

Regulated expression of microinjected DNA in adult *Aedes aegypti* mosquitoes

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Abstract

We have developed a novel molecular genetic approach to investigating gene regulation in adult mosquitoes called whole body transfection (WBT). This DNA microinjection method allows for both constitutive and regulated expression of plasmid vectors in the fat body and midgut of adult mosquitoes within 24 h of injection. Using a luciferase reporter gene containing the *Aedes aegypti* heat shock protein 70 (Hsp70) promoter, we optimized the WBT protocol at various times post-injection and used these parameters to measure the expression of a vitellogenin-luciferase reporter gene in response to blood meal feeding. These studies showed that a 843 bp fragment of the *Ae. aegypti* vitellogenin-C (VgC) promoter caused a greater than 200-fold induction of luciferase activity in a strict tissue-specific manner, and only in response to feeding. Functional mapping of the VgC promoter by WBT identified essential upstream regulatory elements in the region spanning –780 to –182 bp from the transcriptional start site. We also constructed a lipopolysaccharide-regulated expression vector using a 1096 bp genomic fragment of the *Ae. aegypti* cecropin B (CecB) promoter. Our data show that four days after WBT injection, the CecB-luciferase reporter gene could be induced more than 100-fold in the fat body following lipopolysaccharide injection. Moreover, we found that lipopolysaccharide-induction of the CecB reporter gene occurred up to 28 days post-WBT injection. These data suggest that WBT could provide a

novel strategy to express recombinant proteins and RNAi constructs in adult mosquitoes using conventional microinjection methods.

Keywords: luciferase, Hsp70, vitellogenin, cecropin, whole body transfection.

Introduction

The incidences of mosquito borne diseases such as malaria, Dengue, yellow fever and West Nile viruses are on the rise, due in part to a lack of efficient mosquito control strategies. One approach to developing new anti-mosquito strategies is to find ways to inhibit normal metabolic processes associated with larval development or vitellogenesis. For example, by understanding how protease gene expression in the midgut of adult female mosquitoes is coordinately regulated following a blood meal, it may be possible to disrupt this process and thereby block vitellogenesis. Moreover, recent analysis of ammonia metabolism in *Aedes aegypti* mosquitoes has revealed potential control points in glutamine and proline biosynthesis that could be exploited if more was known about related metabolic pathways (Scaraffia *et al.*, 2005).

Two molecular genetic approaches that have been used to investigate biochemical processes in mosquitoes are germline transformation (Adelman *et al.*, 2002) and RNA interference (RNAi) (Sanchez-Vargas *et al.*, 2004; Lu *et al.*, 2006). However, each of these techniques have drawbacks. For example, germline transformation in mosquitoes is inefficient and results can be affected by regulatory sequences flanking the integration sites (Moreira *et al.*, 2002). Moreover, maintenance of transformed mosquito strains is costly and labourious. Likewise, RNAi is not without complications because the effects can be inconsistent and, in some cases, require large amounts of double strand RNA that could promote off-target interference (Boisson *et al.*, 2006). An alternative approach to investigating regulation of mosquito genes has been to perform transient transfection experiments in insect cell lines such as the *Drosophila* S2 cell line (Martin *et al.*, 2001), or immortalized mosquito epithelial cell lines (Gray & Coates, 2004). Results from these cell line transient transfection studies

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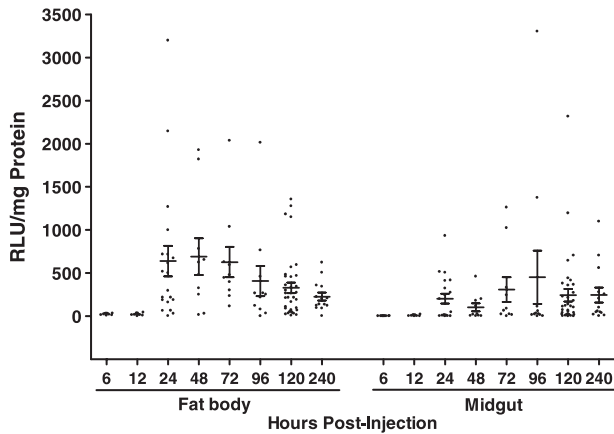


Figure 1. Time course of Hsp70-Luc expression in the fat body and midgut of female mosquitoes following DNA microinjection. Mosquitoes were microinjected within two hours after adult eclosion with 1.0 µg of pHsp70-Luc and luciferase activity was measured in isolated fat body and midgut of individual mosquitoes at various time points. The mean \pm standard error of the mean are shown.

are not always applicable to whole mosquitoes because of differences in the expression of tissue-specific regulatory factors and the inability to study metabolic effects of blood meal feeding.

Based on the success of RNAi microinjection in *Aedes* and *Anopheles* mosquito species (Blandin *et al.*, 2002; Hansen *et al.*, 2004), and the observation that plasmid DNA is transcribed in various mammalian tissues following injection of purified DNA (Zhang *et al.*, 2003; Danialou *et al.*, 2005), we developed a mosquito transient gene expression method we call whole body transfection (WBT). Importantly, the WBT method uses the same microinjection procedures

developed for RNAi studies to deliver plasmid DNA expression vectors directly to the mosquito haemolymph. Potential applications of the WBT procedure to molecular studies in adult mosquitoes using recombinant proteins and short hairpin RNAi are discussed.

