A Hemocyte-like Cell Line Established from the Malaria Vector
Anopheles gambiae Expresses Six Prophenoloxidase Genes*

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Cell lines from the malaria vector Anopheles gambiae have been established as a tool for the study of the mosquito innate immune system in vitro. Here, we describe the first continuous insect cell line that produces prophenoloxidase (PPO). This cell line (4a-3B) expresses constitutively six PPO genes, three of which are novel (PPO4, PPO5, and PPO6). The PPO genes show distinct temporal expression profiles in the intact mosquito, spanning stages from the embryo to the adult in an overlapping manner. Transient induction of larva-specific PPO genes in blood-fed adult females suggests that the developmental hormone 20-hydroxyecdysone may be involved in PPO gene regulation. Indeed, exposure of 4a-3B cells to 20-hydroxyecdysone in culture results in in vitro induction of those PPO genes that are mainly expressed in early developmental stages, and repression of PPO5, which is preferentially expressed at the adult stage. The cell line shows bacteria-induced immune transcripts that encode defensin and Gram-negative bacteria-binding protein, but no induction of PPO transcripts. This cell line most likely derives from a hemocyte lineage, and represents an appropriate in vitro model for the study of the humoral and cellular immune defenses of A. gambiae.

Anopheles gambiae mosquitoes are the principle vectors of Plasmodium falciparum, the parasite causing the most severe form of human malaria. However, within the A. gambiae complex mosquitoes may differ in their efficiency of transmitting malaria (1). Transmission requires completion of a complex sporogonic cycle, which takes place over 2 weeks within the mosquito and can be aborted by innate immune responses, such as the encapsulation of early oocysts soon after invasion of the mosquito (2). Genes responsible for such refractory phenotypes would be candidates for inclusion in a vector control strategy based on genetically manipulated mosquitoes (3, 4). Vertebrate immune defense has been attributed to two general systems, innate and adaptive immunity, which are interconnected (5). In contrast, insect immune defense lacks the adaptive component and therefore an antibody-mediated immune response. Instead, it fully relies on innate immune mechanisms, such as the inducible synthesis of antimicrobial peptides, and the coagulation and melanization cascades (6, 7). The ongoing exploration of immune mechanisms in A. gambiae is hampered by the small size of this insect, limited knowledge of its genetics, and the absence of an efficient method for germine transformation. Insect cell lines were previously shown to exhibit immune properties, including the inducible synthesis of antibacterial peptides (8–10). Therefore, we have taken the approach of establishing A. gambiae cell lines as an in vitro system to analyze the immune mechanisms of the mosquito. We have reported that one such line expresses in an inducible manner a panel of immune marker genes (11).

In the course of establishing a larger variety of immune-responsive A. gambiae cell lines, we identified some that secrete prophenoloxidase (PPO)1 in the culture medium and adopted one such line, 4a-3B, as a standard. No continuous cell lines of this type have been reported previously in any insect. Phenoloxidases (POs) are enzymes that serve multiple tasks in insects, including cuticle pigmentation and sclerotization, wound healing and the melanotic encapsulation of protozoan (plasmodia) and metazoan (filaria) pathogens (12, 13). The POzymogens are known to be synthesized in hemocytes, the cellular component of the insect immune defense, and are activated by trypsin-like serine protease components of the PO activation system, also called the phenoloxidase cascade (see under “Discussion”). The PO cascade is inducible in vitro by microbial cell wall constituents and is considered part of the insect non-self recognition system (14, 15). PO catalyzes the hydroxylation of tyrosine to dihydroxyphenylalanine and the oxidation of dihydroxyphenylalanine and dopamine to their respective quinones, mediators of protein cross-linking, and precursors of the melanin polymer that is a central component of the melanotic capsule (16, 17). In addition, melanin synthesis leads to local increase of toxic quinones and free radicals (16).

Molecular cloning has yielded 12 PPO sequences to date, 1 crustacean, 6 lepidopteran, and 5 from diptera. All the sequences are closely related and show homology to hemocyanin, the arthropod oxygen transporter, which is functionally replaced in insects by the tracheal system. Using degenerate oligonucleotides and cDNA from the PPO producing A. gambiae cell lines as template, we amplified a PCR product consisting of a mixture of six distinct PO sequences. Three of these corresponded to PPO genes found in studies then in press (18, 19); the other three were novel and were designated PPO4, PPO5, and PPO6. Corresponding full-length cDNA clones were isolated and their sequences were determined. Reverse transcrip-

* This work was funded by grants from the John D. and Catherine T. MacArthur Foundation, the Human Frontiers Science Program, and the European Union Training and Mobility of Researchers Network on Insect-Parasite Interactions. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ Supported by a European Union Training and Mobility of Researchers postdoctoral fellowship.

