

Robust gut-specific gene expression in transgenic *Aedes aegypti* mosquitoes

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Genetic modification of the vectorial capacity of mosquito vectors of human disease requires promoters capable of driving gene expression with appropriate tissue and stage specificity. We report on the characterization in transgenic *Aedes aegypti* of two mosquito gut-specific promoters. A 1.4-kb DNA fragment adjacent to the 5' end of the coding region of the *Ae. aegypti* carboxypeptidase (*AeCP*) gene and a corresponding 3.4-kb DNA fragment at the 5' end of the *Anopheles gambiae* carboxypeptidase (*AgCP*) gene were linked to a firefly luciferase reporter gene and introduced into the *Ae. aegypti* germ line by using *Hermes* and *mariner* (*Mos1*) transposons. Six independent transgenic lines were obtained with the *AeCP* construct and one with the *AgCP* construct. Luciferase mRNA and protein were abundantly expressed in the guts of transgenic mosquitoes in four of the six *AeCP* lines and in the *AgCP* line. Expression of the reporter gene was gut-specific and reached peak levels at about 24 h post-blood ingestion. The *AeCP* and *AgCP* promoters can be used to drive the expression of genes that hinder parasite development in the mosquito gut.

malaria | *Hermes* | *mariner* | promoter function

Mosquitoes are responsible for the transmission of major human diseases such as malaria, yellow fever, and dengue. The recent development of transgenic mosquitoes allows for an unprecedented analysis of the molecular interactions between mosquitoes and the pathogens they transmit. Two transposon-mediated germ-line transformation systems have been shown to produce stable integration of DNA into the genome of the yellow fever mosquito, *Aedes aegypti* (1–3). *Ae. aegypti* is a vector for avian malaria, dengue, and yellow fever viruses. A remaining technical hurdle for the genetic manipulation of mosquitoes has been the development of appropriate stage- and tissue-specific promoters to drive the expression of pathogen-targeting gene products. A significant advance was the demonstration of reporter gene expression in the salivary glands of mosquitoes that were transformed with the transposons *Hermes* and *mariner* (*Mos1*) (1, 2).

Mosquito-borne diseases are propagated when the mosquito ingests an infected blood meal. The ingestion of blood leads to a dramatic activation of many genes encoding digestive enzymes (4–6). The availability of gut-specific promoters to drive the expression of antimalarial proteins in the mosquito midgut would open a new frontier to hinder *Plasmodium* development in the mosquito. Here we report that *Ae. aegypti* and *Anopheles gambiae* carboxypeptidase (*AeCP* and *AgCP*, respectively) promoters can be used to drive strong and blood-inducible gene expression in the gut of transgenic *Ae. aegypti*.

Materials and Methods

Plasmid Construction. For *Hermes* transformation, a 3.4-kb *ApaI/SalI* fragment containing ≈ 1.4 kb of the *AeCP* upstream region (7) fused to 2.0 kb of luciferase reporter gene of pGL3 BASIC (Promega) was inserted into p*Hermes* [cn] (ref. 3; gift of M. Q. Benedict, Centers for Disease Control, Chamblee, GA)

into the unique *ApaI* and *XhoI* sites. For *Mos1* transformation, a ≈ 5.4 -kb *NotI/SalI* fragment containing 3.4 kb upstream of the *AgCP* upstream region (8) fused to the same 2.0 kb of luciferase reporter gene was ligated into p Δ M SLFC [cn] (ref. 1; gift of C. J. Coates, Texas A&M University, College Station) into the unique *NotI* and *XhoI* sites.

Embryo Microinjection and Mosquito Rearing. Embryos of homozygous white-eyed *kh^w* mosquitoes (obtained from the University of Notre Dame stock center) were injected as described (1, 9). Embryos were injected with a mixture of the transposon construct (0.5 mg/ml) and either the *Hermes* helper plasmid, pHSHH1.9 (0.3 mg/ml) or the *Mos1* helper plasmid, pKhsp82MOS (0.5 mg/ml), in 5 mM KCl and 0.1 mM NaH₂PO₄ (pH 6.8). Microinjected embryos were exposed to heat shock (42°C for 1 h) to induce the expression of *Hermes* or *Mos1* transposases, which are under the control of the *Drosophila melanogaster* hsp70 and hsp82 gene promoters, respectively. Embryos were kept for 5 days at 27°C and 80% relative humidity before hatching.

