Infectivity-associated Changes in the Transcriptional Repertoire of the Malaria Parasite Sporozoite Stage*

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Kai Matuschewski†‡§, Jessica Ross‡, Stuart M. Brown, Karine Kaiser†, Victor Nussenzweig‡, and Stefan H. I. Kappe‡

From the †Michael Heidelberger Division, Department of Pathology, New York University School of Medicine, New York, New York 10016; §Research Computing Resource, New York University Medical Center, New York, New York 10016, and ‡Department of Parasitology, Heidelberg University School of Medicine, 69120 Heidelberg, Germany

Injection of Plasmodium salivary gland sporozoites into the vertebrate host by Anopheles mosquitoes initiates malaria infection. Sporozoites develop within oocysts in the mosquito midgut and then enter and mature in the salivary glands. Although morphologically similar, oocyst sporozoites and salivary gland sporozoites differ strikingly in their infectivity to the mammalian host, ability to elicit protective immune responses, and cell motility. Here, we show that differential gene expression coincides with these dramatic phenotypic differences. Using suppression subtractive cDNA hybridization we identified highly up-regulated mRNAs transcribed from 30 distinct genes in salivary gland sporozoites. Of those genes, 29 are not significantly expressed in the parasite’s blood stages. The most frequently recovered transcript encodes a protein kinase. Developmental up-regulation of specific mRNAs in the infectious transmission stage of Plasmodium indicates that their translation products may have unique roles in hepatocyte infection and/or development of liver stages.

Malaria transmission occurs by mosquito bite when Plasmodium sporozoites located in the salivary glands of anopheline mosquitoes enter the vertebrate host. Sporozoites invade hepatocytes and differentiate into exo-erythrocytic forms (EEFs)1 that after a few days contain several thousand merozoites. After exiting the hepatocyte, merozoites invade erythrocytes and start the blood stage cycle that causes malaria disease. Salivary gland sporozoites and EEFs are rational targets for immunoprophylaxis and drug prophylaxis because they present a novel means to efficiently identify genes controlling infectivity to the mammalian host. Using a suppression subtractive hybridization screen (13), we identify a number of developmentally up-regulated genes, demonstrating for the first time differential gene expression in sporozoites. The change in transcriptional repertoire could be an important mechanism controlling sporozoite infectivity and therefore transmission success.

EXPERIMENTAL PROCEDURES

Plasmodium berghei Life Cycle—Anopheles stephensi mosquitoes were raised at 28 °C, 75% humidity under a 14-h light/10-h dark cycle and maintained on a 10% sucrose solution during adult stages. 4–5-day-old female mosquitoes were blood-fed on anesthetized young S/D rats or Syrian hamsters that had been infected with the P. berghei strain NK65. Rodents were assayed for high levels of parasitemia and the abundance of gametocyte-stage parasites capable of exflagellation. After the infective blood meal, mosquitoes were maintained at 21 °C, 80% humidity. On day 10 postfeeding, mosquitoes were dissected in RPMI 1640 medium, and isolated midguts were examined for the infection rate. Only mosquito cages having at least 70% of mosquitoes infected were kept for further analysis. Sporozoite populations were separated as described previously (9, 10). Naïve rodents were subjected to blood feeding of infected mosquitoes at day 18 postfeeding to maintain a continuous P. berghei cycle.

Generation of Subtraction Libraries—We dissected four million...
Localization of Plasmodium sporozoite populations within different organs of the mosquito vector coincides with different behavioral phenotypes. Sporozoites are released from an oocyst that lodges between the mosquito midgut epithelium and the basal lamina. They reach the salivary gland by traveling through the mosquito’s hemocoel (Fig. 1A). These sporozoites penetrate the salivary gland cells and migrate through and come to a rest in the lumen of the salivary gland duct (e.g. sporozoites marked by yellow arrow). At this stage, the sporozoites are ready for transmission through mosquito bite. The right panels show the median lobe of the mosquito salivary gland infected with sporozoites expressing the green fluorescent protein (30). In the top right panel (phase-contrast image), the extracellular sporozoites are clearly visible. In the bottom right panel (fluorescence image), extracellular sporozoites and sporozoites within the lobe are visualized.

