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Review

Transgenesis and reverse genetics of mosquito innate immunity

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Summary

In recent years, mosquito molecular biology has been a scene of astounding achievements, namely the development of genetic transformation, characterization of inducible tissue-specific promoters, and acquirement of mosquito genome sequences. However, the lack of a complete genetic tool box for mosquitoes remains a serious obstacle in our ability to study essential mosquito-specific mechanisms. Unlike *Drosophila*, very few null mutations for mosquito genes exist. The development of reverse-genetic analyses based on RNAi and transgenic techniques will help to compensate for these deficiencies and aid in identification of critical genes in important regulatory pathways. The study of mosquito innate immunity is one example and described here. In this study, we combine mosquito transgenesis with reverse genetics. The advantage of transgenesis is the ability to establish genetically stable, dominant-negative and overexpression phenotypes. Using the blood-meal-activated vitellogenin gene (*Vg*) promoter, we have generated transgenic mosquitoes with blood-meal-activated, overexpressed antimicrobial peptides, Defensin A and Cecropin A. Moreover, we have recently generated a transgenic dominant-negative Relish mosquito strain, which after taking a blood meal, becomes immune-deficient to infection by Gram-negative bacteria. The latter accomplishment has opened the door to a reverse-genetic approach in mosquitoes based on transgenesis.

Key words: transgenesis, reverse genetics, mosquito, immunity, defensin, cecropin, Relish, Dorsal, dsDNA, RNAi, *Aedes aegypti*, vitellogenin gene, transposable element.

Introduction

Immunity plays an important role in the interaction between a pathogen and its vector host. Many genes and their products involved in mosquito innate immunity have been identified and characterized either independently or based on their relation to known immune genes of the model organism, *Drosophila melanogaster*. 242 *Anopheles gambiae* genes from 18 gene families have been implicated in innate immunity (Christophides et al., 2002). The mosquito immune genes have diverged widely from those in *Drosophila*, which possibly reflects different selection pressures to a variety of pathogens encountered by these insects' distinct life-styles.

The availability of genomic and EST sequences, together with tools such as DNA microarrays and proteomics, will make mosquitoes powerful model systems for studying innate immunity against parasites and pathogens (Dimopoulos et al., 2002; Christophides et al., 2002). The lack of precise genetic tools, however, has been a serous limitation to the in-depth analysis of the mosquito immune system. Reverse-genetic analyses, based on RNAi and transgenic techniques, will fill these deficiencies in the research of mosquito innate immunity.

RNAi-based reverse genetics

Direct applications of dsRNA

Recently developed double-stranded (ds)RNA/RNA interference (RNAi) technologies have proven to be extremely useful tools of reverse genetics in model organisms, ranging from *Caenorhabditis elegans* and *Drosophila* to zebrafish and mouse (Montgomery et al., 1998; Kennerdell and Carthrew, 1998; Li et al., 2000; Wianny and Zernicka-Goetz, 2000). This technology has also been fruitful in dissecting complex regulatory pathways in cell lines (Clements et al., 2000; Lum et al., 2003). Levashina et al. (2001) used dsRNA knockout in hemocyte-like *An. gambiae* cultured cells to demonstrate the conserved role of a complement-like protein in phagocytosis. Direct injection of (ds)RNA corresponding to a single gene into an organism specifically silences this gene's expression *in vivo*. It has been valuable for studying gene function in organisms that are not amenable to genetic analysis. Blandin et al. (2002) utilized direct injection of dsRNA in order to knock-down the *defensin* gene in *An. gambiae* and demonstrate its function. *Hemolin* gene silencing by injecting dsRNA into *Cecropia* pupae revealed its crucial role for normal development of embryos of the following generation (Bettencourt et al., 2002). Despite its importance as the tool of

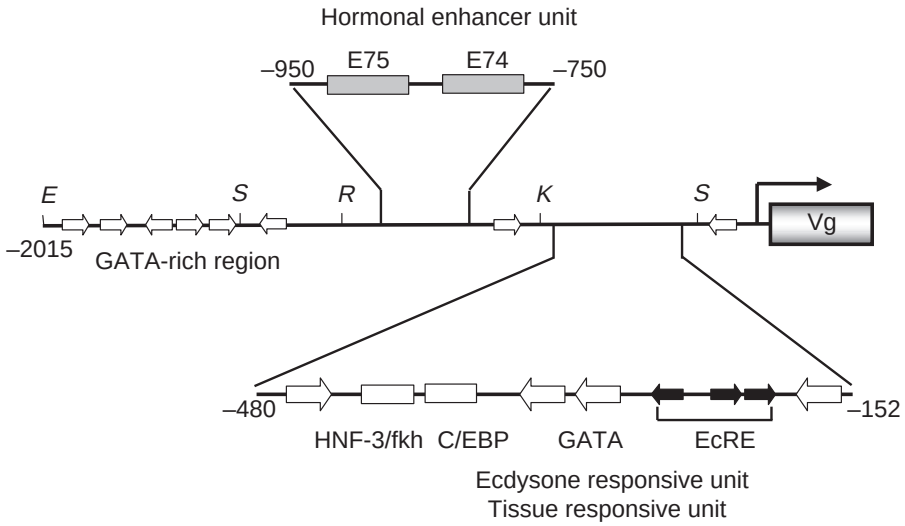

Fig. 1. Schematic illustration of the regulatory regions of *Ae. aegypi Vitellogenin* (*Vg*) gene. Numbers refer to nucleotide positions relative to the transcription start site, and letters refer to restriction enzyme sites: *E*, *Eco*RI; *R*, *Eco*RV; *K*, *Kpn*I; *S*, *Sau*3A. C/EBP, response element of C/EBP transcription factor; EcRE, ecdysteroid response element; E74 and E75, response elements for the respective early gene product of the ecdysone hierarchy; GATA, response element for GATA transcription factor; HNF-3/fkh, response element for HNF/forkhead factor; Vg, coding region of the *Vg* gene. (Reproduced from Kokoza et al., 2001a, with permission.)

reverse genetics, direct injection of dsRNA has the limitation of interfering only transiently with target gene expression. Consequently, it cannot be used to generate heritable gene silencing. In mosquitoes, the current inability of direct dsRNA injection to generate stable heritable knockout dominant negative phenotypes limits its use for the genetic and genomic analysis of immune pathways.

