Parasitol Res (2002) 88: 1–8
DOI 10.1007/s004360100457

ORIGINAL PAPER

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Molecular characterisation and chromosomal mapping of transcripts having tissue-specific expression in the malaria mosquito *Anopheles gambiae*: possible involvement in visual or olfactory processes

Received: 2 May 2001 / Accepted: 11 May 2001 / Published online: 4 August 2001

Abstract We have compared the transcriptional activity of heads, antennae + palps, and carcasses in the mosquito *Anopheles gambiae* by means of differential display PCR (DD-PCR). Three transcripts specifically or preferentially expressed in the heads and in the antennae + palps have been selected. All are very similar to genes related to visual and olfactory mechanisms of several different organisms. They have been named *Ag* arrestin, *Ag* rLDL, and *Ag* dynamin. The potential of the DD-PCR technique in identifying genes involved in mosquito behaviour and the usefulness of the molecular characterisation of these transcripts are discussed.

Introduction

Mosquitoes (Diptera, Culicidae) use visual and olfactory cues in many important behaviours related to different aspects of their natural history, including, among others, finding blood and carbohydrate sources, oviposition sites and mates (Gibson and Torr 1999). Hosts providing blood for ovarian development are found by responsive females through a complex behavioural sequence of responses mediated by chemical properties of the host itself, including its output of carbon dioxide, sweat and skin volatiles, such as short-chain fatty acids (Knols et al. 1997; Costantini et al. 2000), L-lactic acid (Acree et al. 1968), 1-octen-3-ol (Kline et al. 1990), or ammonia (Geier et al. 1999), as well as its physical characteristics such as warm and humid convection currents emanating from the body (reviewed in Takken 1991). The importance of the olfactory information in the performance of behaviours related to host-seeking, landing, and biting is well exemplified by the observation that natural and synthetic repellent chemicals interfere with these mechanisms, thereby preventing mosquitoes from feeding on the treated skin (Davis 1985). Volatile chemicals providing behaviourally relevant information are perceived by olfactory receptors in sensilla found on the antennae and maxillary palps (Sutcliffe 1994). It has been estimated that ≥85% of flagellar neurons carry olfactory signals, underlining the crucial role played by olfaction in the perception of the mosquito environment (McIver 1982).

Mosquitoes generally have specialised breeding habits. Water for laying eggs usually releases odours conveying information on the suitability and trophic properties of the site for any given species (Bentley and Day 1989). For example, several *Culex* and *Aedes* species that breed in waters highly polluted with organic matter respond to compounds derived from bacterial decomposition such as, for example, 3–methylindole (Millar et al. 1992). Moreover, an aggregation pheromone released from the eggs of *Culex quinquefasciatus* has been identified and subsequently synthesised for vector control purposes (reviewed in Pickett and Woodcock 1996). Visual properties of the environment affect habitat selection (Bidlingmayer 1994) and mating (Charlwood and Jones 1980), and integrate chemical information to provide more specific responses. In this respect, the mosquito composite eye is involved in several important functions, among which are the perception of movement, light intensity, luminous reflectance, contrast, shape and patterns (Muir et al. 1992; Allan 1994; Gibson 1995).

Sibling species belonging to the *Anopheles gambiae* complex are important vectors of human malaria in sub-Saharan Africa. *A. gambiae* sensu stricto is distributed over much of the African continent, and it is the most anthropophilic and long-lived among the members of the complex, hence playing the major role in malaria transmission. In West African populations of this species, the analysis of the chromosomal polymorphism of paracentric inversions has led to differentiating several forms having at least limited genetic flow, which are

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adapted to specific ecological conditions (Coluzzi et al. 1979, 1985; Touré et al. 1998; and references therein). These chromosomal forms are identified by specific inversions arrangements: for example, homokaryotypic individuals for inversions *bc* or *u* on the R arm of chromosome 2 belong to the Mopti form. These taxa, whose exact systematic status is still under discussion, are of epidemiological significance because they extend the transmission potential of *A. gambiae* over space and time. In Mali and Burkina Faso, restriction fragment length polymorphisms (RLFPs) discriminate between Mopti and two other chromosomal forms, namely Bamako and Savanna (Favia et al. 1997), whereas in other areas and habitats the correspondence between molecular and chromosomal forms is more complex (della Torre et al., 2001). Analysis of molecular polymorphisms within *A. gambiae* is therefore expected to contribute to better defining of the population structure of this species and to selecting genetic markers that can be used in routine eco-epidemiological work.