P. berghei sporozoites each from midguts and salivary glands of infected A. stephensi mosquitoes. Salivary gland and oocyst sporozoites were purified over a DEAE-cellulose column to remove contaminating mosquito tissue. This crucial step resulted in one million highly purified parasitoids for each population. Poly(A)+ RNA was isolated from these sporozoites, from 50 uninfected salivary glands, and from whole uninfected mosquitoes using oligo(dT) columns (Invitrogen). The poly(A)+ RNA was used as a template for first-strand cDNA synthesis and one subsequent round of amplification using the SMART PCR cDNA synthesis kit (Clontech). Suppression subtractive hybridization was performed with the PCR-Select cDNA subtraction kit (Clontech). The subtracted cDNA population was ligated into vector pCR2.1-Topo (Invitrogen) and transformed into Escherichia coli TOP10 competent cells (Invitrogen). Sequencing was done at a DNA sequencing facility (Invitrogen) and transformed into TOP10 competent cells (Invitrogen) and transformed into pCR2.1-Topo (Invitrogen) and transformed into Escherichia coli TOP10 competent cells (Invitrogen). Sequencing was done at a DNA sequencing facility (Invitrogen) and transformed into E. coli TOP10 cells. The subtracted cDNA population was ligated into vector pCR2.1-Topo (Invitrogen) and transformed into Escherichia coli TOP10 cells. The subtracted cDNA population was ligated into vector pCR2.1-Topo (Invitrogen) and transformed into E. coli TOP10 cells.

Sequence Analysis—The single-pass cDNA sequences from the subtraction libraries tended to be short and contained sequencing errors. It resulted in 500,000 highly purified parasitoids for each population. Total RNA was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA). RNA was treated with RNase I (Invitrogen) to remove contaminating genomic DNA. Twenty ng of RNA for each sporozoite population was used as a template in first-strand cDNA synthesis, using the TaqMan® reverse transcriptase kit (PerkinElmer Life Sciences). Gene-specific oligonucleotide primers were designed using the Primer Express software (PerkinElmer Life Sciences). Sequences for oligonucleotides are as follows: (i) TRAP, 5'-CATCGTACCTCAGAGTAAATATCCAGA-3' (sense) and 5'-TATGATTTACAACGTTAGT-3' (antisense); (ii) CS, 5'-AGGCCAAGAACCTTACCAAGGC-3' (sense) and 5'-GCCAAAGTAACCTGTTAACATATTACCA-3' (antisense); (iii) UIS1, 5'-ATTGT-CAGTATAGATTTTGGTTAGA-3' and 5'-TGGTTGCTTTTCCACCGCCG-3' (antisense); (iv) UIS10, 5'-ACCTCGAACGAGCTGTGCAG-3' (sense) and 5'-TCAACGTGAGATCATCTTATACGACC-3' (antisense); (v) UIS16, 5'-ATCCACAAAGTTCCTACTTT-3' (sense) and 5'-GCCCTTGAAGATATAAGCCAGA-3' (antisense); and (vi) UIS24, 5'-GCATCAAAGCCAAATTACCAGA-3' and 5'-TGGTCTATTACCTTGATCGTTTGCA-3'. Amplicon size for all oligonucleotide primer pairs was kept constant at ~ 200 bp. PCR fragments were cloned into plasmid pCR4 (Invitrogen). Each plasmid construct was used in a 10-fold dilution series (10 copies to 10^6 copies, each in triplicate) to determine a standard curve. The standard curve purity, threshold value of CT, defined as the cycle number at which reporter dye fluorescent intensity increases over background, over plasmid copy number. Absolute transcript copy number for each gene is calculated based on the external standard curve. Real-time RT-PCR amplification was done in a GeneAmp® 5700 Sequence detection System (PerkinElmer Life Sciences) using the double-stranded DNA binding probe SYBR Green I® (PerkinElmer Life Sciences). Reaction were subjected to one cycle of 10 min at 95 °C and 45 cycles of 15 s at 95 °C, 1 min at 60 °C. Real-time RT-PCR experiments were done in triplicate.

