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Validation of novel promoter sequences derived from two endogenous ubiquitin genes in transgenic *Aedes aegypti*

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Abstract

To date, only a limited number of promoter sequences have been described to drive transgene expression in the disease vector *Aedes aegypti*. We sought to increase this repertoire by characterizing the ability of upstream sequences derived from the *Ae. aegypti* *Ub_{L40}* and *polyubiquitin* genes to drive the expression of marker proteins. Both genomic fragments were able to drive robust expression of luciferase in cultured mosquito cells. Following *Mos1*-transformation, the *Ub_{L40}* promoter drove strong expression of a fluorescent marker in early larvae and in ovaries, while the *polyubiquitin* promoter drove robust EGFP expression in all stages of development including constitutive expression throughout the midgut. These promoter fragments provide two new expression profiles for future *Ae. aegypti* genetic experiments.

Keywords

Aedes; promoter; mosquito; ubiquitin; transgenic

INTRODUCTION

Aedes aegypti is the most important mosquito vector of viral disease agents worldwide, responsible for the transmission of viruses which cause dengue fever, dengue hemorrhagic fever, and yellow fever (Halstead 2007). Transposon-based transformation of this species with class II transposable elements is well established (Coates et al. 1998; Jasinskiene et al. 1998; Kokoza et al. 2001), and has allowed investigations into mosquito gene regulation and immunity (Adelman et al. 2008; Antonova et al. 2009; Bian et al. 2005) as well as establishing heritable virus-resistance phenotypes (Franz et al. 2006). These studies are enabled, and limited by, the catalog of promoter elements available to drive transgene expression.

Several endogenously-derived promoter fragments which drive the expression of transgenes in either the *Ae. aegypti* midgut or fat body have been described (Kokoza et al. 2000; Moreira et al. 2000). Each of these promoters is induced following a blood meal, and have been used to generate pathogen resistance phenotypes (Franz et al. 2006; Kokoza et al. 2001) or to study the mosquito innate immune response (Antonova et al. 2009; Bian et al. 2005). Salivary gland promoters derived from the *maltase* and *apyrase* genes have also been described in *Ae. aegypti*, but the utility of these promoters has been limited by their generally weak levels of expression (Coates et al. 1999). Promoters restricted to the ovary (Adelman et al. 2007; Cho et al. 2006) and testes (Smith et al. 2007) have also been

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described. We have recently used the synthetic 3xP3 promoter (Horn et al. 2000) to generate an RNAi sensor strain which can be used to identify genes involved in RNAi (Adelman et al. 2008). The use of tissue or timing-restricted promoters has been useful for the targeted expression or knockdown of genes. However, few tools are available to drive transgene expression in a constitutive, whole body manner which may be desirable for generating gene knockdown or pathogen resistant phenotypes prior to bloodfeeding or in multiple tissues. Thus far, the only whole-body constitutive promoter reported in transformed *Ae. aegypti* is the heterologous *D. melanogaster* Act5C promoter (Pinkerton et al. 2000). While successfully used as a marker for transformation, the activity of this promoter was found to be restricted to the fat body and gut of the developing larvae and to the gonads of the adult (Pinkerton et al. 2000).

Ubiquitin (Ub) is an ancient 76 amino acid protein which is almost perfectly conserved among all eukaryotes. While the best characterized role for ubiquitin is in tagging proteins for degradation by the proteasome, conjugation to ubiquitin has been observed to play a role in numerous other cellular functions such as DNA repair, immune activation and cell signaling (reviewed in Hochstrasser 2009). Due to its importance to cellular functions, ubiquitin genes have served as donors of control sequences to drive gene expression on a global basis. Insect *polyubiquitin (PUB)* gene promoters have been used to drive global transgene expression in *D. melanogaster* (Davis et al. 1995; Handler and Harrell 1999) as well as the red flour beetle *Tribolium castaneum* (Lorenzen et al. 2002). The *D. melanogaster PUB* promoter has also been used in non-drosophilids, successfully driving transgene expression in the caribbean fruit fly, *Anastrepha suspensa*, the screwworm *Cochliomyia hominivorax* and the malaria vector *Anopheles albimanus* (Handler et al. 2009; Handler and Harrell 2001; Perera et al. 2002). While the *PUB* gene has been cloned from *Anopheles gambiae* (Beard et al. 1996), control sequences from this gene have not yet been characterized. Genes encoding ubiquitin or polyubiquitin have not been described as yet for the yellow fever mosquito, *Ae. aegypti*.