Heritable reverse genetics in mosquitoes

Characterization of tissue-specific promoters in mosquitoes

Many of the strategies for developing parasite-resistant transgenic mosquitoes rely on the use of mosquito endogenous promoters to control when, where, and how much an engineered exogenous gene product is expressed. Genes specifically expressed in the midgut, the fat body (which produces most hemolymph proteins) and the salivary glands have been the most studied (Coates et al., 1999; Kokoza et al., 2000, 2001a,b; Moreira et al., 2000). The Maltase-like I (*MalI*; James et al., 1989) and *Apy* (Smartt et al., 1995) genes are expressed specifically in the adult salivary glands. *MalI* encodes an α-glucosidase that is expressed in both males and females, but only in the proximal-lateral lobes of the female salivary gland. The *Apy* gene encodes a potential platelet anti-aggregating factor that is expressed only in the distal-lateral and medial salivary gland lobes in females. Putative promoter fragments have been cloned to the 5′-end of the luciferase gene, placed in *Hermes* vectors, and transformed into mosquitoes (Coates et al., 1999). Both promoters showed the proper sex-, stage- and tissue-specific expressions in transgenic animals, but their expression levels were low, which limits their use for driving effector molecules. In contrast, Moreira et al. (2000) reported a robust gut-specific expression in the 1.4 kb promoter region of the *Ae. aegypti* midgut carboxypeptidase (*CP*) gene in transgenic *Ae. aegypti*. Interestingly, the 3.4-kb *An. gambiae CP* promoter also exhibited a high level of expression in transgenic *Ae. aegypti*. In transgenic *Anopheles stephensi*, the 3.9-kb *An. gambiae CP* promoter also showed high levels of expression (Ito et al., 2002; Moreira et al., 2002). However, no information is available on the composition of the *Ae. aegypti* and *An. gambiae CP* regulatory regions.

Our interest is to explore hemolymph-borne antipathogen factors. We have centered our efforts on the detailed analysis of the *vitellogenin* (*Vg*) gene as a female- and fat body-specific gene that could drive an effector molecule to a high level of expression, but only exclusively after a blood meal. Understanding the internal structure of a promoter for use in transgenic research is essential in order to reduce possible negative effects associated with the engineered gene. Knowledge of the presence and location of precise enhancer elements in the promoter allows its manipulation in order to achieve desired and predicted expression levels. We undertook a detailed analysis of the *Vg* promoter (Kokoza et al., 2001b). This analysis revealed three regulatory regions in the 2.1-kb upstream portion of the *Vg* gene. The proximal region (–121/–619) is required for the correct tissue- and stage-specific expression at a low level. The median region (–619/–1071) is responsible for a stage-specific hormonal enhancement of the *Vg* expression. Finally, the distal region (–1071/–2015) is necessary for the extremely high expression levels characteristic of the *Vg* gene (Fig. 1). The development of the *Vg* gene-based expression cassette that can drive the fat body-specific expression in response to a blood meal permits the testing of numerous effector molecules for their antibacterial and anti-pathogen properties.

Utilization of transgenesis for overexpression of antimicrobial peptides

The sequencing of the *An. gambiae* genome has revealed that *Anopheles* mosquitoes utilize fewer antimicrobial peptide (AMP) families than *Drosophila* (Christophides et al., 2002). The major AMP families in *Anopheles* are Defensins and Cecropins, each consisting of four genes. For *Ae. aegypti*,