Results

Optimization of Hsp70-luciferase reporter gene expression in WBT-injected mosquitoes

We chose to use the *Ae. aegypti* Hsp70 promoter for optimization of the WBT protocol because we found it to be highly expressed in a variety of mosquito tissues (Isoe, unpublished data). For these experiments, we inserted a 659 bp fragment of the *Ae. aegypti* Hsp70 gene containing 159 bp of the 5' UTR and 500 bp of the promoter region and upstream sequences into the promoterless pGL3 firefly luciferase reporter gene construct to create pHsp70-Luc. Newly emerged female mosquitoes were microinjected in the thorax with 500 nL of TE buffer containing 2.0 µg/µL of endotoxin-free plasmid DNA (1.0 µg total). At various times, the fat body and midgut of individual mosquitoes was isolated and used to prepare protein extracts. Luciferase activity in these extracts was measured with a luminometer and expressed as relative light units (RLUs) per mg protein.

As shown in Fig. 1 and summarized in Table 1, fat body expression of the Hsp70-luciferase reporter gene was observed in 40–60% of the injected mosquitoes at 6 and 12 h post-injection (PI) based on an arbitrary cutoff value of 20.0 RLU/mg protein. Note that the level of luciferase activity in mosquitoes injected with the promoterless pGL3 luciferase plasmid averaged only ~2.0 RLU/mg protein, which was 10-fold lower than the cut-off we chose for defining

Table 1. Luciferase activity in pHSP70-Luc and pGL3-Basic Injected mosquitoes

PI (h)	N	Fat body				Midgut			
		Positive	Mean	SEM	Range	Positive	Mean	SEM	Range
pHsp70-Luc¹									
6	10	6	29.2	1.5	23.0–33.5	0	–	–	–
12	10	4	32.8	4.7	21.0–44.0	1	21.0	–	–
24	20	19	672.0	183.5	30.1–3198	11	363.8	72.1	46.8–932.1
48	10	9	765.6	222.9	29.2–1927	5	196.1	72.5	26.1–459.4
72	10	10	626.6	176.0	15.5–2037	8	382.5	171.7	22.8–1262
96	11	10	447.0	186.4	33.6–2014	7	703.7	471.7	22.2–3303
120	36	34	348.0	63.5	22.3–1356	26	332.0	95.3	21.4–2317
120	16	15	204.1	40.9	22.5–591.5	13	605.4	340.1	24.0–3694
240	14	13	243.2	46.3	90.0–622.1	11	308.0	103.3	38.2–1098
1080	7	2	70.2	1.9	68.3–72.1	1	32.7	–	–
Total	144	122	401.0	47.0	21.0–3198	83	397.0	75.0	21.4–3694
pGL3-Basic²									
120 (unfed)	10	0	1.7	0.3	0.2–3.4	0	1.8	0.4	0.0–3.9
120 (fed)	10	0	0.8	0.3	0.0–2.5	0	2.2	0.6	0.0–5.4

¹Calculations use a cutoff of 20.0 RLU/mg protein.

²RLU/mg protein values were calculated for all mosquitoes.

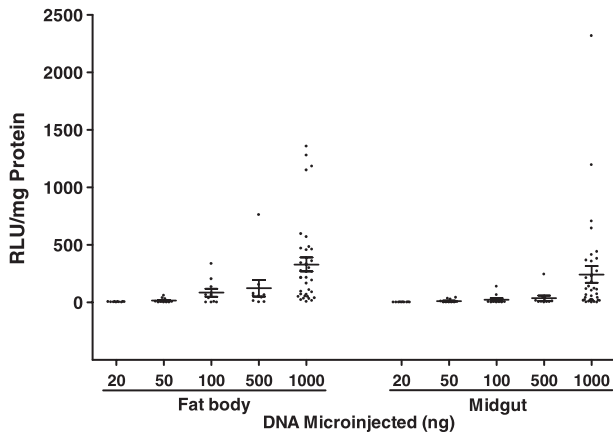


Figure 2. DNA dose–response profile of Hsp70-Luc expression in the fat body and midgut of female mosquitoes. Mosquitoes were microinjected with various amounts of pHsp70-Luc using the same volume of buffer. Luciferase activity was measured in isolated fat bodies and midguts of individual mosquitoes 120 h PI. The mean \pm standard error of the mean are shown. Note that the data shown for 1000 ng in this figure is the same as the 120 h time point in Fig. 1.

WBT-positive mosquitoes (Table 1). By 24 h PI, the mean level of luciferase activity in the fat body increased dramatically to 672.0 RLU/mg protein. This value was 20-fold higher than the level of luciferase activity at 12 h (32.8 RLU/mg protein) and > 300 times higher than the pGL3 plasmid control. Moreover, it can be seen that 95–100% of the injected mosquitoes were positive for luciferase activity in fat body extracts prepared 24–240 h PI, although the level of luciferase activity began to decline after 72 h PI (Table 1). By 1080 h PI (45 days), the number of fat body positive mosquitoes decreased to 28% with an average luciferase activity of 70.2 RLU/mg protein (Table 1).