§ Supported by a European Union Training and Mobility of Researchers Network on Insect-Parasite Interactions.

The abbreviations used are: PPO, prophenoloxidase; 20-HOE, 20-hydroxyecdysone; BAC, bacterial artificial chromosome; FCS, fetal calf serum; PBS, phosphate-buffered saline; PO, phenoloxidase; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR.

1 The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ010193 (PPO4; clone L46), AJ010194 (PPO5; clone A1) and AJ010195 (PPO6; clone A2).

The Journal of Biological Chemistry Vol. 274, No. 17, Issue of April 23, pp. 11727–11735, 1999
tion PCR (RT-PCR) expression analysis using specific primer pairs confirmed that all six PPO genes are expressed constitutively in the same cell line but show distinct expression profiles during mosquito development. Transcription of the genes that are larva-specific is transiently up-regulated in the adult female mosquito following a blood meal. The same genes are up-regulated when our standard cell line is exposed to 20-HOE. Interestingly, the only adult-specific PPO gene, PPO5, shows a converse behavior: it is repressed upon a blood meal in the mosquito and upon 20-HOE treatment of the cell line. Thus, this cell line is a useful in vitro model for the study of hormone-regulated as well as immune-regulated gene expression, phenomena that are frequently connected in insects.

**Experimental Procedures**

**Mosquito Rearing**—The *A. gambiae* strains 4a vr, L3-5, and Sua-koko 2La were maintained at 27 °C and 75% relative humidity on a 12-h light-dark cycle. Larvae were kept in deionized water supplemented with 0.1% marine salt and fed with dry cat food. Adult mosquitoes had access to 20% sucrose solution *ad libidum* and were blood-fed on a volunteer’s arm (H.-M. M.).

**Mosquito Perusions**—Schneider medium (Sigma) supplemented with 20% heat-inactivated FCS, 20 mM EGTA, and 10 mM thiourea was used to perfuse cell cultures. The conditioned medium was transferred in a 20-ml reaction volume of 15 μl. DNA synthesis was primed using the equivalent of 3 μl of oligo(dT)16, magnetic beads (Dynal), washed with distilled water before use. After incubation at 37 °C for 1 h with occasional suspension, reactions were terminated by heating to 95 °C for 5 min. Beads were rinsed several times and resuspended in 200 μl of distilled water. 1.5 μl of bead suspension was used in 20-μl PCRs containing 50 mM KCl, 1.5 mM MgCl2, 10 mM Tris-HCl, pH 8.3, 300 μM dNTP (Amersham Pharmacia Biotech), 0.05 μl of each [α-32P]dATP and [α-32P]dCTP (10 μCi/μl, 3000 Ci/mmol), 0.5 units Taq polymerase (Perkin-Elmer), and 20 pmol of each primer. PCRs were performed in a Perkin-Elmer thermal cyclor (GeneAmp 9600), 1 min at 63 °C, 1 min at 72 °C, and 1 min at 94 °C per cycle. Cycle numbers are indicated in the figure legends. 4 μl of each assay was run on 8% acrylamide gels. Dried gels were exposed on Kodak X-Omat AR films with Ilford Fast Tungstate screens. The ribosomal gene ST2 (22) served as internal control and was used for normalization as follows: the ST2 signals resulting from the initial PCRs of a given set of cDNA samples were quantified with a PhosphorImager (Molecular Dynamics), the ST2 values were equalized, and the template amounts were adjusted accordingly. The ST2 signals obtained from the second round of PCRs were again quantified by phosphorimaging and normalized against ST2 in order to fine-tune the amount of sample loaded on the final gel, resulting in deviations between any ST2 signal in a given experiment of no more than ±5%. The sequences of the primers used in RT-PCR experiments were as follows: ST2-A, 5'-GGCGGATCATCATCTAGCTGT-G-3'; ST2-B, 5'-GTATCGCTGCAATCTGGG-3'; Def-A, 5'-CTGTGCTCTCAGTGAGCACT-3'; Def-B, 5'-CAGACCTCTCTCCCCAGATG-3'; GBNB-A, 5'-GCACGCGAAGAATCTGTACC-3'; GBNB-B, 5'-TAACCACCCAGACCAAGGCG-3'; P01-A, 5'-TTCCATGCTCTTAAACCGGCAGCA-3'; P01-B, 5'-GCGGGATGGTTACCGTGTTACCA-3'; P01-A, 5'-TGCTTGCTGGAAGACCTAGTAAAAGA-3'; P02-B, 5'-CTGCCATACTAAGGTCGTCGGA-3'; P04-C, 5'-GTGCCGCCACGCGTGCGTCA-3'; P04-D, 5'-AGACCTAATTGCTCAGATTACTC-3'; P04-E, 5'-CTGAGTTTTATGTCGTACCAGCA-3'; P04-F, 5'-CTGACGTACGGCAGGCGAA-3'.