In this report we test and validate promoter fragments derived from the *Ae. aegypti* ubiquitin genes *Ub_{L40}* and *PUB*. Both promoter fragments were found to drive robust transgene expression in mosquito cells. In transformed *Ae. aegypti*, the *Ub_{L40}* promoter was found to be highly active in early larvae and ovaries, while the *PUB* promoter was active during all life stages including constitutive expression in the adult female midgut. These promoter fragments can now be used for future studies into pathogen transmission or gene function.

RESULTS and DISCUSSION

Characterization of two *Ae. aegypti* ubiquitin gene promoters in mosquito cells

Putative ubiquitin genes were identified from the *Ae. aegypti* genome through tBLASTn search using the *D. melanogaster* 76 a.a. ubiquitin monomer as a query. Five genes on four supercontigs were identified with an e value of less than 1e-30. Genes AAEL006511 (supercont1.209) and AAEL013536 (supercont1.864) both contained a single ubiquitin monomer and appeared to be homologues of the *Drosophila Ub_{L40}* (Ub52) and *Ub_{S27}* (Ub80) ribosomal fusion genes (Cabrera et al. 1992; Lee et al. 1988). These genes are both well conserved among eukaryotes, and consist of a ubiquitin monomer fused to the ribosomal proteins L40 or S27 (Finley et al. 1989). Genes AAEL003888 and AAEL003877 (supercont1.99) are arranged in tandem, and contain 2 and 14 consecutive ubiquitin repeats, respectively, while the last gene, AAEL000795 (supercontig 1.17), contains 8 repeats. Both the *Ub_{S27}* and AAEL000795 genes appeared to contain intron sequences in the 5' UTR of greater than 10 kb based on the current annotation. As we sought to include all 5' UTR sequence, including any intron sequence, into potential transformation constructs, these genes were not pursued any further. Gene AAEL003888 only contained two ubiquitin

repeats, one of which had an addition of 1 amino acid at the C-terminus. Due to the presence of the much longer ubiquitin gene immediately downstream (14 repeats), we did not pursue this gene any further. Therefore, we restricted our focus to genes AAEL006511 and AAEL003877, hereafter referred to as *Ubl40* and *PUB*, respectively. In order to verify the 5' and 3' start and stop of transcription for each gene, we performed Rapid Amplification of cDNA Ends (RACE). For both the *Ubl40* and *PUB* mRNAs we recovered a consistent 5' end of the mRNA transcript (Fig S1A and C). The 3' end of each transcript exhibited some minor variation in termination; of the seven sequenced *PUB* clones and eleven *Ubl40* sequenced clones, no 3' termination event was recovered more than twice (Fig S1B and D). However, all seven *PUB* clones terminated within 4 nt of each other, and 7 out of the longest 11 *Ubl40* clones terminated within 11 nt of each other. The completed gene structures, using the longest obtained 3'UTRs, are shown in Figure 1 (A + B). Both *Ubl40* and *PUB* genes were found to contain a single small intron in the 5'UTR, with the 3' splice site of each located just upstream of the start codon. To obtain putative promoter sequences from these two genes, primers were designed based on the completed *Ae. aegypti* genome sequence and amplified fragments were cloned upstream of the firefly luciferase open reading frame. The 114 bp upstream boundary selected for the *Ubl40* genomic fragment was due to the presence of a full-length *copia* retrotransposon further upstream. We selected 565 bp of upstream genomic sequence for the *PUB* gene, which represented the entire intergenic space separating *PUB* from the upstream ubiquitin gene AAEL003888. Downstream boundaries of each amplified genomic fragment included sequence corresponding to the entire 5'UTR of each gene, so that the luciferase ORF was placed exactly where ubiquitin translation would normally initiate (Fig1A, B). To determine whether each genomic fragment could drive gene expression in mosquito cells, we performed luciferase assays following plasmid transfection into C6/36 (*Ae. albopictus*) or Aag2 (*Ae. aegypti*) cells (Fig 1C, 1D). As a negative control we transfected a promoterless construct (pGL3), while the baculovirus *IE-1* promoter was used for comparison. All samples were co-transfected with a Renilla luciferase-expressing plasmid to normalize for differences in transfection efficiencies between wells. As shown in Figure 1 (C + D), both the *Ubl40*-derived and *PUB*-derived genomic fragments were able to drive strong luciferase expression in both cell types. While the activity of the *Ubl40* promoter was similar to that of *IE-1*, the *PUB* genomic fragment was found to be at least 10-fold stronger than *IE-1* in all experiments conducted in both C6/36 (Fig. 1C, $p < 0.02$, Students t-test) and Aag2 (Fig. 1D, $p < 0.0001$, Students t-test) cells.