Constitutive Expression of CS and TRAP in Sporozoites—Differential infectivity of salivary gland and oocyst sporozoites may result from a major difference in expression levels of cell surface ligands. We first asked whether the two characterized sporozoite-specific genes CS and TRAP are differentially expressed. In order to compare transcript abundance, we generated amplified cDNAs from poly(A)+ RNA of 1 million purified P. berghei parasites of either late blood stage schizonts, mature salivary gland sporozoites, or oocyst sporozoites, respectively. Next, we hybridized the stage-specific cDNAs with gene-specific labeled probes (Fig. 2A). No significant differences in transcript abundance for either CS or TRAP were observed between the sporozoite stages. As expected, transcripts of CS and TRAP were absent in late blood stage schizonts. To control for similar
sporozoites. The TRAP signal is confirmed by the absence of signal in immunoblotting experiments with the CS antiserum or a monoclonal anti-CS antibody. Specificity of the TRAP signal was confirmed by the absence of signal in the TRAP RT-PCR experiments with total RNA from 5 x 10^6 parasites of the respective stages. The TRAP signal was observed in all stages of development, indicating that TRAP is a constitutive protein that is expressed in all invasive stages of Plasmodium. cDNAs were generated from poly(A)^+ RNA of 1 x 10^7 parasites of the respective stage by RT-PCR and amplified once using the SMART (switching mechanism at the 5' end of RNA transcript) technology. Each experiment was done in triplicate.

FIG. 2. Constitutive expression of the sporozoite-specific genes CS and TRAP throughout sporozoite development. A. cDNA blots of either synchronized late schizonts, salivary gland, or oocyst sporozoites of P. berghei hybridized to labeled probes of the genes encoding the sporozoite invasin TRAP or the major surface protein CS. As a control, cDNA blots were hybridized with a probe for the class XIV myosin gene MyoA, expressed in all invasive stages. cDNAs were generated from poly(A)^+ RNA of 1 x 10^7 parasites of the respective stage by RT-PCR and amplified once using the SMART (switching mechanism at the 5' end of RNA transcript) technology. B. Quantitative real-time RT-PCRs with total RNA from 5 x 10^6 purified P. yoelii sporozoites from either oocysts (O) or salivary glands (S) as templates using gene-specific oligonucleotide primer pairs to CS and TRAP. Transcript quantifiability is represented as the number of copies (+ S.D.) in comparison with an external standard curve generated with gene-specific plasmids. Each experiment was done in triplicate. C. Western blot analysis. Protein extracts of 50,000 wild-type sporozoites from either oocysts, hemocoe, or salivary glands and 50,000 oocyst sporozoites from TRAP− parasites were probed with either polyclonal anti-TRAP antibodies or a monoclonal anti-CS antibody. Specificity of the TRAP signal is confirmed by the absence of signal in the TRAP− sporozoites.

Differential Gene Expression in Plasmodium Sporozoites—To test whether differential gene expression occurs during sporozoite maturation, we wished to compare transcript profiles of infectious salivary gland sporozoites with non-infectious oocyst sporozoites. Sporozoites have to be isolated from infected mosquitoes and are highly contaminated with mosquito tissue. For our experiments, we collected 4 million P. berghei sporozoites from the two respective stages in order to obtain 1 million highly purified salivary gland sporozoites and 1 million highly purified oocyst sporozoites. RNA extraction yielded around 50 ng of poly(A)^+ RNA for each population that was used as a template for cDNA amplification. For all hybridization experiments throughout this work we used the additional amplification step because several attempts to detect a signal from first-strand cDNA synthesized from 50 ng of poly(A)^+ RNA failed. Importantly, the comparison of QRT-PCR experiments with the cDNA blot analysis for CS and TRAP (Fig. 2, A and B) indicated that amplification of cDNAs does not significantly bias gene representation and can be used to compare transcript expression levels of different parasite stages. We could therefore perform a cDNA-based screening technique, suppression subtractive hybridization, with low amounts of starting material. Suppression subtractive hybridization allows selective enrichment of differentially regulated cDNAs of high and low abundance that are present in only one population. This is achieved through a combination of hybridization and PCR amplification protocols that allow simultaneous normalization and subtraction of the cDNA populations (13). We enriched for up-regulated transcripts of P. berghei salivary gland sporozoites by using the sporozoite cDNA as our tester cDNA. The driver was a mixture of cDNAs generated from oocyst sporozoites and uninfected salivary glands (to eliminate possible contaminating salivary gland transcripts). Analysis of P. berghei cDNA sequences isolated by our subtraction screen was facilitated by the genome sequence data from P. falciparum and P. yoelii (PlasmoDB.org) as well as P. berghei genome sequence data (www.sanger.ac.uk). We sequenced 300 cDNA clones and matched their sequences to the genome sequence data bases. None of the sequenced clones was of mosquito origin. Cluster analysis revealed 32 individual genome contigs that were tagged by at least 2 sequenced cDNA clones. Notably, we did not isolate any of the genes that are expressed throughout Plasmodium sporozoite maturation, i.e. MyoA, CS, and TRAP.