The resulting lengths of the corresponding PCR products were as follows: ST7, 460 bp; defensin, 404 bp; GN BP, 511 bp; PPO1, 358 bp; PPO2, 344 bp; PPO3, 343 bp; PPO4, 246 bp; PPO5, 346 bp; and PPO6, 349 bp. Except for PPO2, the primer combinations denoted above do not work on genomic DNA, e.g. on bacterial artificial chromosomes (BACs), as the polymorphic sequence block chosen for the design of the anti-sense primers turned out to span an intron/exon border that occurs in PPO1, which is obviously conserved in all PPO genes except PPO2.

**Preparation of Serum-free Cell Growth-conditioned Medium**—Cells were seeded in 75 cm2 tissue culture flasks (Greiner) containing 25 ml of Schneider medium/10% FCS. After having reached half confluency, cell layers were rinsed several times with protein-free medium (Insect-Xpress, BioWhittaker), 15 ml of protein-free medium was added, and cultures were grown for 2 more weeks at 27 °C. Cell growth was re-tarded in Insect-Xpress medium, although without any sign of deterioration of the cells. The conditioned medium was transferred in a dialysis bag and concentrated on polyethylene glycol 6000, 50,000 pow-der. After dialysis against 10 mM phosphate buffer, pH 6.0, prepara-tions were stored at -20 °C.

**Immune and Hormonal Stimulation of A. gambiae Cell Lines**—Cell cultures were immune-stimulated by adding to a confluent culture for 4 h a mixture of heat-inactivated Escherichia coli 11086 and Micrococcus luteus A270, 1000 bacteria per mosquito cell. 20-Hydroxyecdysone (Sigma) was added yielding a final concentration of 0.5 μg/ml (1 μl), with the ethanol concentration below 0.1%. For the wash-out experiment, 20-HOE-containing medium was aspirated, and cell layers were rinsed several times with Schneider medium before conditioned 20-HOE-free medium, taken from a culture of identical age, was added. Reverse Transcription-PCR Analysis—All molecular techniques were performed as described (21). Total RNA from cell lines and the various mosquito developmental stages was purified using the RNeals PLUS kit (bio101) according to the manufacturer’s instructions. First strand cDNA was synthesized using Moloney murine leukemia virus-reverse transcriptase and supplied solutions (Bethesda Research Laboratories, Inc.) with total RNA in a reaction volume of 15 μl. DNA synthesis was primed using the equivalent of 3 μl of oligo(dT)16, magnetic beads (Dynal), washed with distilled water before use. After incubation at 37 °C for 1 h with occasional suspension, reactions were terminated by heating to 95 °C for 5 min. Beads were rinsed several times and resuspended in 200 μl of distilled water. 1.5 μl of bead suspension was used in 20-μl PCRs containing 50 mM KCl, 1.5 mM MgCl2, 10 mM Tris-HCl, pH 8.3, 300 μM dNTP (Amersham Pharmacia Biotech), 0.05 μl of each [α-32P]dATP and [α-32P]dCTP (10 μCi/μl, 3000 Ci/mmol), 0.5 units Taq polymerase (Perkin-Elmer), and 20 pmol of each primer. PCRs were performed in a Perkin-Elmer thermal cyclor (GeneAmp 9600), 1 min at 63 °C, 1 min at 72 °C, and 1 min at 94 °C per cycle. Cycle numbers are indicated in the figure legends. 4 μl of each assay was run on 8% acrylamide gels. Dried gels were exposed on Kodak X-Omat AR films with Ilford Fast Tungstate screens. The ribosomal gene ST2 (22) served as internal control and was used for normalization as follows: the ST2 signals resulting from the initial PCRs of a given set of cDNA samples were quantified with a PhosphorImager (Molecular Dynamics), the ST2 values were equalized, and the template amounts were adjusted accordingly. The ST2 signals obtained from the second round of PCRs were again quantified by phosphorimaging and normalized against ST2 in order to fine-tune the amount of sample loaded on the final gel, resulting in deviations between any ST2 signal in a given experiment of no more than ±5%. The sequences of the primers used in RT-PCR experiments were as follows: ST7-A, 5'-GGCGGATCATCATCTAGCTGT-G-3'; ST7-B, 5'-GTATCGCTGCAATCTGGG-3'; Def-A, 5'-CTGTGCTCTCAGTGAGCACT-3'; Def-B, 5'-CAGACCTCTCTCCCCAGATG-3'; GBNB-A, 5'-GCACGCGAAGAATCTGTACC-3'; GBNB-B, 5'-TAACCACCCAGACCAAGGCG-3'; P01-A, 5'-TTCCATGCTCTTAAACCGGCAGCA-3'; P01-B, 5'-GCGGGATGGTTACCGTGTTACCA-3'; P04-C, 5'-GTGCCGCCACGCGTGCGTCA-3'; P04-D, 5'-AGACCTAATTGCTCAGATTACTC-3'; P04-E, 5'-CTGAGTTTTATGTCGTACCAGCA-3'; P04-F, 5'-CTGACGTACGGCAGGCGAA-3'.

