

**Exogenously-introduced Homing Endonucleases Catalyze Double-stranded
DNA Breaks in *Aedes aegypti***

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ABSTRACT

Aedes aegypti transmits the viruses which cause yellow fever, dengue fever, and dengue hemorrhagic fever. Homing endonucleases are selfish genetic elements which introduce double-stranded DNA (dsDNA) breaks in a sequence-specific manner. In this study, we aimed to validate a somatic assay to detect recombinant homing endonuclease (rHE)-induced dsDNA breaks in both cultured cells and adult female *Ae. aegypti*. While the cell culture-based two plasmid assay used to test rHE ability to induce dsDNA breaks was inconclusive, assays used to test rHEs in *Ae. aegypti* were successful. Recognition sequences for various rHEs were introduced into *Ae. aegypti* through germline transformation, and imperfect repair at each of these exogenous sites was evaluated. In mosquitoes containing a single exogenous HE site, imperfect gap repair was detected in 40% and 21% of clones sequenced from mosquitoes exposed to I-*PpoI* and I-*SceI*, respectively. In mosquitoes containing two exogenous HE sites flanking a marker gene (EGFP), 100% of clones sequenced from mosquitoes exposed to I-*PpoI*, I-*CreI*, and I-*AniI* demonstrated excision of EGFP. No evidence of EGFP excision or imperfect repair at any HE recognition site was detected in mosquitoes not exposed to a rHE. In summary, a somatic genomic footprint assay was developed and validated to detect rHE or other meganuclease-induced site-specific dsDNA breaks in chromosomal DNA in *Ae. aegypti*.

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Chapter 1

LITERATURE REVIEW

Public health impact

Approximately half of the world's population lives in areas at risk for contracting malaria and dengue fever. Approximately 200 million cases of malaria are diagnosed resulting in two million deaths each year (Gubler, 1998). There are an estimated 50-100 million estimated cases of dengue fever and 500,000 cases of dengue hemorrhagic fever reported yearly (Gubler, 1998). Current methods to control the spread of these diseases, such as insecticides and anti-malarials, at present, have not been sustainable on a large scale for eradication.

As reviewed in Calisher (1994), arthropod-borne viruses (arboviruses) are transmitted by a hematophagous arthropod, mosquitoes often being vectors. Arboviruses are maintained in nature by a transmission cycle that involves an infected mosquito and a susceptible vertebrate host. The mosquito transmits the virus during blood feeding and if the vertebrate host becomes infected and is sufficiently viremic, can infect other mosquitoes and in some cases humans. Humans are usually dead-end or tangential hosts that do not influence the maintenance and dissemination of arboviruses. Two major exceptions are dengue virus and yellow fever virus, both of which are transmitted by *Aedes aegypti* (Monath, 1994). Dengue fever and dengue hemorrhagic fever are caused by four different virus serotypes (DEN 1, 2, 3, and 4). While infection with one virus serotype provides life long immunity, there is no cross protection for other virus serotypes (Rigau-Perez *et al.*, 1998). Yellow fever virus also causes hemorrhagic fever and affects as many as 200,000 each year despite the availability of a vaccine (Monath, 2001).

For both dengue and yellow fever, control of mosquito populations remains critical to prevent the spread and prevalence of these diseases. Insecticide resistance, drug resistance, and lack of funding for surveillance and prevention programs have contributed to the lack of success for control measures. In addition, demographic changes (Gubler, 1998), like the rapid increase in the human population resulting in increased urbanization and a lack of infrastructure in both governments and health care systems also contribute to the continuing spread and increase of vector-borne diseases (Sutherst, 2004). Understanding vectors at a genetic level may help

provide insight on how to control their population. Insecticide discovery, innate immune responses, genome evolution, and genetic manipulation of mosquitoes would all benefit from increased knowledge of vectors at a genetic level. The ability to induce site-specific double-strand DNA (dsDNA) breaks will allow for the study of how dsDNA breaks are repaired, the study of gene function through targeted gene disruption, and finally, as a means for genetic control of mosquitoes.

