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Molecular & Biochemical Parasitology 142 (2005) 76–87
MOLECULAR & BIOCHEMICAL PARASITOLOGY

Transcriptome analysis of *Anopheles stephensi–Plasmodium berghei* interactions

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Received 28 December 2004; received in revised form 23 February 2005; accepted 25 February 2005
Available online 12 April 2005

Abstract

Simultaneous microarray-based transcription analysis of 4987 *Anopheles stephensi* midgut and *Plasmodium berghei* infection stage specific cDNAs was done at seven successive time points: 6, 20 and 40 h, and 4, 8, 14 and 20 days after ingestion of malaria infected blood. The study reveals the molecular components of several *Anopheles* processes relating to blood digestion, midgut expansion and response to *Plasmodium*-infected blood such as digestive enzymes, transporters, cytoskeletal and structural components and stress and immune responsive factors. In parallel, the analysis provide detailed expression patterns of *Plasmodium* genes encoding essential developmental and metabolic factors and proteins implicated in interaction with the mosquito vector and vertebrate host such as kinases, transcription and translational factors, cytoskeletal components and a variety of surface proteins, some of which are potent vaccine targets. Temporal correlation between transcription profiles of both organisms identifies putative gene clusters of interacting processes, such as *Plasmodium* invasion of the midgut epithelium, *Anopheles* immune responses to *Plasmodium* infection, and apoptosis and expulsion of invaded midgut cells from the epithelium. Intriguing transcription patterns for highly variable *Plasmodium* surface antigens may indicate parasite strategies to avoid recognition by the mosquito's immune surveillance system.
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Keywords: *Plasmodium*; *Anopheles*; Gene expression

1. Introduction

Plasmodium, the causative agent of malaria, exploits the female mosquito's need for blood to spread among human hosts. Sets of different digestive enzymes, transporters, structural components, metabolic enzymes and developmental factors are required by the mosquito vector to process the blood and produce eggs. The presence of *Plasmodium* poses additional challenges for the mosquito and triggers transcriptional programs responsible for immune and stress response, apoptosis, tissue healing and other physiological systems that are affected by infected blood components [1,2]. *Plasmodium*

Abbreviations: CSP, circumsporozoite protein; CTRP, circumsporozoite- and TRAP-related protein; EST, expressed sequence tag; NOS, nitric oxide synthase; Nramp, natural resistance-associated macrophage protein; RT-PCR, reverse transcription-polymerase chain reaction; TRAP, thrombospondin-related adhesive protein; WARP, von Willebrand factor A domain-related protein

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doi:10.1016/j.molbiopara.2005.02.013

undergoes a complex life cycle in the mosquito and the temporal accuracy of its gene regulatory program is crucial to avoid exposure of the sensitive parasite stages to the hostile environment of the midgut lumen and the mosquito's immune responses in the epithelium and hemocoel [1].

Malaria development in the mosquito is initiated by gametocyte activation within the first 30 min of blood ingestion, followed by fertilization that leads to the formation of a zygote at approximately 2 h. The zygote transforms into a motile ookinete that traverses the peritrophic matrix using a chitinase that may be activated by a mosquito digestive protease. After entering the ectoperitrophic space approximately 15 h after ingestion, the parasite attaches to, invades and traverses the midgut epithelium. Invasion of the midgut by the rodent malaria parasite *Plasmodium berghei* peaks at around 30 h after infected blood ingestion and proceeds up to 2 days. Large numbers of parasites are killed prior to and during invasion of the midgut epithelium. Invaded midgut cells will undergo apoptosis and finally be expelled from the epithelium. On the basal side the ookinete develops, within a period of approximately 12–20 days, into a mature oocyst that produces thousands of sporozoites [1,3]. Upon the rupturing of oocysts, which is asynchronous and can take several days, sporozoites are dispersed throughout the hemocoel from where they will invade the salivary glands prior to injection into a new host.

Our knowledge of *Anopheles* responses to malaria infected blood and *Plasmodium's* development in the mosquito has experienced a remarkable expansion in the past decade mostly through individual gene analyses for both organisms. Recent gene discovery projects based on subtraction cDNA libraries that were enriched with both *Anopheles stephensi* and *P. berghei* infection stage specific genes, identified numerous new transcripts that are crucial for the interactions between the two organisms [4,5]. However, subtractive hybridization approaches cannot provide accurate temporal transcription patterns of genes across several consecutive time-points, and expression stage specificities of selected genes must therefore be determined by other methods.

To extend our understanding of *Anopheles–Plasmodium* interactions, we have simultaneously assayed gene expression of *A. stephensi* midguts and *P. berghei* at seven time-points after mosquitoes fed on a *P. berghei* infected mouse. Analyses were done with microarrays made from EST clones of subtraction libraries enriched for *Plasmodium* genes expressed in *Anopheles,* and *Anopheles* genes induced by *Plasmodium* infected blood. The analyses reveal processes of *Plasmodium* development, blood digestion and the mosquito's response to infection.

2. Material and methods

2.1. Mosquito rearing and infections

The *A. stephensi* SD500 strain was raised at 28 °C, 75% humidity, under a 12 h light/dark cycle, and maintained on a 10% sucrose solution during adult stages. Female mosquitoes were blood-fed on anaesthetised BALB/c mice. For malaria infections 4-day-old female mosquitoes were fed on anaesthetised BALB/c mice which had been infected with *P. berghei* 4 days previously, and were assayed for high levels of parasitemia and the presence of microgametocytes capable of exflagellation. The mosquitoes were maintained thereafter at 19 °C prior to dissection and extraction of midgut RNAs.

2.2. Dissections and RNA extraction

Midguts were dissected on ice in PBS (0.6 mM $MgCl_2$, 4 mM KCl, 1.8 mM $NaHCO_3$, 150 mM NaCl, 25 mM HEPES, 1.7 mM $CaCl_2$, pH 7) and were immediately frozen on dry ice. Total RNA was prepared from dissected tissues and intact animals using the RNeasy kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions.

