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In vitro Study of *Herves* Transposable Element of *Anopheles gambiae* and Use of RNA Interference (RNAi) in *Culex quinquefasciatus*

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Amandeep Singh Kahlon

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Dissertation Committee:
Dr. Peter W Atkinson, Committee Chairperson
Dr. Sarjeet Gill
Dr. Shou-Wei Ding

The Dissertation of Amandeep Singh Kahlon is approved:

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Committee Chairperson

University of California, Riverside

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DEDICATION

To my family and friends

ABSTRACT OF THE DISSERTATION

In vitro Study of *Herves* Transposable Element of *Anopheles gambiae* and Use of RNA Interference (RNAi) in *Culex quinquefasciatus*

by

Amandeep Singh Kahlon

Doctor of Philosophy, Graduate Program in Cell, Molecular and Developmental Biology
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Dr. Peter W. Atkinson, Chairperson

Transposable elements (TEs) and RNA interference (RNAi) are excellent genetic tools that could help control the incidence and spread of mosquito-borne diseases such as malaria, dengue, yellow fever, etc. This study aimed to 1) understand the RNAi mechanism in *Culex quinquefasciatus*, which will be used to study genes involved in pathogen transmission and will help reveal role of RNAi in antiviral immunity in this species; and 2) characterize the DNA sequences that regulate *Herves* transposase binding, which will help us understand its pre- and post-integration behavior within the host.

By using the *white* eye-pigmentation gene as a marker for RNAi function we demonstrated that introducing dsRNA into embryos of *Cx. quinquefasciatus* induces a specific functional RNAi response, which silenced the *white* gene, consequently allowing a white-eye phenotype to be produced in the hatched larvae and adults. Sequence-specific knockdown of key RNAi components was achieved by introducing the homologous dsRNA into embryos of *Cx. quinquefasciatus*. We found *ago2*-mediated slicing to be more critical than *dcr2*-mediated dicing for the functional RNAi response in *Cx. quinquefasciatus.* Our phylogenetic analysis confirmed the previously reported results

that RNAi components for small RNA biogenesis are present in *Cx. quinquefasciatus*. In addition, we found important differences in the number and the expression of *ago* genes, the predicted domain architecture of Dcr, and the RNAi response between *Cx. quinquefasciatus* and the model organism *D. melanogaster*.

Transposition of Class II TEs is regulated by both *cis*-acting sequences and *trans*-acting host factors. In this study, we used purified *Herves* transposase to characterize the specific DNA-binding sites of the *Herves* transposase. The purified active *Herves* transposase showed site-specific binding to the subterminal and terminal sequences of the L- and R- ends of the element, respectively. Furthermore, the transposase bound strongly with the R-TIR but failed to bind to the L-TIR. We identified an 8bp sequence repeat as the transposase binding motif that is conserved on both the L- and R-end sequences and is critical and sufficient for *Herves* transposase binding.

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General Introduction

1.1 Transposable Elements

Transposable Elements (TEs) are discrete DNA segments that can move from one genomic location to another within a cell. TEs were first discovered by Barbara McClintock in the 1940's (McClintock, 1950) for which she received Noble prize. Although TEs were originally considered "junk DNA", recent studies have indicated their important role in genome evolution and maintenance in various organisms (Dimitri and Junakovic, 1999). They are present in almost all organisms with few exceptions (Hua-Van *et al.*, 2005).

TEs have co-evolved with their respective host genomes and have provided important functions to the host. For example, the non-LTR retrotransposons (*HetA* and *TART*) maintain *Drosophila* telomeres (Biessmann *et al.*, 1990; Danilevskaya *et al.*, 1999; George and Pardue, 2003). Similarly, the V(D)J recombination mechanism in the vertebrate immune system is believed to be adapted from TEs (Zhou *et al.*, 2004). In plants, the DAYSLEEPER (transposase-like protein) is involved in controlling plant developmental genes (Bundock and Hooykaas, 2005).

Depending upon the mechanism of transposition, TEs are divided into two classes: Class I and II TEs. The Class I TEs are mobilized via a RNA intermediate stage, which is then reverse transcribed and inserted into new locations within the genome (Boeke *et al.*, 1985; Craig, 1995; Capy, 2005). The Class II TEs are mobilized via classic 'cut and paste' mechanism, whereby the transposase binds to the inverted terminal repeats and performs both the excision and integration into the target site (Craig, 1995; Craig, 2002).

1.1.1 Class I elements

Class I elements, also called retrotransposons or retroelements, have a significant presence in a wide array of organisms. About 40% of the human genome consists of retroelements (Goodier and Kazazian, 2008). The Class I TEs are further divided into two groups: long terminal repeats (called LTRs) and non -LTRs. The LTRs contains an open reading frame (ORF) that encodes the *gag* and *pol* proteins that are required for transposition (Kazazian, 2004). LTRs include the *Ty* element from *Sacchromyces cervisiae* as well as the *copia* and *gypsy* elements from *D. melanogaster* (Finnegan, 1997).

The Non-LTR retrotransposons consists of autonomous and non-autonomous elements. The autonomous retroelements contain an ORF that encodes various factors sufficient for their mobility, whereas non-autonomous elements lack essential factors and usually depend upon autonomous retroelements for their mobility. The long interspersed nuclear elements (LINEs or L1s) are the autonomous elements that can mobilize non-autonomous elements such as short interspersed elements (SINEs).

1.1.2 Class II TEs

The Class II DNA elements are present in a wide range of prokaryotic and eukaryotic organisms. The DNA elements usually contain the characteristic Terminal Inverted Repeat (TIR) sequences, which flank an ORF (Finnegan, 1992). The ORF encodes the transposase protein, which is responsible for 'cut and paste' transposition of these elements (Craig, 1995). The Class II TEs are classified into various super families, based on the sequence homology, structure of TIRs and target site duplications. Various

super families of DNA elements include *P*, *hAT*, *piggyBac* and *Tc1/mariner* (Finnegan, 1992; Craig, 2002).

1.2 Transposition of Class II TEs

1.2.1 Mechanism of transposition

The underlying transposition reaction includes hydrolysis and trans esterification. During transposition, water acts as a nucleophile thus hydrolyzing the phosphodiester bond of the transposon DNA molecule next to the TIRs (Craig, 1995; Curcio and Derbyshire, 2003). The energy released during the breakage of the bond is stored in the form of a hairpin formed between a 3'-OH and a 5'-P, either at the end of element or the adjacent flanking DNA. In the case of *Tn5/Tn10*, the hairpin formation is on the transposon DNA whereas in case of *hAT* elements such as *Hermes*, the hairpin is formed on the flanking DNA (Craig, 1995; Curcio and Derbyshire, 2003; Zhou *et al.*, 2004). The hairpin formation in *Hermes* is similar to V(D)J recombination, indicating a possible link between the two (Zhou *et al.*, 2004). The exposed 3' –OH subsequently attacks the phosphodiester bond of the target DNA molecule and produces a nick. The hairpin is then resolved, resulting in the element being inserted into the target site. The TE super families make characteristic target site duplications (TSD) upon integrating into a new target region. For example, *hAT* elements create 8bp TSD, whereas the *Tc1/mariner* family makes 2bp TSD and always inserts at TA dinucleotides. Similarly, the *piggyBac* element creates 4bp TSDs and always inserts into the sequence TTAA (Mitra *et al.*, 2008).

1.2.2 *Cis* acting DNA sequences involved in transposition

Tc1/mariner super family

The members of *Tc1/mariner* super family have a remarkably wide host range extending from prokaryotes to eukaryotes, and even to higher vertebrates such as humans (Robertson, 1995; Robertson and Lampe, 1995). The family is named after the well-studied members *Tc1*, from the nematode *C. elegans*, and the *mariner* element from *D. mauritiana*. This super family can be sub-divided into *Tc* like elements (TLEs) and *mariner* like elements (MLEs). A MLE (MITE) has been implicated as a recombination host spot for human disease (Reiter *et al.*, 1996). All members of the *Tc1/mariner* super family share a signature DDE motif, except the *mariner* elements, which have a DDD motif (Hartl *et al.*, 1997).

Tc1/*Tc3* elements

The *Tc1* and *Tc3* elements were isolated from *C. elegans* and are capable of transposition in various systems, including human cell lines (Hartl *et al.*, 1997). These elements contain a single gene that encodes for a transposase protein. The transposase has been established as the only *trans* requirement for successful in vitro transposition (Hartl *et al.*, 1997). The *Tc1* and *Tc3* elements have 54bp and 426bp as their TIR sequences (Fischer *et al.*, 1999). Although *Tc1* and *Tc3* are related elements, both have different *cis* regulatory sequences. The *Tc1* transposase has two binding sites, 7-13bp and 12-26bp, within the TIRs (Vos and Plasterk, 1994). However, the *Tc3* transposase has additional internal binding sites within its TIRs. The *Tc3* transposase (*Tc3*A) binds specifically to bases 9-28 and also to bases 182-203 within the TIRs (Colloms *et al.*, 1994). However, the internal binding sites do not play a role in *Tc3* transposition. It has also been inferred

that the external and internal binding sites of *Tc3* are for the specific and non-specific binding of transposase, respectively (Colloms *et al.*, 1994). This could be the reason that internal binding sites are not imperative for transposition (Fischer *et al.*, 1999).

MosI/mariner

The *MosI* element from *D. mauritiana* is the defining and relatively well-studied element of MLEs subfamily of the *Tc1/mariner* super family. It is similar to other members of its family in that it is 1.3kb long, contains a single transposase-encoding gene and is flanked by 28bp TIRs (Jacobson *et al.*, 1986). It has a wide host range and has been used to transform various organisms such as; *D. melanogaster*, *A. aegypti*, zebra fish, chicken etc. (Coates *et al.*, 1998; Fadool *et al.*, 1998; Sherman *et al.*, 1998; Garza *et al.*, 2001). The *Mos1/mariner* transposase contains a DDD motif, instead of the signature DDE motif in the *Tc1/mariner* super family. The E to D substitution seems to be important to the catalytic activity of the transposase (Lohe *et al.*, 1997). In addition, as opposed to some of the other members of the super family such as *Himar1* and *Sleeping beauty*, *Mos1* does not seem to be regulated by 'overproduction inhibition' (Geurts *et al.*, 2003; Zayed *et al.*, 2004). *Mos1/mariner* elements transpose preferably into TA dinucleotide target sites (Geurts *et al.*, 2003). Interestingly, substituting Mg^{2+} with Mn^{2+} alters the site preference and the transposition frequency (Tosi and Beverley, 2000).

There are conflicting data regarding the *cis* regulatory sequences of *Mos I* elements. Multiple *cis* regulatory sequences have been shown to play an important role in the mobilization of *mariner* elements. Various deletions of internal sequences (referred to as 'critical' sequences) greatly reduce the ability of *Mos I* elements to excise and

transpose. It is not clear if these internal sequences are involved in transposase binding or are important for maintaining essential spacing between binding sites (Lohe and Hartl, 2002). However, in a study by (Pledger *et al.*, 2004), deletion of 'critical' sequences did not affect the transposition efficiency in *E.coli*. Furthermore, recombinant *MosI* with two 3' (right end) TIRs is transpositionally more active than the native *MosI* with the normal 5' (left end) and 3' TIRs. This can be attributed to the higher binding affinity for the 3' end sequences, including 3' TIR sequences.

hAT Super family

Ac Element

The TE *Ac* from *Zea mays* L. was one of the first TEs to be identified and isolated. It is also one of the most extensively studied and founding members of the *hAT* super family of Class II TEs. The *Ac* element is 4.5kb and is composed of characteristic 11bp TIRs and a 3.5kb ORF, which encodes for a transposase protein (Kunze and Starlinger, 1989a). The *cis* regulatory sequences of the *Ac* element have been well characterized. The transposase binds to the subterminal sequences yet fails to bind to the TIRs of the *Ac* element. Studies have identified a transposase "binding box" that extends from 102-157bp and 40-116bp on the 5'*Ac* (left end) and 3'*Ac* (right end), respectively (Kunze and Starlinger, 1989a). Furthermore, a region of six AAACGG motifs or its derivatives, which are repeated in tandem, have been shown to be important for binding. These motifs are considered only a part of the transposase binding motif, as the natural transposase binding site seems to be more complex (Coupland *et al.*, 1989; Kunze and Starlinger, 1989a). Studies have shown that the sequences outside the "binding box" are

also important for transposition of *Ac* elements. The deletion of the terminal 4bp of 3' *Ac* TIRs, a sequence to which transposase does not bind, completely abolishes transposition in tobacco derived protoplasts (Coupland *et al.*, 1989). Furthermore, two independent studies have demonstrated the binding of host nuclear proteins to GGTAAA motif or its derivatives. These nuclear proteins bind cooperatively to multiple sequence motifs, present on the 5'*Ac* and 3'*Ac* subterminal sequences (Becker and Kunze, 1996; Levy *et al.*, 1996).

Tag 1

The *Tag1* transposon from *Arabidopsis thaliana* is another characteristic member of the *hAT* super family (Warren *et al.*, 1994; Frank *et al.*, 1997). It is a developmentally regulated autonomous element (Tsay *et al.*, 1993; Frank *et al.*, 1997). It is about 3.3 kb long and gives rise to one major and several minor transcripts (Tsay *et al.*, 1993; Liu and Crawford, 1998). The major transcript is 2.3kb long and encodes for the transposase (Liu and Crawford, 1998). The *Tag1* element contains several repeat sequences at the 5' and 3' ends, including the 22 bp TIR sequence. Usually the repeats (except TIRs) are present within the subterminal sequence. Unlike other transposons, the 5' and 3' subterminal repeats are different from each other. The 5' subterminal repeat sequences contain a AAACCC motif, whereas, the 3' subterminal sequences contains different sets of TTATT, TATATA and TGACCC motifs (Liu and Crawford, 1998). The DNA binding domain, located at the N terminus, binds specifically to the 5' terminal 98bp sequence, which contains four AAACCC repeats and a terminal 109bp segment at the 3' end that

contains four TGACCC repeats. However, it does not bind to the TTATT and TATATA repeats present at the 3' subterminal sequence.

Hermes

The *Hermes* TE from *Musca domestica*, was discovered due to its ability to cross-mobilize the Drosophila TE *Hobo* TE (Atkinson *et al.*, 1993). *Hermes* is about 2750bp long and has single ORF, encoding a transposase, which is flanked by 17bp TIR sequences (Warren *et al.*, 1994). *Hermes* has been used to transform various insect species such as *D. melanogaster*, *A. aegypti*, and *C. quinquefasciatus* etc. (O'Brochta and Atkinson, 1996; Jasinskiene *et al.*, 1998; O'Brochta *et al.*, 2000; Allen *et al.*, 2001a; Michel *et al.*, 2001).

The mechanism of *Hermes* transposition is similar to RAG1/RAG2 mediated V(D)J recombination of vertebrates and includes transposase-mediated double stranded DNA cleavage at the element ends. This is followed by the hairpin formation and resolution on the flanking donor DNA resulting in insertion into target DNA (Zhou *et al.*, 2004).

Nuclear extracts from *Drosophila* S2 cells expressing *Hermes* transposase bind specifically to the terminal 1-100bp region. More specifically, the *Hermes* transposase binds to 1-30bp and 47-76bp on the left and right ends of the *Hermes* element, respectively (Laver T. *et al*., 2010). Within 1-30bp of the *Hermes* left end, multiple sequences are required for transposase binding. The 11bp binding motif, CAAGTGGCTTA, has been proposed as binding motif on the *Hermes* left end (Laver T.

et al., 2010). A partial sequence variant, GTGGG, of the 11bp binding motif has been indicated to be the binding motif on the right end.

Herves

Herves is an active class II transposable element and belongs to the *hAT* super family (Arensburger *et al.*, 2005). It was originally identified bioinformatically from *An. gambiae*. It has 11bp imperfect TIRs. The *Herves* transposase is the only protein encoded by and required for transposition (Arensburger *et al.*, 2005). The ORF encoding the transposase is flanked by left (*Herves*-L) and right (*Herves*-R) end sequences. The *Herves*-L is unusually long (1478bp) as compared to the *Herves*-R end (421bp), and has three 100bp tandem repeats starting at the 146 nt position (Arensburger *et al.*, 2005). *Herves* is transpositionally active and is capable of transforming *D. melanogaster* at rates comparable to other class II elements such as *P*, *hobo* and *Hermes* (Arensburger *et al.*, 2005).

Population dynamics studies of *Herves* within field populations of *An. gambiae* from Kenya indicate that *Herves* has been recently active (Subramanian *et al.*, 2007). These studies are based on the following facts 1) *Herves* occupied sites are unique and rarely fixed, 2) site occupancy levels are comparable to the reported active transposable elements in *Drosophila*, such as *P* elements, 3) A field isolated *Herves* element is active in *D. melanogaster* 4) The presence of intact *Herves* elements in field populations of *An. gambiae*. Despite the indications that *Herves* was introduced in *An. gambiae* in the distant past, it still has not reached copy-number equilibrium (Subramanian *et al.*, 2007).