We found that the level of luciferase activity in the midgut of these same mosquitoes was only slightly lower than in the fat body; however, in some cases, fewer mosquitoes had midgut luciferase activities that were above the cutoff of 20.0 RLU/mg protein (Table 1). Interestingly, the range of luciferase activity was similar in the fat body and midgut, both showing maximum values > 3000 RLU/mg protein. In addition to the fat body and midgut, we also were able to detect luciferase activity in the ovaries and malpighian tubules of female mosquitoes injected with the Hsp70-Luc plasmid (data not shown).

To determine the optimal amount of microinjected DNA for use in the WBT assay, we injected mosquitoes with 20–1000 ng of pHSP70-Luc and measured luciferase activities in the fat body and midgut at 96 h PI. As shown in Fig. 2, maximum levels of luciferase activity in both the fat body and midgut required 1 μ g of plasmid DNA, although measurable levels of luciferase activity were also observed using 50 ng of DNA. The transfection efficiency was also higher using 1 μ g of DNA for the fat body (80%) and midgut (60%)

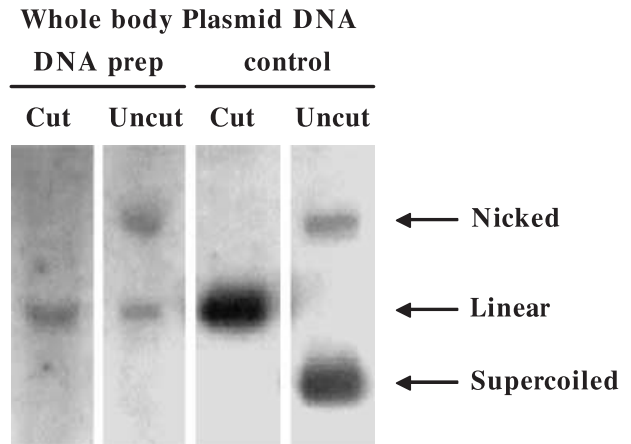


Figure 3. Determination of the fate of injected plasmid DNA in mosquitoes. Southern blot analysis was performed using total DNA isolated from pHsp70-Luc injected mosquitoes 10 days postinjection. The mosquito DNA sample and control plasmid DNA (pHsp70-Luc, 100 μ g) loaded on the gel were *Hind*III-digested or uncut. The blot was hybridized with a luciferase-specific digoxigenin labelled probe.

than it was using 50 ng of DNA (37.5% for both the fat body and midgut).

In order to determine the fate of the plasmid DNA following WBT injection, we injected newly emerged female mosquitoes with pHSP70-Luc DNA and then isolated total DNA from whole mosquitoes 10 days later. Pooled DNA samples were loaded uncut or *Hind*III-digested on to an agarose gel and analysed by Southern blotting using a luciferase-specific probe. As shown in Fig. 3, the only detectable pHSP70-Luc DNA in the mosquito DNA sample was unintegrated circular or linear DNA based on the gel migration pattern of uncut and *Hind*III-digested samples (5.4 kb linear DNA and nicked circles). The presence of linear plasmid DNA in the uncut samples suggests that random nicking of the DNA occurred *in vivo* or during the DNA preparation. We also examined the efficiency of pHSP70-Luc plasmid DNA uptake by quantitative real-time PCR using total DNA from various mosquito tissues and primers directed against the luciferase coding sequence or the endogenous Hsp70 gene. As shown in Table 2, fat body tissue showed the highest level of transfection efficiency with 7.3 times more luciferase template in the sample than that of the endogenous Hsp70 gene. Both the ovaries and malpighian tubules were also transfected with plasmid DNA and accumulated about half as much luciferase template as the fat body. Interestingly, the midgut tissue had the lowest DNA transfection efficiency, even though both the fat body and midgut express similar levels of luciferase enzyme activity (see Table 1).

Finally, we used the pHSP70-Luc reporter construct with WBT to measure luciferase activities in the whole abdomens of male *Ae. aegypti* mosquitoes. As shown in Fig. 4,

Table 2. Transfection efficiency of plasmid DNA by WBT

Tissues	Genomic Hsp70 gene ¹	Plasmid DNA ²	Transfection efficiency ³
Fat body	8.6	62.4	7.3
Midgut	3.5	2.2	0.6
Ovary	1.8	5.4	3.0
Malpighian tubules	1.0	3.9	3.9

¹Relative abundance of genomic DNA based on QPCR results with Hsp70 gene primers.

²Relative abundance of plasmid DNA based on QPCR results with luciferase reporter gene primers. The values are normalized based on the relative genomic DNA abundance of the genomic Hsp70 gene in the DNA preparation from Malpighian tubules.

³Ratio of plasmid DNA to genomic DNA in the tissue samples.

