may provide opportunities for disrupting vector–host interactions, thereby reducing incidence of disease transmission.

As a first step in this process, the cloning and characterization of components of the olfactory signal transduction cascade from *Anopheles gambiae* will facilitate molecular and biochemical studies of this mosquito's olfactory processes. Olfactory signal transduction, which is mediated by G-protein coupled receptors (GPCRs) and their downstream effectors, is widely conserved across a broad spectrum of organisms, including mammals, fish, crustaceans and nematodes [reviewed in Hildebrand and Shepherd (Hildebrand and Shepherd, 1997)]. Indeed, the cloning and characterization of the GPCRs involved in this cascade, known as odorant receptors (ORs) (Buck and Axel, 1991; Ngai *et al*., 1993), has significantly accelerated the molecular analysis of olfaction across a wide array of vertebrate systems [reviewed in Mombaerts (Mombaerts, 1999)]. The first invertebrate organism in which candidate ORs were identified was *Caenorhabditis elegans* through the screening of a genome project for potential signaling molecules (Troemel *et al*., 1995). As is the case for vertebrate ORs, the *C. elegans* ORs are seven transmembrane GPCRs, although the *C. elegans* ORs bear almost no similarity to the vertebrate ORs. Within *C. elegans*, there is only between 10 and 48% identity among the ORs, indicating that they are much more divergent than the vertebrate ORs. Moreover, genes encoding candidate ORs are found in characteristic clusters throughout the mouse (Xie *et al*., 2000) and *C. elegans* (Troemel *et al*., 1995) genomes.

Using a variety of approaches, a large family of candidate ORs was recently identified in *D. melanogaster* (Gao and Chess, 1999; Clyne *et al*., 1999; Vosshall *et al*., 1999). These genes are members of a highly divergent family of receptors, displaying between 10% and 75% identity and bearing no significant homology to any other GPCR family (Smith,

1999). Like ORs from mouse and *C. elegans*, a subset of *Drosophila* ORs have been mapped to several gene clusters within the *D. melanogaster* genome. Furthermore, several studies have used a variety of methods to begin to examine OR–odorant interactions (Zhang *et al*., 1997; Zhao *et al*., 1998; Wetzel *et al*., 1999; Storkuhl and Kettler, 2001; Wetzel *et al*., 2001).

Recently, our group used genomics and molecular-based approaches to identify and characterize four *A. gambiae* odorant receptor (AgOR) genes, AgOr1, AgOr2, AgOr3 and AgOr4, that encode candidate odorant receptor proteins from *A. gambiae* (Fox *et al*., 2001). We have demonstrated that these AgORs display several of the characteristics expected of OR family members. They are all predicted to encode seven transmembrane domains, show significant homology to *D. melanogaster* ORs (DORs), and are selectively expressed in olfactory tissues. Here, we report the cloning and characterization of a fifth OR gene, AgOr5, which is tightly clustered within the *A. gambiae* genome with AgOr3 and AgOr4. Analysis of the developmental profiles of these linked AgORs reveals that, unlike *Drosophila* ORs, AgOrs 3, 4 and 5 display novel and diverse patterns of pre-adult expression.

Materials and methods

Sequencing of *A. gambiae* BAC clones

BAC clone 08K09 was generously provided by Dr Frank Collins (Notre Dame University, South Bend, IN) and directly sequenced or subcloned into pBluescript II KS (+) (Stratagene, La Jolla, CA) prior to sequencing, which was performed with an ABI 377 auto- mated sequencer using Big-Dye chemistry (PE Biosystems, Foster City, CA) using custom primers.

Phylogenetic analysis

Deduced amino acid sequences of AgORs 2–5 were aligned with four representative *D. melanogaster* odorant receptors and one representative *D. melanogaster* gustatory receptor to serve as an outgroup using ClustalX v1.6 (Thompson *et al*., 1997). Phylogenetic analysis was performed using the exhaustive method by PAUP* v4.0b4 (Swofford, 2001), and optimality criterion set at maximum parsimony. Bootstrap analysis was used to assess statistical support for relationships via branch and bound analysis of 1000 pseudo-replicated data sets. Similar trees were obtained using neighbor-joining and heuristic searches (data not shown).