Double-strand DNA breaks and repair of breaks

DNA is continuously exposed to damaging agents that induce dsDNA breaks, such as ultraviolet light, natural and man-made mutagenic chemicals, and reactive oxygen species due to ionizing radiation (Jackson, 2002). If dsDNA breaks are not repaired, cell death will occur (Pastink, 1999). dsDNA break repair may be responsible for genome organization and it has also been speculated that the mechanism of dsDNA break repair used between species may contribute to the evolution of eukaryotic genome size (Kirik, 2000).

There are two well characterized repair mechanisms cells can use to repair dsDNA breaks: homologous recombination or non-homologous end joining. When cells use homologous recombination to repair dsDNA breaks, the corresponding sequence on the other chromosome is used as a template for DNA repair. This process is called gene conversion and alleles on corresponding chromosomes can be duplicated. DNA duplications can contribute to an increase in gene copy number which can provide new combinations of genes capable of different functions (Seoighe, 2000). Insecticide resistance in mosquitoes is an example of how a genetically inherited trait that occurs due to a mutation or gene duplication allows for enhanced detoxification of an insecticide. Selection would increase the survival probability of a resistant genotype (Coleman and Hemingway, 2007). In *Culex*, esterase-based resistance to organophosphates is due to a gene duplication event of the *est α* and *est β* genes which are involved in detoxification (Hemingway and Ranson, 2000).

The second repair mechanism cells can utilize is non-homologous end joining, also known as illegitimate recombination which does not require homologous sequences for dsDNA breaks to be repaired (Moore, 1996). Non-homologous end joining is non-conservative, meaning nucleotides can be deleted or added because free ends are repaired using base pairing between single stranded DNA, resulting in junctions with microhomology (Moore, 1996). During repair of a dsDNA break via non-homologous end joining, the original ends can be ligated together so there is no change in the arrangement on the chromosome; however, if free DNA segments from different chromosomes are ligated, then a chromosomal rearrangement is produced. Rearrangements include deletions, duplications, inversions, or translocations of DNA segments (Griffiths, 1999). Rearrangements can be balanced or imbalanced. In balanced rearrangements genes are rearranged, but DNA is not deleted or duplicated. Inversions, where an internal segment of DNA is cleaved, reversed, and ligated back into the chromosome, and reciprocal translocations which occur when segments from two non-homologous chromosomes switch locations are also types of balanced rearrangements. In imbalanced rearrangements, DNA is deleted or duplicated. Deletions occur when there are multiple breaks and a gene or segment of DNA is lost (Griffiths, 1999) and the two segments flanking the deleted DNA are ligated. Duplications occur when multiple copies of a DNA segment are ligated.

The ability to induce site-specific dsDNA breaks will allow for the study of repair mechanisms since dsDNA break repair pathways in mosquitoes have yet to be characterized. Also, the ability to induce site-specific dsDNA breaks is important for targeted gene disruption (Paques and Duchateau, 2007).

Homing endonucleases

Homing endonucleases are an ancient class of selfish genes which have been identified in Eubacteria, Archaea, and Eukaryota. They are DNA meganucleases (Marcaida *et al.*, 2008) which are able to recognize large target sites (14-40 base pair sequences) and catalyze a dsDNA break at their recognition site (Chen and Zhao, 2005). After a dsDNA break, homing endonucleases use a gene conversion event for self-propagation (Gimble, 2001). An allele containing a homing endonuclease gene cleaves the corresponding allele without a homing endonuclease gene at its recognition site. The dsDNA break is then repaired using homologous

recombination using the homing endonuclease gene containing allele as a template for repair. Homing endonucleases do not appear to offer any advantage for the host organism yet have persevered over millions of years. Homing endonucleases are encoded by group I or II introns or inteins (Jurica and Stoddard, 1999) or can be free-standing (Edgell, 2005). Group I introns and inteins are self splicing and have survived because they are spliced out of the mature mRNA so that the gene is not disrupted (Caprara and Waring, 2005). Group II introns are ribonucleoproteins that mobilize when the intron reverse splices into its target site and is reverse-transcribed by the intron-encoded protein (Lambowitz *et al.*, 2005). They are similar to intron encoded endonucleases except that in the case of an endonuclease, cleavage and insertion sites can be hundreds of base pairs away compared to intron insertion cleavage sites which are usually 2-25 bp apart (Edgell, 2005). Homing endonucleases can be separated into four families: LAGLIDADG, His-Cys Box, GIY-YIG, and HNH, each of which is based on a conserved amino acid sequence motif (Jurica and Stoddard, 1999).