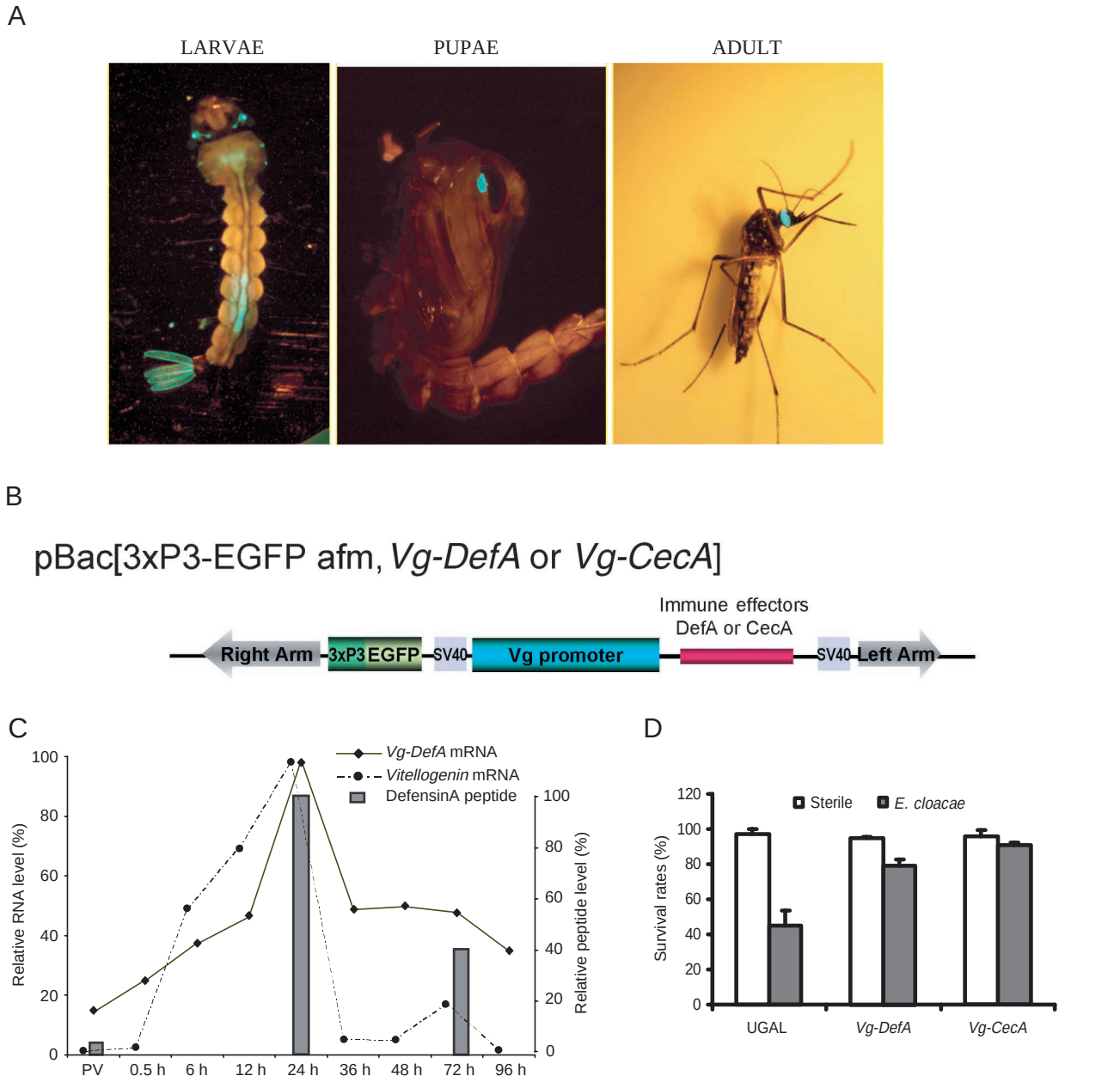

Fig. 2. Transgenic mosquitoes with the 3xP3-EGFP selectable marker and structure of the transformation vector overexpressing the immune effector molecules and its expression in transgenic mosquitoes. (A) Expression of the 3xP3-EGFP selectable marker was observed in the eyes of the larval, pupal and adult stages of transgenic *Ae. aegypti*. (B) Schematic diagram of the pBac[3xP3-EGFP afm, *DefA* or *CecA*] transformation vector that was transformed into the *Ae. aegypti* germ line. (C) Developmental profiles of *Vg-DefA* and *Vg* mRNA expression in the fat bodies of the transgenic mosquitoes. The DefA peptide level of hemolymph was detected by western analysis. PV, previtellogenic stage. (D) The increased resistance to *Enterobacter cloacae* was shown by the survival test of transgenic mosquitoes. Survival rates (%) of the parental wild-type and transgenic blood-fed mosquitoes at 24 h after the injection of *E. cloacae* are shown. UGAL, the parental wild type. *Vg-DefA*, transgenic mosquitoes with the *Vg-DefA* transgene; *Vg-CefA*, transgenic mosquitoes with the *Vg-CefA* transgene.

Defensins and Cecropins also appear to be the major AMPs (Lowenberger, 2001).

Our research has focused primarily on achieving an understanding of the mosquito immune system utilizing the reverse genetic approach through stable transformation. We have elected to focus on *Ae. aegypti* because this species is more amenable to transgenesis. Furthermore, *Ae. aegypti* eggs can be stored over 6 months, which makes it possible to maintain a large genetic stock. *Ae. aegypti* has long been used to study malaria because it can transmit the avian parasite *Plasmodium gallinaceum*. Moreover, *Ae. aegypti g*enomic and EST projects soon will bring abundant information concerning immune genes and effector molecules in this vector mosquito (D. Severson, personal communication). Therefore, *Ae.*