The recognition that sustainable malaria control in Africa will presumably need the development of new vector control tools (Collins and Besansky 1994) has encouraged studies aimed at understanding the mechanisms and the genetic basis of such complex phenomena as the way hosts are found, as well as the genetic basis of the peculiar and specific intrinsic preferences of *A. gambiae* for humans. The molecular analysis of these phenomena can contribute substantially to selecting targets exploitable in transgenic vector technologies such as those proposed by Curtis (1994). In this paper, we have used the differential display PCR (DD-PCR) technique to look at gene transcripts that are preferentially or exclusively expressed in tissues involved in visual or olfactory processes with a view to identifying behaviorally significant genes. Among several that have been identified, we have selected and further characterised three of these transcripts which are here described; furthermore the potential of the DD-PCR technique is discussed.

Materials and methods

Mosquito strain

The *A. gambiae* strain used for molecular analyses and for cytogenetic preparations was the homokaryotypic GASUA reference strain (*Xag, 2R, 2La, 3R, 3L*).

RNA purification

mRNAs were isolated from tissues (antennae and palps, heads, carcasses) by Boehringer-Mannheim mRNA isolation kit following the manufacturer's instructions. mRNAs were at a final concentration of 25 ng/µl and 1 µl was used as template in DD-PCR and RT-PCR reactions.

Differential display PCR

DD-PCR experiments were performed following a previous protocol (Favia et al. 1996) with slight modifications. In this case, the Roche Titan One Tube RT-PCR System was used for the reverse and amplification reactions.

In all experiments performed, reverse transcription reaction and cDNA amplification was done at 45 °C.

Fingerprints were obtained by single-primers amplifications (50 pmol per reaction), sequences of the primers are listed below:

- AC5: 5′GGTTAGCTGGAGTGAGGA3′
- AC3: 5′CGCAGCAACCTCTTCGTCCC3′
- CU1: 5′GCGCCAGGTGGACGGGC3′
- CU4: 5′AACGGTATTCCGCTCGG3′
- SL1: 5′GGTTTAATTACCCAAGTTTGAG3′
- SG3: 5′CTGGACCGCAAGTTCCAG3′
- 005: 5′CCTGTTTCTGTGCGCCCCT3′
- 225: 5′CTGGCGGAGGGGCCGATT3′

Fragment purification

After analysis of the RNA fingerprints, all fragments looking differentially expressed have been excised from the agarose gel, purified with the Gibco BRL Rapid Gel Extraction System and cloned in the Promega pGem Tvector System.

Sequencing

Sequencing was performed by MWG-Biotech.

Nucleotide sequence data reported in this paper are available in the GenBank database under accession numbers:

- *Anopheles gambiae* partial mRNA for arrestin: AJ304409
- *Anopheles gambiae* partial mRNA for LDL receptor: AJ304411
- *Anopheles gambiae* partial mRNA for dynamin: AJ304412

Reverse transcription PCR

RT-PCR experiments were performed by the Roche Titan One Tube RT-PCR System. Reactions conditions wereas follows.

Ag arrestin

30 min at 52 °C, 2 min at 94 °C, then 30 cycles with the following scheme: 30 s at 94 °C, 30 s at 54 °C, 30 s at 68 °C followed by a single step of 7 min at 68 °C.

- Forward primer: 5′CTGTGCGCCCCTAATGG3′
- Reverse primer: 5′GCCCTGCTGCAGCGTG3′

The size of the expected fragment is 369 base pairs (bp).

Ag rLDL

30 min at 52 °C, 2 min at 94 °C, then 30 cycles with the following scheme: 30 s at 94 °C, 30 s at 54 °C, 30 s at 68 °C followed by a single step of 7 min at 68 °C.

- Forward primer: 5′CAGCTGTGCCTGCCCGA3′,
- Reverse primer: 5′GCGCCCCAGTCCGACC3′.

The size of the expected fragment is 486 bp.

Ag dynamin

30 min at 52 °C, 2 min at 94 °C, then 30 cycles with the following scheme: 30 s at 94 °C, 30 s at 60 °C, 30 s at 68 °C followed by a single step of 7 min at 68 °C.