**Screening of A. gambiae cDNA Libraries—Two lambda ZAP cDNA libraries were screened, one was constructed using mRNA isolated from fourth instar larvae of *A. gambiae* G3 (23) and one using mRNA isolated from abdomens of adult female *A. gambiae* Suakoko 2La mosquitoes (24). Both libraries were constructed using the ZAP Express system (Strategene). As probe, the gel-purified PCR product amplified from cDNA of cell line 4a vr using the primers PO-CuA and PO-CuB was enriched with gel-purified POPO4, POPO5, and POPO6 fragments and 32P-labeled via PCR. From a total of 700,000 plaques screened, 200,000 larval and 500,000 abdominal, approximately 60 positive signals were obtained in each library. Before starting the plaque purification procedure, the supernatants of the positive plaque zone of the primary screen were PCR-typed using the set of six specific PPO primers and identified PPO4, POPO5, and POPO6 signals were plaque purified via hybridization, followed by PCR typing.

**Cloning and Sequencing**—The 0.6-kb PCR product amplified from cell cDNA using the degenerate primers PO-CuA (5'-CAICA/TC/T/ GCGA/TC/TGGCGA/TC/CTTGG/GATC/TA/TC/CC/3') and PO-CuB (5'...
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CITGCCAICC(AG)TAAGAAIA(ACT)(CGG/AG/T/CIC/TC/GATCAT3')
(1 min at 94 °C, 1 min at 50 °C, and 1 min at 72 °C, 25 cycles) was
gel-purified and cloned using the TA Cloning Kit (Invitrogen). Plasmid
clones containing PPO cDNA inserts were obtained from the purified
lambda ZAP phage clones using the in vivo excision method according
to the manufacturer’s instructions (Stratagene). The sequenced inserts of
the cloned clones were determined by the EMBL sequencing facility.
The RGD sequence in PPO6 was confirmed by sequencing the corre-
gambiae PPO sequences; furthermore, the amino-terminal sequence of the resulting activated PO (EEATVVPDG) is conserved with only one substitution in A. gambiae PPO1. Cleavage sites at similar positions were determined experimentally in H. diomphalia PPO (31) and Pacifastacus leniusculus PPO (33), located three amino acids before and five amino acids following the M. domestica cleavage site, respectively. There is evidence that the sequential cleavage of both sites in H. diomphalia PPO is necessary for PPO activation (31). The two copper binding sites including the 6 histidine residues that interact with the copper atoms (34) are conserved. PPO2 is the only A. gambiae PPO that has both of the crucial residues in a conserved motif (gCgwQhm) corresponding to a potential thiol ester bridge; this motif was noted in the Manduca sexta PPO (now designated PPO-p2), B. mori PPO2, and Drosophila melanogaster PPO-A1 (29). Among all known PPO sequences, A. gambiae PPO6 is the only one showing the potential adhesive motif RGD.