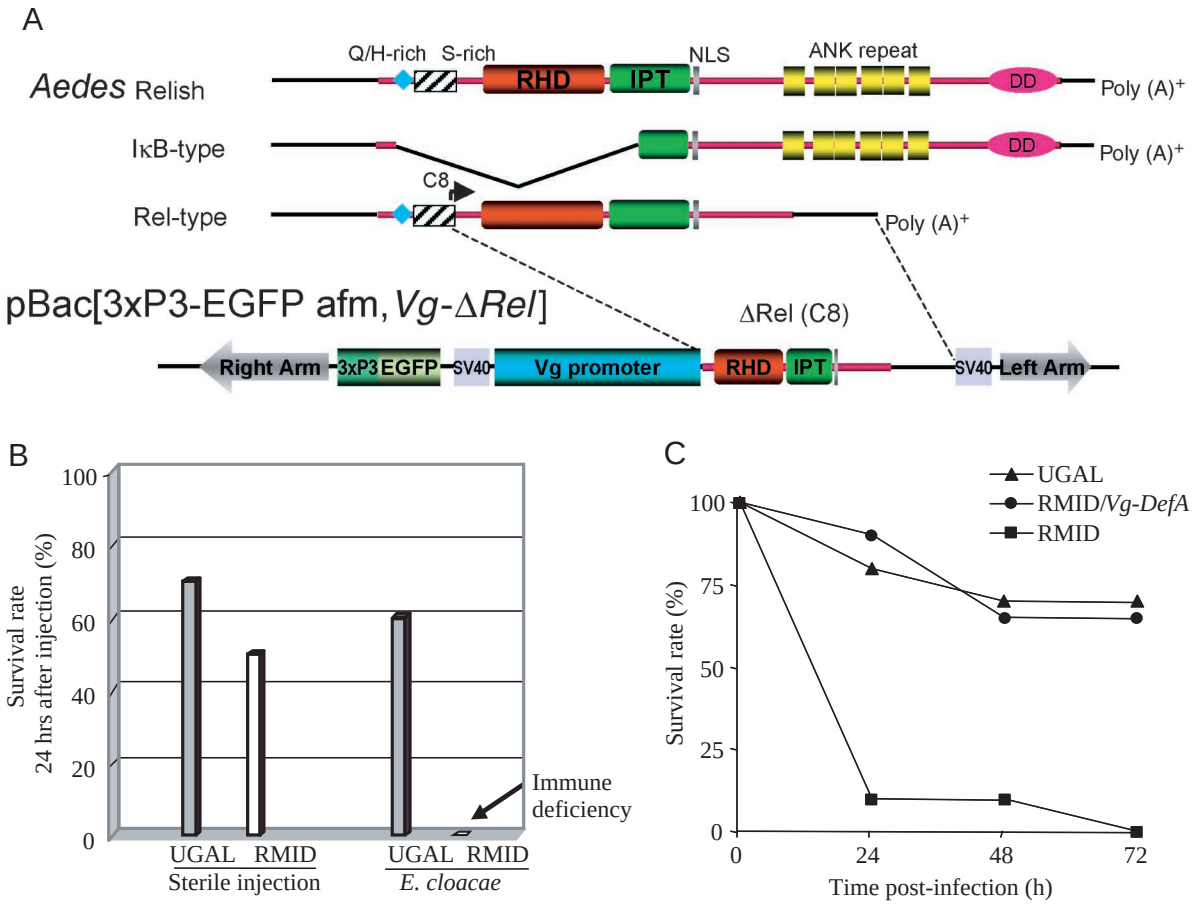

Fig. 3. Structure of the transformation vector for transgenic alteration of the IMD/Relish pathway and its expression in transgenic mosquitoes. (A) Schematic diagram of the pBac[3xP3-EGFP afm, *Vg-ΔRel*] transformation vector that was transformed into the *Ae. aegypti* germ line. Three alternative spliced transcripts of *Aedes* Relish and ΔRel structure are shown. Q/H-rich, glutamine/histidine-rich domain; S-rich, serine-rich domain; RHD, Rel homology region 1; IPT, Ig-like plexin transcription factor domain; NLS, nuclear localization signal; ANK, ankyrin domain; DD, death domain. (B) Marked susceptibility to bacterial infection of the transgenic mosquitoes. Survival rates (%) of the parental wild-type (UGAL) and transgenic blood-fed mosquitoes (RMID) at 24 h after the injection of *E. cloacae* are shown. None of the transgenic mosquitoes at 24 h post-blood meal (PBM) survived more than 24 h, presenting the immune-deficient phenotype. (C) Resistance recovery of immune-compromised mosquitoes overexpressing the Defensin gene. Female RMID transgenic mosquitoes were mated to male *Vg-DefA* transgenic mosquitoes, and their progeny were subjected to survival test with *E. cloacae*. (Reproduced from Shin et al., 2003, with permission.)

aegypti represents an outstanding model system for research on vector-pathogen interactions in mosquitoes.

To express *Aedes* Defensin A, we employed the *Hermes* transposable element as a vector and the *Drosophila cinnabar* gene as a marker to transform the white-eye *Ae. aegypti* host strain (Kokoza et al., 2000). We used the 2.1 kb 5′ upstream region of the *vitellogenin* (*Vg*) gene to drive expression of the *Defensin A* (*DefA*) gene. The *Vg-DefA* transgene insertion was stable and that the *Vg-DefA* transgene was strongly activated in the fat body after a blood meal. The mRNA levels reached a maximum at 24 h post-blood meal, corresponding to the expression peak of the endogenous *Vg* gene. High levels of transgenic Defensin A were accumulated in the hemolymph of blood-fed female mosquitoes and persisted for 20–22 days after a single blood feeding (Fig. 2C). Purified transgenic Defensin A showed antibacterial activity similar to that of Defensin isolated from bacterially challenged control mosquitoes. This work made it possible to use a tissue-specific inducible promoter for the overexpression of immune genes in the center of innate humoral immunity, the fat body.

Further progress has depended on developing an efficient, routine gene transformation for vector insects by using the *piggyBac* transposable vector pBac[3xP3-EGFP afm] and the selectable marker EGFP under the 3xP3 promoter for transformation of *Ae. aegypti* (Kokoza et al., 2001a). Selection was performed from immediately hatched, first-instar larvae of G_1 progeny, and this larval selection, in combination with the use of a vigorous wild-type mosquito strain, significantly improved the efficiency of the labor-intensive transgenic technique (Fig. 2A). Based on developed transgenic techniques, we have successfully generated transgenic mosquitoes containing the AMPs Defensin A and recently Cecropin A (Fig. 2B) (Kokoza et al., 2001a; A. Ahmed, S. W. Shin, I. Lobkov, V. Kokoza and A. S. Raikhel, manuscript in