Generation of transgenic *Ae. aegypti*

As both promoter fragments appeared to be highly active in mosquito cells, we sought to determine their activity in stably transformed mosquitoes. Three *Mos1*-based donor constructs: pMos-3xP3DsRed-RH-*Ubl40*-EGFP (Fig 2A, #1), pMos-3xP3DsRed-*Ubl40*-EGFP-*attP* (Fig 2A, #2) or pMos-3xP3DsRed-*PUB*-EGFP-*attP* (Fig 2A, #3) were injected into *Ae. aegypti* embryos (*kh^w* or Liverpool strains) along with the pKhsp82M helper (Coates et al. 1998). G₁ larvae were screened for eye-specific expression of DsRED as early larvae, with putative transgenic individuals identified from each experiment (Table 1). Preliminary examination of early larvae revealed widespread EGFP fluorescence in *Ubl40*-EGFP lines #18 and #P17A and in all *PUB*-EGFP derived lines, but not in *Ubl40*-EGFP lines #19, P10, P13 and P17B. Southern analysis was used to verify each transgene insertion (Fig. 2B). With the exception of line #19, genomic DNA from each putative transgenic line was subject to restriction enzyme digestion (Fig 2A - *EcoRI*, *XbaI* or *NsiI*) with no target recognition sequence within the transgene in order to reveal the number of *Mos1* insertions (Fig. 2B). Two insertions were observed in lines #18 and P4, while the remaining lines appeared to contain a single *Mos1* element (Fig. 2B). As not all *Ubl40*-EGFP lines appeared to express EGFP, we sought to verify the integrity of each transgene. *Ubl40*-EGFP-derived lines were subject to *SaII* digestion, expected to produce a common ~4 kb hybridization

signal in lines #18 and #19, along with two junction fragments of variable length, or a common ~3kb signal in lines P10, P13A, P17A, P17B, along with two junction fragments expected to vary with the insertion site (Fig. 2A). These hybridization signals were indeed observed in all pools (Fig. 2B). Additionally, we performed PCR on genomic DNA isolated from lines *U_{bL40}*-EGFP #P13A and #P17B and sequenced the resulting amplicons. No disruptions in the *U_{bL40}*-EGFP cassette were found in either line (data not shown). This confirms that the entire gene cassette was integrated intact into the mosquito genome for all *U_{bL40}*-EGFP transgenic lines and suggests that the site of *Mos1* insertion has affected the activity of the *U_{bL40}* promoter in those lines which did not appear to express EGFP. Approximate transformation frequencies for the three experiments were calculated to be 3.2%, 3.2% and 5.5%, assuming a G₀ fertility rate of ~50%, which is similar to previous experiments with *Mos1* (Adelman et al. 2008; Adelman et al. 2007; Coates et al. 1998). While lines #18 and P4 were found to contain two insertions each, we did not include these extra insertions in our determination of the transformation frequency, as they did not appear to segregate from each other following two generations of out-crossing to recipient strain mosquitoes (Table 1).