To further select only those genes that are specifically up-regulated in salivary gland sporozoites, we probed dot-blot of representative cDNAs for each genome contig with labeled amplified total cDNA probes from different stages of the Plasmodium life cycle (Fig. 3). Among the 32 genes recovered, 2 genes (A2 and F4) were abundantly transcribed in all stages tested by dot-blot analysis, thereby identifying them as false positives that could serve as independent internal controls. F4 encodes the P. berghei ortholog of the large subunit of RNA polymerase I of P. falciparum (16), a likely constitutive protein required for rRNA synthesis throughout the Plasmodium life cycle. No function could be assigned to clone A2.

The dot-blot analysis demonstrates that the remaining 30 genes are up-regulated in infective sporozoites isolated from the mosquito salivary glands (termed UIS genes). We conclude that maturation of infectivity in Plasmodium sporozoites is accompanied by dramatic changes in their transcriptional repertoire.

MCP-1 Is Expressed in Two Invasive Plasmodium Stages—One gene (UIS16) displayed a unique expression pattern. It is enriched both in salivary gland sporozoites and blood stage schizonts (clone E3 in Fig. 3). UIS16 encodes the P. berghei ortholog of the previously described P. falciparum MCP-1. MCP-1 is expressed in invasive merozoites and appears to be located initially at the attachment site between merozoites and erythrocytes, and then it migrates backwards around the merozoites during invasion of the red blood cells (17, 18). Invasion proceeds by a “moving junction” between the membrane of the parasite and the membrane of the host cell. It has been proposed that MCP-1 participates in the movement of the junction along the parasite’s cytoskeleton (17). Our findings suggest that MCP-1 has a similar function during invasion of red blood cells by merozoites and invasion of hepatocytes by salivary gland sporozoites.

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Fig. 3. Identification of Plasmodium UIS genes. Thirty-two genes were redundantly recovered in a suppression subtractive hybridization screen designed to enrich salivary gland sporozoite-specific transcripts and further tested for differential expression. Dot-blot of the cDNA inserts were hybridized with labeled total cDNA probes generated from salivary gland or oocyst sporozoites as well as synchronized late blood stage schizonts and mixed blood stages that contain all erythrocytic asexual and sexual forms of the malaria parasite. Two clones (A2 and F4) are false positives and serve as independent internal controls. UIS16 (clone E3) encodes a protein previously described as MCP-1. Note the up-regulation of MCP-1 during sporozoite maturation as well as enrichment in the late schizont stage. The remaining 29 genes are highly up-regulated in virulent salivary gland sporozoites. They are not expressed at significant levels in the parasites blood stages.

Genes That Are Specifically Up-regulated in Infective Sporozoites—Remarkably, the 29 remaining UIS genes are not expressed at significant levels in the parasite blood stages (Fig. 3). Schematic diagrams of the deduced proteins for each UIS are presented in Fig. 4. Overall, we identified novel stage-specific genes that encode putative-regulatory proteins, such as kinases and phosphatases as well as transcriptional regulators. Other UIS gene products have predicted enzymatic activities. In addition, a number of predicted proteins display consensus amino-terminal signal peptides and/or internal hydrophobic segments that could function as transmembrane domains.

The most frequently recovered gene (UIS1, 26% of all sequences) encodes a protein kinase. UIS1 contains the conserved kinase motifs but could not be classified into one specific kinase subfamily. A number of putative secreted molecules are differentially expressed in salivary gland sporozoites. For example, UIS2 (7% of all sequences) encodes a secreted molecule with a region of homology to purple acid phosphatases. In vitro, these enzymes resemble phosphomonoesterases; however, their physiological function remains to be established.