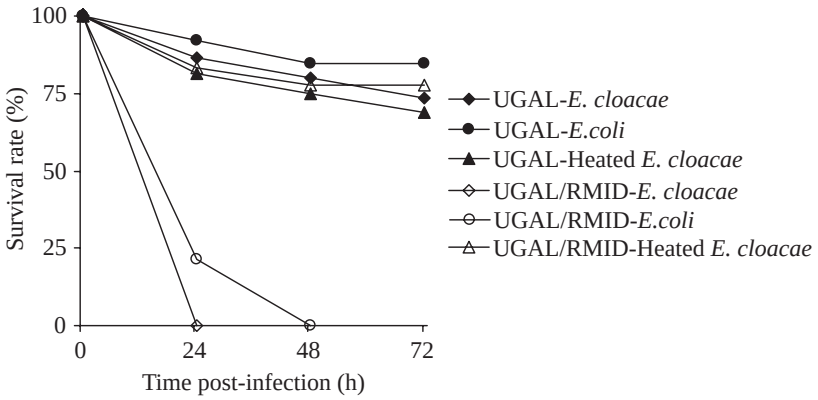

Fig. 4. Genetically dominant phenotype of the *Vg-ΔRel* transgene. Female *Vg-ΔRel* transgenic (RMID) mosquitoes were mated to male wild-type (UGAL) mosquitoes, and their progeny were challenged with bacteria. These hybrid mosquitoes showed a marked susceptibility to live bacteria, *E. cloacae* and *E. coli*. Heat inactivation of *E. cloacae* was performed by incubating the bacterial suspension at 95°C for 30 min. (Reproduced from Shin et al., 2003, with permission.)

preparation). In survival tests, transgenic mosquitoes carrying either *Vg-DefA* or *Vg-CecA* transgenes exhibited resistance to the Gram-negative bacterium *Enterobacter cloacae* that was nearly twice as high as that of the wild-type mosquitoes (Fig. 2D) (A. Ahmed, S. W. Shin, I. Lobkov, V. Kokoza, and A. S. Raikhel, manuscript in preparation).

Anti-malarial activities have been described for the natural cationic AMPs, Cecropins and Defensins. Studies using exogenous Defensins and Cecropins have demonstrated that these antibacterial peptides possess potent anti-*Plasmodium* activity (Shahabuddin et al., 1998; Gwadz et al., 1989). Furthermore, Defensin has been implicated in the local innate immune response of *An. gambiae* midgut to *Plasmodium* infection (Richman et al., 1997; Tahar et al., 2002). These observations suggest that some AMPs could be involved in anti-malarial defense and therefore could be explored as potential effector molecules in transgenic mosquitoes to block transmission of vector-borne diseases. In contrast, dsRNA knock-out of the *Defensin* gene in *An. gambiae* had no effect on the development of *Plasmodium* (Blandin et al., 2002). In our preliminary tests, two independent transgenic *Ae. aegypti* strains overexpressing Defensin A exhibited 65–70% inhibition of *P. gallinaceum* oocyst growth (V. A. Kokoza, M. Shahabuddin, A. Ahmed and A. S. Raikhel, unpublished results). Further studies are required to implicate AMPs in anti-*Plasmodium* activity.

Development of dominant negative knock-out for the IMD/Relish pathway in Ae. Aegypti

In *Drosophila*, three types of Rel regulatory molecules can affect the expression of numerous immune genes, including AMP genes (Hoffmann et al., 1996; Hultmark, 2003). They are involved in two distinct pathways: the Toll pathway, which activates primarily anti-fungal and anti-Gram-positive responses and is mediated by Dorsal-related Immunity Factor (Dif) and Dorsal; and the Imd pathway, which is regulated by Relish and predominantly directed against Gram-negative bacteria.

We have characterized the *Ae. aegypti Relish* gene (Shin et al., 2002). The primary structure of the *Aedes Relish* gene exhibited three unique features compared with *Drosophila Relish*: (1) the mosquito *Relish* gene encodes three alternatively spliced transcripts that give rise to different proteins, (2) a 'Death Domain' is present at the extreme C terminus, and (3) a short His/Gln-rich stretch followed by a long S-rich region is present at the putative N-terminal transactivation domain. *Aedes* Relish transcripts were induced by bacterial injection, and their product bound to κB motifs located within the promoters of insect AMP genes (Shin et al., 2002).

We generated genetically immune-deficient transgenic mosquitoes by overexpression of a dominantly negative construct of *Aedes* Relish (Shin et al., 2003). Relish-mediated immune deficiency (RMID) phenotype was created by transforming an *Ae. aegypti* with the ΔRel driven by the *Vg* promoter using the pBac[3xP3-EGFP afm] vector (Fig. 3A). A stable, transformed strain had a single copy of the *Vg-ΔRel* transgene, the expression of which was highly activated by blood feeding. These transgenic mosquitoes were extremely susceptible to infection by Gram-negative bacteria (Fig. 3B) (Shin et al., 2003). In order to establish whether or not RMID was dominant-negative, we crossed the wild-type (UGAL strain) mosquitoes with the RMID transgenic mosquitoes. The heterozygous RMID/UGAL mosquitoes exhibited the same susceptibility to the *E. cloacae* or *E. coli* infection as the RMID transgenic mosquitoes (Fig. 4A). These experiments clearly showed that the RMID phenotype originated from the *Vg-ΔRel* transgene and that it was genetically dominant. A hypothetical model of how dominant-negative ΔRel is expressed is presented in Fig. 5.

