

Acknowledgements

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Competing interests statement

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Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite

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Malaria is estimated to cause 0.7 to 2.7 million deaths per year, but the actual figures could be substantially higher owing to under-reporting and difficulties in diagnosis¹. If no new control measures are developed, the malaria death toll is projected to double in the next 20 years¹. Efforts to control the disease are hampered by drug resistance in the *Plasmodium* parasites, insecticide resistance in mosquitoes, and the lack of an effective vaccine. Because mosquitoes are obligatory vectors for malaria transmission, the spread of malaria could be curtailed by rendering them incapable of transmitting parasites. Many of the tools required for the genetic manipulation of mosquito competence for malaria transmission have been developed. Foreign genes can now be introduced into the germ line of both culicine^{2,3} and anopheline⁴ mosquitoes, and these transgenes can be expressed in a tissue-specific manner^{5,6}. Here we report on the use of such tools to generate transgenic mosquitoes that express antiparasitic genes in their midgut epithelium, thus rendering them inefficient vectors for the disease. These findings have significant implications for the development of new strategies for malaria control.

When a mosquito ingests a blood meal from an infected host, *Plasmodium* gametocytes transform into gametes that mate and differentiate into zygotes and then ookinetes (elongated motile zygotes). Ookinetes cross the midgut epithelium and differentiate into oocysts, which after 10–15 days liberate sporozoites into the haemocoel. The development of the parasite in the mosquito is completed when sporozoites cross the salivary gland epithelium⁷. The mechanism by which the parasite crosses the mosquito epithelia is unknown, but is suspected to be receptor mediated. *In vivo* selection from a library of bacteriophages displaying random 12-amino-acid peptides led to the identification of a peptide—PCQRAIFQSIQN (termed SM1 for salivary gland- and midgut-binding peptide 1)—that binds specifically to the two epithelia that

are traversed by the parasite: the distal lobes of the salivary glands and the luminal surface of the midgut⁸. Significantly, SM1 strongly inhibited crossing of the two epithelia by the parasites⁸. These results suggest that if SM1 is produced and secreted into the mosquito gut lumen when an infectious blood meal is ingested, then *Plasmodium* development would be blocked.

We searched for a system to drive the expression of genes that inhibit *Plasmodium* development, and found that the carboxypeptidase (CP) promoter and signal sequence has many desirable attributes. The CP promoter is strongly activated by a blood meal, and the CP signal sequence drives secretion of the protein into the midgut lumen, where the initial stages of *Plasmodium* development take place^{6,9}. We constructed a synthetic gene (termed *AgCP[SM1]₄*) consisting of four SM1 units joined by 4-amino-acid linkers attached to the CP signal sequence and driven by the gut-specific and blood-inducible CP promoter (Fig. 1a). This gene was inserted into a *piggyBac* vector and transformed into the germ line of the mosquito *Anopheles stephensi*⁴. Of 394 embryos injected, 63 (16.0%) larvae hatched, yielding 33 (8.4%) adults. The adults were distributed into 14 families, of which 2 (families A and B) yielded green fluorescent protein (GFP)-positive progeny (Fig. 1b). Progeny from two separate mosquito lines from each family were analysed by Southern blot hybridization (Fig. 1c). The results indicate that each of the four lines originated from a different integration event. Northern blot analysis indicated that the *AgCP[SM1]₄* transgene is rapidly and strongly induced by a blood meal in midguts of transgenic mosquitoes with a peak around 3–6 h (Fig. 1d). This pattern is consistent with that previously observed for genes driven by the *Anopheles gambiae* CP promoter^{6,9}. We investigated the synthesis of the *AgCP[SM1]₄* protein by the midgut epithelium by immunofluorescence microscopy. The recombinant protein was detected in the midgut epithelium of mosquitoes dissected at 6 h (data not shown) and 24 h (Fig. 2) after a blood meal, but by 36 h the signal had declined to close to basal level (data not shown). Because ookinetes invade the midgut epithelium