Reverse-transcriptase polymerase chain reaction (RT-PCR)

One hundred mosquitoes were dissected and RNA was extracted (RNeasy, Qiagen, Valencia, CA) and resuspended in 30 µl. Total RNA was reverse transcribed using oligo-dT primers (Roche Molecular Biochemical, Indianapolis, IN) and SuperScriptII reverse transcriptase (Gibco BRL, Rockville, MD). A 10 µl volume of the RNA was used to synthesize cDNA, and 1 µl of each cDNA was used in each PCR reaction. PCR amplifications were carried out with the following forward (f) and reverse (r) primer pairs:

- AgOr3, f5′-GGAAAAGGAGCTGAACGAGA-3′ and r5′-CTAAAACTGCTCCTTCAGTA-3′ (product size: 309 base pairs (bp) cDNA, 367 bp genomic DNA);
- AgOr4, f5′-ATTTACGGCGGCAGTATCTT-3′ and r5′-TCACTGTACATCCATCTTTA-3′ (product size: 450 bp cDNA, 610 bp genomic DNA);
- AgOr5, f5′-TATGTGGTACGCATCAATCA-3′ and r5′-AAACAGTACACCCACGTNTGC-3′ (product size: 561 bp cDNA, 687 bp genomic DNA);
- *rps7*, f5′-GGCGATCATCATCTACGTGC-3′ and r5′-GTAGCTGCTGCAAACTTCGG-3′ (product size: 458 bp cDNA, 610 bp genomic DNA). Optimal annealing temperature, as tested empirically, was 58°C for all AgOR primer pairs and *rps7*.

Mosquito rearing and blood feeding

Anopheles gambiae (G3 strain) embryos were either kindly provided by Dr Mark Benedict (Centers for Disease Control and Prevention, Atlanta, GA) or generated in-house and disinfected with 0.05% sodium hypochlorite prior to hatching in flat plastic pans with distilled water. Larvae were reared on a diet of ground Whiskas Original Recipe cat food (KalKan Inc., Vernon, CA) that was applied to the surface of the water. Pupae were transferred to plastic cups in 1 gallon plastic containers, where newly emerged adults were collected the following morning. Adult mosquitoes were maintained in 1 gallon plastic containers at 27°C with 75% relative humidity under a 12:12 h photoperiod and fed daily with a 10% dextrose solution.

Results

An alignment of the deduced amino acid sequences of three candidate odorant receptors from *A. gambiae*, AgOr3, AgOr4 and AgOr5 is shown in Figure 1A, where a significant degree of sequence conservation is evident among them. Specifically, the strongest pair-wise identity (59%) and similarity (80%) are observed between AgOr3 and AgOr5. AgOr4 and AgOr5 share 29% identity and 64% similarity, while AgOr3 and AgOr4 share 19% identity and 62% similarity, respectively. The relative positions of a subset of introns, as well as the overall length of the deduced proteins (averaging 400 amino acids), are conserved among all five AgORs and also between putative AgORs and DORs. Here we show that AgOr3 and AgOr5 maintain complete conservation with regard to predicted exon/intron positions (Figure 1B), while the positions of the first and second introns of AgOr4 correspond with the first and third introns of AgOr3 and AgOr5, respectively. AgOr4 lacks the last two