- Forward primer: 5′GTCCCGGCGACACACGTTCGG3′
- Reverse primer: 5′GCAGCGCTTCCTTGCACGCG3′

The size of the expected fragment is 488 bp.

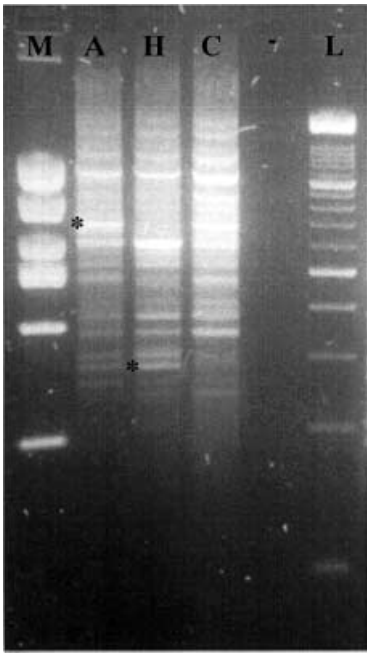

Fig. 1 Differential display PCR fingerprint with single primer: *asterisks* indicate fragments that are "differentially expressed". *M* Molecular weight marker (*Cy1LacZ* plasmid rectricted with *Hin*fIII), *A* antennae/palps complex, *H* heads, *C* carcasses – negative control (no template), *L* ladder 100 bp

In situ hybridisation

In situ hybridisations were performed according to previous protocols (della Torre et al. 1996).

Results and discussion

Our study was based on the application of DD-PCR to identify genes preferentially or specifically expressed in the antennae, palps, eyes and brain of adult *A. gambiae* sensu stricto mosquitoes, hence potentially involved in visual or olfactory mechanisms. The relative transcriptional activity of three tissues was compared: (1) the antennae and palps (hereafter antennae/palps complex); (2) the heads depleted of the antennae and palps; and (3) the remaining carcasses (Fig. 1). So far, we have identified three transcripts that sequence homology analyses (Altschul et al. 1997) indicate are part of the *A. gambiae* coding genes for arrestin, low-density lipoprotein receptor (rLDL), and dynamin (Figs. 2, 3, 4).

Fig. 2 **A** Sequence of the *Ag* arrestin fragment. **B** Comparison of the aminoacidic sequences of the *Ag* arrestin with the corresponding fragment of the *Heliothis virescens* arrestin (P55274)

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                                                                      54
5' CTG TTT CTG TGC GCC CCT AAT GGA AAG GTT ACG CTG TAC ATG GGC AAG CGT GAC
   L   F   L   C   A   P   N   G   K   V   T   L   Y   M   G   K   R   D

                                                                     108
   TTT GTA GAC CAC GTT TCC GGC GTT GAA CCG ATC GAT GGT ATC GTC GTC CTC GAT
   F   V   D   H   V   S   G   V   E   P   I   D   G   I   V   V   L   D

                                                                     162
   GAT GAG TAC ATT CGT GAC AAC CGT AAG GTA TTC GGT CAG ATT GTC TGC AGT TTC
   D   E   Y   I   R   D   N   R   K   V   F   G   Q   I   V   C   S   F

                                                                     216
   CGC TAC GGC CGC GAA GAG GAC GAG GTG ATG GGA CTA AAC TTC CAG AAG GAG TTA
   R   Y   G   R   E   E   D   E   V   M   G   L   N   F   Q   K   E   L

                                                                     270
   TGC CTC GCT TCC GAA CAG ATC TAC CCG CGT CCG GAA AAG TCG GAC AAG GAG CAG
   C   L   A   S   E   Q   I   Y   P   R   P   E   K   S   D   K   E   Q

                                                                     324
   ACC AAG CTC CAG GAG CGA CTG CTG AAG AAG CTG GGT TCG AAC GCC ATC CCG TTC
   T   K   L   Q   E   R   L   L   K   K   L   G   S   N   A   I   P   F

                                                                     378
   ACG TTC AAC ATC TCG CCG AAT GCT CCG TCT TCG GTC ACG CTG CAG CAG GGC GCA
   T   F   N   I   S   P   N   A   P   S   S   V   T   L   Q   Q   G   A

   CAG AAA CAG GA 3'
   Q   K   Q
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A