Table 1 Inhibition of oocyst formation in transgenic mosquitoes

Experiment	Oocyst prevalence*	Oocyst intensity†	Inhibition (%)‡
1. Control	80 (16/20)	80.8 (0–273)	–
B6	53 (9/17)	25.3 (0–186)	68.7
2. Control	86 (18/21)	70.3 (0–225)	–
B3	37 (7/19)	7.3 (0–40)	89.6
3. Control	94 (17/18)	63.8 (0–365)	–
B3	35 (7/20)	7.2 (0–80)	88.7
4. Control	89 (17/19)	64.9 (0–292)	–
B3, B6	41 (9/22)	3.3 (0–19)	94.9
5. Control	89 (17/19)	132.6 (0–328)	–
B3, B6	54 (14/16)	26.8 (0–105)	79.8
6. Control	89 (17/19)	95.1 (0–290)	–
B3, A3	50 (11/22)	22.1 (0–85)	76.8
7. Control	90 (18/20)	83.4 (0–285)	–
A15	33 (7/21)	9.6 (0–98)	88.5
8. Control	90 (18/20)	129.0 (0–250)	–
A15	45 (10/22)	34.0 (0–134)	73.6
9. Control	86 (19/22)	115.2 (0–292)	–
A15	70 (16/23)	30.3 (0–101)	73.7
Average			
Control	88.1 (17.4/19.8)	93.1 (0–365)	–
Transgenic	46.4 (10.0/21.3)	18.5 (0–186)	81.6

For each experiment, transgenic mosquitoes and sibling control (non-transgenic) mosquitoes from the same rearing were fed simultaneously on the same mouse, which was infected with *P. berghei* ANKA 2.34. A3, A15, B3 and B6 indicate the transgenic lines used in each experiment (Fig. 1c). Where two lines are indicated, a mixture of mosquitoes from those lines was used. All transgenic lines were kept as heterozygotes and mosquitoes were fed on mice with 10–15% parasitaemia and 1–1.5% gametocytaemia. Mosquitoes were kept at 21 °C and the number of oocysts per midgut was counted on day 15 after feeding.

*The per cent mosquitoes that had oocysts in their midgut. This value was derived from the number of oocyst-positive mosquitoes over the total number of mosquitoes examined (shown in parentheses).

†The mean oocyst number per midgut. The range of observed values is indicated in parentheses. In all cases, transgenic mosquito values were significantly different ($P < 0.05$) from those of controls, as analysed by the Mann–Whitney *U*-test.

‡Reflects the reduction in the mean oocyst number in transgenic mosquitoes relative to control mosquitoes.

around 24 h after a blood meal, it is important that synthesis and secretion of the recombinant peptide precede the time of parasite invasion.

Previous experiments indicated that when an infectious blood meal was fed along with the SM1 peptide, formation of oocysts, but not of ookinetes, was inhibited⁸. To measure the consequences of *AgCP*[SM1]₄ transgene expression on parasite development, we fed control and transgenic mosquitoes on the same infected mouse and measured the numbers of oocysts formed. In nine experiments, inhibition of oocyst formation ranged between 68.7 and 94.9% (average inhibition 81.6%; Table 1). To ascertain that control and transgenic mosquito lines had the same genetic background, the four transgenic lines were backcrossed in each generation to the wild-type mosquito population. We considered the possibility that the observed effects were caused by the fortuitous disruption of an

endogenous mosquito gene on transgene integration or by some other property of the transposon. Two lines of evidence argue against these possibilities. First, equivalent inhibition of oocyst formation was observed with mosquitoes of three independently derived lines (Table 1). Note that for each line, the transgene integrated in a different position in the mosquito genome (Fig. 1c). Second, development of *Plasmodium berghei* in transgenic *A. stephensi* that express GFP from a Minos-based transposon was indistinguishable from development of *P. berghei* in wild-type mosquitoes (F. Catteruccia, personal communication). Thus, the presence of foreign DNA or expression of GFP by themselves do not affect parasite development. Moreover, the SM1 peptide, but not a control (unrelated) peptide, strongly inhibited parasite development and transmission when administered to mosquitoes⁸. These observations suggest that the sequence of the expressed peptide is