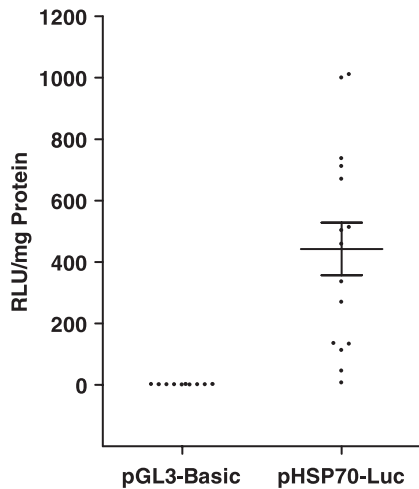


Figure 4. Expression of Hsp70-Luc in abdomens of microinjected male mosquitoes. Mosquitoes were microinjected with 1.0 µg of pHsp70-Luc or 1.0 µg of the pGL3 promoter-less luciferase reporter gene. Luciferase activity was measured in isolated fat body of individual mosquitoes at 96 h PI. The mean ± standard error of the mean are shown.

high levels of luciferase activity were observed in male mosquitoes at 96 h PI (mean value of 474.3 RLU/mg protein). These data demonstrate that the WBT is not limited to female mosquitoes.

Functional mapping of the *Ae. aegypti* promoter by WBT

One of our primary objectives in developing the WBT method was to use it for functional analysis of DNA sequences required for tissue-specific differential gene expression in response to blood meal feeding. To determine if WBT could be used for this purpose, we constructed a reporter plasmid in the pGL3 luciferase expression vector. The *Ae. aegypti* VgC gene has recently been characterized and shown to have the same pattern of differential gene expression in the fat body as the VgA and VgB genes (Isoe & Hagedorn, in press).

For these experiments, we inserted a fragment of the *Ae. aegypti* VgC gene containing 63 bp of the 5' UTR and

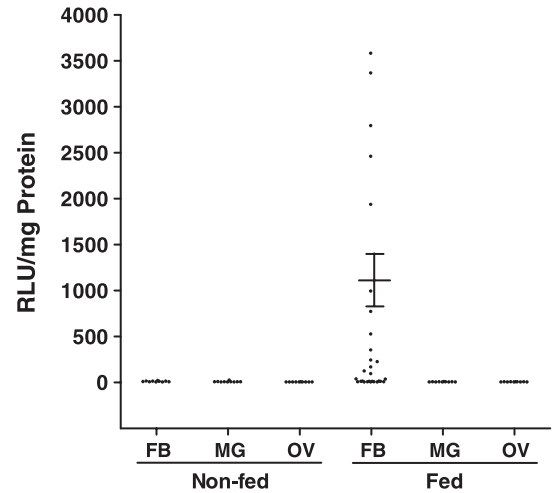
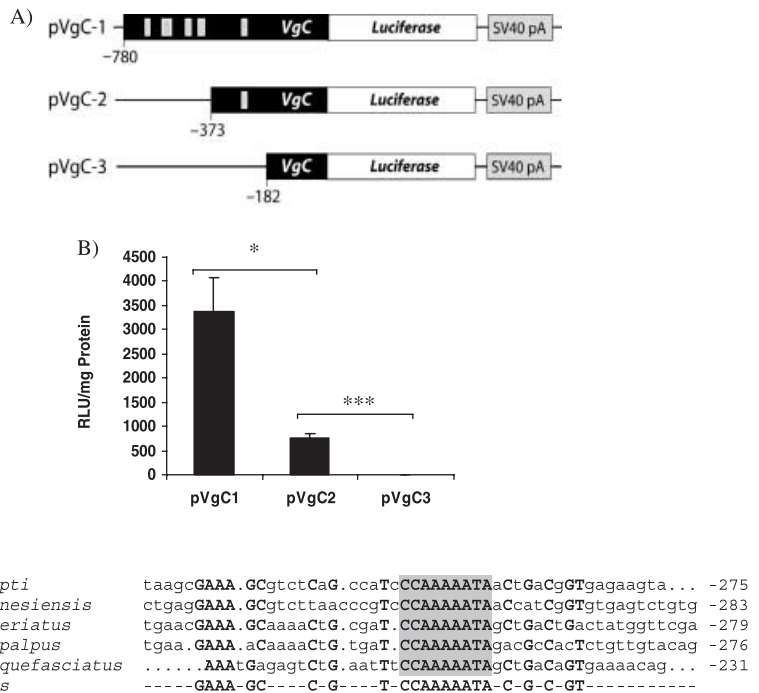


Figure 5. Expression of a vitellogenin-C luciferase reporter gene is restricted to the fat bodies of bloodmeal fed female mosquitoes. Female mosquitoes were microinjected with 1.0 µg of pVgC1-Luc shortly after adult eclosion and dissected 120 h later with or without a bloodmeal 24 h prior to dissection. Luciferase activity in extracts prepared from the fat body, midgut and ovary tissues of individual mosquitoes. The mean ± standard error of the mean are shown. * $P < 0.05$ comparing VgC1 and VgC2, and *** $P < 0.0005$ comparing VgC2 and VgC1 by unpaired Student's *t*-test.