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H.virescens     MVYNFKVFKKCAPNGKITLYMAKRDFVDHISTVEPIDGVVLLDEEYVR-GRKVFGQMVCT 59
A.gambiae       --------LFLCAPNGKVTLYMGKRDFVDHVSGVEPIDGIVVLDDEYIRDNRKVFGQIVCS 53
                        :  ******:****.*******:* ******:*:**:**:* .******:**:

H.virescens     FRYGREEDEVMGLNFYKELFLASEQIYPPPEKRNYELSRTQERLIKKLGDGAIPFRLTVP 119
A.gambiae       FRYGREEDEVMGLNFQKELCLASEQIYPRPEKSDKEQTKLQERLLKKLGSNAIPFTFNIS 113
                ************** *** ******** *** : * :: ****:****..**** :.:.

H.virescens     PGAPGSVILQPGLEDDGEPCGVQYYVKIFVGDSEIDRSHRRSTVALGIRKVQYAPAKPGP 179
A.gambiae       PNAPSSVTLQQGAQKQ---------------------------------------------- 129
                *.**.** ** * :.:
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B

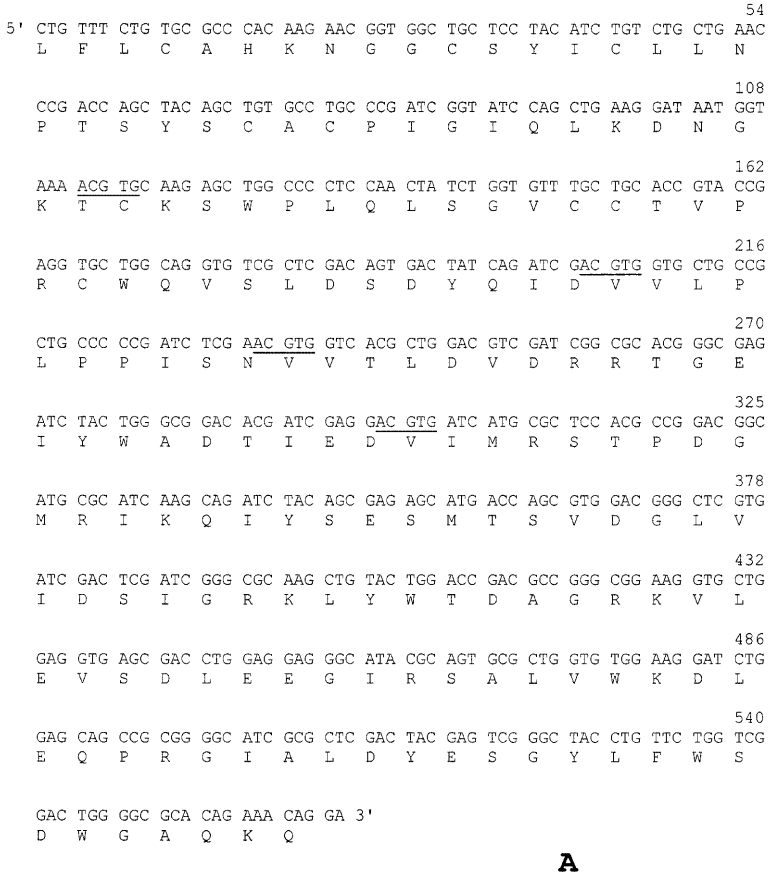

Fig. 3 **A** Sequence of the *Ag* rLDL fragment. The pentanucleotide 5′ACGTG3′ repeats are *underlined*. **B** Comparison of the aminoacidic sequences of the *Ag* rLDL with the corresponding fragment of the rLDL of *Mus musculus* (NP 032540)

Ag arrestin

The putative translational product of one of the identified transcripts shows a very high sequence similarity to several arrestins of different organisms and for this reason we have defined it as the *A. gambiae* (*Ag*) arrestin gene (Fig. 2A).