Reverse genetics and transgenesis in future studies of mosquito innate systemic immunity

The development of a reverse genetic approach as described above has opened a door for further exploration of the mosquito immune system. Using transgenesis has many advantages, the principal one being the establishment of genetically stable, transgenic mosquito lines that can be crossed with other transgenic and wild-type mosquitoes to produce new genetic variations.

With *Drosophila*, the genetic approach was extensively explored in order to identify the functional relevance of

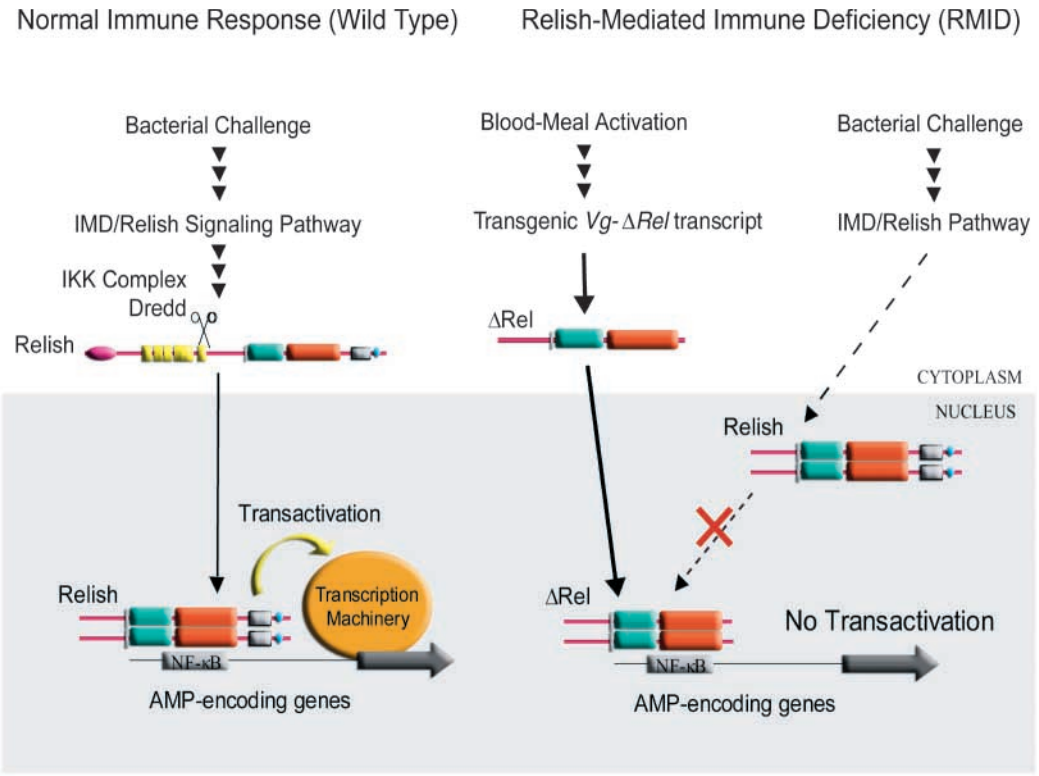

Fig. 5. A schematic representation of dominant-negative action of the *Vg-ΔRel* transgene. During normal immune response (left), bacterial challenge leads to activation of IMD/Relish signaling pathway (details are omitted) that results in proteolytic cleavage of ankyrin inhibitory domain from Relish and its translocation to the nucleus. In the nucleus, Relish binds NF-κB binding sites in anti-microbial peptide (AMP)-encoding genes and activates their expression. In RIMD transgenic mosquitoes (right), the blood meal activates overexpression of ΔRel that lacks the transactivation domain but has the DNA-binding domain and nuclear translocation signal. Upon nuclear translocation, ΔRel binds NF-κB binding sites in AMP-encoding genes. In these mosquitoes challenged with bacteria, ΔRel prevents normal Relish from binding to NF-κB binding sites in AMP-encoding genes and their subsequent activation, causing immune deficiency. In the *Vg-ΔRel*/wild-type hybrid mosquitoes, the *Vg-ΔRel* gene still results in overexpression of ΔRel, preventing transactivation of AMP-encoding genes and thus acting as a dominant-negative gene.

individual AMPs for both IMD/Relish- and Toll-mediated innate immunity (Tzou et al., 2002). The development of reverse genetic tools for *Ae. aegypti* has permitted us to use a similar method for studying mosquito immunity. We generated transgenic hybrids with the *Vg-DefA* and *Vg-ΔRel* transgenes (Fig. 3C). In these hybrid transgenic mosquitoes, *Vg*-mediated activation of Defensin A, which is independent of Relish-mediated activation, restored the resistance to *E. cloacae* (Shin et al., 2003). Thus, the RMID phenotype of the transgenic mosquitoes could be directly linked to the lack of AMP expression. Another hybrid cross that has been established carries two transgenes: *Vg-DefA* and *Vg-CecA*. Both these AMPs are highly expressed in these hybrid transgenic mosquitoes, which exhibited an enhanced resistance to certain microorganisms (A. Ahmed, S. W. Shin, I. Lobkov, V. Kokoza and A. S. Raikhel, manuscript in preparation).