In order to validate our findings, we performed independent expression experiments for selected UIS genes (Fig. 5). To demonstrate that PCR amplification of transcripts had not biased representation, we compared the cDNA blot methodology with expression data generated by QRT-PCR. A blot of amplified cDNA of oocyst sporozoites, salivary gland sporozoites, and late blood stage schizonts hybridized with a UIS1 probe detected no transcripts in P. berghei oocyst sporozoites or blood stage schizonts, whereas transcripts were abundant in salivary gland sporozoites (Fig. 5A). QRT-PCR data generated separately from P. yoelii sporozoites showed an ~24-fold up-regulation of UIS1 transcripts in salivary gland sporozoites when compared with oocyst sporozoites (Fig. 5A), confirming differential expression of UIS1 during sporozoite maturation. Transcript abundance for UIS10 encoding a lecithin-cholesterol acyltransferase was up-regulated ~16-fold during sporozoite maturation (Fig. 5B).

Although subtractive hybridization screens are not exhaustive, and other UIS genes may be identified in the future through additional approaches, we most likely identified a significant portion of genes that are up-regulated during sporozoite maturation. As shown in Fig. 4, UIS1 to UIS13 were recovered frequently in our screen. In good agreement with these data, we observed strong differential expression by either cDNA blot analysis or QRT-PCR or both for UIS3 and UIS4, both of which encode small molecules containing potential transmembrane domains, and UIS5, which encodes a putative aminotransferase class V.

To test whether up-regulation of transcript abundance is still significant for UIS genes recovered only twice in our subtraction screen, we performed QRT-PCR experiments for two representative genes (Fig. 5B). Transcript abundance increased ~8-fold for UIS16/MCP1 and ~6-fold for UIS24/HSP70–3 in salivary gland sporozoites compared with oocyst sporozoites.

DISCUSSION

In this study, we identify the first candidate infectivity genes of Plasmodium sporozoites. We demonstrate that the sporozoites located in the salivary glands differ significantly in their transcriptional repertoire from sporozoites emerging from oocysts. These findings, taken together with the dramatic phenotypic differences (7–10), show that maturation of Plasmodium sporozoite infectivity for the mammalian host follows a developmental program. We found that salivary gland sporozoites up-regulate many genes that are not expressed earlier in development. Among them are potential regulatory proteins, secretory molecules, and metabolic enzymes that probably control sporozoite virulence. Sporozoites residing in the salivary gland are programmed to invade hepatocytes and to continue the life cycle in the mammalian host. The associated phenotypical and molecular changes are probably irreversible because salivary gland sporozoites introduced into the hemocoel of mosquitoes are no longer capable of reentering the salivary glands (11).

Sporozoite transmission during the mosquito bite is one of the bottlenecks in the Plasmodium life cycle (19). A sporozoite within the mosquito salivary gland has to reach the mammalian liver, invade a suitable hepatocyte, and commence development into an EEF. Some of the UIS genes most likely participate in these events. Support for this comes from our finding that UIS16 encodes MCP-1, the only UIS protein characterized previously in invading merozoites. MCP-1/UIS16 localized to the moving junction between merozoites and host red blood cell, leading to the formation of a parasitophorous vacuole (17). MCP-1 expression was tightly regulated. It was only seen in merozoites but not in earlier blood stages. It is likely that MCP-1 performs a similar function in the merozoite and the salivary gland sporozoite. The presence of MCP-1 in the subtraction screen provides support for the identification of genes that control parasite infectivity using differential gene expression profiling.

The noise in our screen is remarkably low. Among 32 selected candidates, we obtained only 2 false positives (A2 and F4 in Fig. 3). We believe that mainly two factors contributed to the success: (i) the purity of the starting material as well as mixing uninfected mosquito material to the driver population, and (ii)
the threshold of at least two hits. Based on the significant up-regulation of UIS genes that just met the threshold level (Fig. 5B), we may recover additional candidate genes that are developmentally regulated among the single-hit cDNAs.