Expression of EGFP reporter in transgenic *Ae. aegypti*

As indicated above, early larvae (1st-2nd instar) from *U_{bL40}*-EGFP line #P18 and *PUB*-EGFP line #P5 transgenic mosquitoes displayed high levels of green fluorescence throughout the entire body (Fig. 3A). Similar expression was observed in *U_{bL40}*-EGFP #17A and in all five other *PUB*-EGFP transformed lines (not shown). While fluorescence remained strong throughout larval development and into pupation for all of the *PUB*-EGFP transformed lines, *U_{bL40}*-EGFP mosquitoes became gradually dimmer, with little fluorescence remaining in pupae (Fig. 3B). Adult females from all *PUB*-EGFP lines remained highly fluorescent, with EGFP easily detectable through the cuticle in the head, thorax and abdomen, while no EGFP could be visualized in adult *U_{bL40}*-EGFP mosquitoes (Fig. 3C). Northern blotting was used to further characterize the expression of EGFP mRNA from transgenic lines *U_{bL40}*-EGFP #18 and P17A, as well as *PUB*-EGFP #P5. Total RNA was extracted from various life stages and tissues and hybridized to a probe corresponding to EGFP or *U_{bL40}* (Fig. 4A) or to the *PUB* 5' UTR present in both the endogenous *PUB* transcript as well as the introduced transgene (Fig. 4B). In both *kh^w* and Liverpool strains, *U_{bL40}* transcript was detected throughout development, with the strongest expression observed in L₁ larvae (Fig. 4A). *U_{bL40}* transcript abundance decreased during larval development but was still detectable in pupae. In adult tissues, *U_{bL40}* mRNA was detectable in the thorax and midgut of the adult mosquito, with the highest transcript amounts detected in the ovaries. Transgenic lines *U_{bL40}*-EGFP #18 (compare to *kh^w*) and P17A (compare to Lvp) displayed similar expression patterns, with high transcript expression in early larvae decreasing through development and adult expression strongest in the ovaries (Fig. 4A). The *D. melanogaster U_{bL40}* gene was also found to be expressed preferentially in ovaries (Cabrera et al. 1992). However, these researchers found that in the fly, *U_{bL40}* mRNA expression remained constant throughout development (Cabrera et al. 1992). Endogenous *PUB* mRNA expression was fairly uniform throughout development and in specific tissues (Fig. 4B). EGFP mRNA expression in line *PUB*-EGFP #P5 was similar (Fig. 4B, bottom), with the exception of ovary tissue, where EGFP mRNA was not readily detectable. EGFP mRNA was not detected in the salivary glands, though this could be due to insufficient RNA. Visual inspection of salivary glands from both *U_{bL40}*-EGFP or *PUB*-EGFP mosquitoes confirmed that EGFP expression in these tissues was not robust (not shown). Interestingly, it appeared that in all but ovarian tissues, the expression of *PUB*-driven EGFP mRNA exceeded expression of the endogenous *PUB* mRNA. Though the reasons for this are not known, it is possible that the genomic context of the inserted *Mos1* element is a more favorable location for *PUB* expression, either through insertion near neighboring activating

elements or via the loss of neighboring repressive elements located in the endogenous *PUB* locus but not included in the transgenic constructs.

As both promoters appeared to have strong activity in the midgut, and the activity of the *U_{bL40}* promoter appeared strongest in the ovaries, we performed dissections to examine the expression pattern of EGFP visible in each of these tissues (Fig. 5). In ovaries taken 24 hr post bloodmeal, *U_{bL40}*-EGFP expression was observed in the nurse cells (Fig. 5A, white arrow), with diffuse fluorescence present in the developing oocyte. By 48 hr the nurse cells had already disappeared and strong EGFP expression was observed throughout the oocyte. By 72 hr, however, EGFP-based fluorescence had largely disappeared from the oocyte, and was mostly visible in the undeveloped secondary and tertiary ovarioles (Fig. 5A, thin white arrows). While some EGFP expression was observed in the *PUB*-EGFP ovaries, this appeared to be primarily associated with accessory tissues (Fig. 5A, 24 + 72hrs), though some EGFP fluorescence was observed in oocytes (Fig. 5A, 48 hr). This situation was reversed in midgut tissues, as robust EGFP expression could be seen throughout the midgut of *PUB*-EGFP mosquitoes (Fig. 5B). Most significantly, this expression did not require a bloodmeal, nor did it appear to diminish in older mosquitoes (not shown). While some EGFP expression was definitely observed in *U_{bL40}*-EGFP midguts, expression was not robust and appeared patchy (Fig. 5B). No EGFP expression was observed in recipient strain (*kh^w*) mosquitoes in either ovaries or midguts under the same conditions (Fig. 5).