2.3. DNA microarray construction

The EST clones that were used for microarray probe preparation originated from four previously described subtraction libraries that represented the transcriptomes of the ookinete stage *P. berghei* (ookinete library: 1–12 h) and the early infected stage (early library: 24–52 h after ingestion of infected blood), intermediate oocyst stage (middle infected midgut library: 4, 6 and 8 days after ingestion of infected blood) and late oocyst stage (infected midgut library: 10, 12 and 14 days after ingestion of infected blood) infected *A. stephensi* midgut and *P. berghei* transcripts [4,5]. Subtraction of cDNAs was done using the Clonetech PCR select subtraction kit (cat. # K1804-1) and the subtracted PCR amplified cDNA fragments were cloned in a pGEMT-easy (Promega) vector prior to transformation into DH5α *Escherichia coli*. The ookinete subtraction library was constructed by subtracting cDNAs from four ookinete stages (6, 12, 18 and 24 h) against blood stage *P. berghei* cDNAs. The early subtraction library was produced by subtracting cDNA from infected midguts at 24, 36 and 52 h after ingestion against cDNAs of blood stage *P. berghei* and cDNAs of non-infected blood fed guts at 24 h after ingestion. The middle subtraction library (representing cDNAs from 4, 6 and 8 days after ingesting infected blood) was subtracted against blood stage *P. berghei* cDNA and cDNAs of non-fed guts, and 24 and 48 h, and 4 days non-infected blood fed guts. To ensure high degree of stage specificity, the middle library had also been subtracted against cDNAs of 24 and 48 h infected guts. The late subtraction library (representing cDNAs from 10, 12 and 14 days after ingesting infected blood) was subtracted against the same cDNAs the middle subtraction library and in addition against cDNAs of infected guts at 4, 6 and 8 days after ingestion. Detailed description of these libraries is provided in previously published work [4,5]. Probes for spotting were amplified from 4987 EST clone bacterial cultures through a two-step PCR amplification with amino-modified T3 and

T7 primers. PCR products were verified through agarose gel electrophoresis. Ethanol-precipitated PCR products were re-suspended in ArrayIt Microspotting solution (Telechem International, Sunnyvale, CA) and spotted on aminosilane-coated glass slides with the Omnigrid microarray spotter (GeneMachines, San Carlos, CA). DNA was cross-linked to the slides by incubation at 60 °C for 3 h and 100 °C for 10 min.

2.4. DNA target preparation and microarray hybridizations

Complementary mRNA (cmRNA) was synthesized with the Ambion MEGAscript T7 RNA synthesis kit (Ambion, Austin, TX) from double-stranded cDNA primed with an oligo d(T)-T7 promoter sequence. Complementary cDNA targets were synthesized and labelled from the cmRNA by incorporation of Cy-3-dUTP and Cy-5-dUTP fluorescent nucleotides through a random primed first-strand reverse-transcription reaction. After removal of unincorporated dNTPs with a Qiagen PCR purification kit (Qiagen, Chatsworth, CA), the targets were combined, lyophilized and re-suspended in hybridization buffer containing 50% formamide, 6× SSC, 0.5% SDS, 5× Denhardt's reagent and 0.5 mg/ml poly(A) DNA. Arrays were pre-hybridized in 6× SSC, 0.5% SDS, and 1% (v/v) BSA at 42 °C for 90 min, hybridized overnight at 42 °C in humidified hybridization chambers, washed twice in 0.1× SSC, 0.1% SDS (30 min), twice in 0.1× SSC (15 min), rinsed with de-ionized H_2O and dried. To assay both *A. stephensi* and *P. berghei* gene expression during the course of infection, Cy-5 labelled cDNA targets made from midgut RNAs of at least 50 dissected mosquitoes at each time point (6, 20 and 40 h, and 4, 8, 14 and 20 days) after feeding on a *P. berghei* infected mouse were hybridized against a Cy-3 labelled reference target made from a pool of an equal quantity of infected midgut RNA from each time point. Four replica experiments corresponding to two independent biological assays (infections), of all analyzed time points, that were hybridized twice to independent microarrays were done.

2.5. DNA microarray analysis

Intensities of hybridized targets were estimated with a GenePix 4000b (AXON Instruments, Foster City, CA) semi-confocal microarray scanner and software. Spots that were poorly hybridized or were covered with hybridization artifacts were removed from the analysis through manual inspection. Spots considered for analysis had median signal to background ratio >3, median background intensity <500 and median signal to noise ratio >3, or showed exceptionally high intensity in one sample. For expression ratio (Cy5/Cy3) normalization, a total intensity normalization method was used; the estimated background corrected ratio of medians was multiplied against the ratio of medians normalization factor for each array (estimated by the GenePix Pro software). The normalized ratio of each clone was then log 2 transformed. To generate the final data set, log 2 transformed normalized ratios of spots corresponding to the same clone and gene were averaged using the GEPAS pre-processing tools. Inconsistent replicates that deviated from the median of replicates by more than a two-fold were not considered for averaging [6,7] (Supplementary data, Table S1). Clustering analysis and graphic presentations were performed using the CLUSTER and TREEVIEW software (available at http://rana.lbl.gov/EisenSoftware.htm) [8].

2.6. EST sequence analysis

Of the 4987 spotted clones, 1273 had been previously sequenced through a random selection of clones and 400 clones were selected for sequencing based on their expression specificity; clones with highly specific expression patterns across only one or two time points were selected. Sequenced clones were classified as *A. stephensi* or *P. berghei* according to their GC content and identity to known genes and genomic sequences [4,5]. Species determination of clones was also done through competitive hybridizations between targets made from a mixture of *P. berghei* blood stage and ookinete stage RNA, and targets made from unfed female *A. stephensi* RNA. In a separate experiment, hybridizations were done between targets made from genomic DNA of *A. stephensi* and *P. berghei* [8,9]. All transcripts originating from the ookinete library were assigned as *P. berghei* specific. Of the total, 3761 clones could be assigned to a species through these criteria and assays, while the remaining 1223 clones, most of which had not been sequenced, did not generate adequate hybridization data for species assignment. Clone inserts sharing a greater than 97% identical nucleotide sequence over at least a 100 bp stretch were assigned to the same sequence clone cluster; this analysis generated 1109 unique sequence clone clusters from the 1673 sequenced genes, suggesting an overall redundancy level of about 66% for the four libraries together. Genbank EST identifiers for sequenced clones are indicated in Supplementary data, Table S3.

