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# Conditional Expression in the Malaria Mosquito *Anopheles stephensi* With Tet-On and Tet-Off Systems

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ABSTRACT

We report successful conditional gene expression in the malaria vector, *Anopheles stephensi*, on the basis of binary systems consisting of gene driver and responder transgenic lines generated by *Minos*-mediated germline transformation. An *A. gambiae* tissue-specific enhancer derived from a serpin (*SRPN10*) gene was utilized to control the temporal and spatial expression of doxycycline (dox)-sensitive transcriptional regulators in the driver lines. The "Tet-Off" driver utilized the tetracycline-controlled transcriptional activator (tTA) that is unable to bind and activate transcription from tetracycline operators (*TetO*) in the presence of dox; the "Tet-on" driver utilized the reverse tTA (rtTA) that, conversely, binds and activates *TetO* operators in the presence of dox. The responder lines carried insertions encompassing a *LacZ* reporter gene, *cis*-regulated by a *TetO-P*-element hybrid promoter. The progeny of crosses between driver and responder lines expressed β-galactosidase under dual, tissue-specific and dox-mediated regulation. In adult rtTA/TetOPlacZ progeny, dox treatment rapidly induced β-galactosidase activity throughout the midgut epithelium and especially in malaria parasite-invaded epithelial cells. Transactivator-dependent, dox-mediated regulation was observed in hemocytes and pericardial cells using both systems. Conditional tissue-specific regulation is a powerful tool for analyzing gene function in mosquitoes and potentially for development of strategies to control disease transmission.

THE functional analysis of genomes and their products is one of the major tasks of current biology. In malaria research, information gleaned from the available genome sequences of the three implicated organisms—human (Venter *et al.* 2001), *Plasmodium falciparum* (Gardner *et al.* 2002), and *Anopheles gambiae* (Holt *et al.* 2002)—may lead to profound advances toward the alleviation of suffering caused by this disease. However, a prerequisite is the ability to discover the *in vivo* functions of the interesting genes that are being identified through high-throughput proteomic and transcriptional analysis (Levashina *et al.* 2001; Christophides *et al.* 2002; Dimopoulos *et al.* 2002; Zdobnov *et al.* 2002). Functional analysis will be most readily achieved through reverse genetic techniques, whereby candidate genes, their mutated forms, or RNAi silencers are conditionally expressed in specific tissues and function is inferred from the consequent physiological and phenotypic alterations. This approach is particularly relevant in species, such as Anopheles, in which the limitations of forward genetic approaches are somewhat counterbalanced by the development of transgenic (Catteruccia *et al.* 2000; Grossman *et al.* 2001) and RNAi techniques (Blandin *et al.* 2002; Brown *et al.* 2003), used in combination with cellular and molecular analysis (Han *et al.* 2000; Dimopoulos *et al.* 2002; Danielli *et al.* 2003).

An important reverse genetic approach is to regulate discrete temporal and spatial expression of transgenes. Two methods are generally used for this purpose. One directs temporal expression by DNA elements such as the heat-shock protein 70 promoter, which achieves high levels of inducibility following temperature elevation (Lis *et al.* 1983). Limitations of this method include "leakiness" of the promoter, difficulty in interpreting phenotypes due to the pleiotropic effects of heat-shock regimes, and the ubiquitous spatial expression of the transgene (Krebs and Feder 1997). The second approach utilizes binary systems such as the GAL4-UAS system, used extensively in Drosophila (Brand and Perrimon 1993), and the widely used "tet" conditional expression system (Gossen and Bujard 1992, 2002). The latter is based on transcriptional activators (TA) responsive to tetracycline or analogs such as doxycycline (dox). These are relatively benign drugs that readily cross lipid bilayers, thus penetrating most compartments of the body (Berens and Hillen 2003). The tet system requires two sets of transgenic lines: driver lines expressing TAs under control of tissue and/or temporally specific promoters and responder lines carrying a cDNA of interest under the transcriptional control of upstream tet operator-derived response elements (*TetO*) to which

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these TAs can bind. Thus, in progeny of crosses between driver and responder lines, the cDNA of interest is under dual regulation: by the specific promoter that controls TA production and by the presence/absence of dox that regulates TA activity. A major advantage is that two alternative TAs exist that are affected by dox in the opposite manner. The original transactivator (Gossen and Bujard 1992) is inactivated by dox, preventing transcription ("Tet-Off" system), whereas the reverse transactivator (Urlinger *et al.* 2000) is activated by dox, leading to rtTA binding to *TetO* and thereby permitting transcription ("Tet-On" system).

We have initially chosen to control production of tet-dependent transactivators using a promoter sequence from the *A. gambiae SRPN10* locus (Danielli *et al.* 2003). Previous analysis established *SRPN10* as a potentially important innate immunity locus, which is activated by parasite invasion of the midgut epithelium and is also expressed in a subset of hemocytes and the pericardial cells (Danielli *et al.* 2003). Transcript and protein analysis also suggested that *SRPN10* is synthesized in larval and pupal stages. Attempts to block parasite transmission by the expression of Plasmodium inhibitors in transgenic mosquitoes would be greatly facilitated by systems that ensure conditional regulation of the transgene in mosquito tissues encountered by the parasite and that respond to invasion.