Regulation of gene expression is probably the major mechanism that underlies the development of sporozoite infectivity. MCP-1 and other UIS proteins may be involved in salivary gland sporozoite invasion of target cells. Others could be important in adapting to new metabolic requirements within the infected hepatocyte. UIS10 is an example of a secretory molecule that is likely to function during EEF replication. It encodes lecithin-cholesterol acyltransferase. Lecithin-cholesterol acyltransferase (20) is the major cholesterol esterifying activity in human plasma, and in addition it facilitates reverse cholesterol transport. The mevalonate pathway of sterol biosynthesis has not been identified in Plasmodium. Therefore, the parasite’s cholesterol is most likely host-derived. The Plasmodium EEFs have a very high demand for membrane biogenesis because they generate thousands of merozoites. Thus, UIS10 may participate in the transport of cholesterol from the hepatocyte into EEFs. UIS10 contains the signature sequence GXSXG that is conserved in the active site of lipases, as well as the loop region that confers binding of lecithin-cholesterol acyltransferase to the surface of lipoproteins (20). In the related apicomplexan parasite Toxoplasma gondii, the mobilization of cholesterol from host cell lysosomes is required for intracellular growth (21), but the underlying enzymatic mechanisms that allow the parasite to utilize an exogenous supply of cholesterol have not been identified. In accordance with a function in EEF, we can detect lecithin-cholesterol acyltransferase transcripts 20 h (at

FIG. 5. Stage-specific up-regulation of selected UIS genes is confirmed by QRRT-PCR. A, comparison of expression analysis of UIS1 by blot of amplified cDNA (P. berghei) and QRRT-PCR (P. yoelii). Both experiments confirm that UIS1 transcript abundance increases dramatically in salivary gland sporozoites (S) when compared with oocyst sporozoites (O). No expression of UIS1 can be detected in late blood stage schizonts by cDNA blot. Compare restriction of transcript expression with the constitutive sporozoite-specific genes TRAP and CSP (Fig. 2, A and B). B, QRRT-PCR analysis of P. yoelii transcript abundance for additional UIS in oocyst (O) and salivary gland (S) sporozoites confirms differentialUIS expression. Shown are the transcript levels of P. yoelii LCAT/UIS10, MCP1/UIS16, and HSP70–3/UIS24. Transcript quantity is represented as the number of copies (±S.D.) in comparison with an external standard curve generated with gene-specific plasmids. Each experiment was done in triplicate.
this time point, all invaded sporozoites have transformed into EEFs) and that the polyamine requirements are differentially regulated in blood stages and EEFs.

UIS genes may encode key regulators themselves. For example, UIS1 encodes a novel protein kinase. Although the function of UIS1 and its product remains to be elucidated by reverse genetics and biochemical approaches, it could participate in the signaling events associated with gliding motility, hepatocyte invasion, and/or the transformation of sporozoites into EEFs. What are the signals that induce UIS transcript up-regulation? One possibility is that Plasmodium sporozoites undergo a time-dependent endogenous developmental program. Thus, independently of external signals, sporozoites gradually increase expression of UIS, and completion of maturation coincides with their entry into the salivary glands. This hypothesis could be tested in the recently developed culture system for P. berghei oocysts (26) that leads to the production of sporozoites that are infective for mice. However, the in vitro-produced sporozoites are ~1,000-fold less infective for rodents when compared with the levels of salivary gland sporozoite infectivity (3, 10). Therefore, the increase in infectivity could be associated with an outside signal that coincides with sporozoite entry of mosquito salivary glands. Recent studies suggest that Plasmodium sporozoites may indeed selectively recognize specific receptors on salivary glands (27–29). Perhaps sporozoite interactions with putative salivary gland receptors trigger the onset of a new gene expression program.

Because transcription of most UIS genes is restricted to salivary gland sporozoites, they can be targeted by reverse genetics. This should reveal those gene products that perform crucial functions for the sporozoite's successful journey from the mosquito salivary gland into hepatocytes and for its subsequent development into EEFs. Functional complementation ofUIS-null mutants with the corresponding P. falciparum sequences will ultimately prove whether the UIS genes identified in this study are true functional orthologs of the human malaria parasite.

The developmentally up-regulated genes identified in this study are important stepping stones for the dissection of the molecular mechanism underlying sporozoite maturation and, as a consequence, infectivity to the mammalian host. UIS genes can serve as a gateway for the identification of parasite genes expressed in the EEF. In fact, we have performed RT-PCR with UIS-specific oligonucleotide primers using RNA isolated from HepG2 cells 20 h after infection with P. berghei sporozoites. Of seven UIS genes tested, six are expressed in EEF at this time point.2

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