2.7. RT-PCR analysis

To assess the accuracy of the microarray assays, at a semi-quantitative level, the diverse expression patterns of five *A. stephensi* genes were independently verified with RT-PCR assays using gene specific primers as previously described (Fig. 2) [10]. The following primer pairs were used: L433/peritrophin A: TTAAAGATAGAAACCAACCTG, B: GCGGCCGAGGTAATTTTT; M855/serine protease A: GTACGAGATCGTCGCAGGT, B: CGGGCAGGTACTATCGAC; M1012/alkaline phosphatase A: GTACGTGCTGTTTGTTGAA, B: ATGTGTACACGCGTCGAT; E517/caspase A: TAATTTCCTCGTCGGTCGTC, B: AAATTCGTATGCCACCTTGC; E65/G12 precursor A: ACTTCAGCCGAGCTCTCGTA, B: GGGCAGGTCGCTAATATTGT.

3. Results

3.1. Complexity and expression specificity of subtraction library clones

The specificity of microarray assays, the library subtraction efficiency and expression patterns of the spotted clones are apparent through clustering expression data of genes represented by the spotted cDNAs (Fig. 1). The majority of assayed subtraction library clones have previously been shown to represent transcripts that are expressed at higher levels in mosquitoes fed on *P. berghei* infected blood and the microarray hybridization strategy provide information on their temporal expression specificity after ingestion of infected blood at several successive time points ([4,5]; see Section 2). Two thousand and eight hundred fifty genes (57% of the 4987 total) are differentially regulated between two or more time points by at least a two-fold (Fig. 1). Approximately half (1215) of these are *P. berghei* transcripts present at the early stages (mostly 6–20 h in expression cluster 1, Fig. 1) after ingestion of infected blood, and derived predominantly from the ookinete stage and early infection stage midgut libraries. The remaining transcripts are mainly *A. stephensi* specific for stages between 20 h and 20 days after ingestion of infected blood, and derived from the middle and late stage libraries (cluster 2, Fig. 1). The remaining 2135 clones represent genes for which our assays did not detect differential expression greater than two-fold between the assayed time points. It is, however, possible that a large fraction of these remaining genes posses significant regulation which is not detectable with the microarray assays due to the hybridization strategy (see Section 2) that tend to suppress the assayed magnitude of differential transcription for genes with elevated expression across several time points. The strong correlation between assayed expression patterns and subtraction library origin of spotted probes (clones) confirms the robustness of the microarray assays and the high degree of specificity of the subtraction procedure that was used for library construction (Fig. 1) [4,5]. Furthermore, the microarray expression data of numerous *A. stephensi* and *P. berghei* genes correlate reproducibly with previously presented expression assays. Examples are mosquito serine proteases the G12 precursor transcripts, a serpin gene and maltase transcripts (Fig. 3) [2,4,5]. For *Plasmodium*, the *myosin A* gene, the ookinete specific *PbS28* and *PbS25* genes, as well as the sporozoite specific *CSP* and *TRAP* genes all confirm accurate microarray-based transcription profiling (discussed below, Fig. 3) [12–15]. To further assess the accuracy of expression assays, selected genes were subjected to semi quantitative RT-PCR assays that confirmed the microarray-assayed patterns (Fig. 2). Stage specificity of the *A. stephensi* EST collection is relatively evenly distributed with a somewhat lower representation of genes expressed at the 6 h time point (Fig. 1). This is most likely due to the selected stages from which the early library was constructed, between 24 and 52 h after ingestion of infected blood [5]. The lower representation of mosquito genes in the early stage library may also reflect a low transcriptional activity of *A. stephensi* genes immediately after blood feeding. In contrast to *A. stephensi*, the great majority of *P. berghei* ESTs are specific for the early time points of 6 and 20 h after ingestion of infected blood. As many as 610 *Plasmodium* genes (33% of the total 1828) show a higher expression level (two-fold or greater) in infected blood against the reference pool RNA target that was made from an equal amount of RNA from each infected mosquito stage (Supplementary data, Table S1) (Fig. 1). A large fraction of these early stage *P. berghei* transcripts appears to be produced by the blood stage parasites as well as the early sporogonic stages such as ookinetes that are mainly present at 20 and 40 h after ingestion. A significant proportion of these early transcripts encode components of the translational machinery, stress response and metabolism; these functions are likely to relate to successive developmental processes and transition from the host to the vector environment that involves a drop in temperature and dramatic change in biochemical environment. The relatively small numbers of *P. berghei* genes in the middle and late stage infected midgut libraries may be explained by the possible presence of a smaller number of sexual and sporogonic stage parasites or a general down-regulation of genes at these later stages. Library origin and expression specificity of clones belonging to the non-determined species group suggest they predominantly represent *A. stephensi* (Fig. 1).

3.2. Anopheles and Plasmodium gene expression during the course of infection and blood processing

A. stephensi and *P. berghei* transcripts with predicted putative functions were clustered separately based on expression specificity, gene function and correlation with specific processes of interaction between the two organisms. Fragments of the parasite's transcriptional program, that directs its development in the mosquito, are presented in *Plasmodium* clusters Pb1–4 and components of the *Anopheles* transcriptome, that is responsible for blood processing and the response to *Plasmodium* infection, is presented in *Anopheles* clusters As1–5.