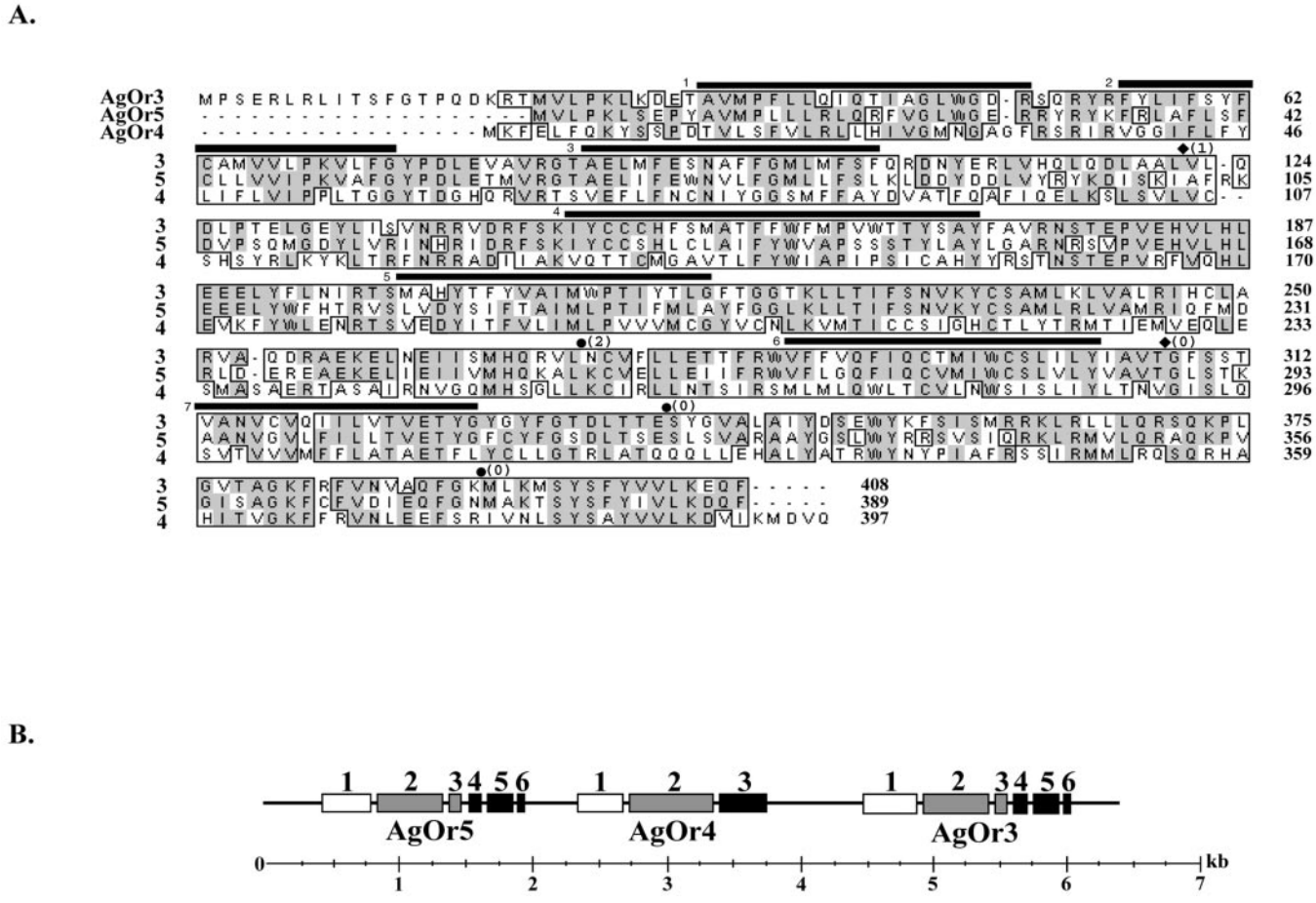

Figure 1 Deduced amino acid alignments and genomic structure of AgOr3, AgOr4 and AgOr5. **(A)** ClustalX (v1.62b) (Thompson *et al*., 1997) alignment viewed in SeqVu (The Garvan Institute, 1995) of AgOr3, AgOr4 and AgOr5. For all alignments, similarity shading is based on an 85% Goldman–Engelman–Steitz scale, and identity shading is based on a 65% scale (Riek *et al*., 1995) using SeqVu. Putative transmembrane domains are designated by black lines above the alignment and are numbered to the left of the lines. Filled diamonds above alignment indicate intron positions conserved among all three AgORs, while filled circles indicate intron positions conserved in AgOrs 3 and 5 only. Numbers in parentheses indicate the phase of each intron. **(B)** Schematic representation of the intron/exon structure and chromosomal linkage of AgOr3, AgOr4 and AgOr5. The position and relative size of exons and introns are drawn to scale as indicated.

introns of AgOr3 and AgOr5 and instead maintains overall amino acid homology by combining exons 4, 5 and 6 of AgOr3 and AgOr5 into a single exon 3. In addition to primary sequence similarity between AgORs and DORs, an analysis of AgOr3, AgOr4 and AgOr5 reveals multiple hydrophobic regions that indicate seven possible transmembrane domains (Figure 2) that are characteristic of this family of GPCRs. Four separate analyses were performed on the predicted protein sequences to estimate the positions of the transmembrane domains [Kyte–Doolittle (Kyte and Doolittle, 1982), Hopp Woods (Hopp and Woods, 1981), and Eisenberg *et al*. (Eisenberg *et al*., 1984)] using DNA Strider, Version 1.2 (Marck, 1988) and TMPRED (www.ch.embnet.org/software/TMPRED_form.html). For all three protein sequences, the majority of the transmembrane domains were predicted by all four algorithms. Even though these types of analyses remain fundamental predictions, the fact that four independent algorithms predicted many of the transmembrane domains significantly increases their reliability.

It is especially interesting to note that AgOr3, AgOr4 and AgOr5 are tightly clustered together within the *A. gambiae* genome (Figure 1B). AgOr5 and AgOr4 are separated by 310 bp while AgOr4 and AgOr3 are separated by 747 bp. It is interesting to note that if ~100 AgORs (see Discussion for rationale for this approximation) were equally spaced along the *A. gambiae* genome (270 Mb), the average distance between each AgOR would be 2.7 Mb, much more distance than separates these clustered genes. Close chromosomal linkage is characteristic of odorant and taste receptor genes from *D. melanogaster* (Clyne *et al*., 1999, 2000; Gao and Chess, 1999; Vosshall *et al*., 1999, 2000), as well as OR genes from *C. elegans* (Troemel *et al*., 1995) and mouse (Xie *et al*., 2000). Taken together, these data are consistent with the classification of these genes as candidate olfactory receptors from *A. gambiae*.