Herves is being maintained in low copy numbers in *Anopheles sp*., as opposed to the high copy number of well-studied *P* element in *Drosophila* (O'Brochta *et al.*, 2006). Transposition of various TEs is often under the control of various host regulatory mechanisms. This often results in deletions, especially in the transposase encoding ORF, which accumulate over time and renders the elements inactive. The fact that despite its extended presence in *An. gambiae*, intact and active forms of *Herves* are maintained indicates the presence of complex interactions and possible evasion of host defense systems.

piggyBac

The *piggyBac* element was isolated from the *Trichoplusia ni* cell line as a result of spontaneous gene disruption in baculovirus plaque morphology mutants (Cary *et al.*, 1989). It is about 2.5 kb long and includes 13bp TIRs, 19bp internal repeats (IRs) and a 2.1kb ORF that encodes for the transposase. The end sequences are asymmetric with 3bp and 31bp spacer sequences between the TR and IR at the 5' and 3' ends, respectively (Cary *et al.*, 1989). *piggyBac* inserts into TTAA sites within the genome and creates target site duplications of the same sequence after insertion into a new genomic location (Cary *et al.*, 1989; Wang and Fraser, 1993; Fraser *et al.*, 1996). The TIR, IR and spacer sequences are important for transposase recognition and cleavage and thus constitute the *cis* regulatory sequences (Li *et al.*, 2001). Studies have indicated that the transposase interacts with the 5' and 3' terminal regions of *piggyBac* as well as the flanking host DNA sequences in order to mediate excision and subsequent insertion via a transposition complex (Elick *et al.*, 1997; Li *et al.*, 2001). Furthermore, the *cis* requirement for

piggyBac differs between plasmid transposition assays and genome transformation. While internal domain (ID) sequences TIRs and IRs are not required for transposition assays, they are indispensable for genetic transformation of *D. melanogaster* (Li *et al.*, 2005).

***P* Element**

P elements were originally discovered in *D. melanogaster* due to the phenomenon of 'Hybrid Dysgenesis' (Kidwell *et al.*, 1977; Kidwell, 1981; Rio, 2002). Hybrid disgenesis includes elevated rates of chromosomal abnormalities and rearrangements, sterility, mutation etc. This phenomenon is only observed in the progeny when males carrying *P* elements (P cytotype) are crossed with females lacking the *P* elements (M cytotype). However, the progeny of a reciprocal cross are normal (without disgenesis) (Rio, 2002; Castro and Carareto, 2004).

The 2.9kb full length *P* element includes 31bp TIRs and 11bp internal repeat sequences, which flank four ORFs encoding for a 87KDa functional transposase. Sequences internal to the TIRs function as transposition enhancers and transposase binding sites (Mullins *et al.*, 1989b).

1.3 RNA Interference (RNAi)

Studies in the 1970s indicated the potential role of foreign RNA molecules in interfering with the expression of homologous genes in animal viruses (Kawade and Ujihara, 1969; Sreevalsan, 1970; Yamamoto *et al.*, 1970). A similar phenomenon of co-suppression was observed in transgenic plants between transgenes and homologous plant genes (vander Krol *et al.*, 1990; Kennerdell and Carthew, 1998). Later on, it was

discovered in *C. elagans* that this process is specifically triggered by dsRNA molecules, and this phenomenon is now termed RNAi or gene silencing (Fire *et al.*, 1998; Montgomery *et al.*, 1998). The phenomenon of RNA silencing is not restricted to just plant and animal viruses, but has been found in a range of organisms from *Neurospora*, to much more complex organisms such as mammals (Cogoni *et al.*, 1996; Fire *et al.*, 1998; Kennerdell and Carthew, 1998; Billy *et al.*, 2001; Elbashir *et al.*, 2001a).

1.4 Mechanism of RNAi

1.4.1 The siRNA pathway

The siRNA pathway is well studied in *D. melanogaster*. It is triggered by long dsRNA molecules, which are subsequently recognized by a RNase III enzyme called *Dicer 2* (*Dcr2*) and cleaved into 21bp fragments known as siRNA (Hannon, 2002). Various dsRNA triggers include viral replicative intermediates, including convergent transcription of DNA viruses, exogenous dsRNA introduction into the cell or endogenous dsRNA. The *Dcr2* then binds to a dsRNA binding protein, *R2D2*, and loads double stranded siRNA molecules into a multi protein complex called the RNA induced silencing complex (RISC) (Liu *et al.*, 2003b). Next, one of the strands of siRNA is removed, which leads to activation of the RISC complex. The activated RISC contains a single strand of the siRNA known as ‘guide strand” and is bound to an endonuclease *Argonaute-2* (*Ago2*) protein which can recognize and catalyze homology- dependent cleavage of target messenger RNAs. It was previously believed that only exogenously introduced long dsRNA elicits the siRNA response. However, recent studies have indicated the presence of an endogenous siRNA pathway. This pathway depends upon a

dsRNA binding protein called *Loquacious* (*Loqs*) instead of *R2D2* (Czech *et al.*, 2008; Ghildiyal *et al.*, 2008; Kawamura *et al.*, 2008; Okamura *et al.*, 2008; Okamura and Lai, 2008).

1.4.2 The miRNA pathway

MiRNAs are 20-23nt long and are the most abundant type of small RNAs in plants and animals. They are transcribed from endogenous primary (pri-miRNA) and pre-miRNA precursor genes (Lee *et al.*, 2003; Bartel, 2004; Aravin and Tuschl, 2005; Borchert, 2006). The pri-miRNA transcripts consist of an imperfect intra-molecular stem-loop structure, which is produced in the nucleus by RNA polymerase (II or III). The pri-miRNAs are processed into about 70nt long pre-miRNA by the RNase III enzyme called *Drosha* (Lee *et al.*, 2003). These pre-miRNA dsRNA molecules are then cleaved into ~22nt miRNA in the cytoplasm by another RNase III enzyme called *Dicer1*(*Dcr1*) and its binding partner, *Loqs*. The 'guide strand' is loaded onto the miRNA-induced silencing complex (miRISC) and helps the miRISC complex to recognize and silence genes primarily via translational repression (Schwarz and Zamore, 2002; Rana, 2007). The miRNA pathway plays an important role in the development of an organism by controlling endogenous gene regulation (Bartel, 2004, 2009).

1.4.3 The piRNA pathway

The Piwi interacting small RNAs (piRNAs) are 24-30nt long small RNAs that interact with the Piwi proteins, which belong to *Argonaute* family of proteins. piRNAs have been specifically shown to prevent the spread of transposons in the germ line cells (Brennecke *et al.*, 2007; Nishida *et al.*, 2007). Unlike other small RNAs (siRNA and

miRNAs), piRNAs are generated through a *Dicer* independent pathway utilizing piRNA gene clusters present in the genome. piRNAs are currently believed to be produced via a "ping-pong" amplification loop, with the help of germline-specific Argonautes, Aubergine, Piwi and *Ago3* proteins (Brennecke *et al.*, 2007; Nishida *et al.*, 2007).

1.5 RNAi based anti-viral immunity in insects

D. melanogaster has served as an excellent model system to study the role of RNAi in antiviral innate immunity (Hoffmann, 2003). Several studies have shown the role of Toll and immune deficiency (Imd) pathways as defenses against invading viruses. However, global expression analysis has revealed up-regulation of additional genes not involved in these pathways (Hoffmann, 2003; Dostert *et al.*, 2005). The same study indicates the role of the Janus kinase-signal transducer and activator of transcription (Jak-STAT) pathway in antiviral immune response. However, the Jak-STAT pathway is required but not sufficient for antiviral immunity in *Drosophila* (Dostert *et al.*, 2005).

RNAi-based antiviral immunity has been described and well studied in *Drosophila* (Li *et al.*, 2002; Galiana-Arnoux *et al.*, 2006; van Rij *et al.*, 2006; Wang *et al.*, 2006). Recent studies have shown the importance of *Dcr2*, *R2D2* and *Ago2* dependent siRNA pathways in mediating antiviral defense in *Drosophila*. *Dcr2* acts as a host sensor and mediates the first step towards antiviral defense by recognizing the viral dsRNAs. It provides host defense against various positive sense single stranded (ss) RNA viruses such as FHV, SINV, and WNV etc (Galiana-Arnoux *et al.*, 2006; Wang *et al.*, 2006; Chotkowski *et al.*, 2008). The FHV infected *Dcr2* mutant *Drosophila* flies have shorter life span and increased mortality, as compared to FHV infected wild type (wt)

flies (Galiana-Arnoux *et al.*, 2006; Wang *et al.*, 2006). These studies have shown that the FHV is able to replicate successfully in the *Dcr2* mutant flies, as is evident from increased expression of FHV RNAs. A similar increases in enhanced disease susceptibility was observed in the case of *R2D2* homozygous mutant flies infected with FHV (Wang *et al.*, 2006).

Virus-vector interactions are complex. Various insect-borne viruses have found ways to evade the insect RNAi-mediated antiviral response (Lu *et al.*, 2005; Sullivan and Ganem, 2005; van Rij *et al.*, 2006). For example, the FHV encodes for a *B2* protein that functions to suppress RNA silencing and is required for its replication inside the vector. The FHV *B2* deletion mutant only accumulates in *Ago2* or *Dcr2* mutant *Drosophila* embryos. In addition, *B2* blocks the *Dcr2*-mediated production of virus-derived small RNAs (viRNA). These results indicate that the *B2* protein acts as suppressor of *Dcr2*/*Ago2* mediated silencing (Li *et al.*, 2002; Li and Ding, 2005; Wang *et al.*, 2006). This indicates the role of *Ago2*/*Dcr2* in the complex virus-vector relationship. However, in contrast, antiviral immunity to DXV has shown to be *Dcr2* independent. This appears to be an exception, however, it might suggest a specificity of RNAi mediated antiviral response.

Studies have also suggested the involvement of miRNA or piRNA genes such as *piwi*, *aub*, *armitage*, *rm62* and *spn-E*, etc. in viral immunity in insects (Zambon *et al.*, 2006; Chotkowski *et al.*, 2008). *Spn-E*, *Ago2*, *Dcr2* and *piwi* proteins are involved in enhanced susceptibility of *Drosophila* against WNV (Chotkowski *et al.*, 2008). However, the mechanism of action of these genes is not known.

RNAi has increasingly been used as a powerful reverse genetics approach in insects (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999; Blandin *et al.*, 2002). Studies done in mosquito cell lines indicate the possibility of a functional RNAi mechanism in mosquitoes (Hoa *et al.*, 2003). In *An. gambiae* cell lines *Dcr2*, *Ago2* and *Ago3* proteins mediate the RNAi pathway. Cell lines pretreated with dsRNA targeted to *Dcr2*, *Ago2* or *Ago3* were equally successful in the rescue of luciferase activity in a cell line treated with dsRNA to *luciferase* (Hoa *et al.*, 2003). Also, it has been shown that expressing a RNAi transgene consisting of inverted repeat sequences to the target gene leads to highly efficient gene silencing in *Anopheles* (Brown *et al.*, 2003b).

1.6 Use of RNAi in control of vector borne diseases

RNAi combined with transgenic technology has been proposed as a control strategy against vector borne diseases. To this end genetically engineered *A. aegypti* expressing an antisense sequence to dengue type 2 virus (DENV2) was resistant to DENV2 infection and replication (Franz *et al.*, 2006a). Furthermore, using tissue-specific promoter sequences to drive virus-targeted RNAi transgenes can lead to significant reduction in virus transmission (Franz *et al.*, 2006a). Studies have further shown that mosquito cells (C6/36) expressing inverted repeat sequence homologous to DENV2 virus are resistant to DENV2 virus infection (Adelman *et al.*, 2002b). RNAi has been shown to mediate an antiviral response in adult *Anopheles* mosquitoes as silencing of the *Ago2* failed to mount a RNAi response against O'nyong-nyong virus (ONNV) (Keene *et al.*, 2004a). This indicates that knockdown of *Ago2* silences the RNAi pathway, otherwise triggered by ONNV. Similar studies were reported against Sindbis virus (SINV) in *Ae.*

aegypti. Transient knockdown of *Ago2* and *Dcr2* transiently increase SINV replication and failed to produce SINV-induced small RNAs (Campbell *et al.*, 2008c).

1.7 Research aims and objectives

Vector borne diseases are a huge burden on global health. Malaria and Dengue fever are among the deadliest of all vector borne diseases. Currently, human drug therapy and chemical control of vectors are the two main strategies for disease control. However, the success rate of these strategies is severely limited by drug resistance or insecticide resistance in the disease agent or vector, respectively. Despite the extensive use of traditional control strategies, the prevalence of many vector borne diseases is increasing. Hence, novel control strategies targeting disease vectors are being sought, including the use of RNAi and TEs.

RNAi is well studied in model organisms such as *D. melanogaster* and *C. elegans*. It is not only an excellent genetic tool but also an important part of antiviral insect immunity. RNAi can be used to combat vector borne diseases and to analyze the wealth of genetic information generated from various genome projects. The *Culex* genome has been sequenced, however its RNAi pathway has not been studied.

TEs have been used as gene vectors to find, isolate and analyze genes and to genetically modify insects for various insect control strategies. However, even successful and widely used transposon-based gene-vector systems are limited by low transposition frequency and unpredictable post-integration behavior. A detailed understanding of the specific regulatory sequences is important to understand the biology and behavior of individual TE.

The research presented here is aimed at 1) Characterizing *cis* regulatory sequences to which *Herves* transposase binds. This will allow us to understand the integration and post integration behavior of *Herves* transposon in its native mosquito *An. gambiae*. 2) Establishing dsRNA mediated RNAi in *C. quinquefasciatus*. This will allow us to use RNAi for functional genetics and to study RNAi as part of the innate immune response in *Culex*.

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Chapter 2:

Identification of the *Herves* transposase binding sites of the *Herves* transposon of *Anopheles gambiae*

2.1 Abstract

Determining the mechanisms by which transposable elements (TEs) move within a genome increases our understanding of how they can shape genome evolution. Class II TEs transpose via a "cut-and-paste" mechanism that is mediated by a transposase that binds to sites at or near the ends of the transposon. *Herves* is a class II TE, isolated from *Anopheles gambiae* Giles, is a member of the *hAT* superfamily and is active in field populations of *An. gambiae*. We identified the specific DNA-binding sites of the *Herves* transposase to determine those *cis*-acting sequences responsible for the successful activity of the transposase. Active *Herves* transposase was purified using an *E. coli* expression system and bound robustly and site-specifically to the sub-terminal and terminal sequences of the L- and R -ends of the element, respectively (15-23 bp and 72-83 bp on the R-end and 28-60 bp at the L-end). It also interacted with the R-TIR but failed to bind the L TIR. We identified a common sub-terminal DNA-binding motif (CG/AATTCAT) that is critical and sufficient for *Herves* transposase binding.

2.2 Introduction

Transposable elements (TEs) exist in nearly all organisms and impact genomic evolution and maintenance (Levis *et al.*, 1993; Dimitri and Junakovic, 1999; Hurst and Werren, 2001; Kidwell and Lisch, 2002; Jiang *et al.*, 2004; Hua-Van *et al.*, 2005). Further, TEs have been used for the delivery of foreign genes into insect disease vectors of medical and agricultural importance and in other biotechnological applications (Coates

et al., 1998; Jasinskiene *et al.*, 1998; Catteruccia *et al.*, 2000; O'Brochta *et al.*, 2000; Grossman *et al.*, 2001b; Michel *et al.*, 2001; Atkinson, 2002; Smith *et al.*, 2006).

An. gambiae is the principal vector of the malaria-causing parasite *Plasmodium* falciparum ands genetic tools, such as those afforded by TEs need to be developed. At present there are only four reports of successful genetic transformation of this mosquito, one using the P element, and three using the piggyBac elements (Miller *et al.*, 1987; Catteruccia *et al.*, 2000; Grossman *et al.*, 2001a; Ito *et al.*, 2002). Transformation remains a low frequency event (Catteruccia *et al.*, 2000; Grossman *et al.*, 2001a; Yoshida and Watanabe, 2006). Isolating active, well-adapted, endogenous elements from *An. gambiae* and understanding their biology is likely to improve genetic transformation in this species. The native active elements are likely to have adapted to overcome or evade the host response.

Herves is a class II TE that was isolated from *An. gambiae* (Arensburger *et al.*, 2005). It comprises a transposase-encoding ORF that is flanked by left (*Herves*-L) and right (*Herves*-R) end sequences (Fig. 1A). The *Herves*-L end is unusually long (1478 bp) compared with the *Herves*-R end (421 bp) and has 3 100-bp imperfect tandem repeats, starting at nt 146 (Fig. 1A). It has 11-bp imperfect terminal inverted repeats (TIRs) on the left (*5′*-TIR) and right (3'-TIR) ends (Fig. 1A). *Herves* is transpositionally active and can genetically transform *D. melanogaster* (Arensburger *et al.*, 2005). Population dynamics studies have observed its recent activity within field populations of *An. gambiae* from Kenya (Subramanian *et al.*, 2007).

TE transpositions often result in the deletion of transposase-encoding ORFs that accumulate, rendering the elements inactive (Engels *et al.*, 1990). The presence of intact forms of *Herves* and other *hAT* TEs, such as *Hermes* and *hobo,* indicate that *hAT* elements are less prone to internal deletions and deletions that arise more slowly (Galindo *et al.*, 1995; Subramanian *et al.*, 2007; Subramanian *et al.*, 2009).

Class II transposase typically binds to the TIRs and nearby internal sequences and mediate transposition to a new genomic location by the classical "cut-and-paste" mechanism (Craig, 2002). Other *cis*-acting elements, however, are also important for proper transposase binding and efficient transposition (Coupland *et al.*, 1989; Liu *et al.*, 2000; Liu *et al.*, 2001; Li *et al.*, 2005). Moreover, transposase-binding *cis* elements are unique to specifc TEs and often consist of repeat sequence motifs (Coupland *et al.*, 1989; Kunze and Starlinger, 1989a). Also, in many cases, native *cis* elements are not optimized for maximal transposition mobility; thus, new and improved gene vectors can be designed by altering these elements to increase or decrease transposase binding (Guynet *et al.*, 2009; Yang *et al.*, 2009).

Characterization of transposase binding sites and specific DNA-binding transposase residues is critical to our understanding of biology and post integration behavior of TEs. This study was aimed at characterizing the DNA sequences of *Herves* elements that are bound by transposase.