Homing endonucleases, in particular I-*SceI* (*Saccharomyces cerevisiae*), have been used extensively in *Drosophila melanogaster* to study dsDNA break repair and also as a tool for gene targeting which is when an endogenous gene is altered using homologous recombination between an exogenously introduced DNA and the endogenous target (Gong and Golic, 2003a). Bellaiche *et al.* (1999) used transgenic *Drosophila* males expressing I-*SceI* in their germ line to induce dsDNA breaks at I-*SceI* recognition sites flanking a gene of interest which resulted in the partial or complete deletion that gene. Rong and Golic (2000) performed gene targeting in *Drosophila* using P-element transformation followed by a site-specific recombinase (FLP) and I-*SceI* to induce dsDNA breaks *in vivo* by generating an extrachromosomal DNA fragment within the gene of interest. Takeuchi *et al.* (2007) developed a system to introduce up to 28 kb of foreign DNA into a transgene through homologous recombination by inducing dsDNA breaks using I-*SceI*.

I-*SceI* has also been shown to successfully induce dsDNA breaks in mouse cells (Rouet *et al.*, 1994) and in *Xenopus* oocytes (Segal and Carroll, 1995). Homing endonucleases I-*PpoI* (*Physarum polycephalum*) and I-*CreI* (*Chlamydomonas reinhardtii*) have been shown to successfully induce dsDNA breaks in plasmids containing their recognition sites and also in the

28S rDNA in human cells, which was the first report of I-*PpoI* being expressed in mammalian cells (Monnat *et al.*, 1999). Windbichler *et al.* (2007) also used a two plasmid based system in which a homing endonuclease was exogenously expressed along with a target plasmid containing homing endonuclease recognition sites to demonstrate that I-*SceI* and I-*PpoI* were able to cleave their respective recognition sites at a high efficiency in *Anopheles gambiae* cells and embryos. They observed both homologous and non-homologous repair at dsDNA breaks and proposed the use of I-*PpoI* as a way in which to bias the mosquito sex ratio towards males, since there is an I-*PpoI* recognition site in the 28S rDNA on the X chromosome (Windbichler *et al.*, 2007).

In addition to homing endonucleases being used to study dsDNA break repair mechanisms, they may serve as a tool targeted gene disruption which requires the induction of site-specific dsDNA breaks. If a homing endonuclease was modified such that it was able to target a pre-existing sequence in a gene, then it may be possible to disrupt the targeted gene. This approach can provide information on the function of genes, a physiological context for how and when genes are used, and a tool for the manipulation of the genome (Porteus and Carroll, 2005). Currently, the success rate of gene targeting in organisms using exogenous DNA is limited, but research suggests that inducing a dsDNA break can increase the frequency of homologous recombination by several orders of magnitude (Porteus and Carroll, 2005). This is especially important for mosquitoes, because currently genes cannot be selectively knocked out in order to study their functions.

Homing endonucleases as a tool for gene targeting

In order for homing endonucleases to be useful as a tool for gene targeting, a pre-introduced target site is required. Alternatively, the homing endonuclease must be modified to recognize alternative sites and induce a site-specific dsDNA break in an endogenous gene. There are many described homing endonucleases, but the probability of a genome containing a recognition site is rare due to the length and complexity of the site. Fusing DNA binding domains to nucleases has been done so that specificity can be given to a nonspecific nuclease (Arnould *et al.*, 2006; Smith *et al.*, 2006; Steuer *et al.*, 2004). An alternative approach is to design an artificial homing endonuclease with a novel recognition site (Beumer *et al.*, 2006; Kim *et al.*, 1996; Lloyd *et al.*, 2005; Moehle *et al.*, 2007; Morton *et al.*, 2006; Porteus and Carroll, 2005; Santiago *et al.*, 2008).