## MATERIALS AND METHODS

**Plasmids and generation of transgenic mosquitoes:** The tTA sequence was removed from pUHD15-1 (Gossen and Bujard 1992) and directly cloned into a modified pBluescript carrying the bovine growth hormone terminator, pbGH. The *SRPN10* promoter (Danielli *et al.* 2003) was introduced into this intermediate vector to produce the "Tet-Off" driver construct, ps10tTA. The "Tet-On" construct, ps10rtTA, was made from this construct by removal of rtTA from pUHrT62-1 (Urlinger *et al.* 2000) and exchange of transactivation domains. The responder plasmid, ptetOP*LacZ*, was constructed by insertion of *LacZ* from pDM79 (Mismer and Rubin 1987) into WTP2 (Bello *et al.* 1998) and removal of the *tetO-P*-element-minimal promoter-*LacZ* fusion from this plasmid and insertion of it into pbGH. The driver and responder sequences were then subcloned into the Actin5C-enhanced green fluorescent protein (Actin5C-eGFP; Catteruccia *et al.* 2000) and Actin5C-discosoma species red fluorescent protein (Actin5C-dsRed1; Brown *et al.* 2003) *Minos* vectors, respectively (Figure 1A). Transgenic lines were generated and established using the fluorescent markers essentially as described (Catteruccia *et al.* 2000). Positive $G_1$ individuals were outcrossed to wild type in small groups (1–10) and inheritance of the fluorescent phenotype was scored in the $G_2$ progeny. In most cases, $G_2$ adults were mass intercrossed, $G_3$ progeny were scored for phenotypic inheritance, and then small groups of homozygous $G_3$ individuals (selection based on fluorescence intensity) were intercrossed to establish pure breeding lines. In two cases, where sex-linked insertions occurred (only $G_2$ females from a male $G_1$ outcross) $G_2$ females were outcrossed and pure breeding lines established from the $G_4$ generation. In two other cases, where a deviation from Mendelian inheritance of single-copy insertions was noted (due to more than one insertion), lines were maintained by enrichment of fluorescent progeny during subsequent generations.

**β-Galactosidase assays and dox treatment:** Larval and adult mosquitoes were dissected in phosphate-buffered saline (PBS) and whole tissues were fixed for 3–5 min in 2% paraformaldehyde/0.2% glutaraldehyde/50 mm phosphate buffer, pH 7.2. Following 2 × 10-min washes in PBS, tissues were routinely incubated at 37° for 2 hr in X-gal solution [10 mm phosphate buffer, pH 7.2, 150 mm NaCl, 1 mm $MgCl_2$, 3.1 mm $K_3Fe(CN)_6$, 3.1 mm $K_4Fe(CN)_6$, 0.3% Triton X-100, and 0.2% X-gal]. For infected midgut staining, midguts were removed and fixed for 1 min in ice-cold 4% paraformaldehyde in PBS, the blood was removed, and the midgut sheets were returned to fixative for 15–30 min. Following 2 × 15-min washes in PBS, the tissues were stained overnight in X-gal solution. Hemocytes were collected from individual mosquitoes by severing the proboscis at its midpoint and applying gentle pressure to the thorax. The droplet of hemolymph emanating from the stub was spotted onto slides (ICN multitest cell culture slides), allowed to dry, briefly fixed (1 min) in 0.5% glutaraldehyde in PBS, and washed for 2 × 5 min in PBS. Hemocytes were stained overnight in X-gal solution (without Triton X-100) and counted under phase-contrast microscopy. For dox treatment, fresh 10 mg/ml stock solutions were brought to pH 7 with 5 m NaOH and diluted to appropriate concentrations in either larval water or 15% sucrose.

## RESULTS

**Generation of tet driver and responder *Anopheles stephensi* lines:** Figure 1A depicts the *Minos* transposons that were used to create the transgenic *A. stephensi* driver and responder lines described herein. The scheme also illustrates how expression of *LacZ* in the progeny of driver and responder line crosses is expected to be regulated by the addition of dox, either negatively in the case of the "Tet-Off" (tTA) driver or positively in the case of the "Tet-On" (rtTA) driver. The tTA driver cassette, marked with Actin5C-eGFP, was co-injected into 123 embryos, from which 21 (17%) larvae were recovered, which in turn gave rise to 5 (4% of injected embryos) adults. Two independent transgenic lines were generated from the surviving adults. Southern analysis demonstrated that lines tTA1 and tTA2 carried one and two insertions, respectively (Figure 1B). Similarly, a *SRPN10* promoter-rtTA expression cassette, carrying the reverse transactivator, was incorporated into the Actin5C-eGFP-marked *Minos* vector and used to generate four transgenic *A. stephensi* lines harboring single insertions (Figure 1B) from an injection series of 186 embryos (larval and adult survival rate: 31 and 14% of injected embryos, respectively). We also generated six independent responder lines carrying the TetOPLacZ minigene and marked with Actin5C-dsRed1 from an injection series of 111 embryos (larval and adult survival rates: 37 and 26% of injected embryos, respectively). Southern analysis indicated that five lines carried single TetOPLacZ insertions, while line TetOPLacZ5 had two insertions (Figure 1B). An accurate estimate cannot be made as to the fertility of individuals, since first-round screening was performed in small groups. However, typically 30–

60% of blood-fed females that were allowed to oviposit in isolation gave viable progeny.

**Pericardial cell and hemocyte-specific expression modulated by dox and blood feeding:** Using appropriate fluoresence filter sets, larvae and pupae that carried driver or responder transposons were readily identified by their green or red fluorescence, respectively; carriers of both markers in the progeny of crosses between driver and responder lines were easily distinguished (Figure 1C). The identification of Actin5C-eGFP- and Actin5C-dsRed-positive adults was reliable, but required practice to distinguish the GFP signal, which is substantially blocked by the adult cuticle; the dsRed signal is obvious at all postembryonic stages. Double carriers showed tissue-specific β-galactosidase expression in both larval and adult mosquitoes, primarily in pericardial cells and a set of cells (typically 5–10 μm in diameter) predominantly found attached to the trachea, but also observed in tissues, including ovaries, fat body, and flight muscle, as well as in isolated hemolymph (which we believe are hemocytes, as discussed below, and refer to them as such herein). Furthermore, this expression was appropriately modulated by the presence or absence of dox (Figures 2–4). This section describes this predominant β-galactosidase expression pattern and its modulation, while a detailed comparison of dox-regulated gene expression, the complex pattern of expression in the midgut, and a low level of background staining in the responder lines are considered later.

Dissected adult progeny of crosses between the tTA1 and tTA2 lines and five different responder lines all showed strong staining in pericardial cells (Figure 2, D and F) and in a subset of hemocytes (Figure 2, E and G, and Figure 3, A–D). The intensity of β-gal staining in pericardial cells was substantially reduced by exposure to dox (Figure 2A *vs.* 2B), as was the percentage of stained hemocytes (Figure 3D). Larvae from the same crosses also showed dox-modulated expression in these two cell types, from the second instar until pupation (example shown in Figure 4A). Interestingly, the intensity of pericardial cell staining (Figure 2C *vs.* 2B) and the percentage of stained hemocytes (Figure 3C) in adult mosquitoes increased significantly after blood feeding, especially at 21°, the temperature that is permissive to *P. berghei* infection (although infection itself did not increase this percentage further).