Mosquitoes were reared by the methods described (10). Individual adult G₁ males with colored eyes were crossed with five virgin noninjected *kh^w* females. Approximately five G₁ virgin females with colored eyes were pooled and mated with 2–4 noninjected *kh^w* males. Females were allowed to feed on blood, and G₁ eggs were collected. Transformants were selected by screening the eye color of adults. This was continued for every generation to establish homozygosity of the transgenic lines.

Southern Blot Analysis. Total genomic DNA was isolated from G₆ transgenic and white-eyed mosquitoes as described (11). DNA digested with *EcoRI* and *BamHI* was separated on a 1% agarose gel, blotted, and hybridized with α -³²P dCTP-labeled luciferase probe (≈ 1.6 -kb fragment from pGL3 BASIC, double digested with *XbaI/NcoI*). To verify DNA loading in each lane, the blot was hybridized with a 0.7-kb *Ae. aegypti* late trypsin probe (12).

Reverse Transcriptase (RT)–PCR. To assay for the presence of luciferase mRNA, total RNA was extracted from guts of transgenic *AeCP* and *kh^w* mosquitoes 24 h after blood feeding. RT (SuperScript II, GIBCO/BRL) reactions were performed after treating the RNA with RQ1 RNase-free DNase (Promega). Primers specific to the *AeCP* coding region (7) (CPRTF: 5'-TTCATTCATACTCTCAACTTCTCTTGTGCCGTAT-3' and CPRTR: 5'-GTCGCAGTTCGTAGGTGTAGGCAATC-TTTA-3') were used together with others specific to luciferase (Lux1105F: 5'-TTTGAAGCGAAGGTTGTGGAT-3' and

Abbreviations: *AeCP*, *Aedes aegypti* carboxypeptidase; *AgCP*, *Anopheles gambiae* carboxypeptidase; RT, reverse transcriptase.

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Lux1462R: 5'-GTCATCGTCTTTCCGTGCTC-3') in a PCR (95°C, 1 min; 55°C, 1:30 min; 72°C, 1 min, 30 cycles).

Northern Blot Analysis. Mosquito guts and carcasses (whole body minus gut) were dissected from 4- to 5-day-old females. Male guts and pupae also were collected, and all tissues were frozen in an ethanol/dry ice bath and immediately stored at -80°C. To determine the temporal profile of luciferase expression in transgenic mosquitoes, guts were dissected at increasing time intervals after a blood meal. Total RNA was extracted with TRI-Reagent (Molecular Research Center, Cincinnati). RNA from ≈10 mosquitoes was separated in each lane of 1.5% agarose-formaldehyde gels and blotted by capillary action to a nylon membrane (Gene Screen, New England Nuclear). Hybridization was performed first with the luciferase probe (described above) and then with a mitochondrial rRNA probe (5, 13) as a loading control.

Western Blot Analysis. Guts were dissected 24 h after a blood meal and homogenized in PBS. The equivalent of 10 guts per lane was analyzed by electrophoresis on a 10% polyacrylamide/SDS gel, followed by electrotransfer to a poly(vinylidene fluoride) membrane. The membrane was incubated with a rabbit antiluciferase polyclonal antibody (Accurate Chemicals), and the signal was detected with a goat anti-rabbit Ig conjugated to alkaline phosphatase (Promega).

Polysome Analysis. Two samples of guts from transgenic mosquitoes, dissected 24 h after a blood meal, were homogenized in 10 mM Tris-HCl (pH 7.0), 5 mM MgOAc, 100 mM KCl, 1% Triton, and 0.5% deoxycholic acid in the presence or absence of 10 mM EDTA (15). The samples were centrifuged for 5 min at 13,600 g, and the resulting postmitochondrial fractions were loaded onto a 10–50% sucrose gradient. Samples were centrifuged at 100,000 g on a SW 28.1 rotor in a Beckman L7–55 ultracentrifuge for 4.5 h. Gradients were analyzed with an ISCO UV-5 monitor with continuous recording at 254 nm. Fourteen fractions were collected. RNA was extracted from each fraction with phenol/chloroform. The RNAs were analyzed by Northern blotting using a luciferase probe.