2.3 Results

2.3.1 Purification of *Herves* transposase

The *Herves* ORF was expressed in *E. coli,* under the control of the arabinose-inducible *araBAD* promoter, at 16°C (Invitrogen) (Zhou *et al.*, 2004). The transposase was purified after cell lysis through *His*-tag purification and dialysis. The identity of transposase was based on its predicted size (70 kDa) by polyacrylamide gel electrophoresis (Fig 1B).

2.3.2 Binding of transposase to the *Herves*-L end

To examine the binding of *Herves* transposase to the *Herves*-L end, we focused on the terminal 100 bp region. The radioactively labeled *Herves*-L 1-100 bp probe was incubated in the presence and absence of purified *Herves* transposase for EMSA. A molar excess (200-fold) of unlabeled homologous and nonhomologous DNA fragments was used as specific and nonspecific competition, respectively.

The transposase interacted robustly with the *Herves*-L 1-100 bp probe and formed three transposase-DNA complexes (Fig. 1C). A homologous competitor competed for the transposase, but the nonhomologous competitor did not affect binding (Fig. 1C), implicating a sequence-specific interaction between the transposase and *Herves*-L 1-100 bp.

To specify the transposase binding site(s) within the terminal 100 bp sequence, overlapping oligonucleotides (~30 bp) were competed with the *Herves*-L 1-100 bp probe for transposase binding. The DNA fragments *Herves*-L 12-48 bp and 28-60 bp competed with the probe in all 3 transposase-DNA complexes, whereas the *Herves*-L 1-30 bp, 48-75 bp, and 76-100 bp fragments had no effect (Fig. 2A). This finding suggests that the *Herves* transposase binds tightly and specifically within the L 12-60 bp region. The

overlapping *Herves*-L 1-30 bp and 48-75 bp fragments did not alter binding, indicating that a binding motif(s) lies in the *Herves*-L 28-48 bp region (Fig. 2A).

We also observed that the L 12-48 bp and 28-60 bp fragments competed partially with the 1-100 bp probe, whereas the homologous 1-100 bp fragment competed fully for transposase binding, implicating the existence of additional binding motifs that act cooperatively with the binding motif(s) in the 28-48 bp region (Fig. 2A).

To confirm these results and determine their binding affinities, each of the unlabeled 30-bp fragments was tested against the *Herves*-L 12-48 bp probe for binding to the transposase. Robust binding to the transposase was observed for this probe (Fig. 2B), which resulted in 2 transposase-DNA complexes. The unlabeled L 28-60 bp fragment competed for binding of the transposase to the level of specific competition (Fig. 2B). *Herves*-L 48-75 bp, 61-90 bp, and 76-100 bp competed partially with the probe, indicating weak transposase binding to these regions. These results suggest that *Herves*-L 12-48 bp and 28-60 bp have strong and equal binding affinities for *Herves* transposase, leading us to believe that the DNA binding motif lay within the *Herves*-L 28-48 bp region.

We performed DNaseI protection assay to confirm the EMSA results and specify the DNA region that was bound by pure tranposase. A terminal 1-100 bp fragment was selectively labeled at the 5' or 3' end. The labeled probes were incubated separately with pure transposase and subsequently with DNase I and analyzed on a denaturing polyacrylamide gel.

Figure 3 indicates the regions that were protected due to transposase binding and hypersensitive sites at the border of the protected regions. Hypersensitive sites often represent DNA regions that are more exposed to DNase I digestion due to structural changes that are caused by the bound transposase. The 5' and 3' end-labeled probes were protected at 25-73 bp and 30-58 bp, respectively (Fig. 3). Increasing amounts of transposase effected greater protection—most of the *Herves*-L 100 bp probe was protected (Fig. 3).

We failed to draw any conclusions on the binding of transposase to *Herves*-L TIRs from this assay, because the terminal regions were not resolved easily on the polyacrylamide gel. Overall, the DNase I protection assay results confirmed the EMSA findings, indicating sequence-specific binding of transposase to the *Herves*-L 28-48 bp region.

2.3.3 Transposase binds to the *Herves*-R end

To investigate the binding of transposase to the *Herves*-R end, the *Herves*-R 1-100 bp fragment was radio-labeled and used in the EMSA experiment as described above. *Herves* transposase interacted specifically with the probe and formed 3 transposase-DNA complexes (Fig. 1C). The unlabeled homologous competitor competed with the probe for transposase.

Notably, the addition of a nonspecific competitor led to the formation of a single, higher-molecular-weight complex (Fig. 1C). The molecular composition of this complex, however, is unknown.

Overlapping 30-bp oligonucleotides were used as probes to identify the transposase binding site(s) within the *Herves*-R 1-100 bp region by EMSA. The 1-30 bp, 15-45 bp, and 61-90 bp fragments elicited specific binding of transposase (Fig. 4A). Fragment 31-60 bp showed weak, nonspecific binding, whereas the 46-75 bp and 91-110 bp fragments failed to bind (Fig. 4A). Two transposase-DNA complexes formed with the *Herves*-R 1-30 bp probe, compared with 1 complex each with the *Herves*-R 15-45 bp and 61-90 bp probes, implicating the existence of 2 transposase binding sites within *Herves*-R 1-30 bp and 1 site within *Herves*-R 15-45 and 61-90 bp each (Fig. 4A).

To determine relative transposase binding affinities, each 30-bp overlapping DNA fragment was allowed to compete against the *Herves*-R 61-90 bp probe for transposase by EMSA. Fragment 15-45 bp partially competed against the probe for transposase, whereas the *Herves*-R 1-30 bp and 31-60 bp fragments had no effect (Fig. 4B). These data suggest that the 61-90 bp fragment has the highest affinity for transposase within the terminal 1-110 bp fragment.

We performed DNase I protection to identify specific binding motifs in the R end of the element. *Herves*-R 1-100 bp fragment, selectively radiolabeled at the 3' end, was incubated with DNase I in the presence or absence of *Herves* transposase as described above. *Herves*-R 1-100 bp was protected at 23-35 bp and 63-92 bp (Fig. 5). Similar to the L end, increased amounts of transposase protected the entire probe.

2.3.4 Mutational analysis of *Herves* transposase binding motif

Because *Herves*-L 28-48 bp showed the strongest binding to transposase, a detailed analysis was performed to define the critical nucleotides for binding. We

analyzed 22 sequence variants for their ability to compete with the *Herves*-L 28-60 bp probe for transposase. Each sequence variant differed from the wild-type sequence by a single nucleotide. Unlabeled wild type *Herves*-L 28-60 bp competed successfully against the probe for transposase, whereas mutating nucleotides *Herves*-L 31-38 and 40-46 abolished this competition, indicating that nucleotides at these positions mediate the binding of transposase (Fig. 6A). We identified a conserved binding motif, CG/AATTCAT, in both regions, suggesting that it constitutes the transposase binding motif.

To confirm these results, we simultaneously mutated this putative motif in both stretches within the *Herves*-L 28-60 bp region and allowed the mutant (*Herves*-L 31-47 mut) to compete against the wild-type *Herves*-L 28-60 bp probe. Mutating both sites abolished the interaction, confirming that CG/AATTCAT is the binding site for *Herves* transposase in the *Herves*-L end (Fig. 6B).

2.3.5 The CG/AATTCAT motif is conserved between the *Herves*-L and R ends

We identified similar binding motifs within the *Herves*-R 15-22 bp and 73-86 bp regions. That the *Herves* transposase also bound to R 1-30 bp and 61-90 bp suggests that the CG/AATTCAT motif mediates the binding of transposase to the *Herves*-R end. Furthermore, the 1-30 bp region also contains the R-TIR, a strong candidate for transposase binding.

To determine whether the R-TIR or the CG/AATTCAT motif mediates the binding of transposase to the *Herves*-R 1-30 bp region, we mutated each region (*Herves*-R TIR mut and *Herves*-R 15-22 mut) and subjected them to EMSA. Mutating each

potential binding site abolished its ability to compete against the wild-type probe, confirming that the CG/AATTCAT motif and R-TIR are both important for the transposase binding to the *Herves*-R end (Fig. 7).

2.3.6 The CGATTCAT motif is sufficient for purified *Herves* transposase binding

We observed that the CG/AATTCAT motif and its derivatives were repeated several times within the transposase-binding regions of the *Herves*-L and R ends (Fig. 9). To determine whether the CG/AATTCAT motif was sufficient for transposase binding, we used a tetramer of the CGATTCAT sequence as a probe to measure binding to the *Herves* transposase. The transposase bound well to the CGATTCAT tetramer and formed 2 transposase-DNA complexes (Fig. 8). Based on the unlabeled specific and nonspecific competitors, the interaction was sequence-specific.

Herves-L 28-60 bp is one of the transposase binding regions, and we used this fragment as a specific competitor for transposase against the CGATTCAT tetramer. *Herves*-L 28-60 bp outcompeted the tetramer for transposase (Fig. 8). Furthermore, splitting the CGATTCAT motif in half abolished the binding (data not shown). These data indicate that the CGATTCAT motif is sufficient for the transposase to bind.
We also tested the ability of unlabeled sequence variants of the CG/AATTCAT motif (CGATTC**T**T/CGATTCA**C**/CG-TTCAT) to compete against radiolabeled CGATTCAT for transposase. None of the sequence variants competed fully with CGATTCAT for transposase, indicating that CGATTCAT is the strongest binding motif (Fig. 8). Nevertheless, CGATTCAC competed partially for transposase, suggesting that this variant is important for the support or proper binding of transposase.

2.4 Discussion

In this study, we purified active *Herves* transposase and demonstrated that it site-specifically binds to end sequences of the *Herves* element. The transposase binds to sub-terminal and terminal sequences on the *Herves*-L and R ends, respectively. Such asymmetrical binding might allow it to differentiate between the *Herves*-L and R end sequences during transposition. The *Drosophila P* element transposase has been shown to distinguish between ends, wherein interchanging the L end sequence with the R end sequence leads to fewer transposition events (Mullins *et al.*, 1989). This phenomenon occurs for the *Ac* element in maize and the *Tag1* element in *Arabidopsis* (Coupland *et al.*, 1989; Liu *et al.*, 2000; Cristancho and Gaitan, 2008).

There is strong transposase binding to the *Herves*-L 12-48 bp and 28-60 bp regions and relatively weak binding to the 48-75 bp region, as shown by EMSA and DNase I footprinting. None of these fragments, however, outcompeted the L 1-100 bp probe for the transposase binding, suggesting that the binding is cooperative between 2 or more regions. Furthermore, the overlapping *Herves-L* 12-48 bp and 28-60 bp fragments have equal transposase binding affinities, indicating that the binding motif lies in the overlapping region in *Herves*-L 28-48 bp. In contrast to the L end, the binding occurs toward the terminal sequences on the *Herves*-R end, during which the transposase binds to the R-TIR, 15-45 bp, and 61-90 bp regions.

The EMSA results with the *Herves*-L 28-60 bp probe and single nucleotide sequence variants indicate that the CGATTCAT motif, or its derivatives, mediates binding of the transposase.

The CGATTCAT transposase binding motif and its derivatives are repeated and conserved in the *Herves*-L and R end sequences. Our results suggest that this motif is important and sufficient for transposase binding, because: 1) mutating the CGATTCAT motif on either end abolishes binding; 2) the transposase binds specifically to a synthetic tetramer of the motif; and 3) splitting the sequence motif in half abolishes binding.

TEs frequently have multiple transposase binding sites (Craigie *et al.*, 1984; Kunze and Starlinger, 1989b, a; Colloms *et al.*, 1994; Vos and Plasterk, 1994). In other *hAT* elements, such as *Ac* and *Tag1,* their respective transposases bind to short sequence repeats (Kunze and Starlinger, 1989b; Liu *et al.*, 2000; Mack and Crawford, 2001).

In many cases, transposase binding sequence motifs differ at the L and R ends (Mack and Crawford, 2001). The *Herves* tranposase-binding motif, however, is conserved at both ends. There are several single nucleotide variants of the CGATTCAT motif, such as CGATTCAC, CGTTCAT, and CGATTCTT, which are conserved in both ends.

Our results suggest that these additional motifs also mediate transposase binding. Although these derivatives are seemingly related to the CGATTCAT motif, their affinities for transposase differ. The transposase binds robustly to CGATTCAT, whereas the CGATTCAC and CGTTCAT motifs bind weakly or assist in transposase binding to other sequence motifs.

It is also possible that the transposase recognizes a family of related sequences in which GATTC or ATTCA is the central sequence. Similar results have been reported for the *Tag1* element, for which the R-TGACCC and L-AAACCC motifs have different affinities for the transposase (Mack and Crawford, 2001).The sequences that flank these

motifs differ, and although they might fail to influence transposase binding, they might regulate transposition (Cui *et al.*, 2002).

Despite making several attempts, we observed no binding to the L-TIR. This finding, however, is not unusual, because several related elements, such as the *Ac, Tag1,* and *Hermes* transposase, do not bind L and R-TIRs (Kunze and Starlinger, 1989a; Mack and Crawford, 2001). This phenomenon raises the possibility that a host factor may also bind to this region. But, nuclear extracts from a *Herves* transposase-expressing *Drosophila* S2 cell line do not bind to L-TIR, either, suggesting that: 1) the *Herves*-L TIRs are not involved in the binding of any host factor, or 2) the homolog of the L-TIR binding protein is absent in *Drosophila*. Nevertheless, pure *Herves* transposase interacts with the R-TIR sequence, the binding between which appears to be cooperative, because the R-TIR and CGATTCAT motif in the 15-22 bp element are important for transposase binding.

2.5 Material and Methods

2.5.1 Plasmid construction

The *Herves* ORF was cloned into pBAD myc/HisA (Invitrogen). The *BspHI* (incorporated into the *Herves* start codon) and native *KpnI* restriction sites were used to amplify a 766-bp fragment of the *Herves* ORF using the *Herves*F-*BspHI* (GATCAATCATGATGGCTCCAACAAACGCAAC) and *Herves*R-*KpnI* (GTTCAAGGTACCTTGAATCCAATTAGCTATATTCTTACC) primers.
The resulting fragment was cloned into *NcoI/KpnI*-digested pBAD myc/HisA to generate pBADHvPCR1. The remaining *Herves* ORF (1118 bp) was amplified using the *Herves*F-

KpnI (CAAGGTACCTTGAACAAATTTGACATAGAGGATAAG) and *Herves*R-*HindIII* primers (TATCAAGCTTTGAACAAATTTGACATAGAGGATAAG) and cloned into *KpnI/HindIII*-digested pBADHvPCR1 to generate pBADHv1.

2.5.2 *Herves* transposase purification

Herves transposase was purified by *His*-tag purification as described (Zhou *et al.*, 2004). pBADHv1-transformed LMG 194 *E. coli* cells were grown overnight at 30°C in LB media that contained carbenicillin (100 mg/ml). The overnight culture was diluted 1:100 in LB and carbenicillin (100mg/ml) and grown at 30°C and 230 rpm to an absorbance of 0.6 at 600 nm. The cultures were then induced with 0.1% L-arabinose and shaken at 16°C for 18 hrs. The cells were harvested and washed by centrifugation with binding buffer (0.5 M NaCl, 20 mM Tris-Cl pH 7.9, 10% glycerol, 10 mM imidazole). The cells were lysed twice using a French press at 20 K psi. The cell lysate was cleared by centrifugation through 0.45-µm syringe filters. Cleared lysate was loaded onto Sepharose (Amersham) chromatography columns that were pre-equilibrated with Ni^{+2}. The columns were washed with 10 ml binding buffer and 6 ml wash buffer (0.5 M NaCl, 20 mM Tris-Cl pH 7.9, 10% glycerol, 50 mM imidazole). His-tagged *Herves* was eluted in 5 1-ml fractions of elution buffer (0.5 M NaCl, 20 mM Tris-Cl pH 7.9, 10% glycerol, 200 mM imidazole). The purified *Herves* transposase was dialyzed overnight in dialysis buffers 1 (0.5 M NaCl, 20 mM Tris base, 10% glycerol pH 8.0) and 2 (0.5 M NaCl, 20 mM Tris base, 2 mM DTT, 25% glycerol pH 8.0) for 3 hrs using a Slide-A-Lyzer dialysis cassette (Thermo Scientific). The dialyzed, purified *Herves* transposase was stored at -80°C.

2.5.3 *In vitro* transposition assay

The *in vitro* transposition assay (IVTPA) was performed with 1X IVPTA buffer (20 mM HEPES, 5% (v/v) glycerol, 5 mM $MgCl_2$, 0.2 ug BSA, 2 mM dithiothreitol), 250 ng of each of the target and donor plasmids, and 5 µl of purified *Herves* transposase. The reaction was incubated at 30ºC for 1.5 hrs, and DNA was extracted by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. The purified DNA was used to transform *E. coli* DH10β competent cells. The transformant was diluted 1:50, and 5 µl of the dilution was plated on LB media that contained ampicillin, IPTG, and X-Gal to obtain the donor titer. The remaining transformed bacterial culture was plated on LB agar plates that contained chloramphenicol, gentamycin, IPTG, and X-Gal to obtain transposition events. Blue colonies were tested for ampicillin sensitivity and the correct restriction digestion patterns.

2.5.4 Electrophoretic Mobility Shift Assay

The DNA fragment (2 pmol) that we tested for transposase binding was end-labeled using T4 polynucleotide kinase and γ32P ATP and purified on a Biospin 30 column (BioRad). The labeled DNA fragment (probe) was incubated at 4°C for 45 min with 1X EMSA binding buffer (16 mM Tris pH 8.0, 0.2 ug BSA, 0.4 ug T3 single-stranded oligo, 0.5 ug polydI-dC, 1 mM DTT, 150 mM NaCl, 0.25% Triton X) and 1.2 µg of *Herves* transposase. Homologous and nonhomologous DNA fragments were used as specific and nonspecific competitors, respectively (if applicable). The reaction was incubated with the probe for an additional 40 min at 4°C. The nonspecific competitors were a 126-bp gDNA fragment (E1) that flanks *Hermes* TE from *Musca domestica* and a

30-bp DNA oligo from *Aedes aegypti β2 tubulin.* The EMSA reaction products were analyzed on a 5% TBE polyacrylamide gel (Bio-Rad).