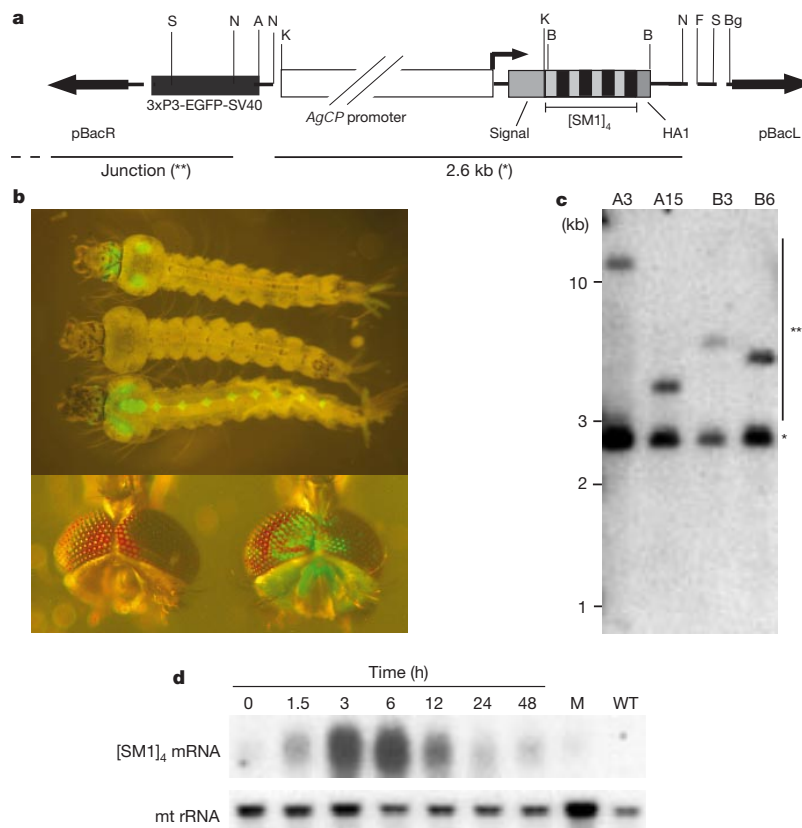


Figure 1 Structure of the *AgCP*[SM1]₄ gene and its expression in transgenic mosquitoes. **a**, Schematic diagram of the *AgCP*[SM1]₄ gene that was transformed into the *A. stephensi* germ line. The construct consists of the *A. gambiae* carboxypeptidase (*AgCP*) promoter (the bent arrow indicates the transcription initiation site), the *AgCP* 5' UTR (line to the right of the promoter), the *AgCP* signal sequence, four units of the SM1 repeat (hatched boxes are the linker amino acids, black boxes are the SM1 peptides), the haemagglutinin epitope (HA1) and the *AgCP* 3' UTR (line to the right of HA1). 3xP3-EGFP-SV40 is the gene that expresses GFP from an eye-specific promoter¹³. The arrows at the end of the construct represent the *piggyBac* arms. Dashed lines represent flanking plasmid sequences. Restriction sites: S, *SalI*; N, *NotI*; A, *AscI*; K, *KpnI*; B, *BamHI*; F, *FseI*; Bg, *BglII*. The lines below the construct show the fragments observed in **c**. The size of the junction fragment is variable and depends on the site of integration in the *A. stephensi* genome. **b**, Detection of *AgCP*[SM1]₄ transgenic mosquitoes by transformation marker-mediated fluorescence. Top, a wild-type (non-transgenic) larva (middle) flanked

by transgenic larvae viewed from the dorsal (top) or ventral (bottom) sides. Note green fluorescence of the ventral nerve cord in the latter, which is similar to marker-mediated fluorescence in *Drosophila*¹³. Bottom, the head of a wild-type (left) and a transgenic (right) mosquito. The entire eye expresses GFP but which facets fluoresce depends on the angle of the incident light. **c**, Southern blot analysis of genomic DNA extracted from mosquitoes from two A and two B transgenic lines, digested with *NotI* and *BglII* enzymes. The probe was a mixture of [SM1]₄ and 3xP3-EGFP-SV40 sequences (compare with **a**). **d**, Time course of [SM1]₄ messenger RNA accumulation after blood feeding. RNAs were extracted from transgenic female mosquitoes at the times after a blood meal indicated on top of each lane. The RNAs were fractionated by electrophoresis on an agarose gel, blotted onto a nylon membrane and sequentially hybridized first with an [SM1]₄ probe and then with a mitochondrial ribosomal RNA (mt rRNA) probe¹⁴ to verify the amount of RNA analysed in each lane. M, RNA from transgenic male mosquitoes; WT, RNA from wild-type (non-transgenic) female mosquitoes extracted 3 h after a blood meal.