The arrestin protein works in the homologous or agonist-activated desensitisation of G-protein-coupled receptors. These receptors can operate the transduction of several extracellular signals as odours, light, hormones, neurotransmitters and neuromodulators. From a functional point of view, the arrestins can be classified as visual or non-visual. Our putative protein shows the maximum degree of similarity (BLAST *P* value = 6e–46) with a non-visual arrestin expressed in the antennae of *Heliothis virescens* (Raming et al. 1993) (Fig. 2B). The major arrestin in the *Drosophila* visual system, arrestin 2 (Arr2), is phosphorylated in a light-dependent manner by a Ca^{2+}/calmodulin-dependent protein kinase and has been shown in vivo to be essential for the termination of the visual signalling cascade. Recently, it has been proposed that phosphorylation of arrestin is necessary for the release of arrestin from rhodopsin, and that sequestering of arrestin to membranes is a possible mechanism for retinal disease associated with previously identified rhodopsin alleles in humans (Alloway and Dolph 1999). Dawson et al. (1993) showed that in the rat, *β*arrestin-2 plays a role as mediator of odorant-induced desensitisation showing that *β*arrestin-2 is enriched in the dendritic knobs and cilia of olfactory receptor neurons in the neuroepithelium, and that antibodies to it prevent desensitisation to odorants in isolated cilia. Localisation experiments revealed that

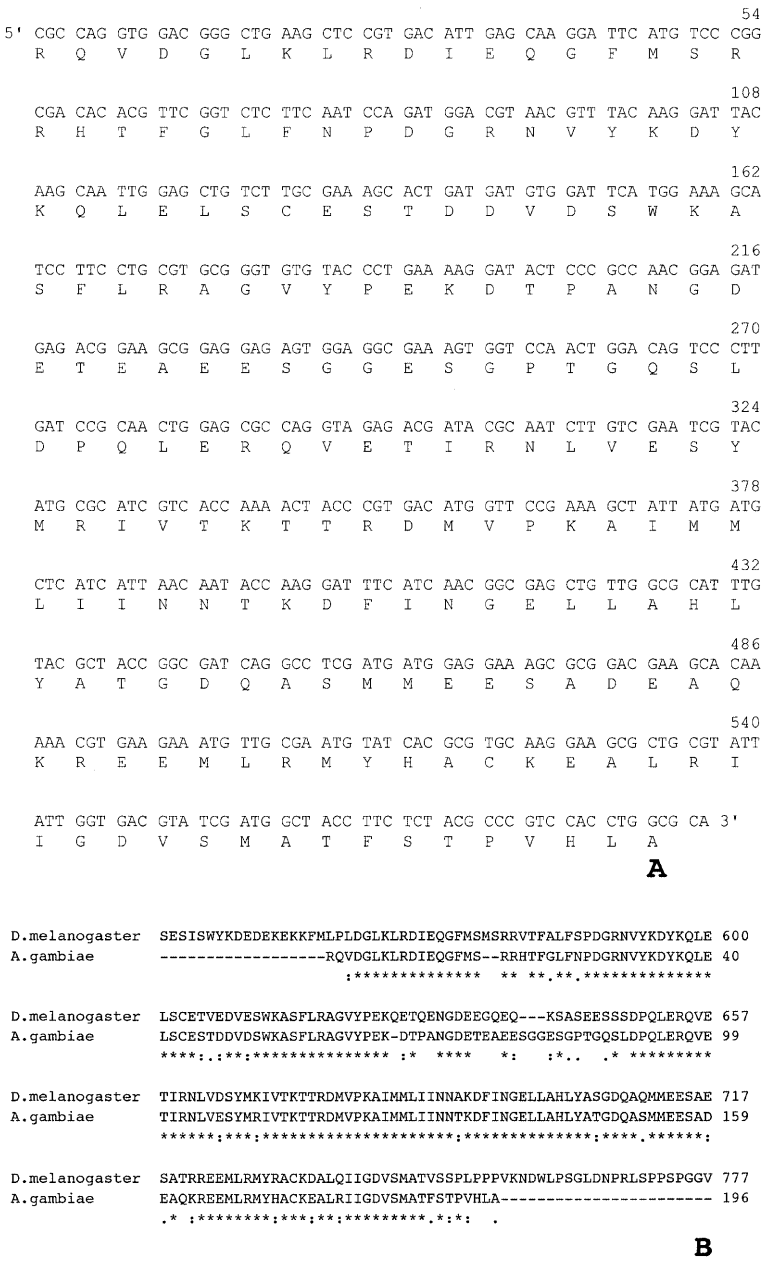

Fig. 4 **A** Sequence of the *Ag* dynamin fragment. **B** Comparison of the amino acid sequences of the *Ag* dynamin with the corresponding fragment of the arrestin of *Drosophila melanogaster* (S15413)

βarrestin-2 is present within the apical dendritic lobes of olfactory receptor neurons, as well as within the cell bodies that express high levels of the protein.