2.5.5 DNase I protection assay

DNA fragments (100 bp each) from the *Herves*-L and R ends, containing an *EcoRV* restriction site at the 5'- or 3'-end, were cloned into pJET 1.2 (Fermentas) to generate pL5'*EcoRV*, pL3'*EcoRV*, pR5'*EcoRV,* and pR3'*EcoRV*. The transferred and nontransferred strands from the *Herves*-L and R ends were selectively radiolabeled at one end by digesting pL5'*EcoRV*, pL3'*EcoRV*, pR5'*EcoRV,* and pR3'*EcoRV* with *XhoI* and *EcoRV* and labeling them with α^{32}P dATP using Klenow (NEB). *Herves* transposase was allowed to bind to 10 fmol single end-labeled DNA fragment (probe) under the same binding conditions as in the EMSA. The DNA probe was subjected to DNase I digestion for 2 min at 4°C. The reaction was stopped by adding stop solution (92% ethanol, 0.7 M ammonium acetate, 0.35 ug tRNA) for 15 min in a dry ice/ethanol bath. DNA was extracted with phenol/chloroform and precipitated with ethanol. The reaction products were analyzed on a 10% denaturing polyacrylamide sequencing gel. The DNA sequencing kit 2.0 (USB) was used to construct a nucleotide ladder that was analyzed with the reaction products on the sequencing gel.

2.6 References

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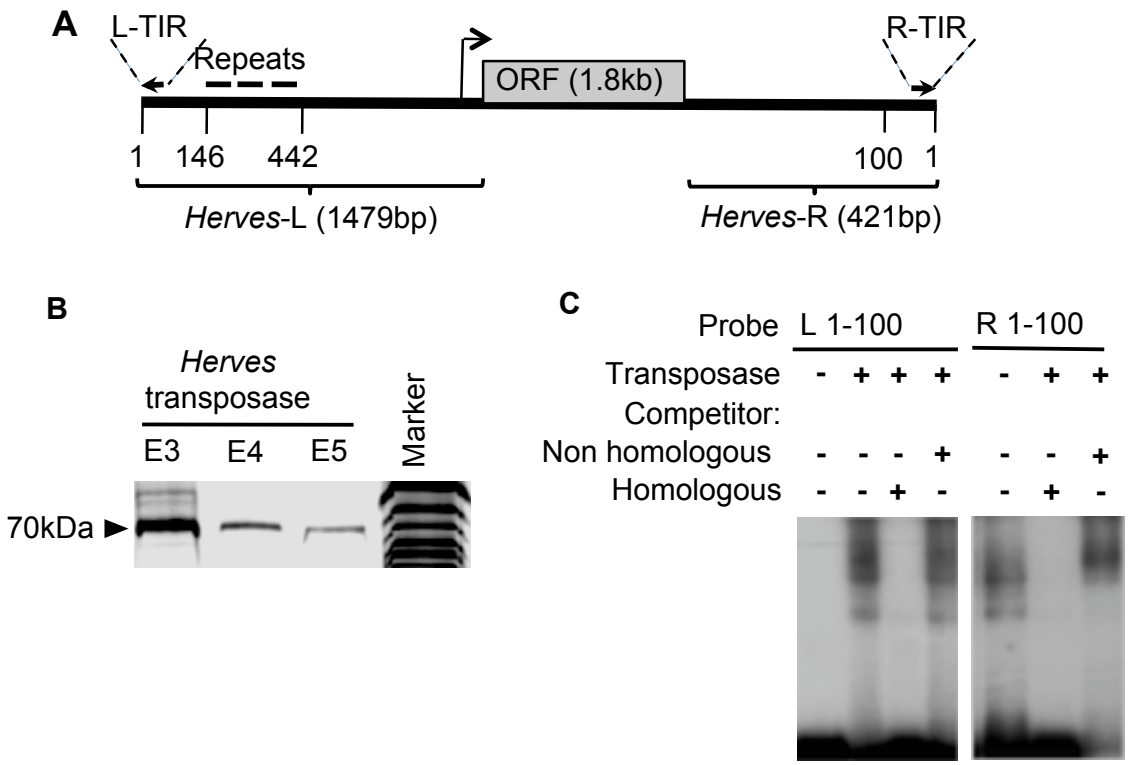


Figure 2.1 Pure transposase binds to the terminal sequences of Land R ends of *Herves* element. A) Schematic representation of *Herves* element. B) SDS-PAGE analysis of purified *Herves* transposase**.** Coomassie stained gel show 70kD purified *Herves* transposase. E3, E4, and E5 represent different elutions obtained during the final step of protein purification. C) Transposase binding to the terminal fragment of *Herves*-L and R ends. EMSA analysis with the *Herves*-L 1-100bp (Lanes 1-4) and R 1-100bp (Lanes 5-7) probes. The DNA fragments were incubated in the presence (+) or absence (-) of pure transposase. A homologous fragment was used as specific competition. The E1 flanking sequence from *Hermes* transposable element was used as nonspecific competition (Warren *et al.*, 1994).The specific and non specific competitors were use at 200-fold molar excess to the probe.

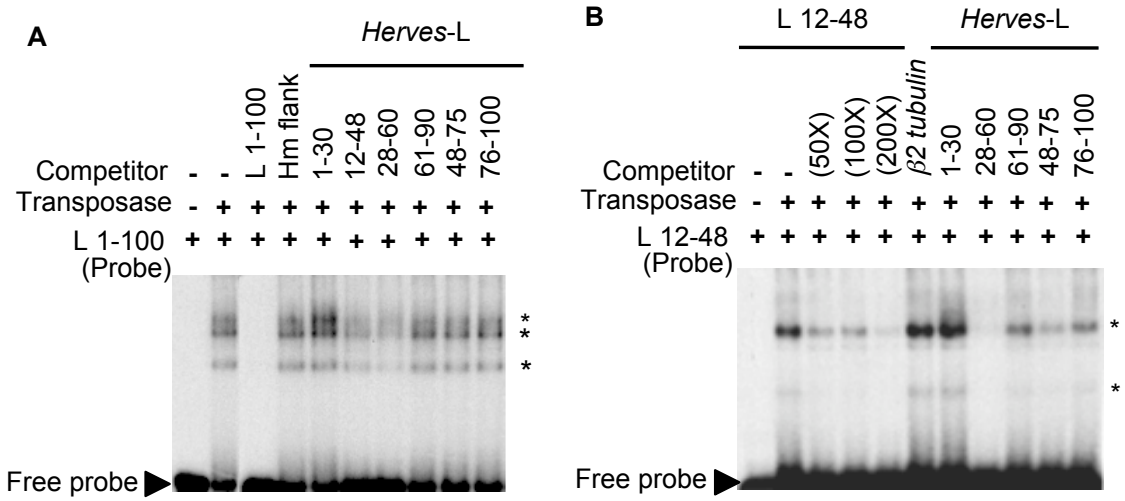


Figure 2.2. The *Herves*-L 12-48bp and 28-60bp are important for transposase binding. EMSA experiment with: A) *Herves*-L 1-100bp and B) *Herves*-L 12-48bp as probes. Overlapping 30bp fragments (mentioned on top) were used as competitors for transposase binding to the probe. The specific and nonspecific competitors were use at 200-fold molar excess to the probe, unless specified otherwise. The star (∗) indicates various protein DNA complexes. A) The specific and nonspecific competition was used as described for Fig. 1; B) A 30bp nonhomologous fragment, from *β2 tubulin* gene of *Ae. aegypti*, was used as a non-specific competition.

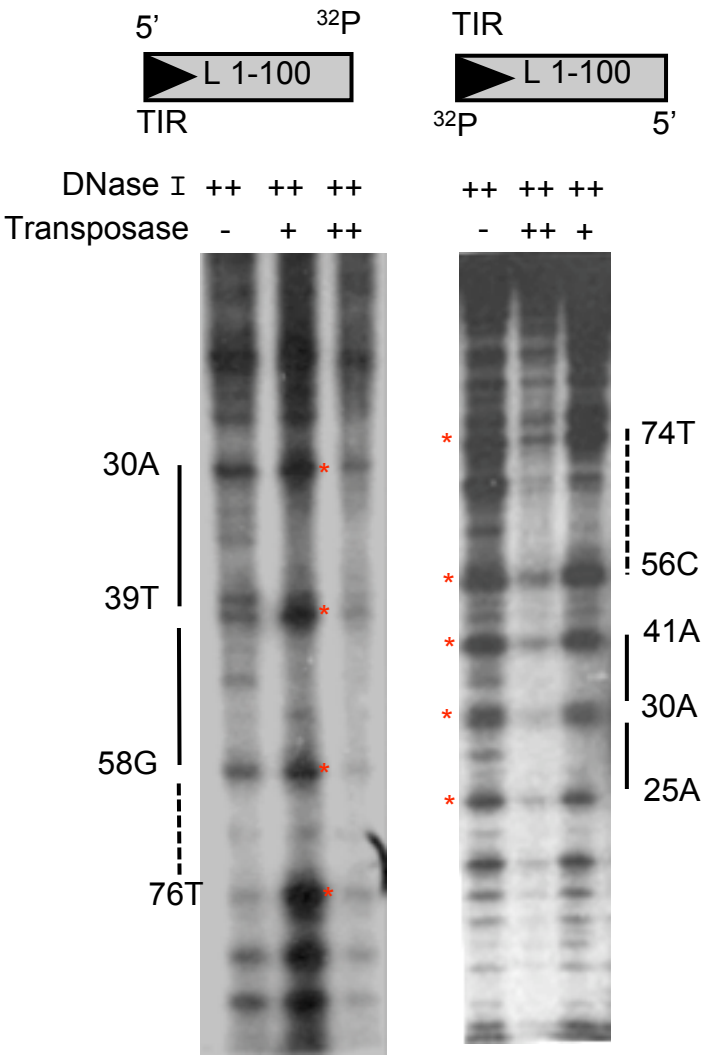
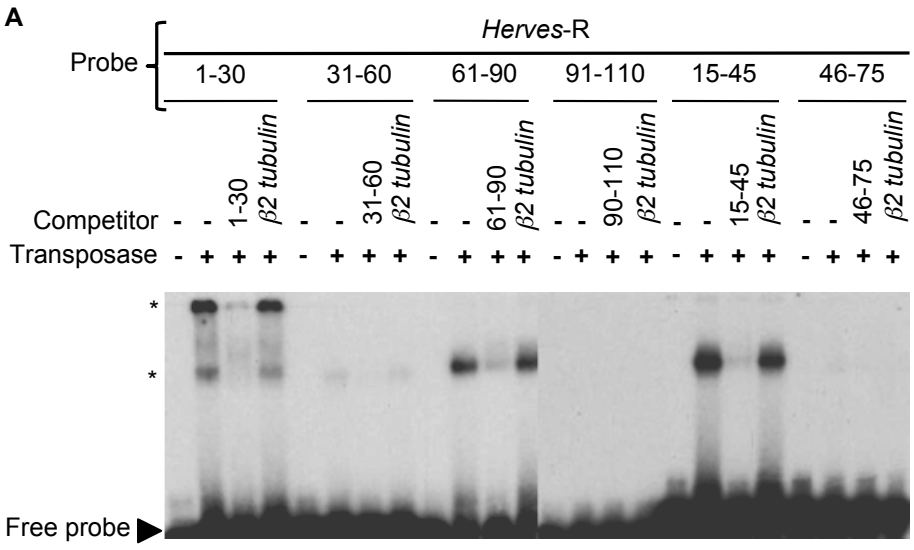
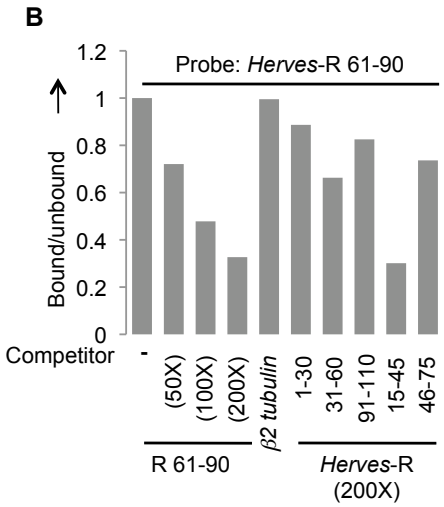


Figure 2.3. The transposase binding analysis to *Herves*-L end sequence.
The single-end labeled *Herves*-L 1-100bp fragment was incubated in presence (+, ++) or absence (-) of DNaseI or the transposase. The ++ indicates double the amount of transposase added to the reaction mix, relative to +. ^{32}P indicates the position where probe was labeled. The solid bars indicate the region that was protected by the transposase from DNaseI degradation, whereas, the dotted line indicates weak transposase binding.


A
Herves-R
Probe
1-30
31-60
61-90
91-110
15-45
46-75
β2 tubulin
Competitor
Transposase
Free probe


B
Probe: Herves-R 61-90
Bound/unbound
Competitor
(50X)
(100X)
(200X)
β2 tubulin
1-30
31-60
91-110
15-45
46-75
R 61-90
Herves-R
(200X)

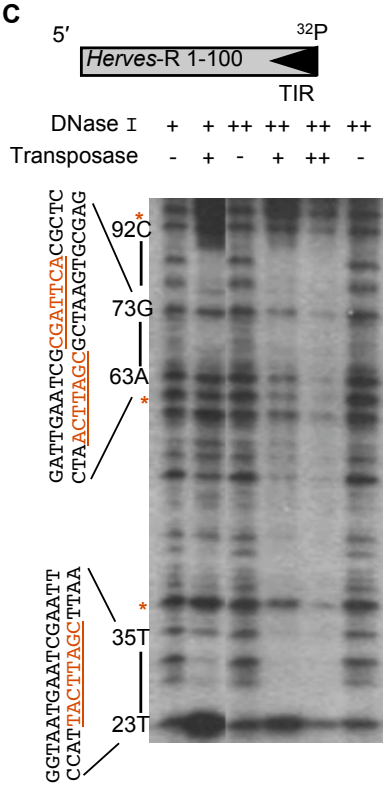

C
5′
32P
Herves-R 1-100
TIR
DNase I
Transposase
GATTGAATCGCGATTCACGCTC
CTAACTTAGCGCTAAGTGCGAG
92C
73G
63A
GGTAATGAATCGAATT
CCATTACTTAGCTTAA
35T
23T

Figure 2.4. Transposase binding to *Herves*-R end
A) The transposase binds to *Herves*-R 1-30bp, 15-45bp and 61-90bp.
EMSA analysis of transposase binding to the overlapping 30bp fragments (1-30bp, 31-60bp, 61-90bp, 91-110bp, 15-45bp and 46-75bp). The specific and nonspecific competition was used as described for Fig. 2B. B) The transposase binding to *Herves*-R 61-90bp. The *Herves*-R 61-90bp fragment was used as a probe in an EMSA experiment. The fraction of the transposase-bound probe was quantified using phosphoimager. A homologous fragment was used as specific competitor at a molar excess of 50, 100 and 200-folds. Overlapping fragments (1-30bp, 31-60bp, 91-110bp, 15-45, and 46-75bp) were used as competitors of transposase binding to the probe, at a 200-fold molar excess. C) DNase I protection assay from *Herves*-R end sequence.
Abbreviations are same as in Figure 3. ^{32}P indicates end of the probe that was labeled. The solid bars on the sides indicate the region of the probe protected by the transposase. The star (∗) indicates hypersensitive sites.