letters to nature

important and that inhibition of *P. berghei* development in the mosquito can be attributed to SM1 expression, not to the transforming vector. SM1 is presumed to bind to a mosquito midgut receptor that is also required for ookinete invasion⁸. It seems that the SM1 tetramer binds to the luminal surface of the midgut (Fig. 2b), inhibiting parasite–epithelium interactions and midgut invasion.

Transgenic mosquitoes were less susceptible to infection (oocyst load) and had fewer sporozoites in their salivary glands than control mosquitoes (Tables 1 and 2). Also, vector competence of transgenic mosquitoes was severely impaired. In two of three experiments, no transmission was detected, and in a third, transmission was reduced by more than twofold (Table 2). In the field, where most mosquitoes carry fewer than five oocysts¹⁰, inhibition of transmission might be very effective. We also note that all experiments were performed with heterozygous mosquitoes that had one copy of the transgene. Inhibition is expected to be even more effective in homozygous mosquitoes that have two copies of the transgene. We were surprised to find that even mosquitoes that had salivary gland sporozoites did not transmit, as indicated by the smaller number of infected mice than infected mosquitoes (Table 2). It is possible that the lightly infected salivary glands had no sporozoites in the duct lumen.

Expression of the SM1 peptide in the mosquito midgut severely reduced vector competence by inhibiting *Plasmodium* development. To our knowledge, this is the first report on the blocking of malaria parasite transmission by a transgenic approach. Preliminary results indicate that the peptide does not alter mosquito fitness (longevity and egg production; unpublished observations). However, many challenges remain to achieve the long-term goal of controlling malaria transmission by genetic modification of the mosquito. A major obstacle will be to devise safe means of spreading

Table 2 Reduction of vector competence in transgenic mosquitoes

Experiment	Sporozoite prevalence*	Sporozoite intensity†	Vector competence‡
1. Control	70 (7/10)	2,320 (0–18,000)	60 (6/10)
A3, B3	13 (1/8)	40 (0–400)	0 (0/8)
2. Control	80 (16/20)	870 (0–4,000)	55 (11/20)
B3, A15	15 (2/13)	62 (0–400)	0 (0/13)
3. Control	80 (8/10)	1,280 (0–3,200)	70 (7/10)
A15	50 (5/10)	240 (0–800)	30 (3/10)

For each experiment, transgenic mosquitoes and sibling control (non-transgenic) mosquitoes were fed on the same mouse, which was infected with *P. berghei*. To measure transmission, single mosquitoes were fed on individual naive mice 25 days after ingesting the infectious blood meal. The salivary gland of each mosquito was dissected immediately after feeding on the mouse, and the number of sporozoites per salivary gland was counted ('sporozoite intensity'). The infection status of each mouse was established by examining a smear of tail vein blood on alternate days. Mice that had no parasites by day 25 were considered not to be infected. A3, A15, B3 and B6 indicate the transgenic lines used in each experiment (Fig. 1c). Where two lines are indicated, a mixture of mosquitoes from those lines was used.

*The per cent mosquitoes that had infected salivary glands. This value was derived from the number of sporozoite-positive mosquitoes over the total number of mosquitoes examined (shown in parentheses).

†The mean sporozoite number per salivary gland. The range of values is indicated in parentheses. This is a minimum estimate because sporozoites from only an aliquot of the salivary gland homogenate were counted. In all cases, infection intensity of transgenic mosquitoes was significantly different ($P < 0.05$) from that of controls, as analysed by the Mann–Whitney *U*-test.