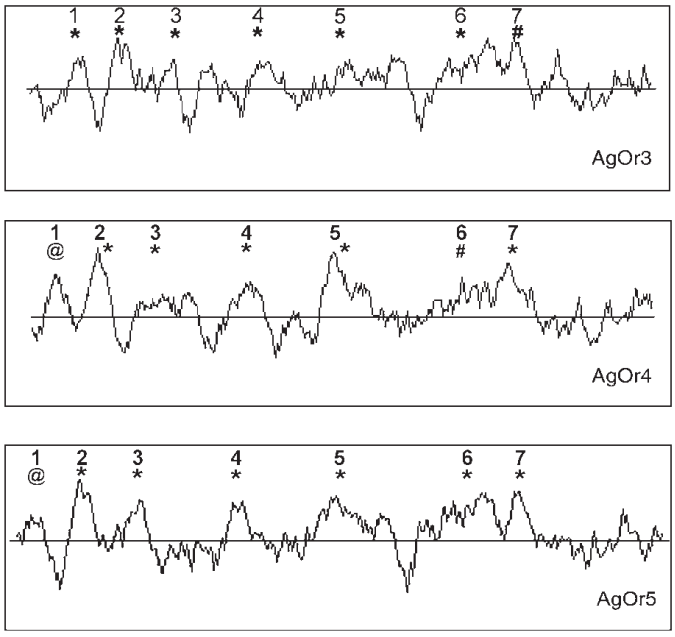

Figure 2 Representative hydropathy plots for AgOr3, AgOr4 and AgOr5 proteins. Hydrophobic peaks predicted by Kyte–Doolittle analysis appear above center lines. The approximate positions of the seven putative transmembrane domains are indicated above each hydropathy plot. The confidence of each transmembrane prediction is denoted as follows: *, predicted by four algorithms; @, predicted by three algorithms; #, predicted by two algorithms.

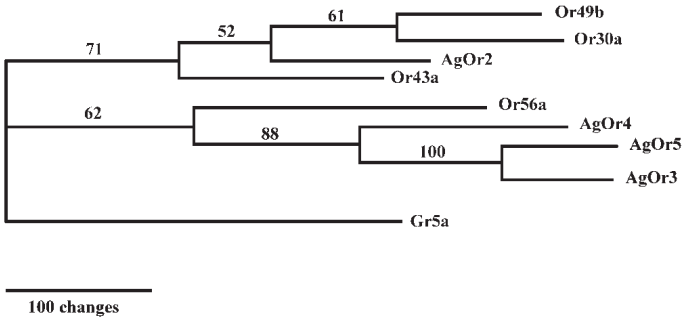

Figure 3 Phylogenetic analysis of AgORs. Phylogenetic tree showing relationships of the three AgORs (bold type) to four ORs from *D. melanogaster*. The tree was rooted with one representative of the *D. melanogaster* gustatory receptor family. The numbers above branches are the percentage of 1000 bootstrap replication trees that branch, with only those above 50% shown. The scale bar indicates 100 changes.

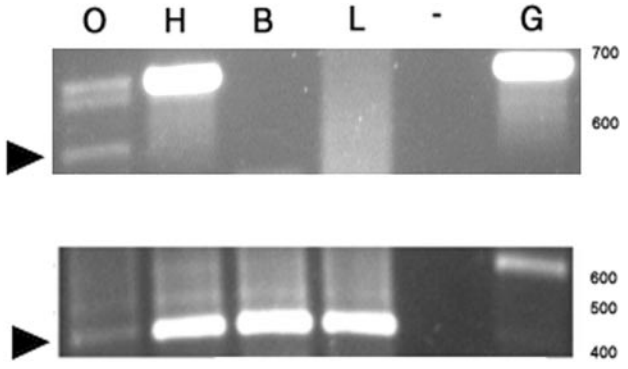

Figure 4 Olfactory tissue specific expression of AgOr5. *Anopheles gambiae* antennae and maxillary palps (O, olfactory tissue), heads stripped of olfactory tissues (H), legs (L) or bodies devoid of appendages (B) were used to generate RNA for reverse-transcription PCR. Reaction products, visualized under ultraviolet illumination after staining with ethidium bromide, represent the amplification of Ag0r5 (561 bp), along with each respective *rps7* control products (458 bp), indicated by arrowheads. A no template negative (–) control ensures the specificity of the amplicons and a genomic DNA template (G) reaction indicates the relative position of PCR product derived from genomic DNA contamination in experimental samples. The position of molecular weight markers (bp) is indicated to the left of each panel.