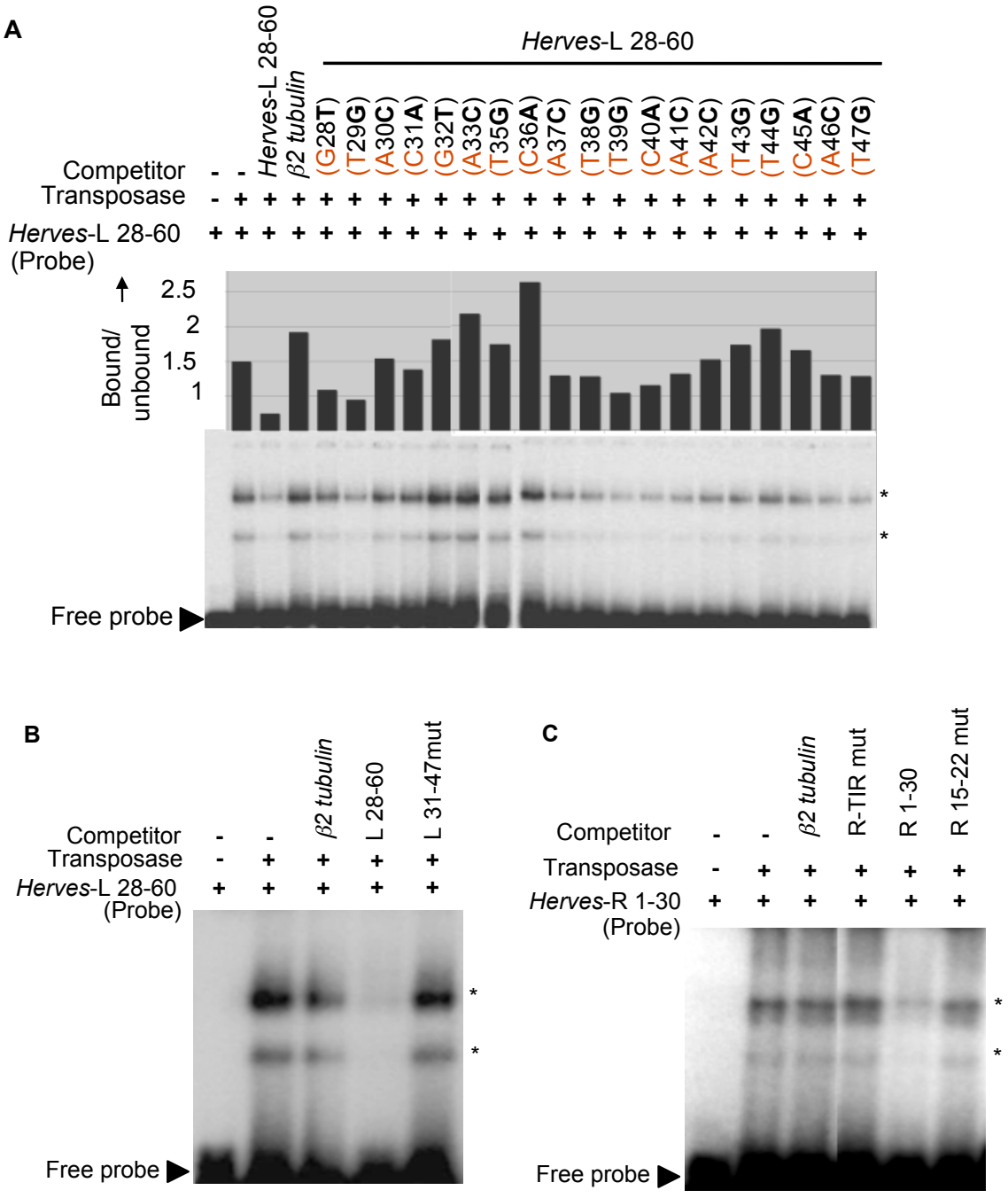

A
Herves-L 28-60
Herves-L 28-60
β2 tubulin
(G28T)
(T29G)
(A30C)
(C31A)
(G32T)
(A33C)
(T35G)
(C36A)
(A37C)
(T38G)
(T39G)
(C40A)
(A41C)
(A42C)
(T43G)
(T44G)
(C45A)
(A46C)
(T47G)
Competitor
Transposase
Herves-L 28-60 (Probe)
Bound/ unbound
2.5
2
1.5
1
*
*
Free probe
B
β2 tubulin
L 28-60
L 31-47mut
Competitor
Transposase
Herves-L 28-60 (Probe)
*
*
Free probe
C
β2 tubulin
R-TIR mut
R 1-30
R 15-22 mut
Competitor
Transposase
Herves-R 1-30 (Probe)
*
*
Free probe

Figure 2.5. The CGATTCAT acts as transposase binding motif.
A) EMSA analysis of transposase binding to *Herves*-L 28-60bp probe and single nucleotide sequence variants (mentioned on the top) as competitors. For example: G28**T** means that G at position 28 was changed to T in *Herves*-L 28-60bp fragment. The fraction of the transposase bound probe, in each lane, was quantified using phosphoimager. Mutations that have no effect on the transposase binding are expected to produce values similar to the specific competition. B) The *Herves*-L 31-47 region is important for binding. The *Herves*-L 28-60bp fragment was used as a probe in EMSA experiment. The *Herves*-L 31-47mut carries mutations at every position within 31-47bp. C) Role of R-TIR in the transposase binding. The *Herves*-R 1-30bp fragment was used as a probe. The *Herves*-R TIRmut and R 15-22mut fragments consist of *Herves*-R 1-30bp sequence with mutations in TIR and 15-22bp, respectively. The probe was incubated in the presence (+) or absence (–) of the transposase or competitors. Unlabelled homologous and non-homologous fragments were used as specific and nonspecific competitors, respectively.

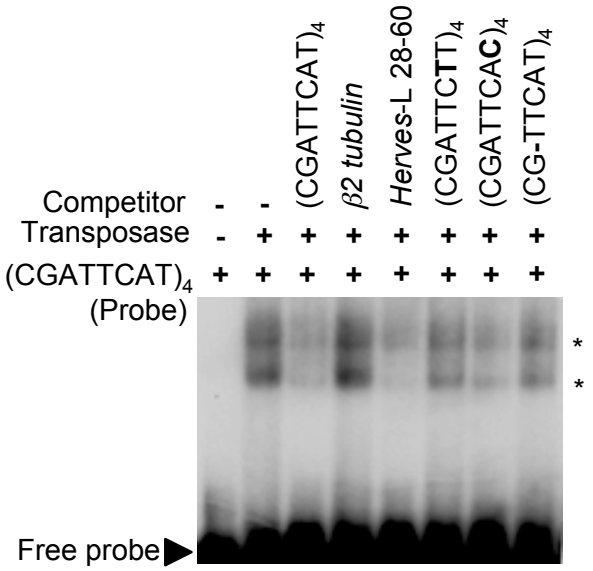


Figure 2.6. The CGATTCAT binding motif is sufficient for transposase binding. The probe $(CGATTCAT)_4$ represents tetramer of CGATTCAT sequence motif. Unlabeled $(CGATTCAT)_4$ and *Herves*-L 28-60bp were used as specific competitor, whereas, *β2 tubulin* was used as nonspecific competitor. The transposase binding was compared between the sequence variants of the binding motif such as CGATTCTT, CGATTCAC and CG-TTCAT (each used as tetramers).

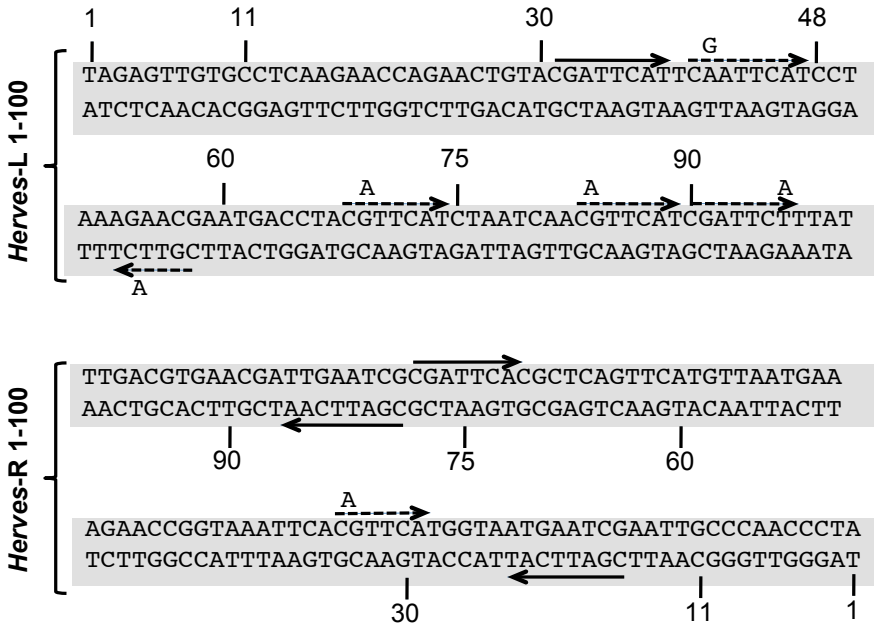


Figure 2.7. Sequence of *Herves*-L 1-100bp and R 1-100bp showing various sequence repeats. The solid arrow indicates conserved CGATTCA transposase binding motif, whereas, the dotted arrow indicates the single nucleotide sequence variants.

Chapter 3:

Double-stranded RNA-mediated interference in *Culex pipiens quinquefasciatus*

3.1 Abstract

RNA interference (RNAi) is an important component of insect antiviral immunity and a powerful tool that has been used to analyze the wealth of genetic information obtained from recent genome sequencing projects. Notably, this information can be used to design disease-vector control strategies. *Culex quinquefasciatus* is an important vector of pathogens such as West Nile virus, St. Louis encephalitis virus and filarial worms. Although the genome of *Cx. quinquefasciatus* has been recently sequenced, RNAi has not been studied in this organism. We targeted the *white* eye pigmentation gene of *Cx. quinquefasciatus* as a marker of RNAi function, which resulted in reduced transcript levels of the *white* gene and reduced eye pigmentation (a white-eye phenotype) in both larvae and adults. In addition, knockdown of the *Cx. quinquefasciatus*-specific *argonaute* (*ago*), *dicer (dcr*) and *r2d2* components of the RNAi pathway was achieved. It was also demonstrated that *ago2*-mediated slicing was more critical than *dcr2*-mediated dicing for functional RNAi in this mosquito. This study also highlights important differences in RNAi between *Cx. quinquefasciatus* and the model organism *D. melanogaster*.

3.2 Introduction

Several species of mosquitoes transmit pathogens that cause human and livestock diseases such as malaria, dengue, yellow fever, lymphatic filariasis, encephalitis and others. Many of these vector-borne diseases do not have an effective treatment or control measures. Traditional control strategies have not been able to provide long-term solutions to these disease-related problems, and new control strategies are needed to reduce the burden of disease on global health (Steven P. Sinkins and Gould, 2006).

RNA interference (RNAi) is triggered by a double-stranded RNA (dsRNA) molecule and leads to silencing of homologous genes either by sequence-specific degradation of mRNA (siRNA pathway) or by blocking the translation of mRNA transcripts (miRNA pathway) (Fire *et al.*, 1998; Carthew and Sontheimer, 2009). The siRNA pathway (referred to as RNAi for this study) has been well studied in *Drosophila melanogaster*, which has provided the basis for its application in various non-model organisms (Blandin *et al.*, 2002; Franz *et al.*, 2006a; Sim and Denlinger, 2008; Kim *et al.*, 2010a). In *D. melanogaster*, the RNAi mechanism is triggered by a dsRNA molecule (from an endogenous or exogenous source) that is cleaved by the Dcr 2 (RNaseIII) enzyme to produce a pair of 22-23 nucleotide (nt) short-interfering RNAs (siRNAs) (Elbashir *et al.*, 2001b). Recently, it has been shown that Dcr 2 first associates with the dsRNA binding protein loquacious (Loqs) to cleave long dsRNA into siRNAs. Subsequently, this complex binds to another dsRNA binding protein, termed r2d2, which loads one strand (guide strand) of the siRNA into the RNA-induced silencing complex (RISC) (Marques *et al.,* 2010). The guide strand-loaded RISC subsequently cleaves homologous mRNA with the help of Ago2 (Kavi *et al.,* 2005).

RNAi has emerged as a powerful tool to identify novel genes and to study gene function (Carpenter and Sabatini, 2004; Nakayashiki *et al.*, 2005; Sim and Denlinger, 2008; Kim *et al.*, 2010a). RNAi-based approaches have recently been used to study medically important disease-vectors, which has resulted in the identification of genes that are important in pathogen transmission (Blandin *et al.*, 2002; Arrighi *et al.*, 2004; Blandin *et al.*, 2004; Osta *et al.*, 2004; Hao *et al.*, 2008). RNAi is also an important part

of antiviral innate immunity in plants and animals (Adelman *et al.*, 2002a; Li *et al.*, 2004; Lu *et al.*, 2005; Voinnet, 2005; Wilkins *et al.*, 2005; Wang *et al.*, 2006). For example, RNAi acts as an antagonist of O'nyong-nyong virus (ONNV) replication in *Anopheles gambiae* (Keene *et al.,* 2004b). Similarly, *D. melanogaster* uses RNAi as a defense against Flock House Virus (FHV) and Cricket Paralysis Virus (CrPV) (Wang *et al.,* 2006).

Genetic transformation technology has been applied to a wide range of insects including mosquito species such as *An. stephensi, Aedes aegypti* and *Cx. quinqueasciatus* (Rubin and Spradling, 1982; Catteruccia *et al.*, 2000a; Allen *et al.*, 2001b; Franz *et al.*, 2006b). RNAi-mediated approaches to gene silencing combined with genetic transformation technology hold great promise for the control of vector-borne diseases (Brown *et al.*, 2003a). Based on this approach, a transgenic *Ae. aegypti* (Carb77) expressing an inverted-repeat (IR) RNA corresponding to the *dengue type 2 virus (DENV-2)* was created and conferred resistance in the mosquito to *DENV-2* virus replication and transmission (Franz *et al.,* 2006b). However, the virus resistance in Carb77 strain was lost overtime primarily due to the reduced transgene expression (Franz *et al.,* 2009). Recent studies have shown that siRNA-mediated gene silencing can be used experimentally to suppress gene expression, however, the mechanism underlying RNAi-mediated inhibition in mosquitoes is not completely understood (Levashina *et al.*, 2001; Adelman *et al.*, 2002a; Blandin *et al.*, 2002; Caplen *et al.*, 2002; Boisson *et al.*, 2006; Campbell *et al.*, 2008d). The *Cx. quinquefasciatus* genome has recently been sequenced and genes involved in the biogenesis of small RNAs identified (Campbell *et al.*, 2008b).

The aim of this study was to establish whether an RNAi approach in *Cx. quinquefasciatus*, which could then be used as a functional genetic tool to identify genes involved in the transmission of pathogens.

3.3 Results

3.3.1 Analysis of the small RNA biogenesis genes of *Cx. quinquefasciatus*

A phylogenetic analysis of Ago, Dcr, Loqs, and r2d2 proteins, the key components of siRNA and miRNA pathways, showed that most proteins organized into monophyletic groups with short internal branches (Fig. 1A). The one exception was the r2d2 protein from *Ae. Aegypti,* which was much more divergent than r2d2 protein from other insects in this analysis (Fig. 1A). For this study, the RNAi genes and proteins in *Ae. aegypti*, *An. gambiae*, *Cx. quinquefasciatus*, *C. elegans*, *D. melanogaster*, and *T. castaneum* will be abbreviated with a prefix Ae, An, Cx, Ce, Dm, and Tc, respectively. There were interesting differences in the composition and structure of RNAi components between *Cx. quinquefasciatus* and *D. melanogaster*. *Cx. quinquefasciatus* has two *ago2* genes (Cx*ago2-1* and Cx*ago2-2*), which is in contrast to *D. melanogaster*, *Ae. aegypti* and *An. gambiae* where there is only one (Fig. 1A) (Campbell *et al.*, 2008b). Similar to *D. melanogaster*, there are two *dcr* genes in *Cx. quinquefasciatus* (Cx*dcr1* and Cx*dcr2*) however, ScanProsite software (de Castro *et al.,* 2006) predicted that the domain structure of Dcr1 and Dcr2 proteins varies between *D. melanogaster* and *Cx. quinquefasciatus*. The CxDcr1 contains an additional N-terminal helicase domain (Fig 1B), which is absent in DmDcr1 (Schauer *et al.*, 2002; Tomoyasu and Denell, 2004). Previous work has shown that the TcDcr1 and CeDcr1 also have this additional N-

terminal helicase domain (Figure 1B) (Tomoyasu and Denell, 2004). Furthermore, DmDcr2 lacks the full length PAZ domain, which is present in CxDcr2, AeDcr2, AnDcr2, and TcDcr2 (Fig. 1B). The presence of two Ago2 proteins and the domain structure of Dcr indicate that the RNAi components in *Cx. quinquefasciatus* more closely resemble *T. castaneum* or *C. elegans* compared to *D. melanogaster*. Also, our phylogenetic analysis independently confirmed results of a previous study, indicating the presence of a functional small RNA pathway in *Cx. quinquefasciatus* (Campbell *et al.*, 2008b). In addition, expression analysis indicated that the key RNAi components (*ago2-1, ago2-2, dcr2* and *r2d2*) were expressed at all developmental stages (embryo, larvae, pupae and adults) of *Cx. quinquefasciatus* (data not shown).

3.3.2 Knockdown of the *white* gene as an RNAi marker

To establish the use of RNAi as a research tool in *Cx. quinquefasciatus*, preblastoderm embryos were injected with dsRNA specific to the *white* gene of *Cx. quinquefasciatus* (ds*white*). The *white* gene belongs to a conserved family of ATP binding cassette (ABC) transporter proteins, which are responsible for transmembrane transport of eye pigments or their precursors (Ames *et al*. 1990; Higgins 1992). We wanted to determine if injecting ds*white* into *Cx. quinquefasciatus* embryos would result in a visible eye phenotype in the hatched larvae and adults, which would be an indication of interference with *white* gene function. Accordingly, the injected ds*white* RNA was expected to act as a trigger for the RNAi-mediated knockdown of the *white* gene. The larval stage of *Cx. quinquefasciatus* has two different types of eyes, which include simple eyes (ocella) and compound eyes while adults have only compound eyes. The same

ommochrome biosynthetic pathway is responsible for eye pigmentation in both developmental stages.

Injecting a 461 base pair (bp) dsRNA of *white* (ds*white*461) into preblastoderm embryos resulted in a white-eye phenotype in 28.4% and 5.2% of the hatched larvae and adults respectively. Primers used to generate dsRNA of *white* gene and to perform gene expression analysis are listed in Table 1 and 2. The larvae with a white-eye phenotype showed a complete reduction of eye pigmentation in the ocella (Fig. 2A) and a visible reduction in the pigmentation of compound eyes (Fig. 2A, black arrow). We observed phenotypes ranging from brown eyes to complete white eyes in corresponding adults (Fig. 2B). RT-PCR analysis verified that the larval and adult white-eye phenotype was due to the reduced expression of the *white* gene (Fig. 2C, D). This indicated that the dsRNA induced a functional RNAi response in a sequence-specific manner in *Cx. quinquefasciatus.*

Various studies have indicated that increasing the length of the dsRNA leads to a more robust RNAi response (Yang *et al.,* 2000b). We undertook a similar study in *Cx. quinquefasciatus* larvae. Increasing the length of ds*white* from 461 bp (ds*white*461) to 935 bp (ds*white*935) led to a significant increase in the frequency of the white-eye phenotype and a corresponding decrease in the quantity of *white* transcripts in larvae (Fig. 2D). This indicated that the dsRNA induced RNAi response proportional to the length of the injected dsRNA.