780 bp of the promoter region and upstream regulatory elements into the pGL3 plasmid to generate pVgC1-Luc. Because expression of the endogenous VgC gene is primarily restricted to the fat body of blood-fed female mosquitoes, we tested if the pVgC1-Luc reporter gene followed the same pattern of expression in WBT-injected mosquitoes. As shown in Fig. 5, with the exception of one marginally positive tissue sample that was obtained from the midgut of an unfed mosquito (20.3 RLU/mg protein), all other luciferase-positive samples were from the fat body of fed mosquitoes. The mean level of luciferase activity in these WBT-positive mosquitoes was 1104 RLU/mg protein and the transfection efficiency was 55%. These WBT data indicate that this 843 bp fragment of the VgC gene fragment recapitulates both the tissue-specificity and blood meal dependence of the endogenous VgC gene.

To determine if WBT can be used for functional mapping of upstream regulatory elements contained within the VgC1 fragment, we constructed two VgC deletion mutants (Fig. 6A). One contained 373 bp of the gene regulatory region (VgC2), while the other had just 182 bp upstream of the start site (VgC3). For these experiments, we measured luciferase activity in four pooled samples each containing fat bodies from five fed mosquitoes. As shown in Fig. 6B, the VgC2 deletant had five-fold lower activity than the full-length VgC1 promoter fragment ($P = 0.011$), suggesting that the four conserved sequence blocks previously identified in the -373 to -780 region (Isoe & Hagedorn, in press) are important for high level blood-meal-induced expression

Figure 6. Use of whole body transfection to functionally map regulatory elements in the vitellogenin-C gene. (A) Functional map of the expression vectors used for these experiments. Three vitellogenin-C constructs were tested with various length of 5' upstream sequence. Note that pVgC1 is the same construct used in the experiments shown in Fig. 5. (B) Results of luciferase assays in which five female mosquitoes were injected with one of the VgC constructs and analysed as a pool. The mean \pm standard error of the mean are shown. Asterisks indicate the significance value of the result. *Significant, $P < 0.05$; ***most significant, $P < 0.001$. (C) Alignment of promoter sequence elements within the -182 to -373 segment of *Ae. aegypti* VgC and other mosquito VgC (accession nos: *Ae. polynesiensis*, AY691320; *Ochlerotatus triseriatus*, AY691323; *Oc. atropalpus*, AY691322; *Culex quinquefasciatus*, accession no.: AY691324). Consensus sequence (> 80% nucleotide identity) is shown below in bold-face. Nine nucleotide A-T rich sequence that is 100% identical to vitellogenin-C promoters from five mosquito species are shaded.



of the VgC gene. None of VgC-luciferase reporter constructs displayed promoter activity in the fat body of unfed female or male mosquitoes, or in the midgut of fed or unfed female mosquitoes (data not shown). Importantly, deleting another 191 bp beyond the 5' endpoint of the VgC2 construct to generate VgC3, resulted in complete loss of promoter activity. These results indicate that this segment of the VgC promoter contains minimal sequences required for expression of the gene in the fat body of fed mosquitoes. The DNA sequence of a highly conserved 49 bp region within the -182 to -373 segment is shown in Fig. 6C. The most striking feature is a nine nucleotide A-T rich sequence that is 100% identical in the VgC promoters of five mosquito species.

Lipopolysaccharide-regulated expression of a cecropinB reporter gene

Because the VgC-derived expression vectors only function in the fat body of blood-fed female mosquitoes, we sought to develop an alternative regulated expression vector that could be induced by external stimuli. Preliminary experiments with the *Ae. aegypti* Hsp70-luciferase reporter gene indicated that it was not responsive to heat shock in WBT-injected mosquitoes using a two hour incubation at 41 °C (data not shown). Therefore, we investigated the possibility of using the 5' regulatory regions of the *Ae. aegypti* cecropinB (CecB) gene which has previously been reported to be induced in mosquitoes by bacterial challenge (Lowenberger *et al.*, 1999) or following injection of purified lipopolysaccharide (Zheng & Zheng, 2002). We first characterized the expression of the endogenous CecB

gene in lipopolysaccharide (LPS) injected mosquitoes using quantitative RT-PCR. As shown in Fig. 7, we observed a > 700-fold induction of CecB expression in LPS-injected mosquitoes by 6 h under conditions in which Hsp70 gene expression was unchanged. Moreover, we found that by 24 h after LPS injection, CecB gene expression had returned to near basal levels.