We have used a library of large A. gambiae genomic DNA fragments (average length, 110 kb) in a BAC vector, kindly provided by Z. Ke and F. Collins, to isolate genomic PPO clones and map them to the polytene chromosomes of the mosquito. For each of the six PPO genes we identified BAC clones via PCR using gene-specific primer pairs specifically designed for this purpose.4

PPO4 and PPO5 were found to be physically linked on one BAC clone (28C11). Similarly, PPO3 and PPO6 were found to be physically linked on another BAC clone (27E19). Both BACs, and therefore PPO3, PPO4, PPO5, and PPO6, map to division 21B on the left arm of chromosome 2 (Fig. 2B). This result was confirmed by in situ hybridization of the corresponding cDNA clones of PPO4, PPO5, and PPO64 and is consistent with an independent mapping of PPO3 cDNA.5 PPO1 and PPO2 are not linked to the PPO3/PPO4/PPO5/PPO6 cluster. They were previously shown to map at different loci: PPO1 at division 13B (19) and PPO2 at division 24B.5

4 C. Blass, unpublished data.
5 M. Gorman, personal communication.
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Analysis of PPO Expression in the Cell Lines at the Level of RNA and Protein—We used RT-PCR analysis to examine the expression of immune markers and of the six PPOs in the cell lines, cultured in normal medium or together with a mixture of heat-killed Gram-positive and Gram-negative bacteria (Fig. 3A). Both lines are immune-inducible, because bacterial challenge significantly increases the signals corresponding to *A. gambiae* PPO genes. B. *subalbatus* and A. *gambiae* polytene chromosomes, of BAC clone 28C11 containing the PPO4 and PPO5 genes. BAC clone 27E19 containing the PPO3 and PPO6 genes yielded the same result; note the characteristic puff adjacent to division 21B (arrow) on the left arm of chromosome 2.

The various PPO sequences correspond to unprocessed polypeptides with calculated molecular masses ranging between 75.1 and 79.2 kDa and isoelectric points ranging between pH 6.1 and pH 6.9. We examined PPO expression in these cell lines at the protein level (Fig. 3B), using antisera generated against recombinant PPO4 and PPO6 fragments expressed in *E. coli*. Our antisera are specific for PPO, as indicated by the total absence of signal in immunoblot of cell line 4a-3A, but detect three major bands in line 4a-3B, centered at approximately 80 kDa, in the size range expected for prophenoloxidases. The same bands, but in different proportions, were found endogenously in hemolymph collected from pupae and female mosquitoes, respectively. A fourth band migrating around 75 kDa, visible in the cell line and pupal lanes, may be derived from proteolytic degradation or processing. Antibodies raised against PPO purified from *M. sexta* (18) and *B. mori* (36) cross-reacted, but detected fewer bands than were seen with the anti-PPO6 serum.3

Both the anti-PPO4 and the anti-PPO6 sera were equally suitable for immunofluorescence experiments, and gave results comparable with those obtained with antibodies against *B. mori* PPO.3 Fig. 4 shows an immunofluorescence experiment using anti-PPO4. The antisera (but not preimmune serum) stained specifically and intensely a subset of presumably differentiated cells (approximately 5%) in the PPO producing line 4a-3B. These cells showed variable morphology, permitting the detection of cell pairs, presumably reflecting relatively recent cell divisions. No staining was detected with the 4a-3A cell line.

Differential Expression of PPO Genes during Development—The expression of the six PPO genes at different developmental stages was assessed using RT-PCR, as shown in Fig. 5A. None of the genes were expressed in freshly laid eggs. Thereafter, different genes showed characteristic temporal profiles of expression. In broad terms and consistent with previous reports (18, 19), PPO1, PPO2, and PPO3 were mostly expressed in embryos and larvae; their expression also persisted in young pupae and, in the case of PPO2, in older pupae and adults as well, albeit at a low level. These genes are not expressed coordinately. For example, in postembryonic life, PPO1 transcripts were most prominent in fourth instar larvae and early pupae, whereas PPO3 expression was maximal in the second instar and declines thereafter. Of the novel genes, PPO4 was expressed in late larvae, young pupae, and adults, somewhat reminiscent of the PPO2 pattern. PPO5 and PPO6 are the only genes that were not transcribed in embryos but mainly in the pupal and adult stages. Relative to the rest, PPO5 was the most adult-specific gene; PPO5 was found with highest frequency among the positive PPO cDNA clones identified in the adult library (see above).