CONCLUSIONS

We have validated control sequences derived from the *Ae. aegypti* *U_{bL40}* and *PUB* genes capable of driving transgene expression in both cultured mosquito cells and in transgenic mosquitoes. *U_{bL40}*-driven expression was strongest in early larvae and in ovaries, though weak to moderate expression was detected in other tissues and life stages. *PUB*-driven expression was robust in all life stages and most tissues, with strong expression observed in the midgut. These constructs provide two new expression patterns which will be of great use in future investigations of *Ae. aegypti* gene regulation and function as well as the generation of virus-resistant phenotypes. The *PUB* control sequence should also be quite useful as an alternative to 3xP3 in driving the expression of marker genes used for identifying new transformation events, as all six 3xP3-DsRED⁺ lines recovered also exhibited strong *PUB*-EGFP expression. As the transgenic strains described here were marked with both, it was readily apparent that the *PUB*-driven fluorescent proteins could be detected at a much lower magnification than required for detection of 3xP3-DsRED. Also, while 3xP3-driven fluorescent proteins are easily visible in white-eyed mutant strains, visualization in wild-type eyes is much more laborious.

Each of the transgene constructs we assembled and tested here also contain a number of features designed to increase the utility of the obtained transgenic strains, such as *loxP*, *attP* and homing endonuclease recognition sites. Excision of the *U_{bL40}*-EGFP or *PUB*-EGFP gene cassettes with *cre* recombinase, a highly efficient process in *Ae. aegypti* (Jasinskiene et al. 2003), would allow the re-introduction of a *U_{bL40}*- or *PUB*-driven gene cassette of interest into a genomic location now shown to be favorable for expression. This would be especially useful for *U_{bL40}*-driven cassettes, as only 1 out of 3 lines appeared to escape repressive position effects. We have also recently shown that the homing endonuclease clusters which were incorporated into line *U_{bL40}*-EGFP #18 and #P17A (also referred to as UUGFP) can be targeted for site specific double-stranded break formation in somatic tissues of *Ae. aegypti* (Traver et al. 2009). Thus, in addition to validating *U_{bL40}* and *PUB* promoter fragments, these transgenic lines will be of great value in studying the genetics of this important disease vector.

EXPERIMENTAL PROCEDURES

Plasmid construction

A promoterless EGFP expression construct was generated by polymerase chain reaction (PCR) of the EGFP-SV40 region from pMos3xP3-EGFP (Horn and Wimmer 2000) using primers F 5'-ctccaccATGGTGAGCAAGGGCGAGGAG-3' and R 5'-tttgaattcAAGATACATTGATGAGTTTGGACAAACCAC-3', with underlined bases indicating restriction enzyme recognition sites added for cloning. This amplicon was digested with *NcoI/EcoRI* and cloned into the *NcoI/EcoRI* sites of pSLfa (Horn and Wimmer 2000), generating pSLfa-EGFP-SV40. Putative promoter regions were amplified from *Ae. aegypti* (Liverpool strain) using Platinum *Pfx* (Invitrogen; Carlsbad, CA) and *Ubl40* primers F 5'-ttttaagcttAGCGTGAACCTTGCATGCGTGC-3' and R 5'-ataccatggCTTGATCAGTCTGTGGAAAAGTCGG-3' or *PUB* primers 5'-ttttacgcgtATCTTTACATGTAGCTTGTGCATTGAATCC-3' and 5'-ttttccatgGTTGAAATCTCTGTTGAGCAGAAAAAGAAACGAGG-3'. PCR conditions were 94°C, 2 min; 94°C, 15 sec; 62°C, 30 sec; 68°C, 1 min; 35 cycles; 68°C, 10 min. Amplified genomic fragments were digested with their respective enzymes and cloned upstream of the EGFP ORF in pSLfa-EGFP-SV40. Two different modified pMos3xP3-DsRED transformation vectors, pMos-3xP3DsRed-RH and pMos-3xP3DsRed-attP were used to test *Ubl40* and *PUB* promoter-based gene cassettes in transformed mosquitoes. Plasmid pMos-3xP3DsRed-RH was generated by inserting a complete duplication of the *Mos1* right hand (RH) downstream of the DsRED gene, as well as a cluster of homing endonuclease recognition sequences (Traver et al. 2009). Plasmid pMos-3xP3DsRed-attP was generated by inserting two clusters of homing endonuclease recognition sites (Traver et al. 2009) and *loxP* target sites flanking the multiple cloning site (MCS), as well as an *attP* recombination site downstream of the MCS. The entire *Ubl40*-EGFP-SV40 or *PUB*-EGFP-SV40 gene cassettes were removed from pSLfa and inserted into the MCS (*AscI/EcoRI*) of the respective *Mos1* vector, generating pMos-3xP3DsRed-RH-*Ubl40*-EGFP, pMos-3xP3DsRed-*Ubl40*-EGFP-attP, and pMos-3xP3DsRed-*PUB*-EGFP-attP (Fig 2A).