To determine whether the 5' regulatory region of the *Ae. aegypti* CecB could function as an LPS-regulated promoter in the WBT assay, we cloned a ~1 kb genomic fragment into the pGL3 reporter gene to generate pCecB-Luc as shown in Fig. 8A. We also constructed an internal control plasmid for these WBT experiments by inserting the Hsp70 promoter into the promoter-less *Renilla* luciferase plasmid to generate pHsp70-Rluc. Newly emerged female mosquitoes were coinjected with equal amounts of both plasmids, and then 3, 13 or 27 days later, the same mosquitoes were injected with LPS. One day later, the fat bodies were dissected and dual luciferase assays were run on the protein extracts. As can be seen in Fig. 8B, in mosquitoes that had been WBT-injected 4 days earlier, the LPS injection led to significant induction of luciferase activity at 8 ($P = 0.046$) and 24 h ($P = 0.0005$). Moreover, even at 14 and 28 days after the WBT-injection, the LPS treatment resulted in an increase in CecB-Luc expression in 20–30% of the injected mosquitoes, indicating that the CecB promoter retains its LPS-sensitivity in some mosquitoes for long periods of time.

We found that LPS-induced expression of the CecB-Luc reporter gene was specific to the CecB promoter since LPS

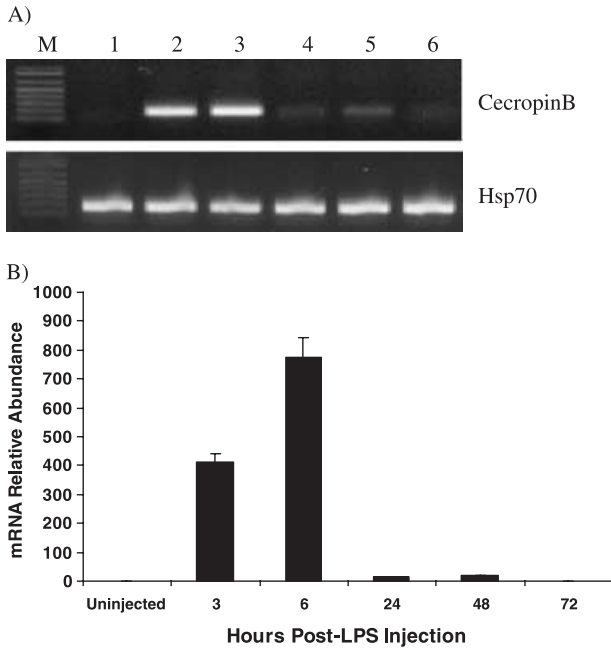


Figure 7. CecropinB expression in LPS-injected mosquitoes. (A) The top and bottom gels show the results for RT-PCR using cecropinB and Hsp70 primers, respectively. cDNA samples were prepared from fat body of non-LPS injected mosquitoes (lane 1), 3 h (lane 2), 6 h (lane 3), 12 h (lane 4), 24 h (lane 5), 48 h (lane 6), and 72 h post-LPS injection (lane 7). (B) Results of quantitative RT-PCR studies showing a time course of lipopolysaccharide-induced expression of the endogenous CecB gene in the same cDNA samples as A. The mRNA relative abundance was determined using a standard curve generated from a 10-fold dilution series using cloned CecB cDNA.

injection had no significant effect on Hsp70-Rluc expression at any of the WBT-injection time points (Table 3). These data confirmed that CecB expression was LPS-dependent as 20–90% of the LPS-injected mosquitoes were positive for firefly luciferase activity (Fluc), while none of the uninjected mosquitoes were found to be Fluc-positive. The lack of pCecB-Fluc expression in the non-LPS injected mosquitoes was not due to an aberrantly low transfection efficiency since 70–95% of the mosquitoes were positive for expression of the control pHsp70-Rluc reporter gene (Table 3).

Discussion

In order to expedite molecular genetic studies of blood meal digestion in mosquitoes we have developed a DNA microinjection method that results in the delivery of transcriptionally competent DNA to mosquito tissues. Optimization experiments using the *Ae. aegypti* Hsp70 promoter demonstrated that WBT can be used routinely to express cloned genes in the fat body and midgut of injected mosquitoes using as little as 50 ng of DNA. Moreover, DNA analysis showed that the injected plasmid DNA was not integrated