Plasmodium Pb1 cluster comprises transcripts that are abundant at the early stages and detectable up to about 20 or 40 h after blood ingestion. They encode metabolic enzymes, replication factors, transcription and translation components, proteasome and heatshock proteins as well as surface antigens. The majority of these transcripts are also present in blood stage parasites. The presence of nine proteasome and heatshock protein transcripts is indicative of a stress response to the transition from the vertebrate host blood in to the mosquito midgut lumen, which involves dramatic changes in both temperature and biochemical environment. *Plasmodium* heatshock proteins have been proven to be essential for growth and play roles in a variety of processes. The presence of several replication machinery components up to 40 h after ingestion is indicative of the major DNA synthetic activity during early stages of oocyst development. A Rab6

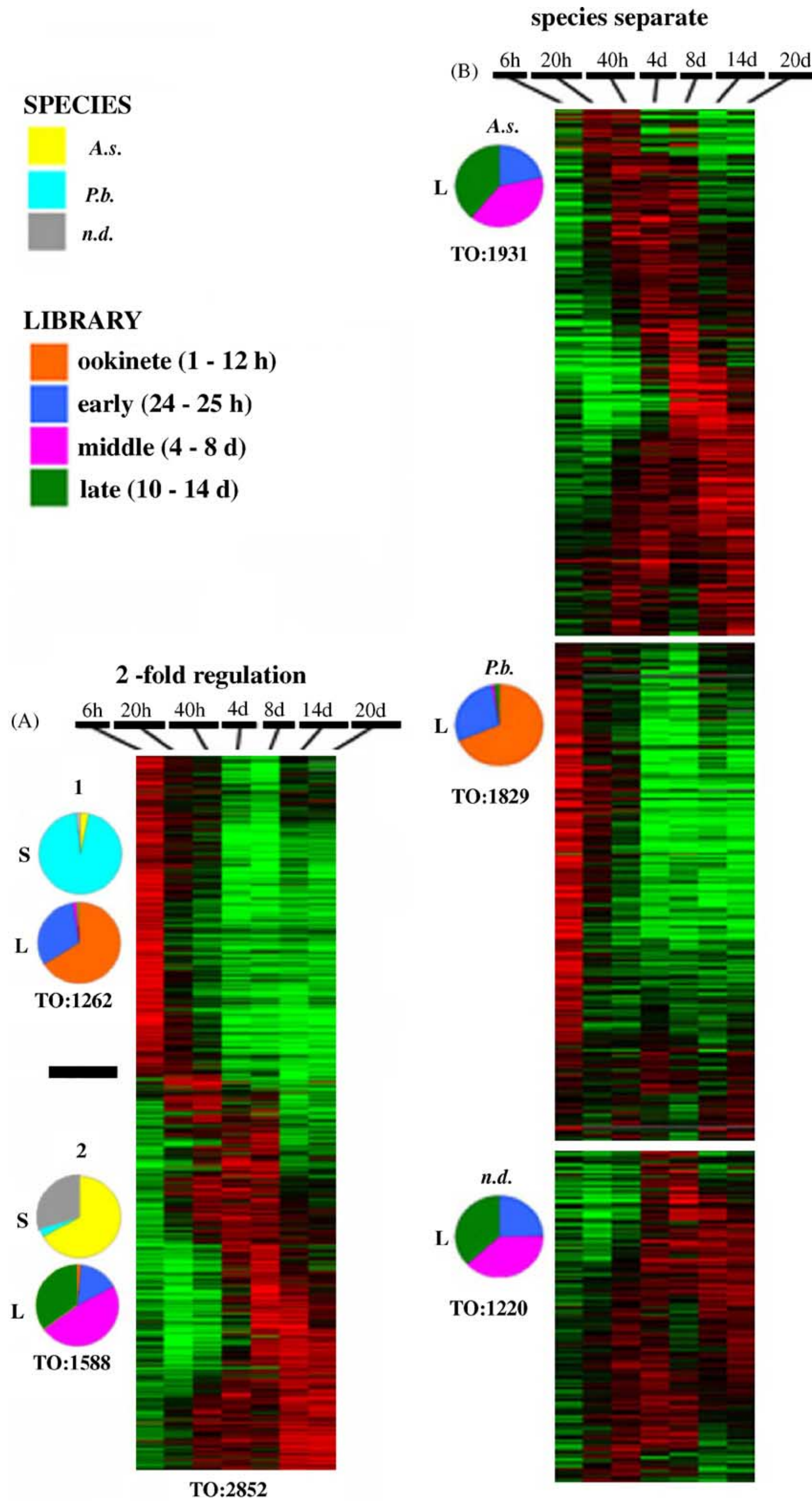

Fig. 1. Transcripts have been ordered in the expression cluster matrices according to a SOM method [11]. Each time point target (6, 20 and 40 h, and 4, 8, 14 and 20 days) has been hybridized against a reference target made from an equal amount of RNA of each time point (see Section 2 for details). Red colour indicate that a gene has a higher expression level than its average expression level across all seven assayed time points. Green colour indicate that a gene has a lower expression level than its average expression level across all assayed time points, and black indicates an equal to the average expression level for each transcript across all assayed time points. Pie charts S indicate proportions of transcripts belonging to *A. stephensi*, *P. berghei* or the non-determined species class. Pie charts L indicate library (ookinete, early, middle and late) of the assayed transcripts. Expression cluster matrix A comprises only those transcripts that have differential expression greater than two-fold between two or more assayed time points, and has been subdivided in two sub-clusters 1 and 2. In expression cluster matrix B, transcripts of each species *A.s.* (*A. stephensi*), *P.b.* (*P. berghei*) and n.d. (non-determined) have been clustered separately. Total (TO) number of transcripts contained in each cluster is indicated.