Results

Germ-Line Transformation. The *AeCP* and *AgCP* genes both are expressed specifically in the mosquito gut and strongly induced by ingestion of a blood meal (7, 8). We investigated whether the upstream regions of the carboxypeptidase genes are sufficient to drive the expression of a transgene with a tissue and temporal pattern similar to that of the endogenous genes. Constructs with a luciferase reporter gene under the control of the putative *AeCP* or *AgCP* promoters were cloned into vectors containing *Hermes* or *Mos1* transposable elements, respectively. These plasmids were injected into *Ae. aegypti* embryos. Six transgenic lines, designated 2T, 13T, 42T, 46T, 64T, and 76T, were obtained for the *AeCP* construct, four of which had dark eye color and two (42T and 64T) had either faint orange eye color or displayed mosaicism (Table 1). One transgenic line, *AgCP*, was obtained with the *Mos1* construct. Mosaicism in *Hermes*-transformed *Ae. aegypti* has been previously observed (3). Although survival was lower for the *AgCP* transformation experiment, transformation efficiency was similar. Transformation efficiency, based on percentage of transgenic lines derived from individual male family founders (4.3% for the *Hermes* and 6.6% for the *Mos1* constructs), are consistent with percentages reported in previous studies (1, 3, 14).

Integration of the constructs into the germ line of the transformed mosquitoes was verified by Southern blot experiments (Fig. 1). Using a luciferase probe, a 2-kb band was detected in five of the six *AeCP* (*Hermes*) transgenic lines and in the *AgCP*

Table 1. Microinjection results of *AeCP* and *AgCP* constructs

	<i>AeCP</i> in <i>Hermes</i>	<i>AgCP</i> in <i>Mos1</i>
Embryos injected	1,594	1,579
Adults emerged	200 (12.54%)*	19 (1.2%)*
G ₀ families started	109	15
Transformed lines established	6 (4.3%) [†]	1 (6.6%) [†]

*Percentage of injected embryos that hatched into adults.

[†]Percentage of the lines derived from individual male family founders that underwent a stable transformation.

(*Mos1*) line. Loading of DNA was verified by probing the same blots with a trypsin probe. Luciferase sequences were not detected in *AeCP* line 64T, which also exhibited mosaic eye color.

The Homologous *AeCP* Promoter Drives Strong Blood-Inducibile Expression of Luciferase mRNA Only in the Midgut. Expression of the integrated genes was measured first by RT-PCR. PCR was conducted with a mixture of two sets of primers: one specific for the sequence of the luciferase transgene, and another specific for the sequences of the endogenous carboxypeptidase gene, to serve as an internal positive control. A carboxypeptidase amplification product was observed from all gut RNA samples (Fig. 2). A luciferase amplification product was observed from gut RNA samples isolated from all but one (64T) transgenic lines. This was expected, because Southern analysis indicated that luciferase sequences were absent in line 64T (Fig. 1). No luciferase product was detected with the nontransformed strain, as expected. The signal from line 42T was consistently weaker when compared with other lines.

Tissue-specific expression was analyzed on Northern blots (Fig. 3). Abundant luciferase mRNA was detected in guts (but not in carcasses) from four of the six *AeCP* transgenic lines. Weak expression was detected in line 42T, and no expression was detected in line 64T and in nontransformed mosquitoes.

Expression from the carboxypeptidase promoters was strongly induced by blood ingestion. The time course of luciferase induction from the *AeCP* promoter (Fig. 4A) closely resembles that of the endogenous carboxypeptidase gene (7).