A majority of individuals from crosses with the tTA2 line showed a greater intensity of staining than those derived from the tTA1 line, but significantly there was the same tissue distribution of expression (not shown). In addition, all individuals from a line made homozygous for both tTA1 and TetOPLacZ6 insertions also

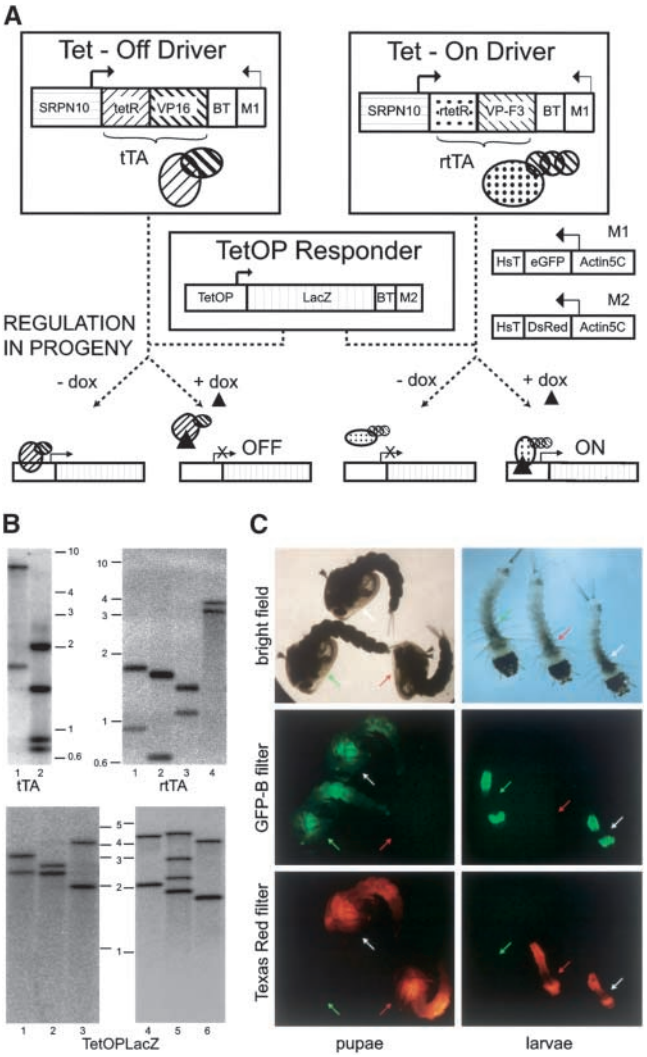



FIGURE 1.—Tet regulatory systems and transgenic line characterization. (A) Scheme of tet regulation. The *SRPN10* promoter (SRPN10) directs the expression of alternative tet-responsive regulators, tTA (tet repressor, tetR, fusion to VP16 activation domain, VP16) and rtTA (reverse tet repressor, rtetR, fusion to three minimal VP16 activation domains, VP-F3) in transgenic *A. stephensi* driver lines marked with the Drosophila Actin 5C promoter-eGFP-Drosophila heat-shock protein 70 termination sequence (HsT) cassettes (M1). A multimeric tetracycline operator sequence fused to the minimal *P* promoter (*TetOP*) *cis* regulates *LacZ* expression in the responder lines, which are marked with a similar Actin 5C-DsRed1-HsT cassette (M2). In the absence of dox, tTA binds to *TetOP* and activates transcription of *LacZ*. By raising progeny from crosses between Tet-Off and responder lines in dox-containing water, *LacZ* expression can be shut off until the antibiotic is withdrawn. Conversely, in Tet-On/responder progeny, rtTA will bind to *TetOP* and activate transcription only in the presence of dox. (B) Southern analysis of transgenic lines. *Hin*dIII-digested genomic DNAs isolated from all 12 transgenic lines were fractionated by agarose gel electrophoresis, transferred to nylon membranes, and probed with the *Minos* inverted repeat. Each insertion yields two bands. (C) Fluoresence analysis of transgenic lines. Individuals expressing GFP (green arrow), dsRed1 (red arrow), or both markers (white arrow) are readily identified in larva and pupa examined with the indicated filter sets. Images were taken with a Nikon SZ1000 stereomicroscope fitted with a UV source, appropriate filters, and a CCD camera (Nikon).

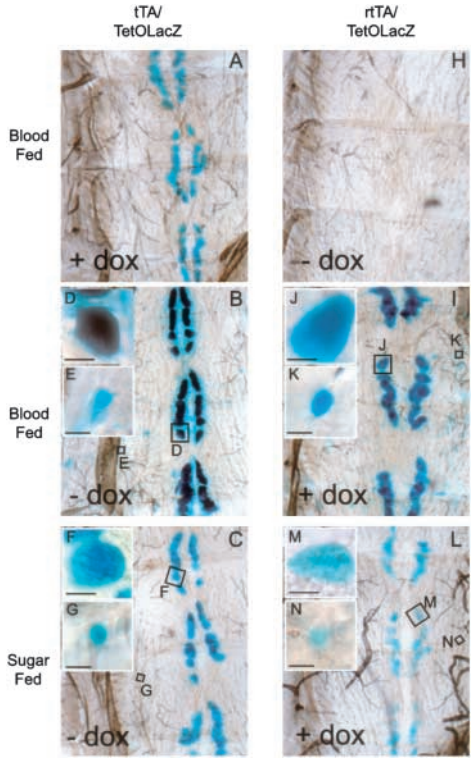



FIGURE 2.—*SRPN10* promoter-mediated regulation of LacZ expression by dox and blood feeding. Adult 2- to 3-day-old tTA/TetOPLacZ (A–G) and rtTA/TetOPLacZ (H–N) progeny were fed on either 15% sucrose (−dox) or 100 μg/ml dox in 15% sucrose (+dox) and were maintained on this regime throughout the assays. After 2 days, they were either blood fed or not, and 48 hr later abdomens were dissected and stained with X-gal. Images shown represent the typical staining patterns observed. Blood feeding increases the constitutive expression observed in the segmental rows of pericardial cells (inset boxes D and F and J and M) and increases the number of stained hemocytes found attached to abdominal structures (inset boxes E and G and K and N). In tTA/TetOPLacZ individuals, the effect of blood feeding is evident (B *vs.* C). Dox treatment leads to inhibition of the increased expression observed in blood-fed, non-dox-treated TA/TetOPLacZ individuals. However, staining is not eliminated by dox treatment. The increased expression observed in dox-treated rtTA/TetOPLacZ individuals following blood feeding (I *vs.* L) is absent when dox is not supplied (I *vs.* H). Bars: D, F, J, and M, 20 μm; E, G, K, and N, 10 μm.

showed the same tissue distribution and greater intensity of staining compared to individuals heterozygous for both insertions (not shown).