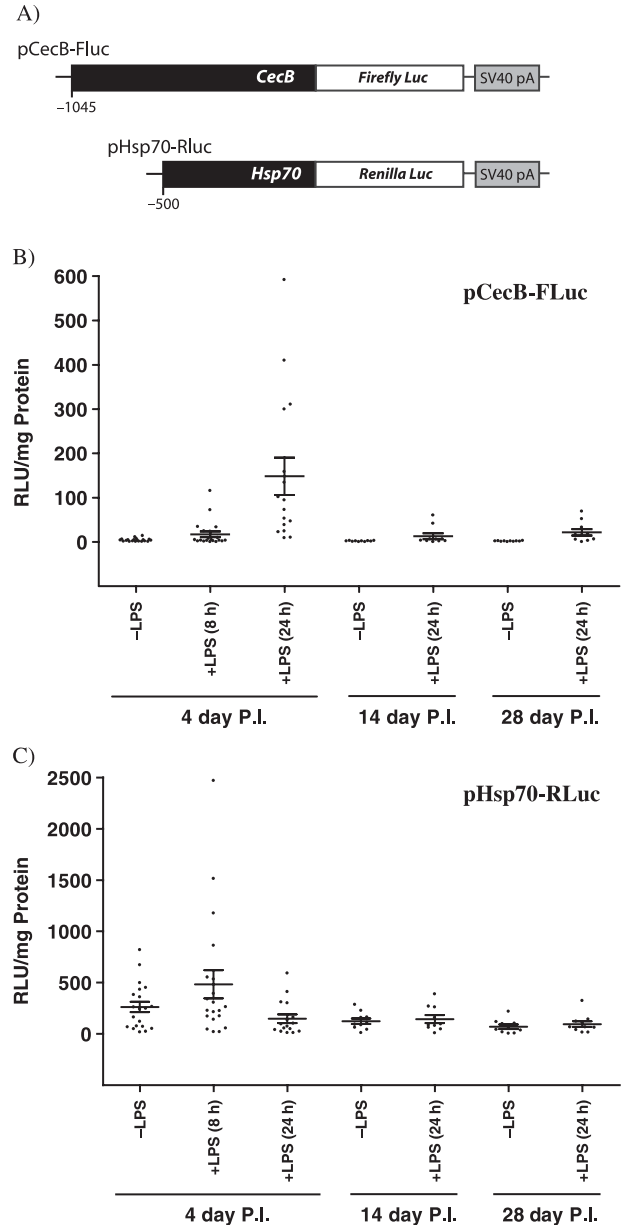


Figure 8. Induced expression of a microinjected cecropinB-luciferase reporter gene with lipopolysaccharide. (A) Map of the pCecB-Fluc and pHsp70-Rluc reporter genes. (B) Results of WBT experiments in which female mosquitoes were coinjected with pCecB-Luc and pHsp70-Rluc shortly after adult eclosion, injected a second time with lipopolysaccharide, and fat bodies were isolated 8 and 24 h later and luciferase activity was measured. Data shown are for Fluc values. (C) Same as in B except Rluc values are shown. The mean \pm standard error of the mean are shown.

into the mosquito genome up to 10 days PI (Fig. 3), and that the fat body accumulates the highest level of DNA (Table 2). Because fat body cells are not thought to be actively dividing in adult mosquitoes, the extended period of expression of injected reporter gene DNA likely reflects non-replicating circular plasmid DNA retained in the nucleus. In addition to being able to study gene expression

Table 3. Induction of pCecB-Luc by lipopolysaccharide

PI (day)	N	LPS	pCecB-FLuc				pHSP70-RLuc Midgut			
			Positive ¹	Mean	SEM	Range	Positive ¹	Mean	SEM	Range
4	20	–	0	–	–	–	19	275.6	51.5	51.1–265.1
4	20	8 h	6	50.5*	14.9	23.4–115.4	18	536.3	146.8	43.5–2470
4	16	24 h	14	386.8**	164.1	34.9–2361	14	168.3	45.8	22.3–591.8
14	10	–	0	–	–	–	9	136.2	26.6	42.2–285.1
14	10	24 h	2	50.8	9.3	41.4–60.1	9	160.0	39.6	45.5–387.7
28	10	–	0	–	–	–	7	95.9	23.6	33.3–218.2
28	10	24 h	3	51.1*	10.5	32.5–68.8	8	115.8	32.0	38.1–322.1

¹Positives are cutoff of 20.0 RLU/mg protein.

* $P < 0.05$, ** $P < 0.01$ by student *t*-test.

in intact animals, we also demonstrated that WBT can be utilized to regulate the expression of cloned genes in a tissue-specific manner by external stimuli. In the case of the VgC1 promoter, the stimulus was blood feeding, whereas the CecB promoter was activated maximally by lipopolysaccharide exposure, mimicking the presence of bacteria in haemolymph. We also tested several other *Ae. aegypti* promoter sequences in luciferase reporter plasmids, including those from the cathepsin L1 and actin 5c genes, however, the promoter activity of these reporter genes was less robust than the pHsp70 promoter (data not shown).

Interestingly, we found that even though WBT is ~70% efficient with regard to the number of mosquitoes containing significant levels of luciferase activity (> 20 RLU/mg protein), the absolute level of luciferase activity between individual mosquitoes varied as much as 100-fold (20–2000 RLU/mg protein) as shown in Table 1. The molecular basis for this variability is currently unknown; however, several possibilities exist. First, it may be related to DNA stability since we have observed that the level of plasmid DNA present in the whole abdomens of individual WBT-injected mosquitoes differed significantly based on Southern blotting analysis (Isoe and Miesfeld, unpublished data). Although we found that DNA-lipid complexes did not improve the transfection efficiency or variability of WBT method (data not shown), it is possible that other DNA binding reagents could be effective. For example, chitosan, a deacetylated form of chitin, has recently been shown greatly to increase the cell transfection efficiency of plasmid DNA through the formation of DNA-chitin nanoparticles (Lavertu *et al.*, 2006).