By RT-PCR, we revealed the expression of *Ag* arrestin in the antennae/palps complex, and in the heads, whereas no expression was detected in the carcasses (Fig. 5). The chromosomal localisation of the fragment has been determined by in situ hybridisation on polytene chromosomes on the 3R chromosome at division 36D.

Ag rLDL

A second fragment identified by DD-PCR, whose putative translational product shows a very high sequence similarity to several rLDLs of different organisms (BLAST *P* value = 8e–34), was defined as the *Ag* rLDL gene (Fig. 3). The LDL receptor gene family encompasses a class of endocytic receptors exhibiting structural similarities to the LDL receptor. LDL receptors are necessary for high-affinity uptake of lipids and proteins that are essential to cell structure and activity. Members of this gene family are present in both vertebrates and invertebrates. Lipoprotein receptors are not only interesting in systemic clearance of lipoproteins, but also in other important biological processes including reproduction, brain development and adipositas (Willnow 1999). Recently, these proteins have also been reported to be involved in signal transduction. The LDL receptor and one of its ligands, i.e., apoprotein E, are known to be synthesised in the central nervous

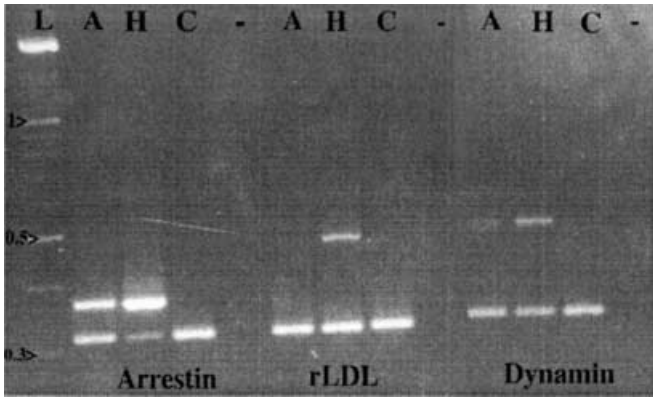

Fig. 5 RT-PCR expression patterns obtained with specific primers (for *Ag* arrestin, *Ag* rLDL, and *Ag* dynamin): *L* 100 bp ladder, *A* antennae/palps complex, *H* heads, *C* carcasses. The lower amplified bands in each panel were obtained with specific primers for the constitutive expressed *Anopheles gambiae* actin gene to evaluate the variation of expression among the different tissues. The fragments' size are expressed in kilobases

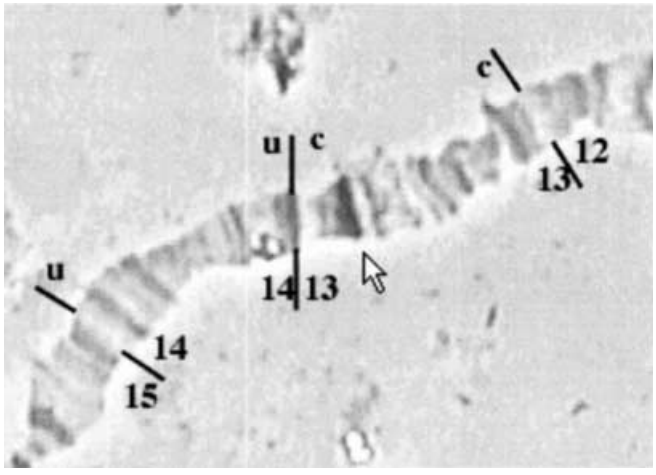

Fig. 6 In situ hybridised with *Ag* dynamin clone on the 2R polytene chromosome of *A. gambiae*. The *arrow* indicates the signal of hybridisation. Paracentric inversions and divisions are also indicated

system (CNS). LDL receptor activity has been demonstrated, among several other tissues, in retinal pigment epithelium (Hayes et al. 1989), and in human and monkey corneal endothelium (Elner et al. 1991). By RT-PCR we revealed that the *Ag* rLDL is expressed at a much higher level in the *A. gambiae* heads (Fig. 5). The nucleotidic sequence of the cDNA is characterised by the repeat 5′ACGTG3′ (Fig. 3). This repeat is present four times along the sequence, and it was identified in the CNS midline transcriptional enhancer of several genes of *Drosophila*. Germ-line transformation experiments established that the specific function of these repeats is to form the core of a specialised element required by the CNS midline transcription (Wharton et al. 1994). The CNS midline element is related to the mammalian xenobiotic response element, which regulates transcription of genes that metabolise aromatic hydrocarbons. The chromosomal localisation of the fragment has been determined by in situ hybridisation on the X chromosome at division 4C.