The Heterologous *AgCP* Promoter Drives Strong Luciferase Expression with the Correct Developmental, Tissue, and Sex Specificity. As for *AeCP*, expression from the heterologous *AgCP* promoter is strongly induced by blood ingestion (Fig. 4B). However, peak induction is reached late (24 h) after a blood meal in transgenic

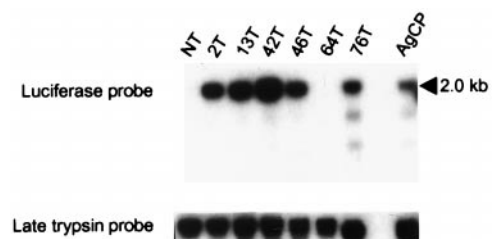


Fig. 1. Verification of transgene integration by Southern blot analysis. Genomic DNA from the nontransformed and transformed mosquito lines was digested with *EcoRI* and *BamHI* and analyzed on a Southern blot with a 1.6-kb luciferase probe. The predicted 2.0-kb luciferase fragment is observed in five of the six *AeCP* transgenic lines and in the *AgCP* line but not in the nontransformed line. To verify loading of DNA in all lanes, the blot subsequently was hybridized with a late trypsin probe (loading control). The source of DNA is indicated at the top of each lane. NT: DNA from *kh^w/kh^w* (nontransformed) mosquitoes. Numbers followed by a T indicate the designation of each transformed mosquito line. *AgCP*: DNA from mosquitoes transformed with the *AgCP* construct.

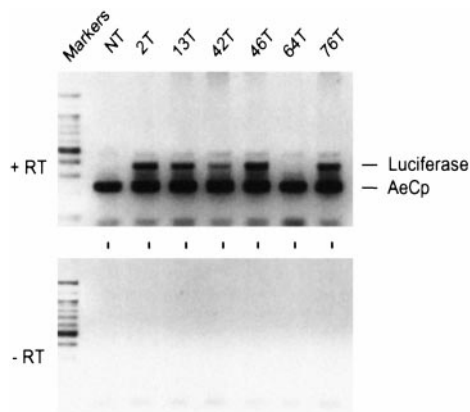


Fig. 2. Assessment of *AeCP* expression by RT-PCR. RT-PCR was conducted with a mixture of two sets of primers: one specific for luciferase that generates a 376-nt fragment (luciferase), and one specific for the endogenous carboxypeptidase that generates a 237-nt fragment (*AeCP*). The template was total RNA extracted from guts 24 h after a blood meal. Luciferase mRNA was detected in five of the six transgenic lines. No luciferase signal was detected with RNA from nontransformed mosquitoes (NT), as expected. The source of RNA is indicated at the top of each lane (see Fig. 1 legend). DNA size markers were run in the first lane of each panel. +RT: PCRs done after RT of the RNA; -RT: RT was omitted.

Ae. aegypti (Fig. 4B) rather than early (3 h) as the endogenous carboxypeptidase promoter in *An. gambiae* (8). Moreover, Fig. 4B provides evidence for the correct tissue (gut) specificity, correct developmental regulation (adults only), and sex specificity (females only) of the *AgCP* promoter.

Robust Luciferase Protein Expression in the Guts of Transgenic Mosquitoes. Assays of gut homogenates from the transformed mosquitoes led to the surprising finding that luciferase enzyme activity was absent from all transgenic lines, despite the presence of abundantly expressed mRNA (see above). Further analysis revealed that because of a cloning artifact a mutation occurred in an early step of assembling the constructs that resulted in a deletion of a T at residue 468 of the luciferase gene. This mutation caused a frame shift that resulted in the premature termination of the protein at amino acid 493. Western blot analysis with a luciferase antibody confirmed this finding (Fig. 5). In addition to several common cross-reacting bands in all samples, a truncated protein of about 55 kDa recognized by the antiluciferase antibody is present only in guts of the *AgCP* and *AeCP* transgenic lines. This is the size predicted from the sequence analysis described above.