For luciferase experiments, *Ubl40* and *PUB* genomic fragments were excised from pMos3xP3-DsRED vectors using *MluI/NcoI* and ligated into the *MluI/NcoI* sites of pGL3 Basic (Promega; Madison, WI) upstream of the luciferase ORF. The baculovirus *IE-1* promoter was amplified using Platinum *Pfx* (Invitrogen; Carlsbad, CA) from the pIE-Hyg vector (Adelman et al. 2002) using primers 5'-ttttggcgcgcgcCGCGTAAAACACAATCAAGT-3' and 5'-ttttccatggGTCAGTTGGTTGTTACGAT-3'. Following PCR, the *IE-1* amplicon was digested with *AscI/NcoI* and ligated into the *MluI/NcoI* sites of pGL3-Basic.

Cell culture expression assays

Aedes albopictus C6/36 cells and *Aedes aegypti* Aag2 cells were maintained at 28°C and 5% CO₂ in Dulbecco's Modification of Eagle's Medium (C6/36) or Schneider's Drosophila Medium (Aag2), 10% FBS, 1% 200mM L-glutamine, 1% Penicillin (5,000 I.U./ml) streptomycin (5 mg/ml). C6/36 cells were transfected using Effectene (Qiagen; Valencia, CA) according to the manufacturer's instructions. Aag2 cells were transfected using Eugene (Roche; Basel, Switzerland) using a ratio of 3μl Eugene reagent to 1μg of DNA (Meredith et al. 2006). Cells were seeded in 12-well plates and were transfected at ~50% cell confluency. C6/36 or Aag2 cells were co-transfected with 500ng of pGL3 construct (no promoter, *IE1*-luc, *Ubl40*-luc or *PUB*-luc) and 25 ng pRL-CMV per well. At 48hrs post transfection, cells were washed twice with Phosphate Buffered Saline (PBS) and scraped with a rubber policeman in 1X Passive Lysis Buffer (PLB), freeze-thawed once and centrifuged briefly to remove cell debris. Lysates were diluted 1:100 in 1X PLB and

luciferase activity was determined using the Dual-Luciferase Reporter Assay System on a GloMax 20/20 Luminometer according to the manufacturer's instructions (Promega; Madison, WI).

Mos1-mediated transformation of *Aedes aegypti*

Aedes aegypti (*kh^w*, Liverpool strains) were maintained at 28°C and 60%-80% relative humidity on a photoperiod of 16 hr light/8 hr dark, as described previously (Adelman et al. 2008). Mosquitoes were maintained by bloodfeeding with defibrinated sheep blood (Colorado Serum Company, Denver, CO) using artificial feeders and parafilm membranes. For transgenic experiments, *kh^w* or Liverpool embryos were microinjected with 0.5 µg/µl Donor plasmid (pMos-3xP3DsRed-RH-*Ubl₄₀*-EGFP, pMos-3xP3DsRed-*Ubl₄₀*-EGFP-*attP*, or pMos-3xP3DsRed-*PUB*-EGFP-*attP*) and 0.3 µg/µl pKhsp82M in 1X Injection Buffer (Coates et al. 1998). Surviving G₀ females were merged into pools of 20-25 and mated to males of the recipient strain. G₀ males were mated individually to 10-15 recipient strain females and pooled prior to bloodfeeding and egg laying. All microinjections were performed using a Leica micromanipulator and a FemtoJet microinjector (Eppendorf; Westbury, NY). G₁ larvae were screened for DsRED⁺ eyes and/or GFP⁺ bodies using a Leica MZ-16FL microscope. All photos were taken using a Canon PowerShot S3 IS digital camera with a constant exposure time of 0.8 seconds.

Southern analysis

Mosquitoes were snap frozen in nitrogen and genomic DNA was isolated from 6 females or 10 males as described previously (Adelman et al. 2008). Genomic DNA was digested overnight with *Sa*II, *Xba*I, *Eco*RI or *Nsi*I, as indicated, followed by gel electrophoresis and capillary transfer to a nylon membrane. A probe corresponding to *Hind*III restriction fragments of the *Mos1* construct was randomly primed and labeled with [α -³²P]-dATP, using the Amersham Megaprime DNA labeling System (GE Healthcare; Buckinghamshire, UK) and purified using an Illustra NICK column (GE Healthcare; Buckinghamshire, UK). Following hybridization overnight at 65°C, membranes were washed and exposed to Kodak BioMax maximum sensitivity film at -80°C.