In order to more fully assess their relationships, four AgOR sequences were aligned with four representative DORs and one gustatory receptor from *D. melanogaster* to serve as an outgroup. From this alignment, phylogenetic trees were generated and bootstrap analysis was used to assess statistical support for the relationships observed. Figure 3 shows that AgOr2 confidently groups with DORs 30a, 49b and 43a. In some analyses, AgOr2 forms a monophyletic group with 43a [data not shown and Fox *et al*. (Fox *et al*., 2001)], while in the tree presented in Figure 2 the monophyletic group of AgOr2, 30a and 49b are branched with respect to 43a with low bootstrap support. AgOr3, AgOr4 and AgOr5 form a monophyletic group with very strong support in all analyses, with AgOr3 and AgOr5 grouping together in every bootstrap replicate (Figure 3 and data not shown). DOR56a is paraphyletic to the AgOr3–5 clade in this analysis with 63% bootstrap support (Figure 3), but it can be placed paraphyletically to the AgOr2 clade in other analyses [data not shown and Fox *et al*. (Fox *et al*., 2001)].

In order to determine whether AgOr5 expression is restricted to olfactory tissues, a characteristic that has been observed for all candidate AgORs to date (Fox *et al*., 2001), RT-PCR-based studies were performed. In these assays, 4-day-old adult mosquitoes were hand dissected into antennae/maxillary palps (olfactory tissues), head (from which olfactory tissue has been removed, but with proboscis attached), body and legs. These tissues were used to generate RNA and, subsequently, cDNA template pools for PCR. Furthermore, as an additional control, all reactions were carried out using oligonucleotide primers that were designed to span predicted introns in order to distinguish between genomic DNA and cDNA templates, as well as oligonucleotide primers against the *A. gambiae* ribosomal protein S7 (*rps7*) (Salazar *et al*., 1993). The *rps7* gene is constitutively expressed at high levels in all tissues of the mosquito and, therefore, provides a control for the integrity of the cDNA templates.

Consistent with its phylogenetic groupings with the other AgORs, olfactory-specific expression of AgOr5 is observed (Figure 4). In these studies, RT-PCR products of the pre-

dicted size are seen exclusively in reactions using antennae/maxillary palp cDNA templates. Importantly, no AgOr5 cDNA products are observed with head/proboscis, body or leg cDNA templates. It is noteworthy that the *rps7* amplifications are more robust for the head, body and leg templates, reflecting the higher template amounts used in these parallel reactions, further demonstrating there is no detectable expression of AgOr5 in non-olfactory tissues. Genomic DNA contamination of cDNA templates prepared from olfactory and head tissue is detectable and, as a result of primer design, is clearly distinguishable from cDNA products. To further verify the specificity of these reactions, the AgOr5 RT-PCR product was subcloned and sequenced, revealing that an AgOr5-specific product had indeed been obtained (data not shown). Lastly, to additionally assure that AgOr5 is not expressed in any tissues other than antennae/maxillary palp, an additional 15 cycles of PCR were added to the control reactions containing head, body and leg cDNA templates. Even under these extremely sensitive conditions, AgOr5 cDNA RT-PCR products are undetectable in non-olfactory tissues (data not shown).

We have made repeated attempts to detect the olfactory-specific expression of AgOr3, AgOr4 and AgOr5 by means of *in situ* hybridization (ISH) but, in each case, obtained inconclusive staining patterns. This is not entirely surprising, given the low expression levels of DORs, of which a sizable subset (30%) are undetectable using ISH methods (Vosshall *et al*., 2000). Furthermore, in two studies involving ISH of candidate *Drosophila* taste receptor genes, only a small fraction was detected (Clyne *et al*., 2000; Scott *et al*., 2001). In light of these studies, it is likely that the expression levels of the three AgOR genes reported here are also beneath the detection threshold for ISH.

In addition, we have examined the expression of the AgOr3, AgOr4 and AgOr5 gene cluster during *A. gambiae* development. As shown in Figure 5, AgOr3 is first detectable in fourth instar larvae and thereafter is maintained into adult stages while AgOr4 is not expressed until reaching sexual maturity in 4-day-old adults. Lastly, AgOr5 is detectable in all stages from first instar larvae through sexually mature adulthood. Moreover, all three AgORs are expressed in both female and male adult mosquitoes.

It is important to note that non-quantitative RT-PCR was performed in these experiments. Therefore, any fluctuations in product amount may not be due to changes in gene expression, and conclusions to relative gene expression levels should not be drawn from these data. These experiments simply examine qualitative aspects of AgOR expression in the various developmental stages.