The pattern of rtTA-driven gene expression was assessed in a similar manner in the progeny of crosses with TetOPLacZ responder lines. Initial tests in adults determined that addition of dox (100 μg/ml–10 mg/ml) to the sucrose food source for 24 hr induced β-gal activity in the pericardial cells and hemocytes (data not shown). Again, blood feeding substantially increased the level of β-gal staining in both cell types (Figure 2I *vs.* 2L; Figure 3D). Strong induction of expression was also detected in the larval progeny of these crosses after addition of dox at 10 ng/ml or higher into the larval breeding water (Figure 4B). No significant difference in the dox-dependent expression patterns was observed in crosses between all the different rtTA lines and two responder lines selected for assay.

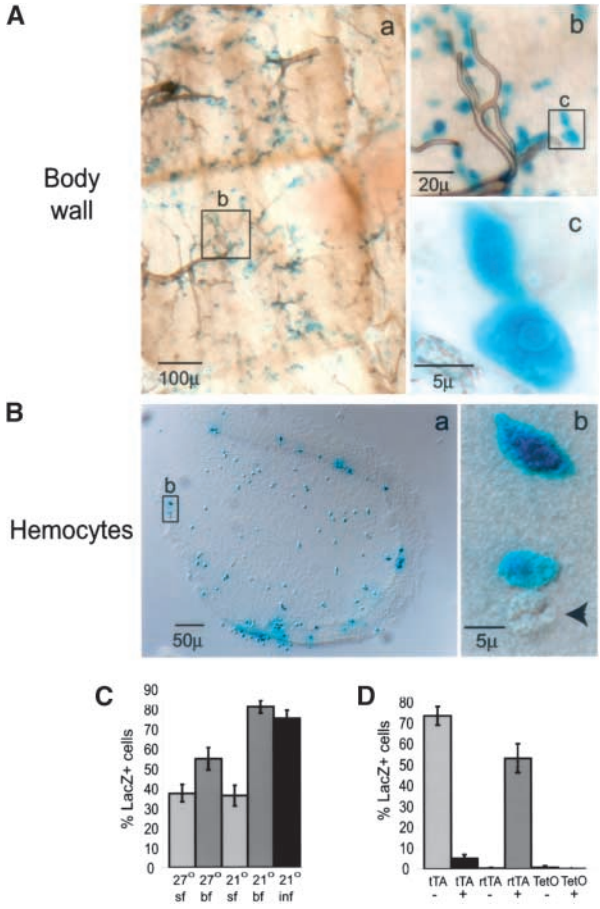
**Background expression:** In the absence of transactivators, only low background levels of X-gal staining were observed in certain tissues of certain responder lines, but not in wild-type controls. Three responder lines showed β-gal staining in the hindgut epithelium and in stripes along the hypodermis in larval stages, but not in the adult. In all but one responder line a small number of sporadic larval and adult midgut cells also stained, as did the imaginal rings (adult precursors) of the posterior (indicated in Figure 5, B and C) and anterior midgut valves (CLEMENTS 1992) in late fourth instar larvae in the absence of transactivators. One TetOPLacZ line failed to show any transactivator-dependent or background staining. Two lines (TetOPLacZ 5 and 6) characterized by the presence or absence of hindgut and hypodermal background were kept for future analysis.

**rtTA-dependent expression in the midgut:** In progeny of crosses between tTA and the selected responder lines, no activity could be detected in the midgut at any stage above the background described above. However, significant midgut-specific staining was observed, superimposed on the background staining, in dox-treated progeny from crosses between each of the rtTA driver lines and the responder lines. rtTA-dependent midgut staining was observed in fourth instar larval midguts following dox treatment either in gastric caecal cells alone (Figure 5A) or additionally in the posterior midgut (Figure 5B) or throughout the midgut (Figure 5C). We interpret this variation as being due to small differences in the timing of progress toward pupation, a process that involves extensive midgut restructuring. This interpretation is substantiated through the observation that larvae displaying prepupal characteristics, including food-free midguts and degenerative caecae, showed the greatest number and distribution of stained cells. Three major morphological cell types have been described in the mosquito larval midgut and their function has been inferred from cytology or immunostaining: columnar (or ion transporting) cells, cuboidal (or resorbing/secreting) cells, and basally located cells that have apical extensions and stain with antibodies to ecdysteroidogenic hormones (endocrine cells; BROWN *et al.* 1985; ZHUANG *et al.* 1999). In whole-mount stainings, two cell types appeared to be $LacZ^+$ in larval midguts. We detected stained cells having the characteristic extensions of endocrine cells throughout the midgut and especially in the caecae (Figure 5, D–F) and strongly staining round cells primarily within the anterior and posterior midgut (Figure 5, G–I). Thin sectioning will be required for detailed characterization of the round cell type.

Following dox treatment of rtTA/tetOPLacZ larvae, newly emerged adults showed β-gal staining in associa-

tion with the meconium formed by the remnants of the larval midgut (Figure 5J; Clements 1992), but virtually none in the new midgut epithelium. In aged adults, specific β-gal activity was undetectable without dox treatment (Figure 6A, a) but was strongly induced following exposure to dox (Figure 6A, b), with kinetics similar to those of pericardial cell staining (Figure 6B).