RNA isolation, RACE and Northern analysis

Ae. aegypti adults, larvae and pupae were snap frozen in liquid nitrogen and stored at -80°C. Adult mosquitoes were dissected in PBS and heads, thorax (without salivary glands), salivary glands, midgut, ovaries (sugar-fed or 4 days post blood meal) were placed into eppendorf tubes and snap frozen in liquid nitrogen and stored at -80°C until RNA extractions were performed. Total RNA was extracted using Trizol reagent (Invitrogen; Carlsbad, CA) according to the manufacturer's protocol. For 5' and 3' RACE, polyA⁺ RNA was purified with the PolyAtract mRNA Isolation System (Promega; Madison, WI) according to the manufacturers' instructions. RACE-ready cDNA was amplified using the SMART RACE cDNA Amplification Kit (Clontech; Mountain View, CA) and Superscript II RNase H-Reverse Transcriptase (Invitrogen; Carlsbad, CA). RACE PCR was performed using the Advantage 2 PCR Enzyme System (Clontech; Mountain View, CA) according to the SMART RACE cDNA Amplification Kit User Manual with one SMART primer and one gene specific primer. Gene specific primers for 5' RACE: 5'-GGATCAGTTCTTTCCCAACGTGTCTCTTCC-3' (*Ubl₄₀*), 5'-GATTCCCTTCACCGAAATGCACTTTCAC-3' and 5'-GGGTTTTGACGAAAATCTGCATTGTTGAAATCT-3' (*PUB*) and 3' RACE 5'-ACTTCAATTTCCCCCGTTCCAAGACCTGG-3' (*Ubl₄₀*) and 5'-GAGAGGCGGACAGTAAAGATGATGTTGAGC-3' (*PUB*). RACE amplicons were cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen; Carlsbad, CA).

For Northern analysis, three micrograms of total RNA was electrophoresed and transferred to nylon membrane. For probes, PCR amplicons derived from the 3'UTR of *Ubl40* (primers F 5'-TGGTGGTATTATTGAGCCATCTCTGCG-3' and 5'-ATAGCAATGCATTGTGACAACCTTCAATTC-3'); the 5'UTR of *Pub* (F 5'-AATCGAATACGTTTCCTAGT-3' and R 5'-GGGTTTTGACGAAAATCTGCATTGTTGAAATCT-3'); or the EGFP coding region (F 5'-TTTTGCGGCCGCCATGGTGAGCAAGGGCGAGGAGC-3' and R 5'-TTTTGGATCCCAAGATACATTGATGAGTTTGGACA-3') were [α - 32 P]-dATP labeled through random-priming. Specific activity ranged from 7.2×10^9 to 5.2×10^{10} counts per minute for *Ubl40* and EGFP probes, 9.4×10^{10} to 2.0×10^{11} counts per minute for *Pub* probes. Membranes were hybridized overnight at 65°C for EGFP/*Ubl40* probes or 55°C for *Pub* probe, washed and exposed to Kodak BioMax maximum sensitivity film.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Ub_{L40} and *PUB* promoters are highly active in mosquito cells. Schematic representation of *Ub_{L40}* (A) and *PUB* (B) gene structure in *Ae. aegypti*. Exons (E) and introns (I) are indicated, with the predicted mRNA, including 5' and 3' untranslated regions (UTRs) and open reading frame (ORF) are shown below each gene. Numbers indicate length in base pairs. Gray boxes indicate the 76 amino acid ubiquitin monomer. Bars above each gene represent putative promoter regions utilized in (C, D) and all further experiments. (C, D) Relative luciferase activity from *IE-1*, *Ub_{L40}* and *PUB* promoters compared to a no promoter control plasmid (pGL3) at 48hrs post-transfection in C6/36 (C) and Aag2 cells (D). Each experiment (Exp) was performed in triplicate. Statistical significance (*) was determined by Students t-test. *Ub_{L40}* and *PUB* promoter/gene sequences have been deposited in Genbank (accession #s GU179017 and GU179018).