3.3.3 Silencing of individual RNAi genes

Targeting RNAi components has been shown to silence the RNAi pathway. This method has been used to study genes involved in the RNAi pathway and the role of RNAi in antiviral defense (Dudley *et al.*, 2002; Tomoyasu and Denell, 2004; Wang *et al.*, 2006). To use similar approach in *Cx. Quinquefasciatus*, first we targeted the individual RNAi genes (*i.e.*, *ago2-1, ago2-2, dcr2* and *r2d2* genes) by using dsRNA-mediated knockdown, to test the sequence-specific knockdown of each gene. Primers used to synthesize dsRNA of each gene and the mRNA regions targeted are listed in Table 1 whereas the primers used to perform qPCR expression anaysis Table 2. Introducing corresponding dsRNA into *Cx. quinquefasciatus* embryos led to a sequence-specific reduction in the transcript levels of *ago2-1, ago2-2, dcr2* and *r2d2* in the hatched larvae (Fig. 3). The quantitative PCR (qPCR) indicated that the targeted transcript levels were reduced up to 10-fold compared to the control (Fig. 3).

3.3.4 Functional role of *dcr2* and *r2d2* in RNAi

To validate the functional roles of the *dcr2* and *r2d2*, we co-injected preblastoderm embryos with ds*white*935 and dsRNAs homologous to *dcr2* or *r2d2*. Knockdown of any one of these genes was expected to make *white* RNAi less efficient and reduce the frequency of the white-eye phenotype (Fig. 4A). We found that co-injecting dsRNA for *dcr2* or *r2d2* with ds*white*935 did not reduce the frequency of white-eye phenotype as compared to injecting ds*white*935 alone (Fig. 4B). Examination of transcript levels by qPCR showed that the *white* transcript levels were still reduced (Fig. 4C) despite the knockdown of *dcr2* or *r2d2* (Fig. 4D), in the coinjection experiment. This

indicated that the dcr2 and r2d2 are dispensable for RNAi mechanism in *Cx. quinquefasciatus*.

3.3.5 Functional role of *ago2* genes in RNAi

There are two *ago*2 genes (*ago2-1* and *ago2-2*) in *Cx. quinquefasciatus* (Fig. 1). The amino acid sequences of *ago2-1* and *ago2-2* are 49% identical. Furthermore, the *ago2-1* has the two alternatively spliced isoforms: *ago2-1A* and *ago2-1B* (Fig. 5A). First, we wanted to determine whether both of these isoforms were expressed in *Cx. quinquefasciatus* and if they displayed development-specific differential expression. We designed oligos to produce 769 bp and 865 bp RT-PCR amplicons specific to *ago2-1A* and *2-1B*, respectively. However, RT-PCR results only produced the 865 bp amplicon corresponding to *ago2-1B* in the developmental stages tested (embryo, larvae, pupae and adults) and *ago2-1B* expression varied at each stage (Fig. 5B). Notably, *ago2-1A* expression was not detected in any of the developmental stages tested.

We were especially interested in the roles that the two *ago2* genes may play in mediating RNAi response. We co-injected preblastoderm embryos with ds*white*935 and dsRNAs homologous to *ago2-1* or *ago2-2*. We found that co-injecting dsRNA for *ago2-2* and ds*white935* led to a significant reduction (2.5-fold) in the frequency of the white-eye phenotype as compared to injecting ds*white*935 alone (Fig. 4B). In contrast, co-injecting dsRNA for *ago2-1* with ds*white*935 did not affect the white-eye phenotype (Fig. 4B). Examination of transcript levels confirmed that the *white* RNAi was still functional (Fig. 4C) even with the knockdown of *ago2-1* (Fig. 4D). In contrast, there

was a 3-fold suppression of the *white* RNAi in ds*ago2-2* and ds*white*935 co-injected embryos (Fig. 4C, D).

3.3.6 Expression analysis of two *ago2* genes in *Cx. quinquefasciatus*

The qPCR based gene expression analysis indicated 2-fold higher expression of *ago2-1* relative to *ago2-2* in the larvae and adult males (Fig. 5C). We observed 6- and 11-fold higher expression levels of *ago2-1* over *ago2-2* in pre- and post-bloodmeal ingested adult females, respectively (Fig. 5C). Furthermore, ovaries were dissected from adult females (2 days post blood meal) to determine whether the relative over-expression of *ago2-1* (compared to *ago2-2*) within adult females was germ-line specific. We found that *ago2-2* had a higher expression level (relative to *ago2-1*) in the ovaries. In contrast, *ago2-1* was expressed at a higher level (relative to *ago2-2*) in the carcass (Fig. 5D).

3.4 **Discussion**

Our phylogenetic analysis confirmed previously reported results indicating that genes involved in the siRNA and miRNA pathways were present in *Cx. quinquefasciatus* (Campbell *et al.*, 2008b) . Overall the small RNA biogenesis genes of *Cx. quinquefasciatus* resemble those of *D. melanogaster* however; there are some interesting differences. There are two *ago2* genes in *Cx. quinquefasciatus* (*ago2-1* and *ago2-2*) and only one *ago2* gene in *D. melanogaster*. Both *ago2-1* and *ago2-2* are expressed at all developmental stages in *Cx. quinquefasciatus*, however, *ago2-1* is expressed at relatively higher rate, than *ago2-2*, in the larvae, adult males, and females. Also there is an increase in a*go2-1* expression after a blood meal. Interestingly, this increased *ago2-1* expression is restricted to the somatic cells as the dissected ovaries from bloodfed females showed

higher expression of *ago2-2* instead of *ago2-1*. The exact reasons for this differential expression of *ago2* genes are not known, however, we speculate its role in regulating RNAi-mediated immune response in the *Cx. quinquefasciatus* females against possible viruses in the bloodmeal. Previously, *T. castaneum* is also reported to have two *ago2* genes where both play a role in RNAi (Campbell *et al.*, 2008b; Tomoyasu *et al.*, 2008).

The ScanProsite predicted domain architecture of Dcr1 and Dcr2 also differs between *Cx. quinquefasciatus* and *D. melanogaster*. The DmDcr1 lacks a N-terminal helicase domain, which is present in CuDcr1. Previous studis have shown that the same helicase domain is also present in TcDcr1, and CeDcr1. The presence of two ago2 genes and the domain architecture of Dcr proteins indicate that *Cx. quinquefasciatus* may have a different dsRNA-mediated RNAi response than in *D. melanogaster*. Similarly, it is also possible that *Cx. quinquefasciatus* RNAi components are more closely related to *T. castaneum* and *C. elegans* than to *D. melanogaster* (Tomoyasu *et al.,* 2008). However, a phylogenetic analysis indicates that the two TcAgo2 and CxAgo2 proteins grouped separately and thus may not be related. Overall, this might have important implications in the parental RNAi response in *Cx. quinquefasciatus.* Both *T. castaneum* and *C. elegans* have parental RNAi whereby dsRNA injected into haemocoel of the mother can silence expression of the zygotic genes (parental RNAi) (Bucher *et al.,* 2002). It would be interesting to test for the presence of parental RNAi response in *Cx. quinquefasciatus*, which can open new possibilities in functional genetic studies in this species.

We decided to target the *white*-eye pigmentation gene as an RNAi marker to determine if there was a functional endogenous RNAi pathway present in *Cx.*

quinquefasciatus. The *white* gene serves as an important genetic marker for many *Diptera* such as *D. melanogaster* and *Ae. aegypti*. It has been specifically used as a marker to study RNAi pathway in *D. melanogaster* (Misquita and Paterson, 1999; Kim *et al.*, 2007). In *D. melanogaster*, it is a common practice to introduce dsRNA into embryos to study genes involved in embryonic development. However, this same approach is lacking in mosquitoes and so limits the use of RNAi to study early developmental genes. We introduced dsRNA corresponding to the *white* gene of *Cx. quinquefasciatus* (ds*white*461) into the preblastoderm stage of *Cx. quinquefasciatus* embryos and screened hatched larvae and adults for any visible indication of interference of the *white* gene. We observed a white-eye phenotype at the larval and the adult stages of *Cx. quinquefasciatus*. Also, the frequency of the white-eye phenotype increased significantly with increases in the size (bp) of the ds*white* RNA used for microinjections. Previous studies in *D. melanogaster* have demonstrated that the frequency of the RNAi phenotype was proportional to the size of the dsRNA introduced to the embryo (Misquitta and Paterson, 1999; Yang *et al.*, 2000a). Furthermore, expression analysis confirmed that the white-eye phenotype was due to reduced levels of the *white* transcript. These results clearly indicate that injection of a sequence-specific dsRNA can induce the RNAi pathway in *Cx. quinquefasciatus*. Even though the frequency of the white-eye phenotype was low in adults, it was comparable to previously reported studies in *D. melanogaster* (Misquita and Paterson, 1999).

Mutagenesis and silencing of RNAi biogenesis genes has been used to study the functional role of RNAi genes and the role of RNAi in insect innate immunity (Dudley *et*

al., 2002; Keene *et al.,* 2004a; Wang *et al.,* 2006). We achieved sequence-specific knockdown of *ago2-1, ago2-2*, *dcr2* and *r2d2* using their respective dsRNAs for microinjection as determined by qPCR. To determine whether knockdown of RNAi genes could silence the RNAi pathway in *Cx. quinquefasciatus*, we co-injected ds*white*935 along with dsRNAs directed against *ago2-1, ago2-2*, *dcr2* or *r2d2*. Our results showed that knockdown of *ago2-2* reduced the frequency of the white-eye phenotype by ds*white*935. This indicated that *ago2-2*-mediated slicer activity was critical and indispensable to the RNAi pathway in *Cx. quinquefasciatus*.

In *D. melanogaster*, *dcr2* and *r2d2* are required for initiating the RNAi response brought about by exogenously introduced dsRNAs, and depleting either one can silence RNAi (Lee *et al.*, 2004; Wang *et al.*, 2006; Marques *et al.*, 2010). However, the same is not true for *Cx. quinquefasciatus*. The knockdown of *dcr2* and *r2d2* failed to reduce the efficiency of *white* RNAi in *Cx. quinquefasciatus*. The exact reason for this is not known. However, it is possible that *dcr2* or *r2d2* were not completely depleted in *Cx. quinquefasciatus* and that the residual activity of each was sufficient for proper RNAi activity (Liu *et al.,* 2003b).

There are two *dicer* genes in *D. melanogaster* and *Cx. quinquefasciatus*: *dcr1* (miRNA pathway) and *dcr2* (siRNA pathway). The *dcr1* gene product functions in processing pre-miRNA into mature miRNA (Lee *et al.,* 2004) while *dcr2* serves two functions, which include the dicing of long dsRNA into short siRNA and the loading of guide siRNA to the RISC complex (Lee *et al.,* 2004; Pham *et al.,* 2004). However, there appears to be functional redundancy between *dcr1* and *dcr2*, whereby *dcr1* can

independently load guide siRNA to RISC in the absence of *dcr2* (Lee *et al.,* 2004). It is possible that *dcr1* also plays a role in the siRNA pathway (especially in the absence of functional *dcr2*) (Lee *et al.,* 2004; Pham *et al.,* 2004). Furthermore, *C. elegans* contains only a single dicer protein (CeDcr1) that is involved in both miRNA and siRNA pathways (Bernstein *et al.,* 2001). The fact that the domain architecture of CpDcr1 is more similar to CeDcr1 than DmDcr1 suggests that CpDcr1 may also be involved in the RNAi pathway.

dsRNA binding proteins *r2d2* and *Loqs* associate with *dcr2* at various stages of the RNAi pathway (Liu *et al.*, 2003a; Pham *et al.*, 2004; Marques *et al.*, 2010). Depleting *r2d2* transcript levels in *Cx. quinquefasciatus* did not result in any differences in *white* RNAi efficiency. The exact reasons for this are not known. However, we propose the following two possible explanations: 1) *dcr2* acts independent of RNA binding proteins (*i.e.*, *r2d2* and *Loqs*) in processing and loading pre-siRNA into RISC; 2) another dsRNA binding protein (or *Loqs* working alone) takes over the functional role of loading guide siRNA to RISC (Liu *et al.,* 2003a).

3.5 Experimental procedures

3.5.1 Phylogenetic analysis of *Cx. quinquefasciatus* specific RNAi components

The *Cx. quinquefasciatus* genome has been sequenced and is publically available (www.vectorbase.org). Peptide sequences of RNAi genes were aligned from the following four insect species: *D. melanogaster*, *An. gambiae*, *Ae. aegypti* and *C. quinquefasciatus* (Campbell et al. 2008). Sequences were aligned using the T-Coffee software package (Notredame et al. 2000). The tree of phylogenetic relationships was

generated using the PHYLIP package (Felsenstein 1993). The computer-predicted ORFs of *ago2-1, ago2-2*, *dcr2, r2d2* and *white* genes were aligned to the genomic sequence. The position of introns/exons was mapped using BLAST (www.ncbi.nlm.nih.gov) and VectorNTI (www.invitrogen.com), which were confirmed by RT-PCR and PCR analysis.

3.5.2 dsRNA synthesis and microinjections

A colony of *C. quinquefasciatus* was reared according to published protocols (Pinkerton *et al.*, 2000; Allen *et al.*, 2001b). To synthesize dsRNA corresponding to *Cx. quinquefasciatus white* (ds*white*461 and ds*white*935), *dcr2*, *r2d2* and *ago2* (*ago2-1 and 2-2*) transcripts, gene-specific RT-PCR-amplified products were cloned into pJET 1.2 vector (Fermentas) using blunt end cloning. PCR primers with an attached T7 promoter sequence were used to generate a linear template, which was used to synthesize dsRNA using the MEGAscript RNAi kit (Ambion) according to the manufacturer's instructions. Approximately 100 pl of various dsRNAs (1.5ug/ul) were microinjected into preblastoderm embryos as described (Allen *et al.*, 2001b), except that the embryos were aligned horizontally and briefly incubated on a piece of filter paper. Injected embryos were released into water and allowed to develop to larval or adult stages under standard rearing conditions. The embryos co-injected with ds*white* and dsRNA to RNAi genes of interest were harvested 6-8 days after hatching (at the larval stage) for gene expression analysis using RT-PCR/qPCR. The *Cx. quinquefasciatus* embryos were injected with the dsRNA to the *luciferase* gene (ds*luc*) or *GFP* (ds*GFP*) as mock controls. The larvae or adults resulting from embryo microinjections were examined for any change in eye

pigmentation (at the larval or adult stage) as compared to uninjected (wild-type) or the mock-injected embryos.

3.5.3 RNA extraction and expression analysis

Total RNA was extracted from the hatched larvae or adults using TRIzol reagent (Invitrogen). A total of 2 µg of total RNA was reverse transcribed to complementary DNA (cDNA) using an oligodT primer and a SuperScript RT kit (Invitrogen) according to the manufacturer's instructions. Equal amounts of cDNA were used in each PCR amplification reaction, which used 2.5U *Taq* polymerase and the Triplemaster PCR system kit (Eppendorf). RT-PCR amplified products were fractionated on 1% agarose gels and stained with ethidium bromide. Real time (qRT-PCR) was performed using equal amounts of cDNA and SYBR green supermix (BioRad), according to previously published methods (Nolan *et al.,* 2006). qRT-PCR data were analyzed according to a previously validated technique (Pfaffl, 2001). All of the gene-specific primers used for RT-PCR and qPCR based expression analysis were designed outside of the region used to synthesize the dsRNA of interest.