Discussion

In this study we have identified and characterized an additional gene, AgOr5, encoding a candidate OR from the malaria vector mosquito, *A. gambiae*. AgOr5 is highly

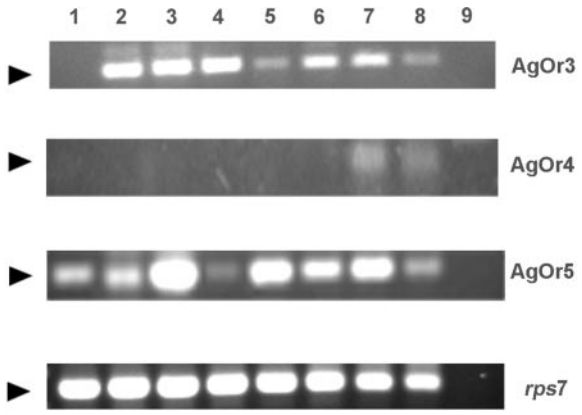

Figure 5 Developmental expression of AgOr3, AgOr4 and AgOr5. Reverse-transcription PCR was used to examine the developmental expression of AgOr3, AgOr4 and AgOr5. Lanes are: (1) first instar larvae, (2) fourth instar larvae, (3) early pupae, (4) late pupae, (5) 1-day-old (d.o.) female olfactory tissue, (6) 1 d.o. male olfactory tissue, (7) 4 d.o. female olfactory tissue, (8) 4 d.o. male olfactory tissue, (9) no template negative control. cDNA products are indicated by arrowheads. The sizes of the cDNA products are as follows: AgOr3: 309 bp; AgOr4: 450 bp; AgOr5: 561 bp; and *rps7*: 450 bp, amplified as a control.

similar to two previously identified (Fox *et al*., 2001) candidate OR genes, AgOr3 and AgOr4. Importantly, in keeping with the paradigm established for the other AgORs previously described (Fox *et al*., 2001), AgOr5 is similar to several DORs, displays approximately seven transmembrane domains, and is selectively expressed in olfactory tissues of the mosquito. In addition to these OR characteristics, AgOr5 contains a subset of introns whose positions are conserved in DORs and AgORs, and maintains the general conservation of protein length of ~400 amino acids that has been observed for AgORs and DORs. The fact that AgOr3, AgOr4 and AgOr5 are clustered within the *A. gambiae* genome is in keeping with the characterization of many ORs from mouse (Xie *et al*., 2000), *C. elegans* (Troemel *et al*., 1995) and *D. melanogaster* (Clyne *et al*., 1999; Vosshall *et al*., 1999). It is intriguing to speculate as to the underlying principle behind this apparent conservation of linkage among some ORs. In this regard, the ability (see below) to generate a large gene family such as the ORs through duplication events (that would tend to favor close chromosomal linkage) is an especially appealing rationale.

Phylogenetic analyses of several AgORs confidently groups AgORs 3, 4 and 5 together as a monophyletic lineage on the resulting tree. The monophyly of these chromosomally adjacent genes is consistent with their origin through two intra-chromosomal duplication events: the first resulting in AgOr4 and the ancestral gene copy of AgOr3 and AgOr5, and the second yielding the latter gene copies. The sequences and exon/intron structures of AgORs 3 and 5 further supports this scenario and suggest these duplications to be relatively recent events. The positions of two introns are absolutely conserved among all three AgORs. The

positions of the remaining three introns of AgOr3 and AgOr5 are also exactly conserved and their sequences are less divergent (35–50% identity; data not shown) than the two absolutely conserved introns (>25% identity; data not shown). The placement of AgOr4 between the more highly homologous AgOr3 and AgOr5 copies is suggestive of some role for recombination during or following the second duplication event, although the precise mechanism is not of central concern for the present investigation. It is also possible that additional AgORs will be found in this cluster pending the completion of the *A. gambiae* genome sequencing project.

AgOr3, AgOr4 and AgOr5 do not have readily apparent orthologs within the family of DORs (Figure 3). These three AgOR genes might therefore represent a class of receptors associated with behaviors that are unique to insects such as *A. gambiae.* It is intriguing to note that these behaviors include complex activities such as responses to ovipositional and host preference cues. Host preference cues for an anthropophilic mosquito such as *A. gambiae* are of critical importance for establishing the insect's vectorial capacity and might be expected to consist largely of human-specific odorants.

While our data show that AgOr3, AgOr4 and AgOr5 are detectable in both male and female olfactory tissues (Figure 5), there is little reason to preclude the possibility that these genes could play a role in sex-specific olfactory behaviors such as blood meal host preference selection that are present in hematophagous insects such as *A. gambiae.* In support of this, male mosquitoes have been shown to respond to vertebrate host-specific odorants in the vicinity of the host (Takken and Knols, 1999), probably to facilitate mating in proximity to host. Further study is required to determine the precise behaviors these AgORs underlie.