‡The per cent mosquitoes that transmitted the parasite to a naive mouse. The number of infected mice over the total is given in parentheses.

foreign genes across mosquito populations in the field. Another potential obstacle is the genetic diversity and mutability of *Plasmodium*. Because development of the parasite in transgenic mosquitoes is not completely blocked, the possibility exists that 'resistant' variants will be selected. To address this concern, it will be important that mosquitoes be modified with multiple genes, each of which inhibits parasite development by a different mechanism. Work in progress in our laboratory, and in others, is seeking to identify such additional 'effector genes'. Although considerable efforts are needed to respond to these many challenges, the potential payoff is large.

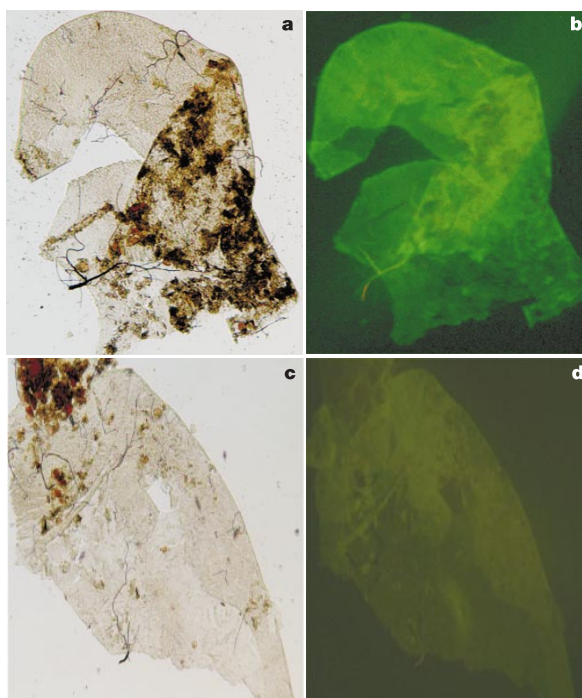


Figure 2 Detection of mosquito-synthesized [SM1]₄ protein. Midguts were dissected 24 h after a blood meal, opened into a sheet, fixed and incubated with an anti-HA1 antibody (Boehringer Mannheim; 1:4,000 dilution), followed by incubation with a fluorescent secondary antibody. **a, b**, Midgut from a female heterozygous for the

AgCP[SM1]₄ gene. **c, d**, Midgut from a wild-type (non-transgenic) female. In each case, a differential interference contrast microscopic image (left) is paired with a fluorescent image (right) of the same midgut.

Genetic manipulation of mosquito vector competence of the type reported here would add a new weapon to the arsenal (drugs, insecticides and perhaps vaccines) for our war against malaria. □

Methods

Transformation vector

For [SM1]₄, a synthetic gene coding for four units of the SM1 peptide (PCQRAIFQSQICN) separated by 4-amino-acid (GSPG) linkers was constructed as follows. Two oligonucleotides, SM1⁺ (5'-C CCGTGCCAGCGCGCCATCTTCCAGTGCATCTGCAA CGGCTCGCCGGG-3') and SM1⁻ (5'-GCCCGGGAGCCGTTGCAGATCGACTGGAA GATGGCGCGCTGGCAGG-3'), were annealed, phosphorylated and self-ligated. The ligation products were fractionated by gel electrophoresis and the 4-repeat unit was excised from the gel to yield [SM1]₄. Two adaptors, 5' and 3', were added to [SM1]₄. The 5' adaptor was obtained by annealing 5'-CGGATCCCCGGG-3' and 5'-GCCCGGGGA TCCGGTAC-3', and the 3' adaptor by annealing 5'-CTACCCTACGACGTGCCCGAC TACCCCG-3' and 5'-GATCCGGGTAGTCGGGCACGTCGTAGGGGTA-3'. The 3' adaptor codes for the HA1 influenza haemagglutinin epitope.

For 5' Cp, a 1.8-kilobase (kb) *KpnI-KpnI* fragment containing the *A. gambiae* CP 1.7-kb promoter, 5' untranslated region (UTR) and signal peptide down to nucleotide +125 (ref. 9) was obtained by PCR with T7 (5'-GTAATACGACTCACTATAGGGG-3') and AgCPKpn (5'-GGTACCCTCGGCGGCTTCGACACT-3') primers using the pBluescript AgCP genomic subclone⁹ as a template, followed by digestion with *KpnI*.