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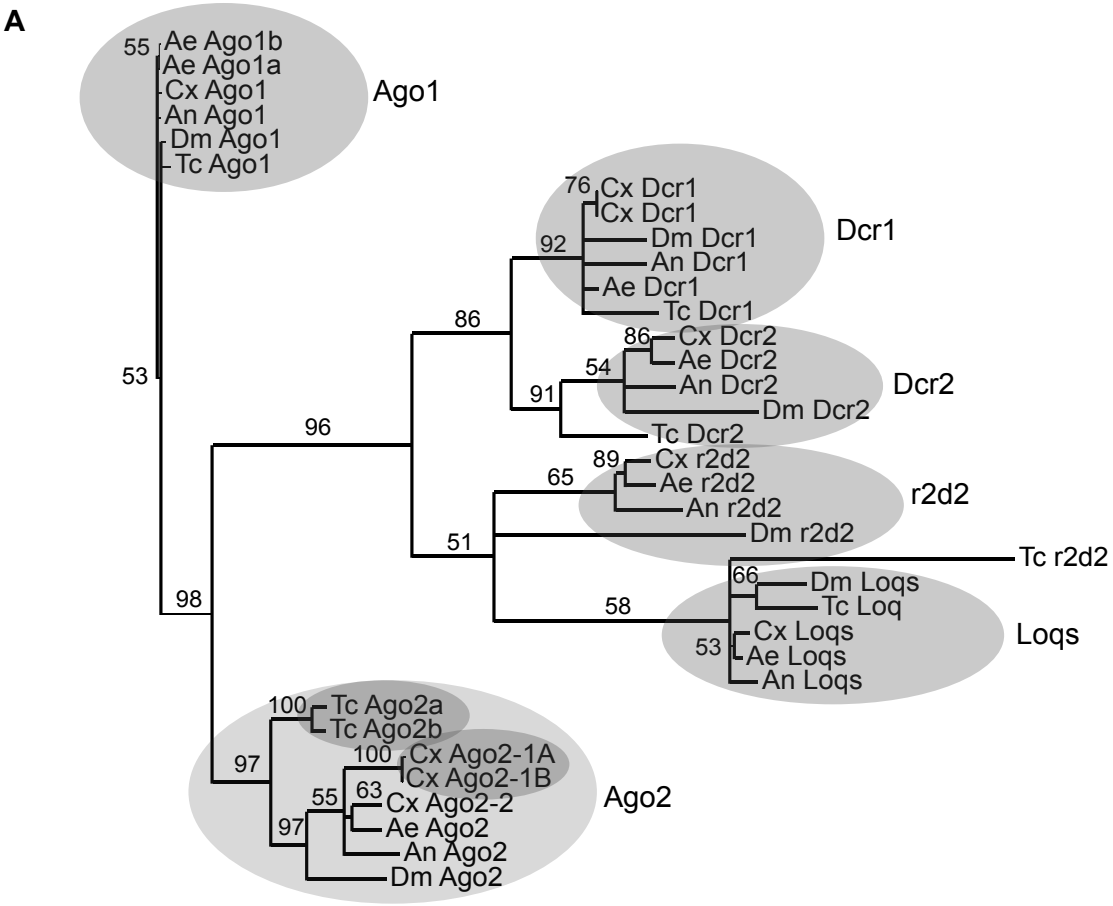
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Ago1
Cx Dcr1
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An Dcr1
Ae Dcr1
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Dm Dcr2
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Tc r2d2
Dm Loqs
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Ae Loqs
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Tc Ago2b
Cx Ago2-1A
Cx Ago2-1B
Cx Ago2-2
Ae Ago2
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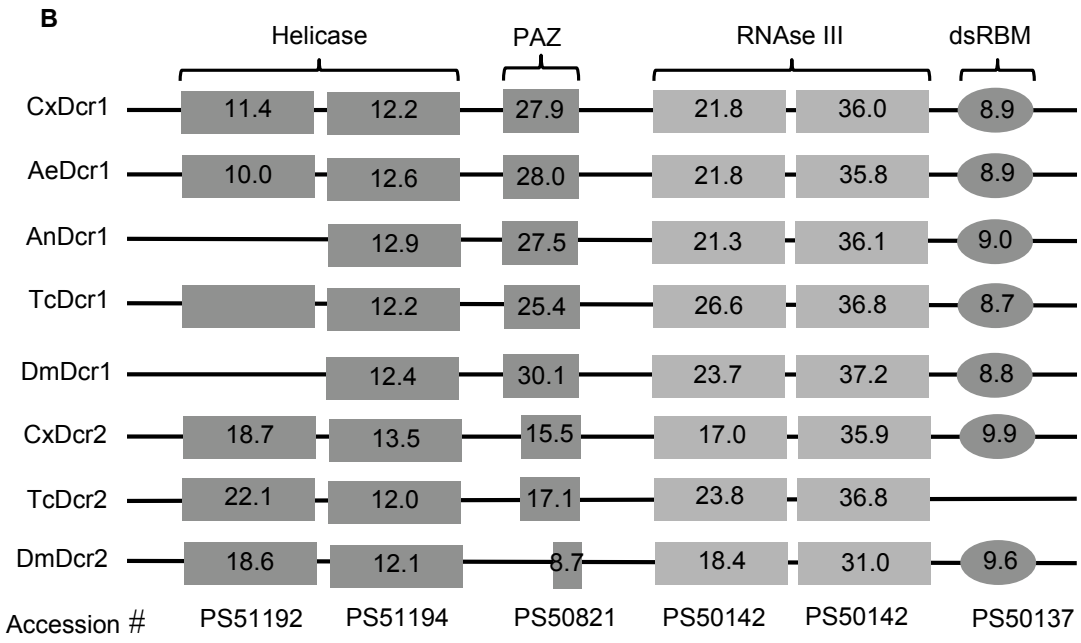

Figure 3.1. A phylogenetic analysis of the RNAi components in *Culex*. (A) Maximum likelihood tree of amino acid sequences of selected RNAi proteins from four insect genomes: *D. melanogaster*, *Ae. aegypti*, *An. gambiae,* and *C. quinquefasciatus*. Sequences were aligned using the T-Coffee software package (Notredame et al. 2000), and the tree of phylogenetic relationships was evaluated using TREE-PUZZLE (Schmidt et al. 2002). Numbers next to most nodes represent the percentage support of 100 bootstrap replicates. Bootstrap support of nodes supporting the Ago1 clade were too small to be drawn and are not shown. (B) The domain architecture of *Culex* Dcr1 (CpDcr1) has a N-terminal helicase domain and is more closely related to *Aedes* Dcr1 (AeDcr1) and *Tribolium* Dcr1 (TcDcr1) than to *Drosophila* Dcr1 (DmDcr1). The corresponding ScanProsite scores for each domain are shown.

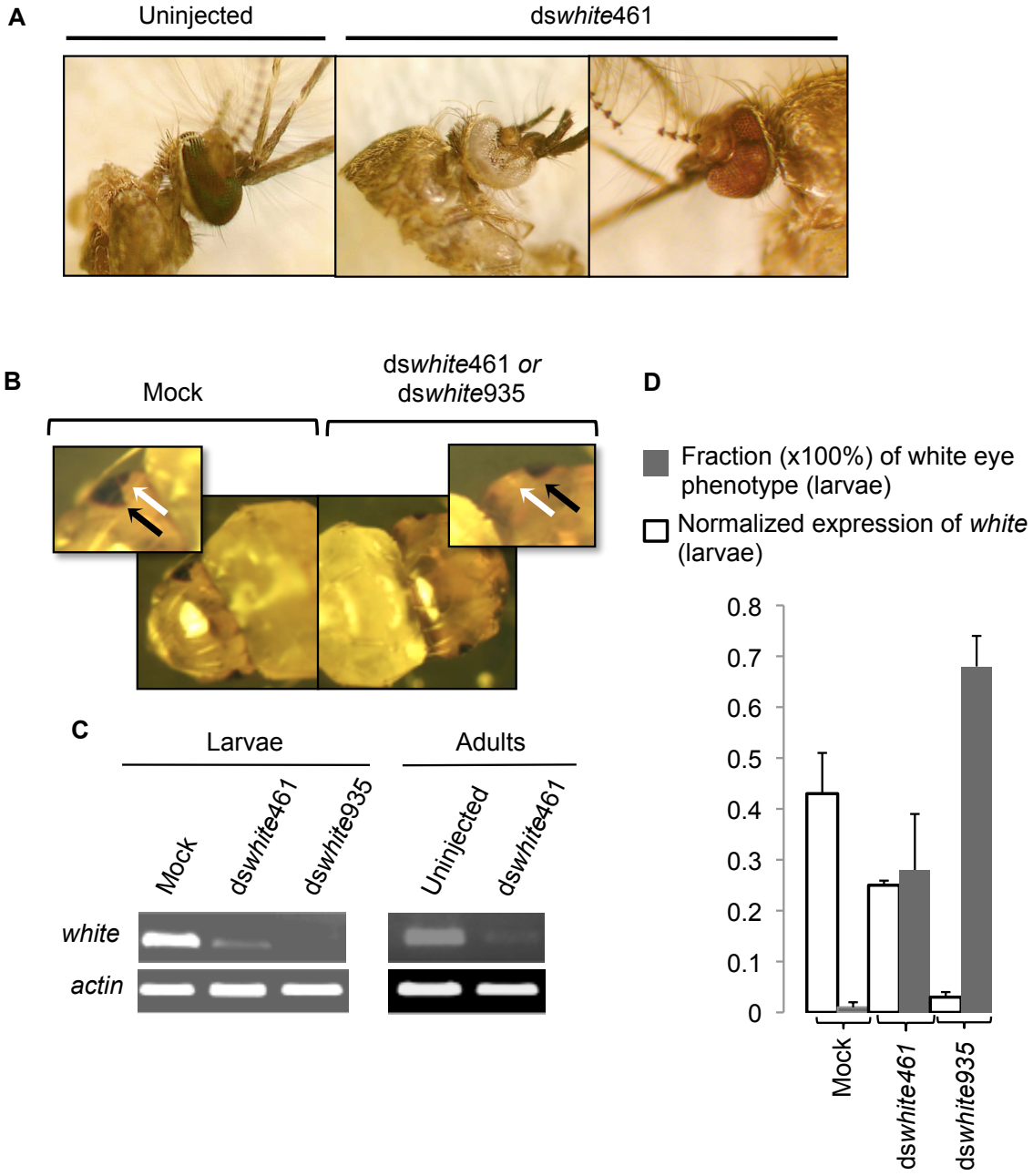
A
Uninjected
dswhite461
B
Mock
dswhite461 or
dswhite935
C
Larvae
Adults
Mock
dswhite461
dswhite935
Uninjected
dswhite461
white
actin
D
Fraction (x100%) of white eye phenotype (larvae)
Normalized expression of white (larvae)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Mock
dswhite461
dswhite935

Figure 3.2. Use of the *white* gene as an RNAi marker in *Culex*.
The *white* dsRNA (ds*white*461 or ds*white*935) injections into the preblastoderm embryos induced a white-eye phenotype in the: (A) larvae, and (B) adults. White and black arrows indicate simple eyes (ocella) and compound eyes of *Culex* larvae, respectively. The dsRNA of *luciferase* (ds*luc*) was injected as mock control. (C) Semi-quantitative RT-PCR confirmed the knockdown of the *white* gene in larvae and adults, which showed the white-eye phenotype. The *Culex actin* gene was used as a control. (D) Percentage of larvae showing the white-eye phenotype was increased when injected with ds*white*935 (935bp long dsRNA) as compared to ds*white*461 (461bp long dsRNA). The qPCR confirmed the corresponding decrease in expression of the *white* gene, in the ds*white*935 injections as compared to the ds*white*461 injections. The ds*luc* was injected as mock control. Gene-specific primers were used to perform Real-time RT-PCR and the data was normalized to *actin*. Error bars represent the standard deviation (SD) to the mean of three biological replicates.

Table 3.1. Primers used for dsRNA synthesis.

Gene	Primer	Sequence (5'-3')	Size (bp)	Region
ago2-1 (CPIJ014791)	Forward Reverse	GCATAGCTCCATCGGCAGCATAATG CAACTATCTCGCGCTGAACTTGACAAG	865	PAZ domain
ago2-2 (CPIJ009898)	Forward Reverse	CGGAACGAACCACGTGCTGAAG GTACGTGACCGATTGCAGCTCGTC	640	PIWI domain
dcr1 (CPIJ003169)	Forward Reverse	CAATCTGGTCACGCAGCACAGC GGTATTGTTGACCACCGCAGATCG	647	RNase III domain
dcr2 (CPIJ010534)	Forward Reverse	CACATGGAAGCAGGACCGTTGG GATTCACGCTTGTCCAGCTCAAACTG	654	Helicase
r2d2 (CPIJ011746)	Forward Reverse	CTGTCACGGAACTGCAGGAAATTTGC CGCTTGCAGTGTTTTCAAGATCAACATCTCG	594	RNA binding domain
White (CPIJ005542)	Forward Reverse	AACACCTGCTGAAAGAACGTGACCG CAGCAGATGCGGATCGGATCGGTGAG	461	ABC transporter
	Forward Reverse	CGCTACAGCTCCAGCAGCTACCC TCCGCCATCAGCAGTATCTTGTCG	935	ABC transporter

Table 3.2. Primers used for qPCR expression analysis.

Gene	Primer	Sequence (5'-3')	Size (bp)
ago2-1	Forward Reverse	CGATGGAGCTATGCCAGATT GTTCCATGATTTTTCGCTTCC	126
ago2-2	Forward Reverse	CAGCTGCTGCAATTCAAGAC CACTCTGGATCGCGTTCAGT	124
dcr2	Forward Reverse	CTTCTGTCATCCACAAGCAA ACCGTGTTTTCCGGTGTTAG	124
r2d2	Forward Reverse	CTGTCACGGAACTGCAGGAAATTTGC CCAGCTTCTCTGATGTCCGAGTTTTG	464
white	Forward Reverse	GTTCCTCTTCCTGACCAACATGAC CAAATAGTGGACGTAACCTGACTTGAG	232
actin	Forward Reverse	GTATCGTTCTGGATTCCGGAGATG CAGTGTTGGCGTACAGATCCTTACG	445

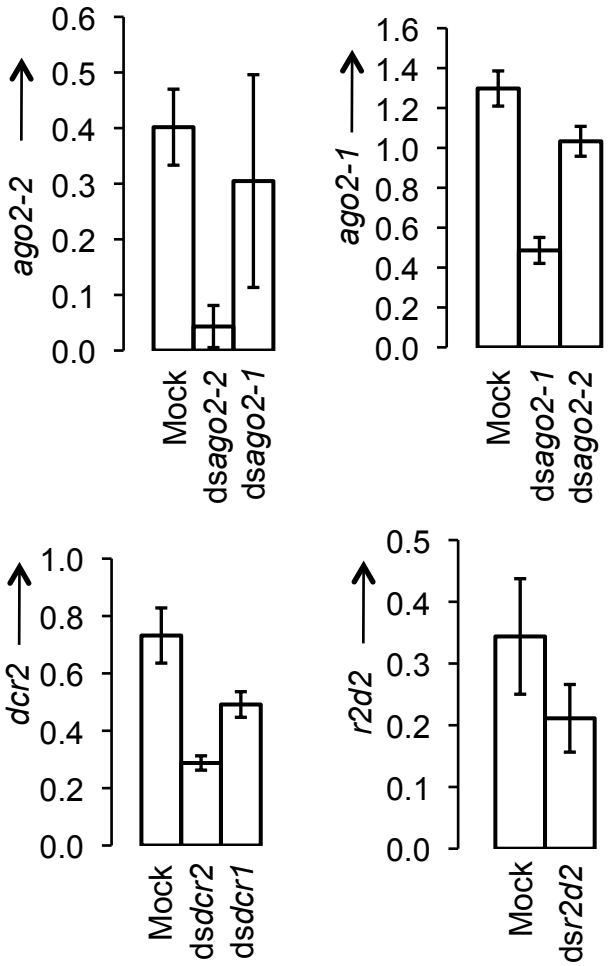

Figure 3.3. The reduced expression of *ago2-1*, *ago2-2*, *dcr2*, and *r2d2* genes following the injections with dsRNAs of *ago2-1*, *ago2-2*, *dcr2*, and *r2d2*, respectively. The expression of each gene was determined using qPCR and compared to the ds*luc* (mock)-injected control and normalized to *actin.* In each of the graphs, the y-axis represents the relative expression levels of the indicated RNAi genes.

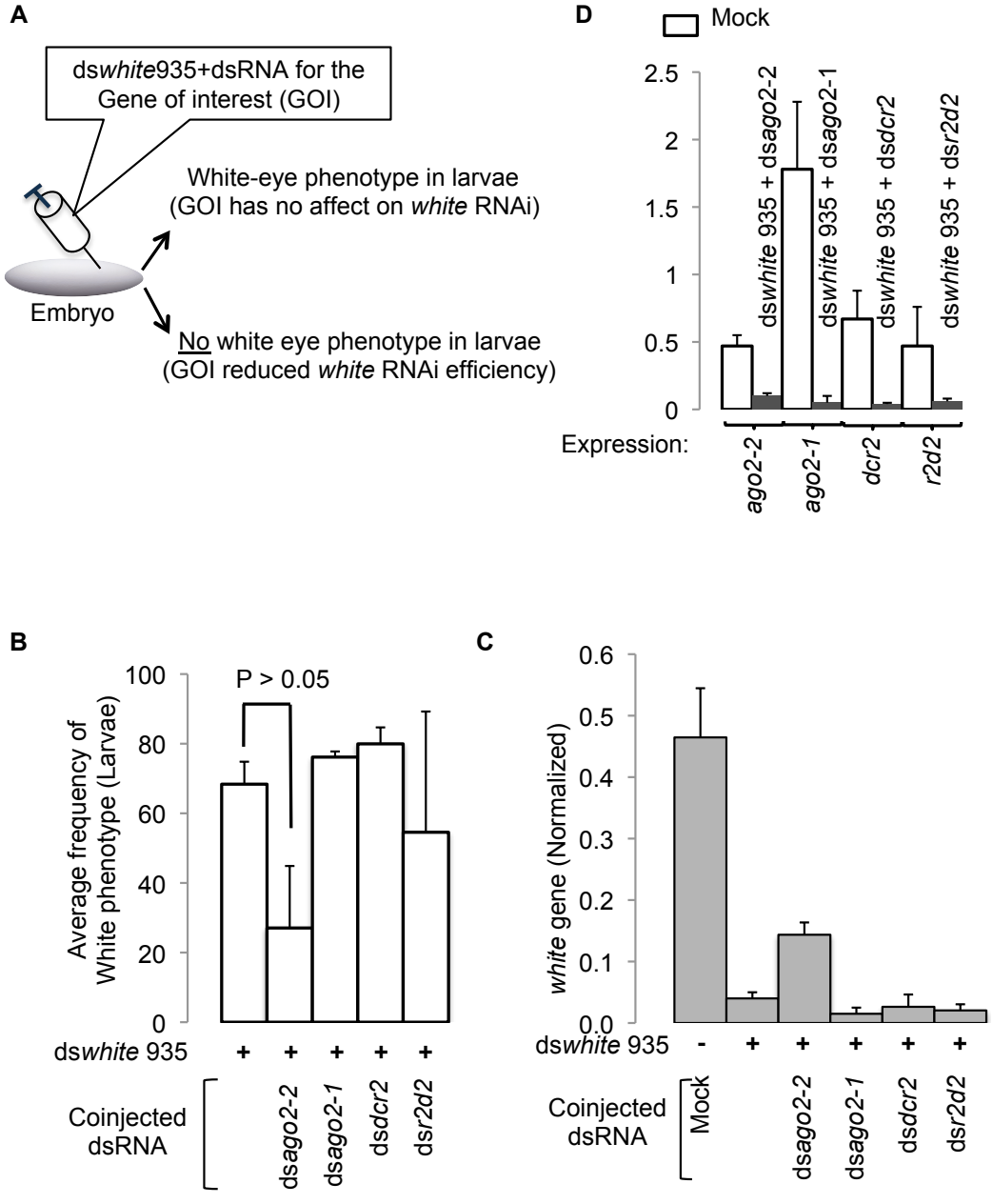
A
dswhite935+dsRNA for the Gene of interest (GOI)
White-eye phenotype in larvae (GOI has no affect on white RNAi)
Embryo
No white eye phenotype in larvae (GOI reduced white RNAi efficiency)
D
Mock
2.5
2
1.5
1
0.5
0
dswhite 935 + dsago2-2
dswhite 935 + dsago2-1
dswhite 935 + dsdcr2
dswhite 935 + dsr2d2
Expression:
ago2-2
ago2-1
dcr2
r2d2
B
P > 0.05
Average frequency of White phenotype (Larvae)
100
80
60
40
20
0
dswhite 935
Coinjected dsRNA
dsago2-2
dsago2-1
dsdcr2
dsr2d2
C
white gene (Normalized)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
dswhite 935
Coinjected dsRNA
Mock
dsago2-2
dsago2-1
dsdcr2
dsr2d2

Figure 3.4. Functional role of RNAi genes
(A) A schematic representation of the experimental approach used to assay for the RNAi genes. (B) Coinjecting ds*white*935 along with ds*ago2-2* led to reduction in frequency of the white-eye phenotype in the larvae. (C) This graph confirmed the suppression of *white* knockdown (suppression of RNAi) after coinjection of ds*white*935 and ds*ago2-2.* The y-axis represents the expression levels of the *white* gene determined by qPCR.
(D) The reduced expression of *ago2-1, ago2-2, dcr2 and r2d2* genes, following coinjection of ds*white* 935 and dsRNA specific to the RNAi genes (as indicated). Embryo injection of ds*luc* was used as the mock control. Gene-specific primers were used to perform qPCR and the data was normalized to *actin*. The graphs (B, C, and D) represent mean values from three biological replicates and SD is shown as error bars.