Induced Transcription of Larval PPO Genes in Response to Blood Feeding—Blood feeding has a tremendous impact on mosquito physiology, for example, stimulating digestive enzyme production and resulting in a transient increase in ecdysteroid concentration. The hormone 20-HOE is produced by the ovaries following the blood meal and stimulates the production of vitellogenin and other serum proteins by the fat body cells (37). Fig. 5B shows that the four PPO genes that are mainly expressed in the embryonic and larval stages, PPO1 through PPO4, were induced after a blood meal. Their transcripts show maximal accumulation 24 h after feeding, a decline toward the end of blood digestion at 48 h, and approximate basal levels 2 days later. In clear contrast, PPO6 shows no significant change after blood feeding, and PPO5 is transiently repressed at 24 h at the mRNA level.

20-Hydroxyecdysone Effects on PPO Expression in Cell Line 4a-3B—The transient induction of the larval PPOs following a blood meal suggested the possibility of hormonal regulation by the molting hormone 20-HOE. The experiment shown in Fig. 6 confirmed this supposition and revealed that the effects of the hormone on the cell line are gene-specific, paralleling the effects of a blood meal in the intact mosquito (Fig. 5B). The larval specific PPO4 gene shows strong in vitro induction that began by 8 h, peaked at 16 h, and was still detectable at 40 h after hormone addition; PPO1, PPO2, and PPO3 yielded similar results.3 PPO6 also showed induction that was unusually persistent. In contrast, the adult-specific PPO5 gene is repressed by hormone treatment as early as 2 h after hormone addition. The effects of the hormone were gradually reversed by removal of hormone from the medium (“washout experiment”). If exter-
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Fig. 3. PPO expression in cell line 4a-3B on RNA and protein levels. A, RT-PCR analysis of cell lines 4a-3A (3A) and 4a-3B (3B), nonstimulated (n) or immune-stimulated (i) with a mixture of heat-inactivated E. coli and M. luteus bacteria 4 h prior to RNA extraction. The primer pairs used were specific for the ribosomal protein S7 gene (rpS7-), (15 cycles), the immune-inducible genes defensin (def) (20 cycles) and Gram-negative bacteria-binding protein (GNBP) (20 cycles), and the six PPO genes (PPO1–PPO6) (20 cycles each). Radioactively labeled PCR products were separated on native 6% polyacrylamide gels. Note that the 4a-3B line expresses all six PPO sequences but that the 4a-3A line expresses no PPO transcript; both lines are immune inducible with respect to defensin and A. gambiae Gram-negative bacteria-binding protein. B, immunoblot analysis, using an antisera against PPO6, of proteins in serum-free medium conditioned by the growth of cell lines 4a-3A (3A) and 4a-3B (3B) and of hemolymph proteins obtained from A. gambiae pupae (Pu) and adult females (Ad). Proteins were separated under reducing conditions by 7% SDS-polyacrylamide gel electrophoresis. Samples loaded per lane corresponded to 50 μl of medium, the hemolymph of half a pupa or of one female mosquito. Molecular mass standards are indicated in kDa.

DISCUSSION

In the present report we have isolated three members of the PPO gene family in the most important malaria vector mosquito, A. gambiae; we have illuminated the evolutionary relationship and chromosomal arrangement of this gene family; we have documented the disparate developmental expression profiles of the six PPO genes and their differential regulation by the hormone 20-HOE; we have shown that they are expressed widely within the body, associated with expression in the hemocytes; and we have isolated stable PPO-expressing and nonexpressing cell lines, which provide an unprecedented tool for molecular fine analysis of gene regulation and enzyme activation in this important gene family.