Ag dynamin

The third and last fragment identified by DD-PCR has been defined as the *Ag* dynamin on the basis of the high similarity (TBLAST N value = 1e-22) of its DNA sequence to that of the *shibire* gene of *Drosophila*, a gene coding for a dynamin protein. Moreover, its putative translational product shows a very high sequence similarity to several dynamins of different organisms (BLAST *P* value = 4e–74) (Fig. 4).

Dynamin is a neuronal phosphoprotein and a GTPase enzyme mediating late stages of endocytosis in both neural and non-neural cells. This protein is involved in many biological processes such as phosphorylation, protein–protein interactions, and phospholipid binding. Dynamin I is an isoform of the enzyme primarily located in the CNS and peripheral nervous system, where it is enriched in areas of abundant synaptic contacts, and is phosphorylated in vivo by protein kinase C and dephosphorylated on depolarisation and calcium influx into nerve terminals (McClure and Robinson 1996). From the *shibire* locus in *D. melanogaster*, six different isoforms of dynamin are generated by alternative splicing (Staples and Ramaswami 1999). By RT-PCR we revealed the preferential expression of the *Ag* dynamin in mosquito heads (Fig. 5). The chromosomal localisation of the fragment has been determined by in situ hybridisation on polytene chromosomes on the 2R chromosome at division 13F.

Summary and outlook

Despite recent progress in the molecular genetics of mosquito vectors, not much is known of the molecular mechanisms by which visual and olfactory stimuli can trigger a behavioural response. A molecular approach to clarify how mosquitoes respond to and process visual and olfactory inputs is much needed. By means of DD-PCR we attempted to identify transcripts that are specifically or preferentially expressed in selected tissues to isolate genes potentially involved in visual or olfactory processes of the major afro-tropical malaria vector *A. gambiae*. Our pilot experiment has led to the identification of three peptides which could play a significant role in those mechanisms. The potential of the technique in allowing large screening of tissue-specific transcripts indicates that a larger number of such transcripts can be identified and characterised. Cloning of the complete cDNA sequences of the three selected fragments and the determination of their patterns of expression in different sexes, life stages, physiological

states, and anatomical districts will contribute to defining the biological function of the corresponding proteins. Moreover, the localisation of *Ag* dynamin determined by in situ hybridisation on polytene chromosomes appears particularly interesting as the fragment maps at the 13F division of the 2R chromosome (Fig. 6) near the breakpoint of the 2R*c* inversion (Coluzzi et al. 1979). This localisation suggests that this fragment could be used as a suitable probe for genomic walking aimed at cloning the breakpoint of this inversion. The importance of sequencing this breakpoint stems from the role of the 2R*bc* arrangement as a diagnostic marker for the Mopti chromosomal form of *A. gambiae* in the Sudan savanna belt of West Africa, where a molecular marker could be usefully employed in routine eco-epidemiological work. The molecular characterisation of the corresponding full-length genes and proteins among different species of mosquito vectors and that of non-blood feeding insects should contribute to a better understanding of the behavioural properties of such genes. Given the great behavioural and ecological diversity of the member species of the *A. gambiae* complex, their comparative analysis for the pattern of expression of these transcripts could be a first step in this direction. As pointed out by Carlson (1996), in *Drosophila* the olfactory system overlaps and is supported to a large extent by the visual system: in this context a finer characterisation of the *A. gambiae* arrestins could be particularly meaningful.

Acknowledgements We wish to thank Professor Mario Coluzzi for his advice during the research, Dr. David Modiano, Dr. Stefano D'Amelio and Dr. Bruno Arca' for their helpful suggestions and critical reading of the manuscript and Dr. Alessandra della Torre for help with the figures.

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