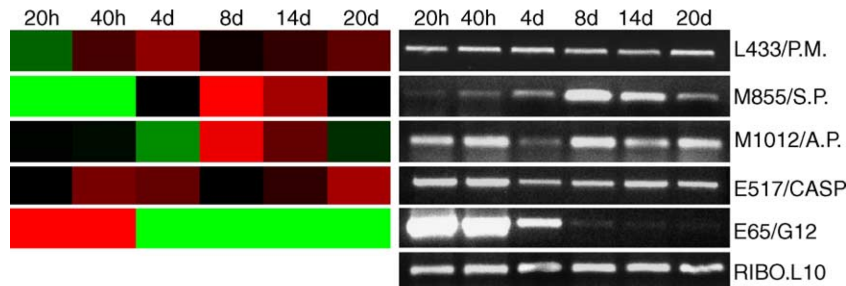

Fig. 2. Semi-quantitative RT-PCR analyses (right panel) of expression of selected genes at the time points that were assayed in the microarray analyses are compared to microarray data from 1 replica assay (left panel). cDNAs used for RT-PCR assays were normalized using an *A. stephensi* ribosomal L10 gene. For microarray analyses, hybridization strategy and colour scheme is the same as in Fig. 1. Differences between RT-PCR assayed and microarray assayed expression patterns is likely to relate to differences in normalization methods; microarray assays measure the expression level of each gene at each time point against its average (median) expression level across all assayed time points, while RT-PCR assays measure transcript abundance of genes in RNA of each time point that contain an equal amount of the ribosomal L10 gene transcripts. S.P., serine protease; P.M., peritrophic matrix; A.P., alkaline phosphatase; CASP, caspase; G12, G12 precursor; RIBO.L10, ribosomal protein L10.

transcript, L491, is up-regulated at early time points and at later time points during sporozoite differentiation (14 days after blood ingestion). A *P. falciparum* Rab6 is associated with the Golgi apparatus of the blood-stage parasite as well as at the later sporozoite stages [5,16] Expression of Rab6 may reflect a preparative up-regulation of Golgi-trafficking and protein sorting for upcoming increase in protein secretions. A highly conserved 14-3-3 transcript, E446, previously characterized in asexual blood stage parasites, is abundantly expressed in the sporogonic stages. The function of the 14-3-3 proteins is not precisely known, but has been linked to signal transduction and cell cycle control [17].

Plasmodium Pb2 cluster has similar expression specificity to Pb1 but comprises a variety of surface protein transcripts known to be implicated in interaction and invasion of vector and host cells and immune evasion. Three putative HSP70 transcripts, O81, OE484 and E1042, are expressed up to 40 h, when ookinetes invade the epithelium, and also at 14 days and later when sporozoites are dispersed in the hemolymph and invade the salivary glands, suggesting a specialized role in motility and invasion. HSP70 has indeed been shown to play a role in *P. falciparum* actin polymerisation as a modulator of capping activity, and hence the regulation of invasion which is dependent on local actin filament growth [18]. Two putative integral membrane protein genes (IMP), E936 and E647, are expressed at significantly higher levels at 6 h after ingestion and are therefore likely to play roles in the early sporogonic stages. The circumsporozoite- and TRAP-related protein (CTRP) gene is transcribed throughout the parasites development in the mosquito with a peak at 20 h where it is implicated in ookinete invasion of the epithelium [19]. Pbs28 and Pbs25 are expressed at the ookinete stage and earlier and encode for GPI anchored surface proteins that play multiple roles during parasite development in the midgut [12]. The E893 transcript shares high similarity to von Willebrand factor A domain-related protein (WARP) and has peak expression at the ookinete stages as well as at the late sporozoite stage. WARP is a micronemal adhesive protein implicated in *Plasmodium*–midgut interactions [4,20]. A myosin A gene, O123, is expressed at the ookinete and at the sporozoite stages where it has been described to play a role in locomotion and invasion of vector and host cells [13]. Two *Plasmodium* actins are also co-regulated with myosin A and are likely components of the ookinete and sporozoite locomotive machinery. A berghepain-2 encoding transcript, O423, encodes a cysteine protease with likely function in haemoglobin degradation and possibly also other functions during development in the mosquito. A chitinase has an expression peak at 40 h after ingestion and is also expressed at later stages. A *Plasmodium* chitinase has previously been implicated in traversal of the peritrophic matrix after activation by an *Anopheles* trypsin [21]. Proteins encoded by three transcripts, O312, E436 and O17, share similarity to erythrocyte binding membrane proteins (EMP) that belong to the highly variable bir (*P. berghei* immunovariant) family that have been studied in *P. vivax*. The O17 transcript is expressed in early midgut stages while O312 and E436 are also transcribed at the later oocyst and sporozoite stages. These proteins have a modular structure with domains that are implicated in interaction with a variety of host cell ligands and evasion of immune responses in the vertebrate [22]. A microtubule binding protein transcript, O292, is highly expressed during oocyst stages where it is likely to play a role in the morphogenesis of the developing sporozoite [15].

The Plasmodium Pb3 cluster is enriched with components implicated in interactions with the vector and host and is more specific for the sporozoite stage time points at 14- and 20-days after ingestion. Transcripts encoding the sporozoite-specific surface proteins TRAP and CSP are highly expressed at the sporozoite stage. Both proteins have been implicated in mosquito salivary gland invasion and hepatocyte invasion [23]. Transcription pattern of TRAP correlates with the previously observed gradual increase of TRAP protein content on the sporozoite surface that peaks at the mature sporozoite stage, while the CSP gene is highly expressed already at 14 days in developing oocysts [14,18]. Interestingly, both TRAP

and CSP are also transcribed at significant levels by blood stage parasites, even though production of their encoded proteins by these stages has not been validated with means other than immunohistological assays [24]. A putative erythrocyte binding protein-like (EBP-like), L194, has a transcription peak at the late sporozoite stage (20 days), suggesting a potential role in interaction between the sporozoite and the vertebrate host cells. A promising vaccine candidate, the hepatocyte erythrocyte protein 17 kDa gene (HEP17), is encoded by the transcript E1149 and is highly expressed at the sporozoite stages in the mosquito, although earlier studies have documented detection of its encoded protein first at 6 h after hepatocyte infection [25]. A transcript, E1143, with significant similarity to the rhoptry protein gene family, is expressed throughout the parasite's development in the mosquito with peaks at 20 h and 20 days. Rhoptry proteins are implicated in the binding of infected erythrocytes to host endothelial cells, and merozoite–erythrocyte interactions [26,27]. Expression of the E1143 encoding putative rhoptry protein suggests a role in the interactions with the vector and host. Two other rhoptry-like transcripts, O1124 and O51, are expressed at the early stages up to 20 h in cluster Pb1. Components of the parasite antioxidant defence system, encoded by a thioredoxin related transcript L37 and a glutathione peroxidase transcript L382, are expressed at elevated levels in the sporozoite stage and in the ookinete and sporozoite stages, respectively. Their presence is likely to indicate an oxidatively more challenging environment in the midgut and hemocoel [1].