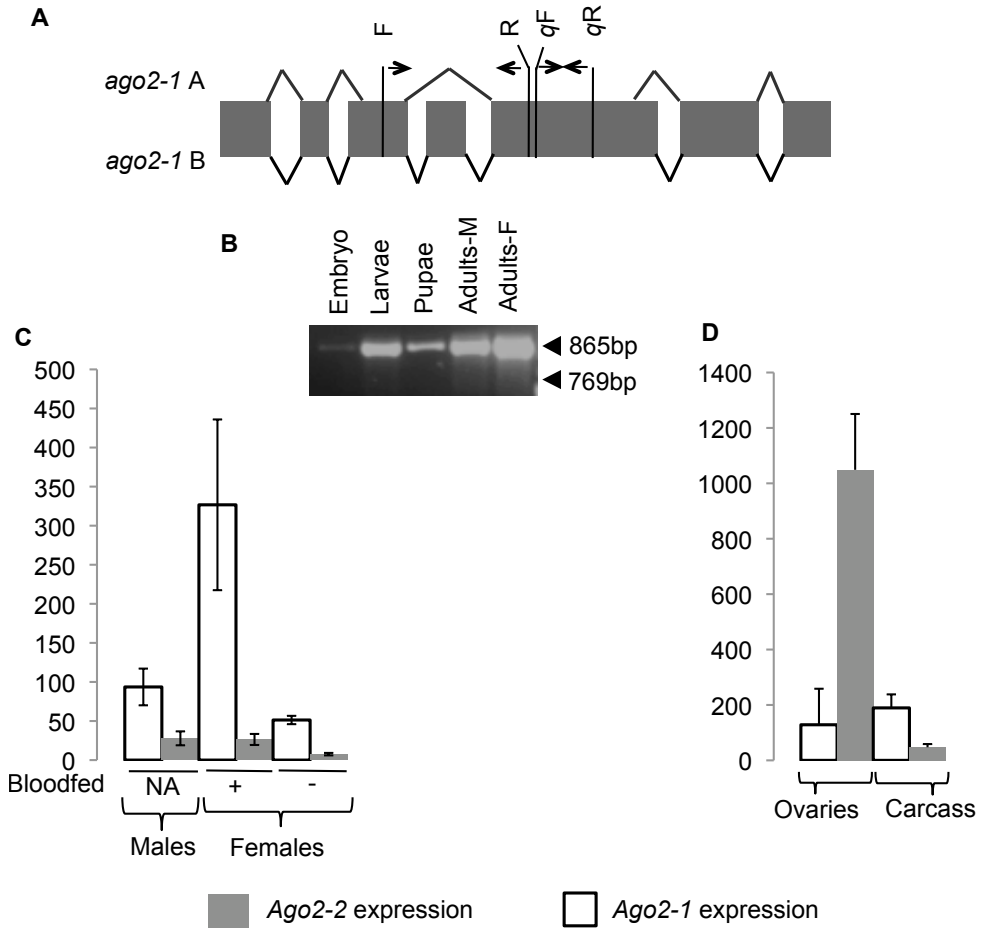

Figure 3.5 (A) Schematic representation of two alternatively spliced isoforms of *ago2-1* (*2-1A* and *2-1B*) in *Culex*. Forward and reverse primers used for RT-PCR (F/R) and qPCR (qF/qR) are indicated. (B) Expression of *ago2-1B* at various developmental stages: embryo larvae, pupae, adult males (M), and females (F)) of *Culex*, as indicated. The *ago2-1* specific (isoform non-specific) primers were used for RT-PCR. The position of expected *ago2-1A*- and *ago2-1B*-specific RT-PCR amplicons is indicated. The reaction products were analyzed on ethidium bromide stained 1.2 % agarose gel.
(C) The relative expression of *ago2-1* and *ago2-2* in the *Culex* larvae, adult males, bloofed- and non-bloodfed adult females. (D) The relative expression of *ago2-1* and *ago2-2* in the dissected ovaries and carcass. In each of the graphs, the y-axis represents the expression level of the indicated genes. The expression levels were determined using qPCR and the data was normalized to *actin.* Each graph is drawn from the mean value of two biological replicates and the error bars indicate SD.

Chapter 4:

Summary and Conclusions

The aims of this study were to 1) characterize the *cis*-acting elements that regulate *Herves* transposase-binding, 2) investigate the functionality of RNA interference (RNAi) in *Culex pipiens quinquefasciatus.*

Herves transposase binding to the _Herves_-L and R end sequences

We know that transposable elements (TEs) regulate genomic evolution and maintenance in various organisms (Hurst & Werren, 2001; Kidwell & Lisch, 2002) and that they serve as a 'genetic toolbox' for genome manipulation in insects. However, their potential usefulness is limited by their non-canonical integrations and low transformation rates (Atkinson, 2002; O'Brochta *et al.*, 2003; Arensburger *et al.*, 2005). In fact, the desirable features of TEs depend on their intended use. For example, post-integration genetic stability is essential for gene therapy and sterile insect technique (SIT), whereas the property of high mobility is desirable when TEs are used in insertional mutagenesis or as part of the gene drive system. In order to optimize TEs for various uses, it is critical to characterize the specific residues of the transposase that interact with the element and also the specific motifs to which the transposase binds.

The transposition process is regulated both by the *cis*-acting nucleotide sequences of the element as well as *trans*-acting host factors. However, the native *cis*-acting elements are not always optimized for higher transposition activity (Guynet *et al.*, 2009; Yang *et al.*, 2009). Investigation of this problem provides an opportunity to design new and improved gene vectors.

In this study we purified the *Herves* transposase using a bacterial expression system, according to the method of Zhou *et al.*, (2004). The purified transposase was then

used to investigate the *cis* elements that regulate *Herves* transposase binding. We found that the *Herves* transposase bound strongly to the subterminal and terminal sequences on the *Herves*-L and R, respectively. We observed the formation of three protein-DNA complexes on the *Herves*-L 1-100bp fragment and two complexes on the *Herves*-R 1-100bp fragment. The transposase specifically bound to *Herves*-L 28-48bp and R 15-23bp and 72-83bp.

In addition, mutational analysis identified the CGATTCAT sequence repeat as the most important transposase-binding motif. This conclusion is based on experimental results, which demonsrate that: 1) when we mutated the CGATTCAT sequence motif, the transposase no longer bound to the *Herves*-L and R ends of the element; 2) a synthetic tetramer of the CGATTCAT sequence motif was also sufficient for transposase binding; 3) the intact form of this sequence motif showed the binding, but splitting the motif in half eliminated transposase binding. This sequence motif was repeated and retained on both *Herves*-L and R end sequences of the element. Also, we found several sequence variants of the CGATTCAT sequence motif that appeared to regulate transposase binding.

We observed that none of the short (30bp) fragments, from either *Herves*-L or R, outcompeted the transposase binding to *Herves*-L or R 1-100bp probe. This indicates that there is cooperative binding between more than one motif on both *Herves*-L and R end sequences. Furthermore, we observed that the transposase failed to bind with L-TIR. An EMSA experiment with *Herves*-L 1-30bp fragment containing the L-TIR failed to produce any band of retarded mobility that would indicate transposase binding. It is

possible that an unidentified host factor can bind to the L-TIR. However, nuclear proteins from *Herves* transposase-expressing *D. melanogaster* S2 cells also failed to bind with L-TIR. This could suggest two possibilities: 1) The L-TIR sequence does not by itself interact with the *Herves* transposase or any of the host factors, or 2) The host factor that interacts with L-TIR is absent in *D. melanogaster*. Although it did not bind to L-TIR, the transposase does bind strongly to R-TIR. The *Herves*-R 1-30bp fragment produced two protein-DNA complexes, possibly due to two potential transposase-binding sites within the *Herves*-R 1-30bp fragment: the R-TIR and CGATTCAT binding motifs present at 15-23bp. Mutating the R-TIR or CGATTCAT binding motif eliminated binding, which indicates that cooperative binding is occurring between these regions.

Host factors also play an important role in regulating TEs (Sewitz *et al.*, 2003; Zayed *et al.*, 2003). Various host factors in *D. melanogaster*, *Ae. aegypti* and *An. gambiae* have been shown to interact with *Herves*-L and R end sequences (Perumalsami *et al.,* 2010). Some of the host proteins that interact with *Herves* include CHK-like proteins and Cyclophilin B (Perumalsami *et al.,* 2010). However, the exact binding motifs for these host proteins are not known. *Herves* has an unusually long L end sequence (relative to its R end sequence) with several different short sequence-repeats. We can expect that these sequence-repeats within the L and R ends serve as the motifs for host factor binding. Previous studies have shown that short sequence repeats are responsible both for transposase-binding and for binding with host factors.

The large native size of an element is known to cause lower transposition rates. Thus, carefully pruning the size of an element to minimum *cis* requirement can lead to

increased transposition rates (Li *et al.*, 2001). Similarly, the relatively longer size of *Herves*-L can possibly be the reason for its low rate of transposition. This study provides a basis for determining the minimum *cis* requirement for *Herves* transposition and for investigating the possibility of increasing its transposition rate. It would be interesting to design deletion derivatives according to these binding motifs and then test them for increased transposition mobility in *An. gambiae.* However, determining the minimum *cis* requirement of an element is complex; it needs careful consideration of the sequencing and spacing between the relevant binding motifs (Li *et al.*, 2005).

RNA interference (RNAi) in *Culex quinquefasciatus*

With recent breakthroughs in sequencing technology, more and more insect genomes are being sequenced (Holt *et al.*, 2002; Nene *et al.*, 2007). Efficient functional genetic tools are needed to analyze the wealth of genetic information from these sequencing projects. RNAi has proven to be a powerful tool for studying functional genetics in various organisms. Furthermore, the RNAi has also shown to play an important role in antiviral innate immunity in insects and can be manipulated to disrupt the vector-virus interaction to control spread of vector-borne diseases.

RNAi has been well studied in model organisms, such as *D. melanogaster*, and has increasingly been applied to mosquitoes (Blandin *et al.*, 2002; Brown *et al.*, 2003; Carthew, 2003; Carthew and Sontheimer, 2009; Kim *et al.*, 2010). However, our understanding of the RNAi mechanism in mosquitoes is so far limited.

Many mosquito species, including *Cx. quinquefasciatus*, transmit human parasites that cause diseases such as lymphatic filariasis, and Saint Louis encephalitis. Although

the *Cx. quinquefasciatus* genome has been recently sequenced, RNAi mechanism has not been studied. The research presented here addresses this issue and highlights important differences in dsRNA-mediated RNAi response between *Cx. quinquefasciatus* and *D. melanogaster*.

Previous studies have shown and our phylogenetic analysis has confirmed that RNAi genes are present in *Cx. quinquefasciatus* (Campbell *et al.*, 2008). Generally, *Cx. quinquefasciatus* RNAi genes are similar to those of *D. melanogaster*. However, in contrast to *D. melanogaster*, *Cx. quinquefasciatus* has two ago2 genes: *ago2-1* and *ago2-2* (Campbell *et al.*, 2008). We found that the *ago2-1* gene has two alternatively-spliced isoforms: *ago2-1A* and *ago2-1B*, but only *ago2-1B* is expressed in all developmental stages (embryo, larvae, pupae, and adult) of *Cx. quinquefasciatus*. Expression of *ago2-1B* varies at each developmental stage. Real-time PCR analysis shows higher expression of *ago2-1* as compared to *ago2-2* in the larvae, adult males, and females. The expression of *ago2-1* increases in females after a blood meal; however, the *ago2-2* is expressed more (relative to *ago2-1*) in the ovaries of blood-fed females. Overall, our expression analysis indicates that key RNAi components (*ago2-1B*, *ago2-2*, *dcr2* and *r2d2*) are expressed at all developmental stages of *Cx. quinquefasciatus* (embryo, larvae, pupae and adults). These results strongly suggest the presence of a fully functional RNAi mechanism in *Cx. quinquefasciatus*.

ScanProsite analysis showed that the domain architecture of CxDcr is different from DmDcr in that an additional N-terminal helicase domain was present in CxDcr1. Also, DmDcr2 lacked a fully functional PAZ domain whereas, based on the ScanProsite

result, it is present in CxDcr2. This indicates that the dsRNA-mediated RNAi response may differ between *Cx. quinquefasciatus* and *D. melanogaster* and that further validation of these results is required. Our experimental data do show that in contrast to *D. melanogaster*, *dcr2* and *r2d2* are not required for the RNAi response in *Cx. quinquefasciatus* (see below).

Previous studies have shown that *T. castaneum* also possesses two *ago2* genes (ago2-1A and ago2-1B) and that both *T. castaneum* and *C. elegans* have an additional N-terminal helicase domain. This indicates that the RNAi components in *Cx. quinquefasciatus* are more similar to *T. castaneum* than to *D. melanogaster*. However, at present there is no experimental data to support these claims.

To further investigate whether RNAi can be used as a tool for functional genetic studies, we used the *white* eye color gene as an RNAi marker. The *white* gene belongs to the ATP-binding cassette (ABC) transporter protein family, which is responsible for trans membrane transport of eye pigments and hence for proper eye pigmentation (Ewart & Howells, 1998; Mackenzie *et al.*, 1999). Double-stranded RNA to the *white* gene (ds*white*) was injected into the preblastoderm embryos. The injected ds*white* was expected to trigger an RNAi-mediated knockdown of the *white* gene, which could be seen as a discoloration of eye pigments in the hatched larvae and adults (the white-eye phenotype). Introducing ds*white* into the embryos led to the white-eye phenotype in the hatched larvae and adults, as expected. In the larvae the white-eye phenotype consisted of complete discoloration of pigmentation in the ocella whereas in adults the *white* RNAi phenotype produced eye color varying from brown to completely white. The frequency of

the white-eye phenotype in the larvae was proportional to the size of *white* dsRNA injected. Similarly, previous studies in *D. melanogaster* have shown that RNAi efficiency depends upon the size of the dsRNA trigger (Yang *et al.*, 2000). These results, along with the expression analysis of the *white* gene, confirmed that the white-eye phenotype is specifically due to knockdown of the *white* gene.

Silencing components of RNAi pathway can reduce the efficiency of the RNAi response. This approach has been used to study RNAi pathway and also to understand its role in innate immunity of insects (Dudley *et al.*, 2002; Wang *et al.*, 2006). To determine the functional role of *Cx. quinquefasciatus*-specific *ago2-1*, *ago2-2*, *dcr2* and *r2d2* in RNAi, we coinjected ds*white*935 (a 935bp long ds*white*) along with dsRNA to each of the RNAi genes into *Cx. quinquefasciatus* embryos. We found that coinjecting ds*ago2-2* led to significant (2.5-fold) reduction in white-eye phenotype frequency. On the other hand, coinjecting ds*ago2-1*, ds*dcr2*, and ds*r2d2* failed to reduce *white* RNAi efficiency. Our quantitative expression analysis indicates that the knockdown of *ago2-2* reduced the *white* gene knockdown effect, suggesting that *ago2-2* is critical for RNAi functioning. Overall, the results presented here indicate that the key components involved in small RNA (siRNA) biogenesis are expressed in all developmental stages of *Cx. quinquefasciatus*. Also, the knockdown of *ago2-2* effectively silences RNAi and thus is indispensable for dsRNA-mediated RNAi response. This maks this gene a key target for manipulation of the RNAi pathway. This study also highlights some of the important differences between the RNAi response in *Cx. quinquefasciatus* and *D. melanogaster*; these differences need further experimental validation.

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