Phenoloxidases are indeed of crucial importance for the physiology and defense reactions of insects and other arthropods. They are intimately related to the periodic production of an effective exoskeleton barrier during the life cycle, functioning in sclerotization and pigmentation of the new cuticle after each moult (41). They also function in wound healing by cross-linking and melanizing temporary barriers that are established at cut surfaces by hemolymph clotting (42). Phenoloxidase-associated local melanization is an important defense response of insects, which is triggered by non-self recognition and frequently is associated with physical encapsulation of invading parasites (13). In A. gambiae, melanotic encapsulation of young Plasmodium oocysts is a prime example of a refractoriness mechanism that can prevent completion of the malaria life cycle in the mosquito (2, 43). In general, the reactions set in motion by phenoloxidase activity result in chemical as well as physical protection, because the oxidations leading to melanin formation also generate free radicals and toxic quinone intermediate radicals (16). By the same token, the activity of these enzymes must be readily elicited but also finely regulated to avoid damage to the insect itself. Rapid local and fine regula-
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preimmune serum (recent cell divisions were marked. 4a-3B cells (the anti-PPO4 antibodies, detected by a fluorescein isothiocyanate-counterstained with DAPI and the corresponding cells recognized by that they are amplified by our gene-specific primers. 

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gambiae ers, we suspect that the six genes that we now know in total of 68 cDNA and PCR clones with PPO gene-specific prim-

phenoloxidase family. Based on the results of rescreening a 
sion and activation reactions reminiscent of those that control blood clotting (15). In sum, phenoloxidases serve multiple and fundamental mechanisms of insect developmental physiology, non-self recognition and defense, which are frequently interconnected. Nevertheless, relatively little is known at the molecular level about the mechanisms that regulate prophenoloxidase production and activation.

Full-length sequence analysis of a small number of prophenoloxidases from insects and a crayfish has only been achieved recently. The present work, together with the recent studies of Brey and co-workers (19) and Kanost and co-workers (18), make A. gambiae a favorite system for the study of the phenoloxidase family. Based on the results of rescreening a total of 68 cDNA and PCR clones with PPO gene-specific primers, we suspect that the six genes that we now know in A. gambiae may well constitute the complete phenoloxidase family, the only one that has been fully elucidated in any insect. If additional PPO genes exist, they are either nonexpressed, highly divergent in sequence, or so similar to the known genes that they are amplified by our gene-specific primers.

The dendrogram of the sequence relationships (Fig. 2A), which is rooted with the crayfish PPO sequence as outgroup, suggests that the anopheline PPO family has arisen by five gene reduplications. On the assumption of a constant molecular clock, and considering the present chromosomal locations of the genes, a plausible model of the evolution of this gene family is as follows. An ancient gene duplication and a subsequent gene dispersal gave rise to the ancestor of PPO1 (at chromosomal division 13B) and the common ancestor of the other five genes (at division 21B). In turn, the latter reduplicated without gene dispersal to give the ancestor of PPO4/PPO5, linked to the ancestor of PPO6/PPO3/PPO2. The latter underwent a third reduplication, and one of its products translocated to division 24B, ultimately becoming PPO2. Both genes remaining at 21B finally reduplicated in situ, with the fourth duplication giving rise to the PPO4 and PPO3 pair, and the fifth producing the PPO6 and PPO3 pair. As a result, division 21B currently consists of a four gene cluster of the composition (PPO4/PPO5)- (PPO6/PPO3/PPO2). The precise order and spacing of the genes in the cluster is currently unknown, but we do know that the most closely related genes are proximate neighbors. Even the most recent gene duplications were followed by regulatory diversification: the paired PPO4 and PPO5 genes (72.2% identical at the protein level) differ substantially in terms of developmental specificity, as do the paired genes PPO3 and PPO6 (81.8% identical). Highly specific antibodies that discriminate individual members of the family will be required to study the tissue distribution of individual genes and to facilitate further analysis of their regulation.

Complete recovery, sequence determination and expression analysis of the PPO families in additional insects will be required to determine when the gene duplications, and the regulatory diversification, occurred relative to the phylogeny of insects. Currently, we know two widely diverged PPO genes in each of the three moths, B. mori, M. sexta, and Hyphantria
In each case, one of the genes is located on the same branch of the dendrogram as \textit{PPO1} of \textit{A. gambiae}, suggesting that at least the first of the postulated duplications, giving rise to \textit{PPO1}, antedates the last common ancestor of diptera and lepidoptera. At the other extreme, the sequence similarity of \textit{PPO6} and \textit{PPO3} to the presently known \textit{PPO} gene of the mosquito \textit{Armigeres subalbatus} (a culicine mosquito belonging to the tribe Aedini, like \textit{Aedes}) indicates that the third postulated reduplication occurred prior to the separation of anophelines and culicines. It is likely that the second model mosquito, \textit{Aedes aegypti}, will prove to have several \textit{PPO} genes as well.