Figure 2. Southern analysis of *Ub_{L40}*-EGFP and *PUB*-EGFP insertions in *Mos1*-transformed *Ae. aegypti*. **(A)** Schematic representation of hypothetical transgene insertions for each of the donor constructs used. Block arrows represent the right (R) and left (L) arms of the *Mos1* transposon. Bar indicates the size of the entire insertion and the dashed line represents mosquito genomic DNA. Restriction enzyme sites *EcoRI* (E), *SalI* (S), *XbaI* (X) and *NsiI* (N) are indicated. **(B)** Genomic DNA from each of the families identified as DsRED⁺ was digested with the indicated enzymes and hybridized with a probe corresponding to the *Mos1* arms as well as the DsRED-SV40 gene cassette. The recipient strain *kh^w* is included as a negative control for all hybridizations. Molecular weight markers are shown at left (in kbp).

Figure 3.

Ub_{L40} and *PUB* promoters drive EGFP expression in transformed *Aedes aegypti*. EGFP expression in L₁ larvae (A) and pupae (B) for *PUB*-EGFP #P5, *kh^w* and *Ub_{L40}*-EGFP #P17A. (C) White light, EGFP and DsRED images of adult female *kh^w*, *Ub_{L40}*-EGFP #18 and *PUB*-EGFP #P5.

Figure 4.

Northern analysis of *UbL40*-EGFP and *PUB*-EGFP expression in transformed *Ae. aegypti*. (A) Northern blot of endogenous *UbL40* (*kh^w*, *Lvp*) mRNA and EGFP mRNA in transgenic lines *UbL40*-EGFP #18 and #P17A. Blots were probed with either the L40 portion of the *UbL40* ORF (last 159bp) along with its 3'UTR or for EGFP. (B) Northern blot of *PUB* and *PUB*-EGFP expression in *kh^w* and *PUB*-EGFP #P5 transgenic mosquitoes. A probe corresponding to the *PUB* 5'UTR was used for hybridization to each blot, with ribosomal RNA shown as a loading control below. Individual lanes include: first through fourth instar larvae (L1-L4), male pupae (σ P) female pupae (φ P), adult whole body (WB), head (H), thorax without salivary glands (T), salivary glands (S), midgut (M), sugar-fed ovary (Os), and ovaries at 4 days post blood meal (Ob).

Figure 5.

Ub_{L40} and *PUB* promoters drive robust transgene expression in *Ae. aegypti* ovary and midgut tissues. (A) White light and EGFP images of *kh^w*, *Ub_{L40}-EGFP #18* and *PUB-EGFP #P5* ovaries at 24 hr, 48 hr and 72 hrs post blood meal. Thick white arrow indicates concentrated EGFP expression in nurse cells, thin white arrows indicate undeveloped secondary and tertiary ovarioles. (B) White light and EGFP images of midgut tissue obtained from *kh^w*, *Ub_{L40}-EGFP #18* and *PUB-EGFP #P5* mosquitoes.

Table 1
Mos1-mediated transformation of *Ae. aegypti* with *Ubi_{L40}*-EGFP or *PUB*-EGFP gene cassettes.

<i>Mos1</i> Donor	# embryos injected	# <i>G</i> ₀ survivors (%)	# <i>G</i> ₁ progeny screened	Pools with <i>DsRED</i> ⁺ progeny (#)	<i>G</i> ₁ ⁺ /Total (%)	<i>G</i> ₃ ⁺ /Total (%)
<i>Ubi_{L40}</i> (exp1)	1347	126 (9.4%)	24	7358	#18 (3+) 5/18 (28%)	23/49 (47%)
					#19 (2+) 84/304 (28%)	338/626 (54%)
<i>Ubi_{L40}</i> (exp2)	1825	248 (13.6%)	17	13503	#P10 (3+) 18/51 (35%)	-
					#P13A (53+) 131/234 (56%)	-
					#P17A (7+) 232/342 (68%)	40/76 (53%)
					#P17B (5+) 9/74 (12%)	-
<i>PUB</i>	1902	218 (11.5%)	7	16391	#P2 (34+) 102/200 (51%)	205/354 (58%)
					#P3 (38+) 144/300 (48%)	309/591 (52%)
					#P4 (13+) 42/200 (21%)	135/246 (55%)
					#P5 (13+) 357/728 (49%)	157/305 (51%)
					#P6 (10+) 117/223 (52%)	172/329 (52%)
					#P7 (18+) 136/306 (44%)	131/259 (51%)