Alteration of homing endonuclease cleavage specificity

The use of “designer” homing endonucleases is a growing field of interest. If a homing endonuclease could be designed to recognize a sequence in an endogenous gene, then an induced dsDNA break at the sequence could be used for site specific gene disruption. Doyon *et al.* (2006) developed a high throughput screen to identify alternative homing endonuclease recognition sites. Using their system, they identified a mutant I-*SceI* recognition site that was favored over the wild-type recognition site. I-*CreI* variants have been engineered so that the specificity was altered to recognize a novel sequence by altering the DNA binding domains within the conserved core structure of the protein (Smith *et al.*, 2006). Other research has focused on single amino acid residue changes that lead to altered sequence specificity (Seligman *et al.*, 2002). Arnould *et al.* (2006) found that novel homing endonucleases could be derived from wild-type homing endonucleases while maintaining high specificity and a high frequency in cutting by changing the whole subdomain. Ashworth *et al.* (2006) redesigned the specificity of I-*MsoI* (*Monomastix sp.*) cleavage resulting in binding and cleaving of a redesigned target site 10,000 times more efficiently compared to the wild-type I-*MsoI*. Using a computational model to incorporate packing, hydrogen bonding, and other interactions involved with sequence specificity and DNA binding, each of the side chains of I-*MsoI* was altered so that the redesigned I-*MsoI* could recognize a new target sequence.

Other protein engineering approaches focus on fusing different homing endonuclease DNA binding domains. I-*DmoI* (*Desulfurococcus mobilis*) and I-*CreI* domains were fused to create E-*DreI* (Chevalier *et al.*, 2002) and also *DmoCre* (Epinat *et al.*, 2003). In both cases, the chimeric proteins were able to cleave as efficiently and frequently as the wild-type enzyme. Epinat *et al.* (2003) also used an engineered single chain I-*CreI* variant (scI-*CreI*) which had similar enzymatic properties as wild type I-*CreI*. Exchange of DNA binding domains from different nucleases has also been used successfully. The binding domain from the *Candida tropicalis* (Ctr) VMA1 intein, which is inactive and has no nuclease activity, was exchanged with the binding domain from PI-I-*SceI* resulting in an active Ctr endonuclease (Steuer *et al.*, 2004).

Zinc finger nucleases

Zinc fingers were first described in 1986 (Diakun *et al.*, 1986) and are DNA binding domains contained within the most common family of transcription factors in eukaryotes (Porteus and Carroll, 2005). Zinc-finger nucleases use heterologous zinc-finger DNA binding domains designed to bind a target recognition site that is fused to the *FokI* nuclease domain (Santiago *et al.*, 2008). Each zinc finger binds a 3-bp target site independent of one another. Zinc fingers can be altered so that each finger recognizes specific 3-bp targets to allow for a change in the binding specificity. Additionally each nucleotide interacts with a single amino acid side chain and altering each amino acid residue could change the specificity for each individual finger (Porteus and Carroll, 2005). *FokI* has been fused to the *Drosophila melanogaster* homeobox domain, with a zinc finger DNA binding domain and with the yeast Gal4 DNA-binding domain, all of which demonstrated that DNA cleavage could be altered using a chimeric nuclease (Kim *et al.*, 1996; Kim and Chandrasegaran, 1994).

Zinc-finger nucleases have been used as an efficient method for targeted mutagenesis in a variety of organisms such as *Arabidopsis* (Lloyd *et al.*, 2005), humans (Moehle *et al.*, 2007; Porteus and Baltimore, 2003), CHO-S Chinese hamster cells (Santiago *et al.*, 2008), *Drosophila* (Beumer *et al.*, 2006), *Caenorhabditis elegans* (Morton *et al.*, 2006), and *Xenopus laevis* oocytes (Bibikova *et al.*, 2001). Maeder *et al.* (2008) also used zinc finger nucleases as a tool for targeted gene disruption and developed a publicly available platform for engineering zinc-finger arrays called OPEN (Oligomerized Pool Engineering).