In crustaceans and insects, \textit{PPO} is synthesized in the hemocytes (33, 44–47). In lepidoptera, it has been shown at the RNA and protein levels that insect \textit{PPO} is synthesized in a specialized type of hemocytes, the oenocytes (44, 46). In contrast to crustaceans, \textit{PPO} is already present in the cell-free plasma of noninfected insects (47, 48), and in the case of \textit{B. mori}, it has been shown that \textit{PPO} is transported to the cuticle (42). In the dipteran species \textit{D. melanogaster}, the components responsible for melanin synthesis are located in the crystal cells (49). However, within the family Culicidae (mosquitoes), no general classification of hemocyte types has been achieved as yet (39, 50, 51). According to a recent analysis of the \textit{domino} mutation in \textit{D. melanogaster} (52), secondary sites of \textit{PPO} synthesis cannot be ruled out. Indeed, in \textit{Anopheles} \textit{PPO} was localized in the midgut (36, 53) and the salivary glands (36). Whether these epithelial organs are minor sites of \textit{PPO} synthesis or take up \textit{PPO} synthesized in hemocytes remains to be determined. The low level of \textit{PPO} RNA detected in the \textit{A. gambiae} midgut, relatively enriched in \textit{PPO1} RNA (Fig. 5A), is supportive of the first alternative.

\textit{In A. gambiae}, our perfusion studies have shown the presence of \textit{PPO} in hemocytes but not fat body cells. The wide distribution of transcripts of each \textit{PPO} gene in head, and thoracic and abdominal body parts is consistent with the current view that these genes are largely expressed in the widely dispersed hemocytes. Future \textit{in situ} hybridizations and immunofluorescence studies (with gene-specific antibodies that are not yet available), should reveal all the sites of \textit{PPO} synthesis and...
protein localization, complementing in the spatial dimension our current knowledge of temporally specific expression of individual genes. It will be interesting to know whether PPO gene transcription or translation in the mosquito is immune-responsive at any time during development or whether PPO regulation in response to immune challenge only engages the activators and inhibitors of the phenoloxidase cascade.

The two A. gambiae cell lines that we have described here, which were generated from the same mosquito strain, show intriguing properties. Both of these cell lines are immune-responsive, in terms of induction of the defense and Gram-negative bacteria-binding protein genes after challenge by bacterial constituents. Therefore, both lines presumably possess pattern recognition receptors and the capacity to respond to activation of these receptors. However, one of the lines (4a-3A) is incapable of expressing the phenoloxidase genes, whereas the other (4a-3B) expresses all six of these genes in a constitutive manner. In the latter line, PPO transcript levels are unaffected or decrease slightly after bacterial challenge. In contrast, these levels are strongly regulated by 20-HOE in a reversible and gene-specific manner. Immunofluorescence analysis detects PPO protein in only a subset of approximately 5% of the cells in this line; the positive cells tend to be large and morphologically variable. Yet PPO expression is a stable property of this cell line, and the PPO-positive cells are frequently found in morphologically similar pairs, suggesting recent cell divisions. Our current interpretation is that the positive cells represent a differentiated state, in which infrequent cell divisions continue. This inference will need to be confirmed by cloning the cell line and subsequently investigating what exogenous signals might lead nonexpressing cell progenitors to turn on the PPO genes. It will be interesting to investigate whether all PPO genes can be turned on simultaneously or whether cell differentiation might recapitulate the developmental succession of PPO genes in the whole animal. To date, we only know that the various PPO transcripts in the cell line are subject to hormonal regulation, in a gene-specific manner that is related to their presumed hormonal regulation in the intact adult female mosquito, following a blood meal. Clearly, these unique cell lines are highly promising tools for the study of phenoloxidase regulation in insects.

Acknowledgments—We thank P. Brey for making available the PPO1 cDNA sequence and M. Kanost for making available the PPO2 and PPO3 cDNA sequences prior to publication. We thank M. Ashida and M. Kanost for providing antibodies against B. mori PPO and M. sexta PPO, respectively. We are grateful to F. Collins and Z. Ko for providing the A. gambiae BAC library and to M. Gorman and M. Kanost for sharing the mapping data for PPO2 and PPO3.

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