Surprisingly, the published genome sequences of *An. gambiae* do not carry a Dif ortholog. Dif is important in the activation of many *Drosophila* immune genes and its apparent absence in mosquitoes raises a

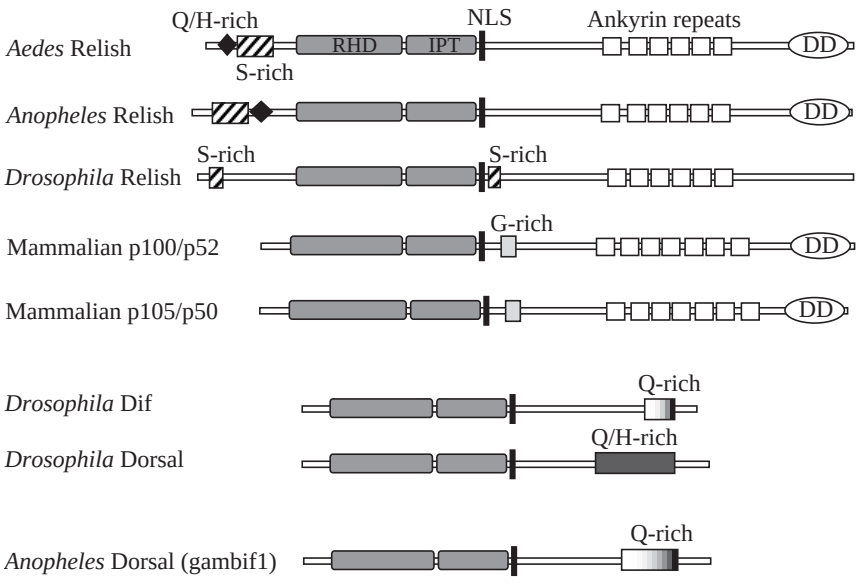

Fig. 6. Domain structure comparison of mosquito and *Drosophila* Rel-related factors with mammalian p105 and p100. Q/H-rich, glutamine- and histidine-rich region; Q-rich, glutamine-rich region; S-rich, serine-rich region; G-rich, glycine-rich region; RHD, Rel-homology domain 1; IPT, Ig-like plexin transcription factor domains (Rel-homology domain 2); NLS, Nuclear localization signal; DD, death domain.