Genetic control- population suppression

While meganucleases have been used in other organisms for studying dsDNA break repair and as a means for targeted gene disruption or knock down, they could also be used in a mosquito as a part of a mechanism for gene drive of anti-pathogen genes into a mosquito population. Taking advantage of gene conversion initiated by homing endonuclease-induced dsDNA breaks could provide a new genetic control strategy. The idea of genetic control of insects is not a new concept and was first proposed in the 1940's and picked up momentum in the 1950's when the United States Department of Agriculture (USDA) started an eradication program against the screwworm fly by releasing millions of sterile males (Gould, 2004). The idea behind release of

sterile males, a procedure called the sterile insect technique, is that females will mate with a male but will not produce any viable progeny so the target insect population will decrease. Knipling (1955) recognized that releasing sexually sterile males would be difficult and expensive but could prove to be advantageous under certain circumstances, especially when eradication of a pest species is desirable to prevent the spread and increased density of the target organism.

Knipling (1955) also proposed five different criteria to determine whether releasing sterile males is appropriate. 1) ability to rear millions of insects 2) adequate dispersal of sterile males so they are able to find virgin females 3) a sterilization method that does not have a negative effect on the fitness of males 4) females of the target insect mate once during their lifetime and 5) the population density of the target insect has to be low, whether naturally low or reduced in some other way for a possible release of sterile males to cause a significant decrease in the population.

While the sterile insect technique for eradicating the screwworm fly was successful, in part due to the geography of Mexico and Central America, using the sterile insect technique to control mosquito populations in continental Africa would be more difficult. Though four of the criteria mentioned above seem plausible for mosquitoes, the criteria requiring a low population density is most problematic. Nevertheless, small scale sterile insect technique programs have been successfully used against mosquitoes in the 1970s in El Salvador and on Seahorse key near the Florida coast (Alphey and Andreasen, 2002). Large scale control methods were also attempted in India and El Salvador with some success, but were ended due to political opposition (Alphey and Andreasen, 2002).

Genetic control- population conversion

A second type of genetic control is population conversion. To prevent the spread of vector-borne diseases, transgenic mosquitoes refractory to a pathogen would be released to spread an anti-pathogen gene and convert the natural vector population into a pathogen resistant population (Marrelli, 2006). If a vector mosquito could be altered so that it was resistant to a pathogen resulting in a decrease in vector competence, then the spread of disease would also decrease (Collins and James, 1996). Competent vectors are susceptible to pathogen infection and efficiently transmit pathogens, whereas incompetent vectors are resistant to infection and do not efficiently transmit pathogens (Beaty, 2000).

Requirements for population conversion

Currently, researchers have proposed three possible requirements that a population conversion genetic control strategy would need to meet in order to be considered successful (Alphey *et al.*, 2002). The first is the discovery of anti-pathogen genes. The second is the ability to genetically modify or transform a mosquito. Finally, the third is the ability to spread, or drive, the anti-pathogen gene throughout the population. Research has focused on the first two requirements, anti-pathogen genes and transformation capabilities of mosquitoes; however, little progress to date has been made on how to effectively introduce an anti-pathogen gene into a natural mosquito population.

The first requirement for a population conversion genetic control strategy is the availability of effector molecules that confer resistance to an organism. Examples of effector molecules include parasite ligands such as single-chain antibody fragments (scFv) that can interfere with parasite development (Yoshida *et al.*, 1999); molecules that target tissue recognition receptors in mosquitoes that could be blocked to prevent parasite development (Nirmala and James, 2003) such as artificial peptides (Ito *et al.*, 2002; Nirmala and James, 2003); insect immuno-molecules (Kokoza *et al.*, 2000; Moreira *et al.*, 2002); toxic proteins, like scorpine, that inhibits parasite development (Renaud *et al.*, 2000); and RNA interference (Olson *et al.*, 2002).