In a dramatic departure from *Drosophila* ORs where expression is restricted to pupal and adult stages, AgOr3 and AgOr5 display robust expression as early as the first larval instars. While the limited number of AgORs examined in this study makes it difficult to put this difference in developmental expression in precise biological context, it is nevertheless worthwhile noting the radically different life-cycles these two dipterans undergo. For example, in contrast to *D. melanogaster* where the pre-adult stages are entirely terrestrial, the equivalent stages of the *A. gambiae* life-cycle are aquatic. It is reasonable to speculate that such differences in environmental constraints might very well result in the utilization of AgORs in chemosensory-based behaviors at unique times relative to the academic model insect *D. melanogaster*.

Our studies indicate that AgOr3, AgOr4 and AgOr5 exhibit different developmental expression profiles during the mosquito's life cycle. This may be indicative of the presence of several unique regulatory sequences capable of directing distinct temporal expression within or close to the borders of this locus. Furthermore, the ability to detect distinct AgOR expression patterns during development is consistent with the hypothesis in which the presence of a particular OR or novel combinations of OR genes might be correlated with a unique set of behavioral objectives. For example, AgOr4 is only expressed in sexually mature (4 day old) adult mosquitoes, but not in immature (1 day old) mosquitoes. It is intriguing to speculate that AgOr4 may be involved in behaviors restricted to adults capable of mating, perhaps playing a role in mating itself. In addition to indicating the roles these AgORs might play in mosquito behavior, investigating the developmental expression of AgORs may lend insight into what types of odorants these putative ORs interact with, i.e. odorants that are specific for larvae, adults or both. Information regarding developmental expression may also indicate at which life stage potential antagonists or other novel treatments directed against particular AgORs might be applied within the context of olfaction-based mosquito control programs.

With the identification of five members of a family of candidate OR genes in *A. gambiae*, biochemical, behavioral and transgenic studies may now begin to determine the specific classes of odorant ligands that activate these receptors. Approximately 5% of the *A. gambiae* genome has been screened to date for the presence of AgORs. This leads to an estimate of ~100 AgORs, a number on the order of current estimates for DORs (*Drosophila* Receptor Nomenclature Committee, 2000). Identification and characterization of the whole family of AgORs may indicate potential mosquito attractants and/or repellants. Furthermore, comparative studies of putative ORs from hematophagous and non-hematophagous insects, as well as between anthropophilic and zoophilic species of Anopheline mosquitoes, may provide information concerning the molecular basis for host preference selection among these insects. This information could lead to novel disease-control strategies targeting vector–host interactions.

Acknowledgements

We acknowledge the work of Dr Hugh M. Robertson (University of Illinois at Urbana–Champaign) for his collaborative efforts to elucidate and characterize the AgOR gene family. We would like to thank Patricia Russell for mosquito care and C. Elaine Merrill for helpful discussions. Special thanks to Dr Daniel J. Funk (Vanderbilt University) for help with phylogenetic analysis and discussion. BAC clones were generously provided by Dr Frank Collins. This investigation received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR V30/181/208 to L.J.Z.) and by grants from the Division of Integrative Biology & Neuroscience of the National Science Foundation (IBN 0075338 to L.J.Z.) and the National Institute of Deafness and Other Communication Disorders (1 R01 DC04692-01 to L.J.Z.).

References

Buck, L. and **Axel, R.** (1991) *A novel mulitgene family may encode odorant receptors: a molecular basis for odor recognition*. Cell, 65, 175–187.

Clyne, P.J., Warr, C.G., Freeman, M.R., Lessing, D., Kim, J. and **Carlson, J.R.** (1999) *A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in* Drosophila*. Neuron, 22, 327–338.*

Clyne, P.J., Warr, C.G. and **Carlson, J.R.** (2000) *Candidate taste receptors in* Drosophila. Science, 287, 1830–1834.

Collins, F.H. and **Paskewitz, S.M.** (1995) *Malaria: current and future prospects for control*. Annu. Rev. Entomol., 40, 195–219.

***Drosophila* Odorant Receptor Nomenclature Committee** (2000) *A unified nomenclature system for the* Drosophila *odorant receptors*. Cell, 102, 145–146.

Eisenberg, D., Weiss, R.M. and **Terwilliger, T.C.** (1984) *The hydrophobic moment detects periodicity in protein hydrophobicity*. Proc. Natl Acad. Sci. USA, 81, 140–144.

Fox, A.N., Pitts, R.J., Robertson, H.M., Carlson, J.R. and **Zwiebel, L.J.** (2001) *Candidate odorant receptors from the malaria vector mosquito* Anopheles gambiae *and evidence of down-regulation in response to blood feeding*. Proc. Natl Acad. Sci. USA, 98, 14693–14697.

Gao, Q. and **Chess, A.** (1999) *Identification of candidate* Drosophila *olfactory receptors from genomic DNA sequence*. Genomics, 60, 31–39.

Hildebrand, J.G. and **Shepherd, G.M.** (1997) *Mechanisms of olfactory discrimination: converging evidence for common principles across phyla*. Annu. Rev. Neurosci., 20, 595–631.