We also examined midguts taken from mosquitoes fed on *P. berghei*-infected mice to determine whether the *SRPN10* promoter fragment used is responsive in cells invaded by the parasite. As previously shown (Danielli *et al.* 2003), expression of the endogenous *SRPN10* gene is strongly induced in midguts taken from Plasmodium-infected mosquitoes and especially in parasite-invaded epithelial cells. These cells are cytologically distinguishable by a number of markers and morphologically distinguishable by their protrusion toward the gut lumen. Figure 6C illustrates typical midguts of infected rtTA/tetOPlacZ mosquitoes. Following dox treatment (Figure 6C, b), strong staining is associated with the characteristic invaded cells (rounding up and bulging away from the epithelium toward the lumen; Han *et al.* 2000; Danielli *et al.* 2003), while the surrounding cells stain more weakly. In the absence of dox (Figure 6C, a) only a few invaded and noninvaded cells showed very weak β-gal activity, comparable to the background seen in responder lines.




**Dox regulation of tTA and rtTA:** Since the *SRPN10* promoter is active in both larvae and adults we were able to compare the ability of the two tet systems to switch transactivation at different developmental stages. For analysis of dox-regulated expression in larvae, we characterized the effects of various dox concentrations in the breeding water. Continuous exposure to 1 μg/ml dox caused reduced growth rate and increased failure to eclose, whereas no detrimental effect was observed with exposure to <100 ng/ml (data not shown). In tTA/tetOPLacZ fourth instar larvae kept under continuous dox treatment, pericardial cell staining indicated a concentration-dependent inhibition of tTA transactivation by dox (Figure 4A). The inhibition was detectable at 0.1 ng/ml and complete at 1 ng/ml. Removal of dox at the second instar stage led to expression by the fourth instar, indicating significant clearance of dox within 4 days.

To regulate expression in adults, dox was added to the sucrose pad used for feeding. Initial experiments indicated that adults could tolerate up to 1 mg/ml continuous exposure without obvious detrimental effects. Above this concentration, increased death rate was observed (not shown). Most experiments with adults were thus performed using a concentration of 100 μg/ml. Exposure of tTA-bearing adults to dox at this concentration for 48 hr prior to blood feeding did not eliminate but reduced substantially (>12-fold) the percentage of stained hemocytes (Figure 3D) and the intensity of staining in pericardial cells relative to nontreated controls

Figure 3.—β-Galactosidase activity regulated by tTA and rtTA in hemocytes. (A, a) Constitutive strong staining of hemocytes from a tTA/TetOPLacZ mosquito. (b) Magnified image from square in a. Hemocytes are mainly found attached to the tracheae in the abdomen (and ovaries and flight muscles, not shown). (c) Magnified image from square in b. (B, a) Example of hemocyte-specific staining in a drop of hemolymph collected from an individual adult (tTA/TetOPLacZ) spotted onto slide and stained with X-gal. (b) Magnified image of square in a, showing two stained and one unstained hemocyte (arrowhead). (C) Percentage of stained hemocytes (*cf.* B) from 7- to 9-day-old tTA/TetOPLacZ adults 48 hr after transfer to the indicated temperature and following various treatments. sf, sugar fed; bf, blood fed; inf, *P. berghei*-infected blood meal. Error bars indicate standard errors calculated from pooled samples taken from two independent experiments containing at least 26 mosquitoes per sample. Student's *t*-tests indicate that blood feeding has a significant effect compared to sugar feeding at $P < 0.05$ for either temperature, but feedings on infected or noninfected blood are comparable. (D) Percentage of stained hemocytes collected from tTA/TetOPLacZ (tTA) and rtTA/TetOPLacZ (rtTA) individuals 48 hr following an infective blood meal and maintenance at 21° in the presence (+) or absence (−) of 100 μg/ml dox in 15% sucrose. Percentages from the responder line controls are also shown. The actual mean percentages from left to right are 71.2, 5.1, 0.4, 51.6, 0.71, and 0.2. Error bars indicate standard errors calculated from pooled samples taken from two independent experiments containing at least 17 mosquitoes/sample.

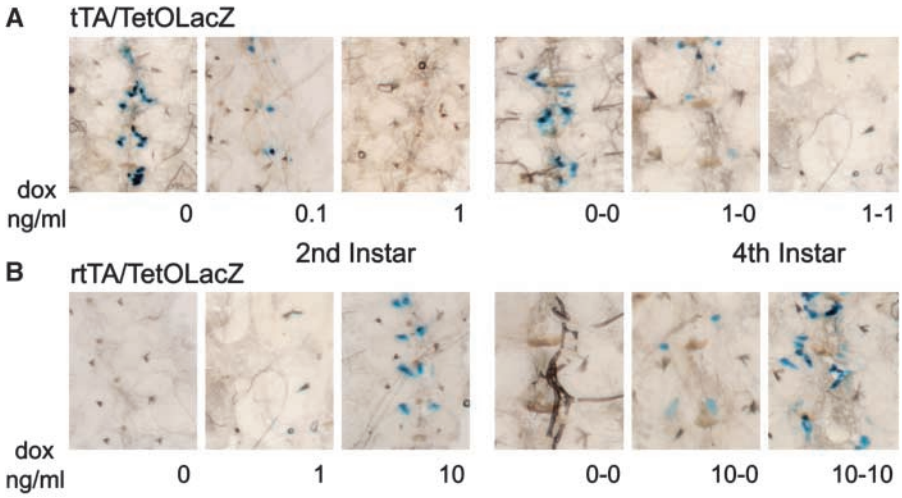



FIGURE 4.—Dox treatment "tightly" regulates expression in larvae. Newly hatched larval progeny (A) tTA/TetOPLacZ and (B) rtTA/TetOPLacZ were exposed to indicated concentrations of dox in the breeding water. Second instar samples were dissected and stained with X-gal (left three panels). The remaining larvae were maintained at various dox concentrations as shown (*e.g.*, 1-0 means larvae were kept at 1 ng/ml dox until second instar and then in its absence). At the fourth instar, further samples were dissected and stained with X-gal. Note that both tTA and rtTA are precisely regulated (*i.e.*, lack of background expression when transactivator is switched off) in the presence or absence of dox, respectively, and that higher dox concentrations are required to activate rtTA than to inactivate tTA.

(Figure 2A *vs.* 2B). The intensity of pericardial cell staining in sugar-fed and blood-fed tTA/tetOPLacZ mosquitoes was further reduced by continuous exposure to dox (100 μg) over a further 3-day period compared to the respective non-dox-treated controls, but was not completely eliminated in either group (not shown).