A second explanation for the variability in WBT transfection efficiencies could be inherent biological differences between individual mosquitoes, such as undetected differences in developmental staging or stress responsiveness to the microinjection procedure. A third explanation is that the variability arises from stochastic events affecting transcriptional competence of the injected DNA, even between closely matched mosquitoes. There are several examples

of transcriptional 'noise' occurring in identical twins or cloned organisms that appear to arise from differences in transcription factor abundance or activity as a result of intrinsic or extrinsic signals (Raser & O'Shea, 2005). Perhaps the transcriptional competence of injected DNA in individual mosquitoes is determined by stochastic events occurring in the haemolymph shortly after injection. For example, depending on which proteins bind first to the injected plasmid DNA, the DNA could become more or less transcriptionally competent or silenced (or degraded).

Applications of the WBT method for investigating regulatory processes in mosquitoes include using it for promoter-driven short hairpin RNA (shRNA) expression studies. Recently, the utility of using a U6 promoter-driven shRNA was demonstrated in *Drosophila* S2 cells (Wakiyama *et al.*, 2005). In addition, *Drosophila* actin 5C promoter-driven synthesis of long hairpin RNA has also been shown to be effective for RNAi-mediated knockdown in germline transformed *An. stephensi* (Brown *et al.*, 2003). Studies in mammalian systems have used RNA polymerase II promoter constructs to express shRNA sequences that are linked to microRNA sequence to generate RNA substrates that are efficiently processed into active RNAi molecules (Stegmeier *et al.*, 2005). One possibility would be to use the WBT method with RNA polymerase II promoter constructs, such as the VgC or CecB promoters, to direct shRNA-microRNA expression to specific mosquito tissues or in response to external stimuli (feeding or lipopolysaccharide injection).

It should also be possible to use the WBT method for transient expression of dominant negative or constitutively active proteins. This could include the use of WBT for *in vivo* protein interaction studies using epitope-tagged coding sequences and immunoprecipitation. Finally, while we have so far only tested WBT in mosquitoes (*Ae. albopictus* and *An. albimanus*; Isoe, unpublished results), it is reasonable to expect that it may work in other insect species that have been shown to be amenable to RNAi studies using microinjection. This could include *Manduca sexta*, *Bombyx mori* and *Apis mellifera* insect species.

(Promega) and allowed to lyse for 15 min at room temperature. After one freeze-thaw cycle, lysates were cleared by centrifugation at 2 700 g for 2 min. Lysate was then added to Luciferase Assay reagent (LAR) and the fluorescence was measured using a Model TD-20/20 luminometer (Turner Designs, Sunnyvale CA). Relative Luciferase Units (RLU) were measured per milligram protein in individual mosquito samples based on average protein concentration values for pooled tissues. For experiments using individual mosquitoes, the amount of protein used in each assay was 10 µg of total protein. In the CecB co-transfection WBT experiments, the Dual Luciferase Assay (Promega Stop & Glow) was used to measure both firefly luciferase (LAR II) and *Renilla* luciferase in the same samples.

Genomic Southern analysis

To determine the fate of injected plasmid DNA, pHsp70-Luc plasmid DNA was microinjected into newly emerged female mosquitoes as described above. Total DNA was isolated from injected mosquitoes 10 days PI. One mosquito equivalent of DNA pooled from five mosquitoes was used for standard Southern blot analysis using uncut or *Hind*III-digested DNA (Sambrook *et al.*, 1989). A digoxigenin-labelled DNA probe encoding the open reading frame of luciferase was amplified by PCR using pGL3-Luc ORF primers shown in Table 4. Hybridized probes were detected with CSPD chemiluminescent substrate according to manufacturers instructions (Roche Molecular Biochemicals, Indianapolis, IN). The control plasmid DNA (100 pg) used for this analysis was *Hind*III-digested or uncut pHsp70-Luc plasmid DNA.

Tissue distribution of injected plasmid DNA

To determine the transfection efficiency of mosquito tissues by WBT, pHsp70-Luc plasmid DNA was microinjected into female mosquitoes described above. Total DNA was isolated from fat body, midgut, ovary, and malpighian tubules 5 days postinjection. DNA from each tissue was subjected to real-time quantitative PCR using two primer sets; pGL3-Luc ORF and *Ae. aegypti* Hsp70 (Table 4).

Quantitative RT-PCR

The mRNA expression levels of endogenous *Ae. aegypti* CecB and Hsp70 genes were examined by quantitative real-time reverse transcriptase-polymerase chain reaction (QRT-PCR) as described before (Scaraffia *et al.*, 2005). Briefly, the fat body was dissected from non-injected and post-LPS injected mosquitoes at 6, 12, 24, 48, and 72 h. Total RNA was extracted from the tissues using Trizol reagent (Invitrogen), and 1.0 µg total RNA was reverse transcribed using oligo-(dT)₂₀ primer and reverse transcriptase (New England Biolabs, Beverly, MA). QRT-PCR amplifications were carried out using the 7300 Real-Time PCR System in a 96-well microtiter plate as described before (Scaraffia *et al.*, 2005). Gene-specific primers for CecB and Hsp70 are shown in Table 4. Each sample was run in triplicate for each gene. Data were analysed using ABI Prism 7300 SDS Software (version 1.2.2, Applied Biosystems).

Statistical analysis

An unpaired Student's *t*-test was performed using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). A *P*-value of less than 0.05 was considered significant.

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