Hopp, T.P. and **Woods, K.R.** (1981) *Prediction of protein antigenic determinants from amino acid sequences*. Proc. Natl Acad. Sci. USA, 78, 3824–3828.

Kyte, J. and **Doolittle, R.F.** (1982) *A simple method for displaying the hydropathic character of a protein*. J. Mol. Biol., 157, 105–132.

Marck, C. (1988) '*DNA Strider': a 'C' program for the fast analysis of DNA and protein sequences on the Apple Macintosh family of computers*. Nucleic Acids Res., 16, 1829–1836.

Mombaerts, P. (1999) *Molecular biology of odorant receptors in vertebrates*. Annu. Rev. Neurosci., 22, 487–509.

Ngai, J., Dowling, M.M., Buck, L., Axel, R. and **Chess, A.** (1993) *The family of genes encoding odorant receptors in the channel catfish*. Cell, 72, 657–666.

Riek, R.P., Handschumacher, M.D., Sung, S.S., Tan, M.J., Schluchter, M.D., Novotny, J. and **Graham, R.M.** (1995) *Evolutionary conservation of both the hydrophilic and hydrophobic nature of transmembrane residues*. J. Theor. Biol. 172, 245–258.

Salazar, C.E., Mills-Hamm, D., Kumar, V. and **Collins, F.H.** (1993) *Sequence of a cDNA from the mosquito* Anopheles gambiae *encoding a homologue of human ribosomal protein S7*. Nucleic Acids Res., 21, 4147.

Scott, K., Brady, R., Jr, Cravchick, A., Morozov, P., Rzhetsky, A., Zuker, C. and **Axel, R.** (2001) *A chemosensory gene family encoding candidate gustatory and olfactory receptors in* Drosophila. Cell, 104, 661–673.

Smith, D.P. (1999) Drosophila *odor receptors revealed*. Neuron, 22, 203–204.

Storkuhl, K.F. and **Kettler, R.** (2001) *Functional analysis of an olfactory receptor in* Drosophila melanogaster. Proc. Natl Acad. Sci. USA, 98, 9381–9385.

Swofford, D.L. (2001) PAUP*: Phylogenetic Analysis Using Parsimony (and Other Methods), version 4.0b4. Sinauer, Sunderland, MA.

Takken, W. (1991) *The role of olfaction in host-seeking of mosquitoes: a review*. Insect Sci. App., 12, 287–295.

Takken, W. and **Knols, B.G.** (1999) *Odor-mediated behavior of Afrotropical malaria mosquitoes*. Annu. Rev. Entomol., 44, 131–157.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and **Higgins, D.G.** (1997) *The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools*. Nucleic Acids Res., 24, 4876–4882.

Troemel, E.R., Chou, J.H., Dwyer, N.D., Colbert, H.A. and **Bargmann, C.I.** (1995) *Divergent seven transmembrane receptors are candidate chemosensory receptors in* C. elegans. Cell, 83, 207–218.

Vosshall, L.B., Amrein, H., Morozov, P.S., Rzhetsky, A. and **Axel, R.** (1999) *A spatial map of olfactory receptor expression in the* Drosophila *antenna*. Cell, 96, 725–736.

Vosshall, L.B., Wong, A.M. and **Axel, R.** (2000) *An olfactory sensory map in the fly brain*. Cell, 102, 147–159.

Wetzel, C.H., Oles, M., Wellerdieck, C., Kuczkowiak, M., Gisselmann, G. and **Hatt, H.** (1999) *Specificity and sensitivity of a human olfactory receptor functionally expressed in human embryonic kidney 293 cells and* Xenopus laevis *oocytes*. J. Neurosci., 19, 7426–7433.

Wetzel, C.H., Behrendt, H., Gisselmann, G., Storkuhl, K.F., Hovemann, B. and **Hatt, H.** (2001) *Functional expression and characterization of a* Drosophila *odorant receptor in a heterologous cell system*. Proc. Natl Acad. Sci. USA, 98, 9377–9380.

Xie, S.Y., Feinstein, P. and **Mombaerts, P.** (2000) *Characterization of a cluster comprising approximately 100 odorant receptor genes in mouse.* Mamm. Genome, 11, 1070–1078.

Zhang, Y., Chou, J.H., Bradley, J., Bargmann, C.I. and **Zinn, K.** (1997) *The* Caenorhabditis elegans *seven-transmembrane protein ODR-10 functions as an odorant receptor in mammalian cells*. Proc. Natl Acad. Sci. USA, 94, 12162–12167.

Zhao, H., Ivic, L., Otaki, J.M., Hashimoto, M., Mikoshiba, K. and **Firestein, S.** (1998) *Functional expression of a mammalian odorant receptor*. Science, 279, 237–242.

Accepted February 25, 2002