The switching of gene expression by rtTA, as monitored by β-gal staining, is clearer. In the absence of dox, no rtTA-dependent reporter gene expression could be detected in either larvae or adults, yet Tet-On switching was effective after dox application at both stages (Figures 2–4). Interestingly, as shown in Figure 4, higher concentrations of dox in second instar larvae were required to activate the rtTA driver (10 ng/ml) than to inhibit tTA (1 ng/ml). We also determined that removal of dox from the water during the second instar of rtTA/tetOPLacZ larvae significantly reduced the staining intensity observed at fourth instar, as compared to those larvae under continuous exposure (Figure 4B).

The kinetics of appearance of β-gal activity in adult rtTA/tetOPLacZ female mosquitoes was followed after supplementing the sucrose used for *ad libidum* feeding of the adults with dox solution. Detectable β-gal activity in the pericardial cells and midgut was absent at 2 hr but observed after 7 hr of exposure to dox, while at later times (24 and 48 hr) substantially higher accumulation of activity was observed in both tissues (Figure 6B).

## DISCUSSION

**Specificity of the *SRPN10* promoter and regulation of transactivators by dox:** We have demonstrated that a 0.7-kb promoter fragment of the *A. gambiae SRPN10* gene locus (DANIELLI *et al.* 2003) is able to confer tissue-specific and inducible expression to two alternative tetracycline-dependent transactivators (tTA and rtTA) both in a large fraction of hemocytes and in pericardial cells. In addition, active rtTA is expressed in late fourth instar larval and adult midguts: expression was strong with all four rtTA lines, but very low in the two tTA lines. Our results establish that rtTA indeed permits very precise regulation of transactivation in the mosquito and imparts a pattern of reporter gene expression in hemocytes, pericardial cells, and epithelial midgut cells that reflects the overall endogenous SRPN10 expression pattern previously determined by immunoblot and immunohistochemical analysis (DANIELLI *et al.* 2003).

A possible but unlikely explanation for the lack of active tTA expression in the midgut might be position effects on all tTA insertions. A more reasonable explanation, however, is the difference in the composition of the alternative transactivators. At the outset of this work we used the original tTA, which functions efficiently in Drosophila (BELLO *et al.* 1998) and carries the original, unmodified bacterial sequence and complete VP16 activation sequence (GOSSEN and BUJARD 1992). The rtTA, introduced later in the work, was synthetically "restructured" according to human codon usage and carries three copies of the short activation domain of VP16 (URLINGER *et al.* 2000). This transactivator is significantly more stable than previous versions (URLINGER *et al.* 2000) and less prone to transcription factor "squelching" and toxicity, which occur after Gal4 or VP16 overexpression in some organisms or tissues (GILL and PTASHNE 1988; DUFFY 2002). These modifications may make the rtTA specifically more active than tTA in the mosquito midgut. If so, we would expect pronounced midgut expression by the use of the next generation tTA (URLINGER *et al.* 2000), which has been similarly modified.

In the progeny of crosses between driver and responder lines, small cells (typically 5–10 μm) expressing LacZ, under dox regulation, have been found attached to tissues including trachea, fat body, ovaries, and epidermis, as well as circulating in the hemolymph. In this report we have constantly referred to these cells as

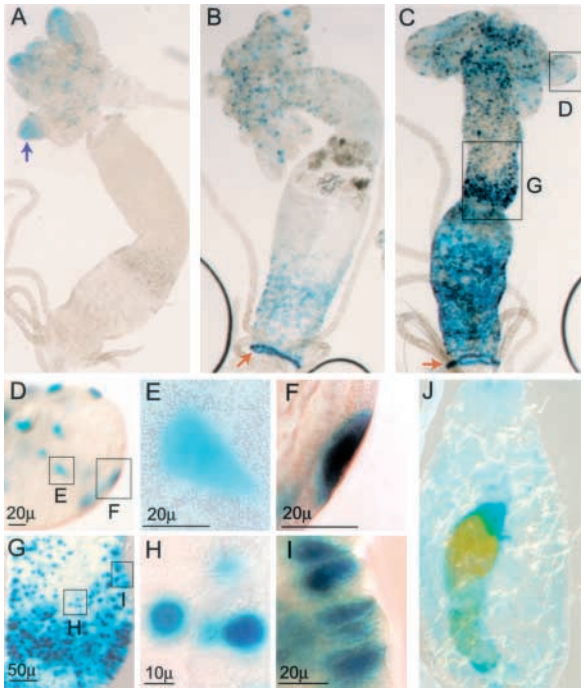



FIGURE 5.—Midgut-specific *LacZ* expression in rtTA/TetOPcLacZ larvae. rtTA-dependent midgut epithelial cell staining is observed only in late fourth instar rtTA/TetOPcLacZ larvae after exposure to dox (10 ng/ml). A–C illustrate the variation in staining distribution in individual midguts from the same cohort examined at the same time, which we interpret as representing larvae progressively closer to pupation. D–I are magnifications of inset boxes to indicate the different cell types stained. D is a cecal lobe and E and F highlight the endocrine (displaying typical "neck" structures) cells that are predominant in ceca and are present in the anterior midgut. G–I highlight the cell types predominant in the anterior and posterior midgut. Thin sectioning is required to determine the precise cell type. The diffuse staining (blue arrow) in the cecal lumen (A) is also observed in wild-type mosquitoes (not shown) and probably represents bacterial activity in the lumen. The posterior ring structure seen in B and C (red arrows) is the imaginal ring of the pyloric valve; they are stained in all responder lines in the absence of the TA alleles (not shown) but not in wild type, indicating leakiness of the TetOPlacZ responder. (J) A midgut taken from a newly emerged adult that displays β-gal activity in association with the meconium (yellow body) derived from the degenerating larval midgut.