For 3' Cp, a 555-base pair (bp) fragment containing the CP 3' region (including the stop codon and 3' UTR; nucleotides +1,337 to +1,880) was obtained by PCR with the primers AgCP3BH (5'-GGATCTGGAAGTCTCTCTACCGG-3') and AgCP3Sc (5'-CCGCGGTAAGGCTAGCATTGCCA-3') using the AgCP pBluescript genomic subclone as a template, followed by digestion with *Bam*HI and *Sac*II. The three fragments, 5' Cp, [SM1]₄ with adaptors, and 3' Cp, were combined and sub-cloned into pGEM-T Easy vector (Promega), then digested with *Nor*I and inserted into the *Nor*I site of pSLfa1180fa (ref. 11). This construct was digested with *Fse*I and *Asc*I, and inserted into the *Fse*I-*Asc*I site of pBac[3xP3-EGFPam] plasmid¹¹ to yield pBacAgCP[SM1]₄.

Germline transformation

Germline transformation of *A. stephensi* embryos was as previously described⁴ with modifications. Briefly, embryos were treated with 0.2 mM *p*-nitro phenyl *p'*-guanidinobenzoate (Sigma) and microinjected with quartz needles pulled on a P-2000 puller (Sutter). The construct pBacAgCP[SM1]₄ (0.5 mg ml⁻¹) was mixed with the helper phsp-pBac (0.3 mg ml⁻¹)¹². For each founder family, 1–4 adult mosquitoes originating from the injected embryos (G₀) were mated with 5–10 wild-type mosquitoes of the opposite sex. In the next generation (G₁), transgenic mosquitoes were screened by searching for larvae displaying green fluorescence (Fig. 1b). In each generation, mosquitoes were propagated by crossing transgenic males with virgin non-transgenic females from the population that was used to create the transgenic lines. This ensured that the genetic background of all transgenic lines was the same as that of the wild-type control mosquitoes.

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HDAC6 is a microtubule-associated deacetylase

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Reversible acetylation of α -tubulin has been implicated in regulating microtubule stability and function¹. The distribution of acetylated α -tubulin is tightly controlled and stereotypic. Acetylated α -tubulin is most abundant in stable microtubules but is absent from dynamic cellular structures such as neuronal growth cones and the leading edges of fibroblasts^{1,2}. However, the enzymes responsible for regulating tubulin acetylation and deacetylation are not known. Here we report that a member of the histone deacetylase family, HDAC6, functions as a tubulin deacetylase. HDAC6 is localized exclusively in the cytoplasm, where it associates with microtubules and localizes with the microtubule motor complex containing p150^{Glued} (ref. 3). *In vivo*, the overexpression of HDAC6 leads to a global deacetylation of α -tubulin, whereas a decrease in HDAC6 increases α -tubulin acetylation. *In vitro*, purified HDAC6 potently deacetylates α -tubulin in assembled microtubules. Furthermore, overexpression of HDAC6 promotes chemotactic cell movement, supporting the idea that HDAC6-mediated deacetylation regulates microtubule-dependent cell motility. Our results show that HDAC6 is the tubulin deacetylase, and provide evidence that reversible acetylation regulates important biological processes beyond histone metabolism and gene transcription.

Extensive studies of histone acetylation, a process controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs), have firmly established a role for reversible acetylation in transcriptional regulation and histone metabolism⁴. At least 11 proteins predicted to be members of the HDAC family have been identified on the basis of homology within the catalytic domain^{5,6}. The sequences outside the catalytic domain are highly divergent, indicating that these enzymes might have different biological functions and a broader substrate repertoire beyond histones. Indeed, recent studies reveal that many non-histone nuclear transcription factors, such as p53, E2Fs and myoD, are regulated by acetylation^{7–9}. Furthermore, there are also cytoplasmic proteins that are subject to modification by acetylation (reviewed in ref. 10). The most notable of these is α -tubulin.