Plasmodium Pb4 cluster is expressed throughout the parasites sporogonic development with small differences in expression levels between assayed time points, and represent genes of diverse functions. Several kinases, represented by transcripts O120, O173, O194 and O111, are significantly expressed during the sporogonic stages and some of them are likely components of the developmental signalling cascades that are responsible for triggering transitions between the different parasite stages [28]. Several essential metabolic genes are found in this cluster that is generally biased to constitutive housekeeping functions, and therefore lack significant differential regulation between assayed time points.

Anopheles As1 cluster is specific for the early stages (6 h–8 days) and is functionally biased to digestion, comprising four serine protease, four aminopeptidase and two maltase transcripts. Aminopeptidases M714 and L667 are similar to *A. gambiae* neutral zink metallopeptidases while E352 is a carboxypeptidase with likely implication in the digestive process [29]. Serine protease transcript E275 is up-regulated at 6–40 h while L238, E485 and L293 (serine protease group a; Fig. 3) have a more narrow expression window from 20 to 40 h, and are therefore most likely responsible for the later stages of blood digestion (in contrast to serine proteases found in cluster As5; discussed below). Serine proteases L238 and E485 are similar to *A. gambiae* serine proteases ENSANGG00000018576 and ENSANGG00000018603 that have not been characterized, while serine protease L293 is similar to the *A. gambiae* chymotrypsin 2 precursor which has a documented induction at 12–60 h after blood feeding [30]. A caspase transcript, E497, is up-regulated at 6 and 20 h, possibly representing a mosquito immune or apoptotic response to *Plasmodium* infected blood. Two maltase transcripts, L557 and L610, are induced between 40 h and 8 days that most likely indicate a switch to sugar source at this stage after blood ingestion. Transcripts, E904 and E117, share significant similarity to a previously cloned G12 gene of unknown function that encodes a putative secreted protein of 211 amino acids. The tight co-regulation of G12 with blood digestive serine proteases and peptidases suggest a functional role in a related process.

Anopheles As2 cluster comprises seven tightly co-regulated transcripts with an expression pattern that is characterized by two up-regulated peaks; the first at 20 and 40 h and the second at around 8 days after ingestion of infected blood. Transcripts M925 and M1012 are alkaline phosphatases that can hydrolyse a variety of phosphate compounds and are implicated in diverse functions, and transcripts M1058, L958 and M147 share similarity to sphingomyelin phosphodiesterases (ceramidases) have been linked to functions that may be of major importance to *Plasmodium* infection [31]. Sphingomyelin phosphodiesterases are utilized by some pathogenic bacteria for their entry into epithelial cells; they are also essential in the phagolysosomal defence against intracellular pathogens and are furthermore implicated in the metabolism of ceramide that regulates key effector systems including apoptosis [32–34]. Another transcript, M62, encode a 3-hydroxykynurenine transaminase that plays a crucial role in mosquito tryptophan catabolism by detoxifying 3-hydroxykynurenine to xanthurenic acid that is an essential trigger for *Plasmodium* gametocyte activation in the midgut [35,36].

Anopheles As3 cluster is enriched with components that respond to *Plasmodium* invasion of the midgut, which in turn is mediated by components of *Plasmodium* cluster Pb2 (described earlier). A serpin, E1028, and a nitric oxide synthase (NOS) transcript have expression peaks at 40 h. In agreement to a previous study, two isoforms KRAL and RCM of the genomic locus SRPN10 from *A. gambiae*, which codes for four alternatively spliced serine protease inhibitors of the serpin superfamily, have shown to be strongly up-regulated in female mosquitoes in response to midgut invasion by *P. berghei* ookinetes [37]. NOS has also been demonstrated to be linked to *Plasmodium* killing in the midgut [38]. Expression peaks of the E517 caspase transcript and a bax-like inhibitor of apoptosis transcript, L756, coincide with the invasion of the midgut epithelium and release of sporozoites in the hemocoel at around 40 h and 14–20 days, respectively. These components are most likely linked to the *Plasmodium* induced apoptosis of epithelial cells and immune responses [1,3,39]. Upon traversal of *Plasmodium* ookinetes across the midgut, the invaded epithelial cells will induce apoptosis and become expelled from the epithelium through a mechanism where actin filaments are redistributed to the basal side of cells to cause a constriction that will essentially result in the