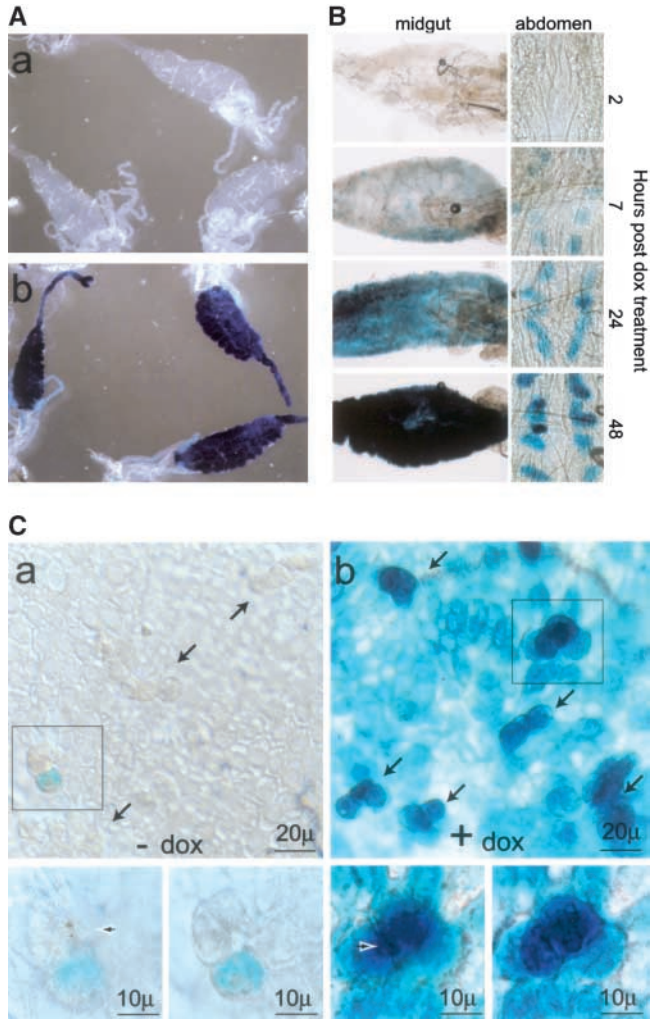



FIGURE 6.—Midgut-specific *LacZ* expression in rtTA/TetOPcLacZ adults. (A, a and b) Midguts dissected from adults 24 hr after treatment with 15% sucrose or 15% sucrose containing 10 mg/ml dox, respectively. (B) Kinetics of dox-induced LacZ expression in sugar-fed adults. Midguts and abdomens were dissected and stained with X-gal at the indicated times following exposure to sucrose containing 100 μg/ml dox. Note partial staining of both pericardial cells in the abdomen and midgut 7 hr after exposure and progressive accumulation of β-gal at later times. (C, a and b) Midguts dissected from adults 26–28 hr after feeding on a *P. berghei*-infected mouse. a had been fed only sucrose, whereas b had been exposed to 100 μg/ml dox in sucrose 48 hr prior to blood feeding and shows many stained epithelial cells. Arrows indicate characteristic invaded cells that stain intensely and bulge away from the epithelium. Inset boxes are magnified below each large image at different focal planes: the left image is focused on the parasite with red granules (arrowhead), and the right image is at the apical plane of invaded cells protruding above the monolayer. The weak staining observed in single sporadic invaded cells in the absence of dox is also observed in the responder lines, but not in wild type (not shown).

hemocytes. Cells of the same morphology and distribution have also been previously reported as hemocytes in mosquitoes (CLEMENTS 1992; HILLYER and CHRISTENSEN 2002) and are described as belonging to the plasmatocyte class of hemocytes, thought to be professional phagocytes in Drosophila (MEISTER and LAGUEUX 2003). Cells of this description in the mosquito have been shown to express proteins homologous to those found in hemocytes of other insects or associated with immune responses (MULLER *et al.* 1999; DANIELLI *et al.* 2000, 2003; LEVASHINA *et al.* 2001; BLANDIN *et al.* 2004; OSTA *et al.* 2004). Significantly, *SRPN10* gene products are included among these proteins (DANIELLI *et al.* 2003).

An intriguing finding has been the increase in the percentage of β-gal-expressing hemocytes observed following blood feeding, which is enhanced at 21° in the tTA lines that have been most extensively examined. This change is not affected by a parasite burden and appears blood-meal specific as the percentages are not

significantly different between sugar-fed mosquitoes at alternative temperatures. Blood-meal ingestion by female mosquitoes triggers a series of physiological responses among which the best studied are probably digestion and vitellogenesis. We have previously reported a complex organization of the *SRPN10* promoter, according to Genomatix Matinspector analysis (Danielli *et al.* 2003), which indicated the presence of multiple putative binding sites for transcription factors controlling morphogenesis and other processes. The promoter fragment used in this study carries putative binding sites for Dorsal, activator protein 1 (AP1), c-REL, CCAAT enhancer binding protein, ADF-1, and GATA factors. The latter participate in numerous morphogenetic and physiological events, including hematopoiesis (Meister and Lagueux 2003), through synergistic interactions with other transcriptional activators (Merika and Orkin 1995; Brodu *et al.* 1999; Watanabe *et al.* 2000). In *Aedes aegypti* it has been shown that the concentration of GATA activity in the fat body increases dramatically following an infective blood meal, contributing substantially to the extremely high levels of vitellogenin expression (Kokoza *et al.* 2001). Therefore, we cannot exclude the possibility that the blood meal triggers similar regulatory circuits in the hemocytes and that the higher fraction of hemocytes expressing LacZ following a blood meal correlates with increased GATA activity. However, there is no further evidence to substantiate these suggestions. It is also not clear why the increase in the percentage of LacZ-expressing hemocytes should be greater after blood feeding and maintenance at the lower temperature, yet it is reasonable to speculate that physiological and molecular responses affecting hemocyte gene expression during metabolism of the blood meal may also be induced by changes in environmental temperature.

In adult midgut cells invaded by *P. berghei*, transcriptional induction from the *SRPN10* locus correlates with morphological manifestations of apoptotic cell death, suggesting a possible implication of SRPN10 in this process (Danielli *et al.* 2003). In midguts isolated from infected *A. stephensi*, these invaded and apoptotic cells are easily identified by their characteristic rounded shape and are found bulging away from the midgut epithelium. In dox-treated, *P. berghei*-infected rtTA/tetOPLacZ mosquitoes, clearly higher reporter gene activity was detected in these cells compared to the rest of the midgut epithelium, indicating that the promoter fragment used reflects, in addition, this aspect of SRPN10 regulation.