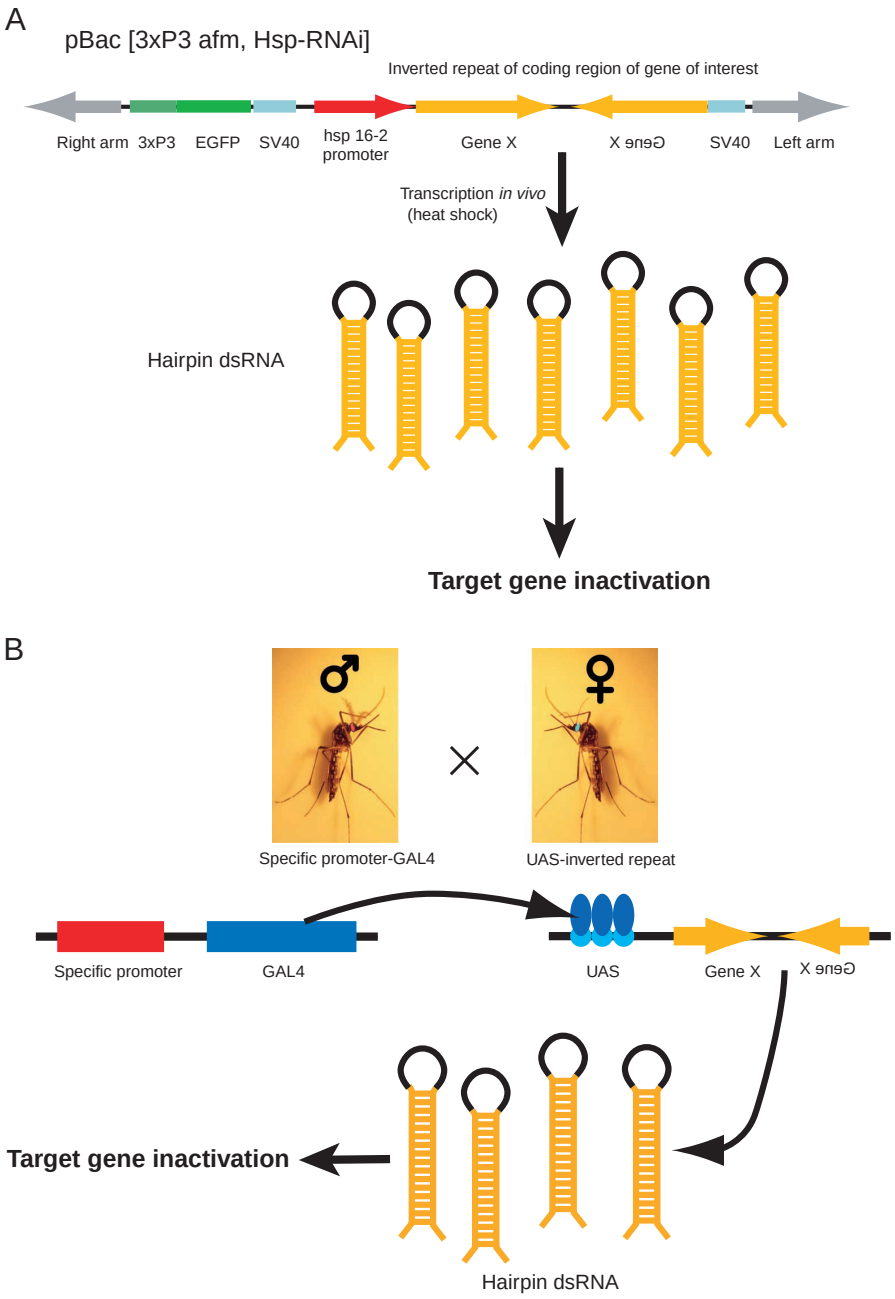

Fig. 7. Strategies for generating heritable and inducible RNAi. (A) A strategy for generating transgenic mosquitoes utilizing a strong inducible promoter. The construct containing a strong inducible promoter fused with an inverted repeat of a target gene is placed in the *piggyBac* transposable vector pBac[3xP3-EGFP afm], and transgenic mosquitoes are generated using an established transformation technique. Upon activation of these transgenic mosquitoes, transcripts are generated which fold back to form double-stranded RNA in all cells or tissues where the inducible promoter is expressed. (B) An alternative strategy utilizing a binary GAL4/UAS system. The inverted repeat is placed downstream of the upstream activating sequence (UAS) promoter, and a transgenic strain utilizing the pBac[3xP3-EGFP afm] vector is generated. A tissue-/stage-specific inducible promoter controlling GAL4 is transformed using the pBac[3xP3-DsRed afm] vector. Crossing two strains with different color selectable makers allows easy selection of hybrids in the F_1 generation. The F_1 hybrids contain both GAL4 and UAS genes. Tissue-specific activation of the GAL4-UAS transgene results in production of hairpin-loop RNA and consequently of RNAi.

question about the difference between their immune responses and that of *Drosophila*. The translated primary sequences of Relish from *Ae. aegypti* and *An. gambiae* (str. PEST, agCP3820, and agCP3732) show more complicated structures when compared with *Drosophila* Relish or mammalian NF-κB/I-κB compound proteins, p100 and p105 (Fig. 6). Due to the absence of Dif in the mosquito immune repertoire, complex alternative splicing and the additional N-terminal structure of mosquito Relish may in part function as substitutes for Dif. In order to initiate studies of Toll-mediated innate immunity in *Ae. aegypti*, we cloned the gene homologous to *Drosophila dorsal* (S. W. Shin, unpublished data). *Aedes dorsal* has two isoforms that show high structural similarity to *Drosophila* Dl and Dl-B splice variants (Gross et al., 1999).

Establishing inheritable and inducible expression of dsRNA using transgenesis and specific promoters is a challenging but important goal in the development of reverse genetics of mosquitoes. Model organisms, such as *C. elegans* and *Drosophila*, provide a roadmap for how this can be achieved (Kennerdell and Carthew, 2000; Lam and Thummel, 2000; Tavernarakis et al., 2000; Lee and Carthew, 2003). Double-stranded RNA can be expressed as an extended hairpin-loop RNA transcribed from a DNA construct encoding inverted dyad symmetry (IR) of the target gene. In order to stably transform this IR construct, it is inserted into a transformation vector under the control of a tissue- and stage-specific promoter (Fig. 7A). Transgenic mosquitoes can then be generated by a well-established methodology (Kokoza et al., 2001a,b). Advantages of this approach are: (1) an established stably transformed strain can be maintained indefinitely in a homozygous state; (2) large numbers of uniform knockout mosquitoes can be used in genetic and genomic analyses; (3) RNAi-mediated gene suppression is driven by a promoter in a tissue and stage-specific manner. The availability of promoters that strictly control IR expression in a tissue- and stage-specific manner is crucial for extending this technique to the study of mosquito immunity. For example, it is important to identify

promoters acting in the fat body after the cessation of vitellogenesis in order to drive immune factor-specific dsRNA expression beyond temporal activity of the *Vg* gene. Universal inducible promoters such as *heat shock* (*hsp*) can be used only for systemic temporal activation of the IR expression. In addition, *hsp* promoters are 'leaky', and in the case of their direct utilization (as described above) they could create undesirable effects during organism development.

To circumvent this problem, another transgenic RNAi technique has been developed in *Drosophila* in which dsRNA-producing transgenes are expressed through a binary GAL4/UAS system (Kennerdell and Carthew, 2000; Lam and Thummel, 2000; Lee and Carthew, 2003). In this binary system, the target gene linked to UAS remains silent. The activator, *hsp* or a selected tissue/stage-specific promoter, is coupled to GAL4 without a target gene to activate. Therefore, two independent parental transgenic strains can be maintained without possible adverse effect of a target gene expression. After crossing the two parental stains, the target gene is activated *via* GAL4-UAS system in the progeny and the phenotypic expression of its expression can be studied (Brand and Perrimon, 1993). Efficiency of RNAi action has additionally been improved by insertion of a short intron sequence between inverted repeats of RNAi construct (Kennerdell and Carthew, 2000; Lee and Carthew, 2003). Recently, we have demonstrated that addition of a short intron from an unrelated *A. aegypti* gene to the anti-GATA factor RNAi construct improves its performance in *Ae. aedypti* (G. Attardo, S. Higgs, K. A. Klingler, D. L. Vanlandingham and A. S. Raikhel, submitted). The development of an heritable and inducible dsRNA-mediated system based on the binary system in mosquitoes will offer additional advantages in dissecting immune pathways and mosquito-pathogen interactions. The proposed strategy for generating of transgenic RNAi based on the binary GAL4/UAS system is presented in Fig. 7B.

Mosquito innate immunity clearly has an important role in vector–parasite interactions, particularly in the immune surveillance of the malaria parasite. The limitation of parasite replication may depend upon the anti-malarial activity of immune effector molecules. Two immunological activation pathways in *Drosophila*, the IMD/Relish pathway and the Toll/Dif pathway, have been shown to activate numerous immune genes, and the activation and regulation of the melanization process seems to be closely linked to these two pathways (Ligoxygakis et al., 2002). The development of transgenic immune-deficient mosquitoes to generate knock-out mutants in the mosquito immunological activation pathways, and consequent studies of immune-deficient vector–parasite interactions, will provide insight into the development of alternative disease-control strategies to block parasite transmission by mosquitoes.

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