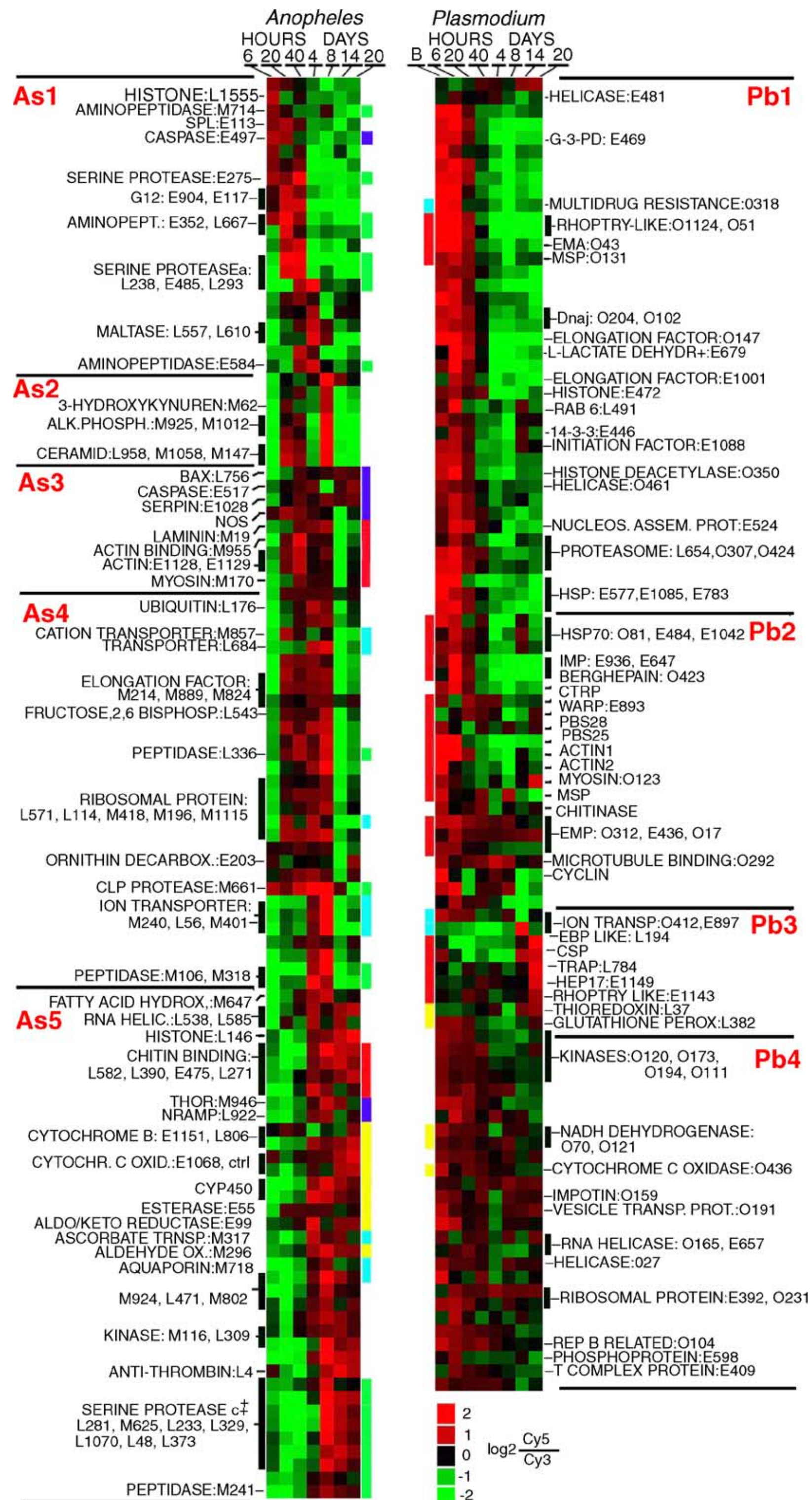

Fig. 3. *A. stephensi* genes of known function and with differential regulation magnitudes greater or close to two-fold between at least two time points have been clustered with a hierarchical cluster algorithm, and manually arranged in five distinct clusters As1–5 in the left panel. *P. berghei* genes of known function but regardless of differential expression level have been clustered in four distinct clusters Pb1–4 in the right panel. Hybridization strategy and colour scheme is the same as in Fig. 1. Colour bars on the right side of *A. stephensi* cluster matrix and left side of the *P. berghei* cluster matrix indicate genes of specific functional classes. For *A. stephensi*, red = structural and cytoskeletal, green = digestive, light blue = transporters, dark blue = immunity and apoptosis and yellow = oxidoreductive and detoxification. For *P. berghei*, red = structural, cytoskeletal and surface proteins, blue = transporters and yellow = oxidoreductive and detoxification. A complete list of all genes presented in this figure with their expression values is presented in Supplementary data, Table S2. Genbank EST identifiers for microarray assayed clones are indicated in Supplementary data, Table S3.

cells budding off into the midgut lumen [3,40]. Several components in the As3 cluster are likely components of this expulsion mechanism; two actin transcripts, E1128 and E1129, a putative actin binding calponin-like transcript, M955, and a myosin transcript, M170, that are up-regulated between 20 h and 8 days after ingestion (Fig. 3). Induction of a laminin transcript, M19, between 40 h and 8 days is most likely reflecting the expansion of the epithelium and basal lamina and possibly implication in a healing mechanism of the basal lamina that also may involve other extracellular matrix proteins (Fig. 3) [41]. Strong *Plasmodium* infection responsiveness has been documented for these transcripts in separate experiments (data not shown).

Anopheles As4 cluster has similar expression specificity to As3 but is highly enriched with transcripts implicated in translation, indicating a major biosynthetic activity at these stages. This cluster also comprises several peptidases, L336, M106 and M318, with likely implication in processes such as blood digestion, tissue remodelling and wound healing. Expression of several ion transporter transcripts, M857, M240, L56 and M401, most likely relate to a larger blood meal when feeding on infected blood, a detoxification process or the disruption of osmotic balance caused by ookinete invasion.