Strong reporter expression in late fourth instar midguts is also consistent with the hypothesis that the *SRPN10* promoter is regulated in cells fated to undergo cell death. In mosquitoes the larval midgut epithelium is destroyed during morphogenesis and replaced by adult tissue during pupation (Clements 1992). Cytologically distinct alterations first occur during the prepupal stage in the gastric cecum before spreading to the posterior and then the anterior midgut (Clements 1992). In Drosophila, it is known that the extensive midgut remodeling begins with programmed cell death triggered by the ecdysone pulse that marks the onset of larval-to-pupal metamorphosis; cell death is also first apparent in the ceca ~4 hr before pupal formation and then spreads to the entire epithelium at pupation (Jiang *et al.* 1997). The β-gal staining patterns that we observe correlate well with the onset of morphogenesis of the mosquito midgut and suggest a general role for SRPN10 in programmed cell death.

Transactivation by both Tet-On and Tet-Off systems can be switched by dox supplements in a broad developmental range from second instar larvae to adult stages. The very low effective concentrations used to switch expression suggest that dox penetration in larvae is highly efficient. Dox intake through feeding is also efficient in the adult, where addition of dox to the sucrose food source is sufficient to control pericardial cell, hemocyte, and midgut expression. Higher dox concentrations are needed to "switch on" rtTA than to "switch off" tTA-dependent transcription of the same transgene. This is consistent with the *in vitro* binding characteristics of the two activators with dox and the empirical results of their use in mammalian cells (Gossen and Bujard 2002). The regulation imparted by rtTA is "tighter" operationally (*cf.* Figures 2 and 3D) in that β-galactosidase activity is undetectable in the absence of dox, but is rapidly induced in its presence. However, it is not known to what extent the observed difference between the systems may simply reflect the slow turnover of β-galactosidase, since in testing the tTA system the kinetics of β-gal degradation will be a major factor in determining the efficiency of observed regulation. The 1- to 2-day half-life of β-gal shown in mammals (Smith *et al.* 1995), if applicable to mosquitoes, would make the total elimination of staining difficult to achieve in the time course of these experiments. Later time points (not shown), up to 7 days after continuous dox treatment, show a continuing loss of β-gal activity relative to nontreated controls. Despite this continued inhibition, we cannot be sure that repression is complete in the adult stage. Nevertheless, it must be borne in mind that during larval stages, where dox can be given before LacZ expression is evident, it is possible to eliminate β-galactosidase activity throughout mosquito development. In future applications of the systems, specific characteristics such as protein half-life and transcriptional strength of the requisite driver construct will be important parameters that may need to be assessed for each gene under study. At this stage we can say that both systems are suitable for use in the mosquito since dox concentrations needed for regulation are below levels that affect growth and eclosion.

**Background expression:** The limited background *LacZ* expression in hindgut, hypodermis, imaginal rings,

and sporadic midgut cells in responder lines in the absence of transactivators indicates that there is some "leakage" from the TetOPLacZ expression cassette. Three of six responder lines with independent insertions all show similar background expression patterns, suggesting that sequences within the expression cassette can promote expression in certain contexts. Similarly, specific transient fluorescence has been detected in the hindgut following injection into wild-type embryos of a plasmid carrying yellow FP under regulation by the TetOP promoter (our unpublished results). Moreover, hypodermis and hindgut-specific expression has also been observed in stable transgenic *A. gambiae* lines carrying alternative promoter-GFP fusions (J. R. Clayton and G. J. Lycett, unpublished results). The present report is the first to examine mosquito promoter function by specific localization of reporter gene activity in mosquitoes and further work is required to determine whether the background patterns are common.

The hybrid promoter used in the present study and in Drosophila (Bello *et al.* 1998) consists of a heptamer of tetO-binding sites fused to the *P* transposase minimal promoter. In pilot experiments on transfected *A. gambiae* cell lines, this promoter showed lower background activity than a TetO-heat-shock protein 70 promoter alternative (data not shown) and was thus the choice for this study. However, analysis of a broad range of alternative minimal promoter sequences is often necessary to achieve low background and specific expression in certain cell types (Hoffmann *et al.* 1997; Leuchtenberger *et al.* 2001). Clearly, this may be of critical importance in certain applications of the system to mosquitoes. It must be emphasized, however, that despite a low level of background expression being observed, we have succeeded in identifying those transgenic responder lines that give minimal background yet retain high levels of responsiveness to dox.

**Utility of the tet systems in Anopheles:** Undoubtedly, numerous genes must play key roles in mosquito development, innate immunity, and parasite transmission. This supposition has recently been experimentally supported by RNAi silencing approaches in the mosquito (Blandin *et al.* 2002, 2004; Osta *et al.* 2004). Rigorous characterization of the function of these genes will depend on conditional expression systems such as those demonstrated in this article. The major advantages of conditional binary systems include the ability to regulate gene expression by an exogenous molecule such as doxycycline (and so monitor phenotypic changes within the same genotypic background), the ability to target gene expression to specific tissues or stages, and their logistic efficiency (a matter of importance because of the labor intensity of Anopheline strain maintenance). Rather than generating multiple "unitary" lines anew, it would be possible with the tet systems to generate a collection of reusable drivers, each specifying a precisely defined expression pattern. The phenotypes caused by expression or silencing a gene of interest in the available driver patterns could then be assessed by crossing these with a newly generated line bearing the appropriate responder construct.

Apart from their importance for the functional analysis of genes, the tet systems may represent enormously valuable enhancements of the sterile insect techniques envisioned for mosquito control (Thomas *et al.* 2000; Benedict and Robinson 2003) or even localized species eradication (Krafsur *et al.* 1986). Such methods involve mass release of males (either sterile or carrying female-specific dominant lethal genes) to reduce the effective breeding population. The logistics of generating males for release would be greatly facilitated by a genetic sexing method, in which the breeding stocks needed would be routinely maintained and then switched to strictly male-only mass production by addition or removal of a simple compound to food, such as doxycycline. The efficacy of this approach has been demonstrated by utilizing the tTA system in Drosophila (Thomas *et al.* 2000; Horn and Wimmer 2003) and the present report makes its adoption feasible in Anopheles.

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