Translation of the recombinant luciferase mRNA in the guts of transgenic mosquitoes was independently verified by measuring its association with polysomes. We found that the vast majority of the luciferase mRNA is polysome-associated (Fig. 6A). This association was EDTA-sensitive, indicating that asso-

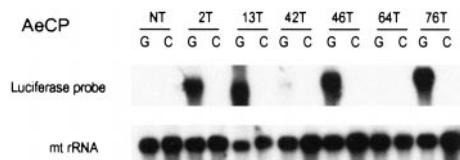


Fig. 3. Northern blot analysis of luciferase expression in mosquitoes transformed with the *AeCP* construct. RNA extracted from dissected blood-fed guts (G) and carcasses (C) (nongut tissues) was analyzed by Northern blotting with a radioactive luciferase probe. RNA loading in each lane was verified by hybridization of the same blot with a mitochondrial rRNA (mt rRNA) probe (5).

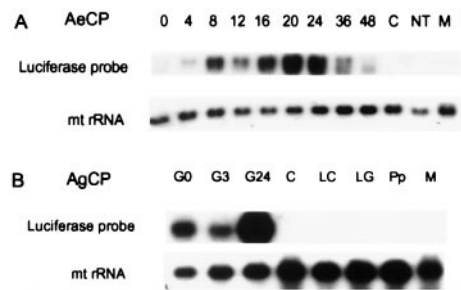


Fig. 4. Analysis of transgenic mRNA accumulation. (A) Time course of luciferase mRNA accumulation after a blood meal. Total RNA extracted from guts (or carcasses) dissected at different times after a blood meal was fractionated by electrophoresis on agarose gels and blotted to a nylon membrane. The membrane was first hybridized with a radioactive luciferase DNA probe and then with a mitochondrial rRNA probe, used as a loading control. Numbers above the lanes refer to times after a blood meal when *AeCP* transgenic mosquitoes were dissected. 0: Guts from sugar-fed mosquitoes; C: carcasses (nongut tissues); NT: guts from nontransformed mosquitoes dissected 24 h after a blood meal; M: male gut. (B) Assessment by Northern blot analysis of luciferase expression in mosquitoes transformed with the *AgCP* construct. RNA was extracted from different tissues, developmental stages, or sexes and analyzed by Northern blotting with a radioactive luciferase probe. RNA loading was verified as above. G: Guts (numbers indicate hours after a blood meal); C: carcass; LC: larval carcass; LG: larval gut; Pp: pupae; M: male gut.

ciation of the luciferase mRNA was functionally significant and probably not caused by the cosedimentation of aggregated particles.

Discussion

Using the transposable elements *Hermes* and *Mos1*, we generated transgenic *Ae. aegypti* mosquitoes capable of driving robust sex-, tissue- and stage-specific mRNA and protein expression in the midgut epithelium. Carboxypeptidase promoters from *Ae. aegypti* and the distantly related *An. gambiae* both were functional. Furthermore, both promoters were strongly induced by blood ingestion. This finding is significant because *Plasmodium* ookinetes reach the midgut epithelium at a time of peak transgene expression. Considering that the midgut epithelium is also the source of the transgenic protein, this is an ideal temporal

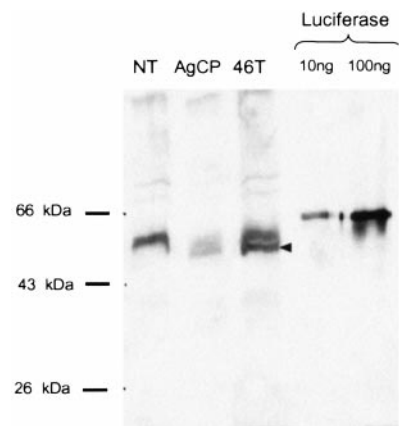


Fig. 5. Western blot analysis of luciferase protein expression. Guts from the nontransformed recipient mosquito line (NT), the *AgCP* line, or an *AeCP* line (46T) were dissected from mosquitoes 24 h after a blood meal. Gut homogenates were analyzed by Western blotting with an antiluciferase antibody. The two lanes on the right contain recombinant luciferase (10 and 100 ng), used as a positive control. Migration of molecular weight markers is indicated on the left. The arrowhead indicates the position of migration of the predicted truncated protein (see text), detected only in the transgenic lines.