Anopheles As5 cluster is enriched with components that are required immediately upon ingestion of blood; seven protease transcripts, L281, M625, L233, L329, L1070, L48 and L373 (in the serine protease c group; Fig. 3), are most likely implicated in blood digestion immediately after ingestion, and are down-regulated prior to the 20 h time point. Sometime between 4 and 8 days they are up-regulated again, enabling the mosquito to digest the next blood meal. Of these serine proteases, L233 and L1070 are similar to an *A. gambiae* infection responsive serine protease ISPR13 [42] and L48 is similar to *A. gambiae* trypsin 3, which is induced at 28 h after blood ingestion [43]. Serine proteases L281 and M625 show only limited identity (55 and 46%) to *A. gambiae* serine proteases and L373 is highly identical to the chymotrypsin-like ENSANGG00000010749. An anti-thrombin factor, L4, is expressed similarly to the serine proteases and is most likely preventing blood clotting at the early stages immediately after ingestion. Four transcripts, L582, L390, L271 and E475, share significant sequence similarity to the infection responsive *A. gambiae ichit* gene that encodes two chitin binding domains and is a likely component of the peritrophic matrix that separates the blood meal from the epithelium and constitutes the first structural barrier to *Plasmodium* [10]. Three peritrophins, E613, M203 and E451, and two transcripts, E464 and E1049, sharing similarity to chitinases, are not significantly differentially expressed (<2-fold) between assayed time points but are, however, somewhat stronger transcribed at the later stages of *Plasmodium* differentiation (not shown in Fig. 3; Supplementary data, Table S1). The peritrophic matrix is assembled immediately upon blood ingestion and transcription and translation of some of its structural components is therefore to a large extent taking place constitutively prior to feeding [44]. A transcript, M946, encoding a translational regulator of immune response, Thor, is up-regulated at 4 days and later [45]; this is likely to indicate a possible regulation of *Plasmodium* infection response at the translational level. A gene with significant similarity to the natural resistance-associated macrophage protein (Nramp) gene, L922, is up-regulated at 8 and 14 days after ingestion. Nramp, a *D. melanogaster* malvolio homologue, is immune inducible and has thereby been linked to a role in the fly's defense system [46]. Detoxification factors represented by an esterase, E55, an ascorbate transporter, M317, several cytochromal transcripts and an aldehyde oxidase transcript, M296, are significantly induced at 4 days and later. This is likely to reflect a late detoxification response to infected blood [47]. Mosquitoes fed on infected blood have previously been shown to induce an oxidative stress-like response [3,48]. Ion pump and transporter transcripts, M924, L471 and M802, and an aquaporin, M718, are also found in cluster As5 and are most likely linked to nutrition uptake and restoration of osmotic balance.

4. Discussion

The study has exploited the effectiveness of microarray-based transcription profiling of anonymous cDNA clones to characterize EST library complexity and to discover genes with biological functions that are associated with highly specific expression patterns. Gene discovery approaches based on sequencing of randomly selected cDNAs of stage specific libraries alone do not provide detailed information on expression specificity at specific time points. The study has furthermore validated the feasibility of analyzing *Anopheles* and *Plasmodium* gene expression simultaneously, during the course of the parasite's development in the vector mosquito, with microarrays containing targets from both organisms. This approach not only generates information on the biology of the two interacting organisms but also on the qualitative and temporal dynamics of the interactions themselves. Expression clusters Pb2 and As3 in Fig. 3 represent *Plasmodium's* invasion of the midgut epithelium and the mosquito's physiological response to the invasion; it is predictably more revealing to study the biology of infection from the point of the pathogen and host in parallel. Surprisingly, only four transcripts belonging to known *Anopheles* immune gene classes were identified among the clones presented here and in the earlier studies where similar subtraction libraries were analyzed [4,5]. This is in sharp contrast to the robust immune responses that have been documented in *A. gambiae,* where numerous immunity genes belonging to diverse classes are induced by *P. berghei* infection at the time of midgut invasion [8]. It may reflect a weaker immune response of *A. stephensi* to *P. berghei* that in turn may explain the higher permissiveness of this mosquito species to *P. berghei* infection. Novel putative immune genes that have not been studied in the context of *Anopheles–Plasmodium* interactions previously, have

been identified and include a translational regulator Thor and a Nramp homologue [45,46]. Elevated expression of histone transcripts and several ribosomal transcripts may also indicate roles in immune defence for these proteins [49]. Such proteins have indeed been linked to anti-microbial activity in previous studies [50,51]. Subtraction library enrichment of components linked to hematophagy is likely to relate to the larger blood meal when feeding on the less viscous anaemic blood, and indicate some of the physiological impacts infected blood imposes on the mosquito, other than the immune responses [4,5]. Numerous novel *Plasmodium* gene expression patterns have been discovered, several of which involve genes encoding surface and secreted proteins that previously had been classified as vertebrate infection-stage specific. Transcription of blood stage genes in the sporogonic stages has been documented previously and is intriguing, suggesting additional roles in the interactions with the *Anopheles* vector [52]. Members of the vir gene family (homologues of the bir family in *P. berghei*) have been suggested to be implicated in immune evasion in the vertebrate host [21]. These proteins may play similar roles in the vector mosquito where they can complicate the adaptation of the immune surveillance system towards their recognition. Protein expression of these transcriptionally active stages remains to be proven. Our analyses provide evidence for the implications of mosquito and parasite genes in specific processes linked to the interaction of the two organisms, however, functional assays will be required to validate these findings and to provide details on the actual mechanisms. A significant fraction of clones represented by the microarray has yet not been sequenced, or do not share similarity with known genes, and therefore constitutes a valuable source for infection-stage specific *Anopheles* and *Plasmodium* gene discovery. The use of microarrays comprising the complete transcriptomes, of both organisms, will in the future provide more information on the processes of the development, interactions and infection responses at a higher resolution and detail, to further elucidate the complex mechanisms that enable the transmission of malaria. At present, such microarrays for *P. berghei* and *A. stephensi* are not available to the broader scientific community. One obvious challenge for performing similar analyses on the existing *A. gambiae–P. falciparum* genechip is the significantly lower infection levels of the human malaria parasite in its natural vector. The present and future analyses on *Anopheles* and *Plasmodium* gene expression will be essential for the identification of mosquito factors that can influence *Plasmodium's* development and mosquito stage and tissue specific promoters that can drive candidate anti-Plasmodium effector genes when and where they are needed. Assessment of *Plasmodium* gene expression can identify candidate pre-erythrocytic vaccine targets, that are present on the sporozoite surface at the time of transmission from the vector to the host, as well as essential *Plasmodium* components that can be targeted by inhibitory peptides expressed by transgenic mosquitoes or inhibitory compounds exposed to the mosquito in its natural environment.

Acknowledgements

We would like to thank the EMBL microarray facility for assistance with microarray construction, Ms. C. Hsieu for help with data processing and Dr. N. Mohammed for the CMMI microarray facility management. This work has been supported by funding from the European Commission Research Training Networks Grants HPRN-CT-2000-00080, grants from the National Institute of Allergy and Infectious Diseases, the Imperial College London and The Johns Hopkins Malaria Research Institute, Ellison Medical Foundation and the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2005.02.013.

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