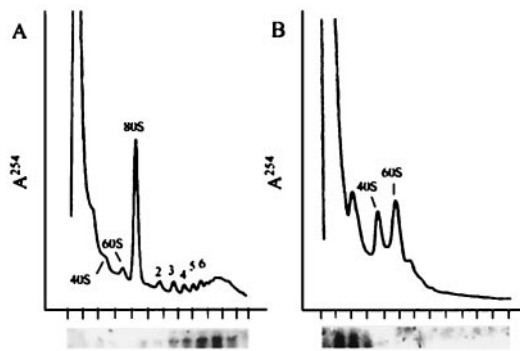


Fig. 6. Association of the luciferase mRNA with polysomes. Guts from mosquitoes transformed with the *AeCP* construct were dissected at 24 h after a blood meal, homogenized, and divided into two equal aliquots. One was analyzed on a sucrose gradient without further additions (A), and the other was analyzed after dissociation of polysomes by addition of EDTA to 10 mM (B). The upper part of each panel shows the A^{254} profile of the gradient after centrifugation, and the lower part shows the Northern blot analysis of each fraction of the gradient by using a radioactive luciferase probe. The short vertical marks below the abscissae indicate the position of each fraction. The numbers above the absorption profiles indicate the position of migration of the ribosomal subunits (40S and 60S), whole ribosomes (80S), or the number of ribosomes per polysome.

pattern for driving the expression of antimalarial gene products that target ookinetes. Thus, the promoters characterized in this work constitute a valuable tool for the genetic modification of mosquitoes.

The strength of the promoters was measured not only by the abundance of mRNA that accumulated in the midgut epithelium, but also by abundant protein expression. Based on the comparison of the signal of the transgenic luciferase on Western blots with that of a reference enzyme, it can be calculated that approximately 2 ng of luciferase protein accumulated per gut at 24 h after a blood meal. Based on a 1- μ l volume in the midgut lumen, that would yield a concentration of 2 μ g/ml. Concentration at the periphery, near the midgut epithelium, is probably much higher if diffusion of the secreted protein across the peritrophic matrix and into the blood bolus is slow. It is therefore feasible that a protein expressed from the carboxypeptidase promoter will accumulate at sufficiently high concentrations in

the midgut lumen to act as an effective inhibitor of *Plasmodium* development.

Ae. aegypti and *An. gambiae* bear only a distant evolutionary relationship. These mosquitoes last shared a common ancestor more than 140 million years ago (16). Thus it was impossible to predict whether the *AgCP* promoter, which does not contain any apparent sequence similarity to the *AeCP* promoter, would drive gut-specific expression in the *Ae. aegypti* gut. There are precedents for such functional conservation of tissue-specific promoters (17–19). Although most properties of the *AgCP* promoter were maintained in transgenic *Ae. aegypti*, one significant difference was observed. Although induction of the *AgCP* promoter by a blood meal is rapid (3 h) in *An. gambiae* (8), it was much slower (24 h) in *Ae. aegypti* (Fig. 4B). Possibly *Ae. aegypti* transcription factors may not recognize the appropriate *An. gambiae* promoter regulatory sequences. Alternatively, some promoter sequences may be missing from the \approx 3.4-kb fragment used for these experiments. In any event, transformation of anopheline mosquitoes, which are the relevant mosquitoes for human malaria transmission, recently has been achieved (20). Analysis of the carboxypeptidase promoters in anopheline mosquitoes should clarify these issues. The early induction of the *AgCP* promoter in anophelines would allow transgene expression at a much earlier stage of parasite development, before maturation of the peritrophic matrix (which may be a physical barrier to transgenic protein diffusion) and before the induction of the main digestive enzymes (which may promote degradation of transgenic products). Ideally, expression of an antimalarial gene product would be driven by both promoters, thus maximizing protein production during most of the parasite development in the mosquito midgut.

In summary, we report on the functional characterization of two mosquito gut-specific promoters. They are able to drive robust expression of mRNA and protein in a blood-inducible manner. These properties make the carboxypeptidase promoters ideal for use in the genetic modification of mosquitoes to refractoriness to the malaria parasite.

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