The second requirement for using a genetic control strategy is the ability to successfully transform mosquito vectors. In the past ten years mosquito transformation has become routine. *Aedes aegypti*, a vector of yellow fever virus and dengue viruses and *Anopheles stephensi* and *Anopheles gambiae*, vectors for malaria, have been transformed using *Mariner*, *Hermes*, *Minos*, and *piggyBac* transposable elements, respectively (Catteruccia *et al.*, 2000; Coates *et al.*, 1998; Grossman *et al.*, 2001; Jasinskiene *et al.*, 1998). Use of transgenic mosquitoes has been gaining momentum due to advances in understanding the genomics of mosquitoes. Available genome sequences for important vector mosquito species, identification and use of transposable elements for germ line transformation, transformation markers, standardization of microinjection techniques, characterization of promoters that can drive expression of genes in specific tissues and life stages, and identification and characterization of effector molecules (Marrelli, 2006) have all contributed to advancing transformation capabilities.

The final requirement for a population replacement genetic control strategy is a mechanism for introduction of anti-pathogen genes into a natural population. Gene drive refers to the ability of a gene to be inherited at rates faster than Mendelian genetics would predict, so the frequency of a gene increases in each generation until a desired allele is fixed, meaning the frequency of the allele is at 100 percent in the population. For a genetic drive strategy to be successful, certain criteria have been suggested as key points (Coleman and Alphey, 2004; Sinkins and Gould, 2006). The gene drive mechanism has to be able to spread effector genes to fixation on a human timescale; the drive mechanism and the effector gene must remain linked; should be safe so that there are no unwanted side-effects such as change in host preference or changes in feeding frequency; should target multiple vector species because often viruses have multiple vectors; should have a minimal impact on the fitness of the mosquito (Sinkins and Gould, 2006); should be capable of recall to eliminate a harmful gene from the population; should be replaceable, re-useable, and generic so that one drive mechanism could be used to drive different effector genes; and finally, specific to the target species (Coleman and Alphey, 2004).

Gene drive systems- transposable elements

After the discovery of transposable elements, also known as transposons and jumping genes, in *Drosophila*, researchers believed that DNA could be inserted within a transposon and used to introduce and drive new genes into populations (Gould, 2004). Retrotransposons, or class I transposons require an RNA intermediate using reverse transcriptase whereas class II transposons do not require an RNA intermediate and use a transposase. The transposase is responsible for a “cut and paste” mechanism that moves the target transposon from one location in a genome and inserts itself randomly into another location (Karp, 2002). Following transposition, if homologous recombination is used to repair the gap, then there would be a duplication of the transposon sequence. If an anti-pathogen gene was “loaded” into a transposon, the transposon could spread and drive an anti-pathogen gene into a mosquito population. If the transposition event took place in the germline, then it is possible that the transposon and anti-pathogen gene will be heritable (Karp, 2002); however, transposon based systems have a few drawbacks. One potential drawback is that the rate of transposition can vary based on the size of the transposable element, a second is that transposons insert randomly and could potentially disrupt genes. There is also a risk of horizontal transfer, meaning a transposon with a gene could

jump into a different species (Sinkins and Gould, 2006), and finally, it is possible for a transposon to lose a “loaded” gene and spread successfully without the anti-pathogen gene (Gould, 2006).

Gene drive systems- homing endonucleases

Another possibility for gene drive is a homing endonuclease (Burt, 2003). If an anti-pathogen gene was linked to a homing endonuclease gene, then every time there was a homing endonuclease induced dsDNA break that was repaired using homologous recombination, the homing endonuclease gene and linked gene would be copied. In this gene conversion event, a homozygote expressing an anti-pathogen gene could be generated. Homing endonucleases offer several advantages over transposon based gene drive systems. One advantage is that homing endonucleases have a specific recognition sequence that direct where a dsDNA break is induced. Also, transposable elements could insert numerous times resulting in many copies throughout a genome, while homing endonucleases would be limited to two copies within a genome (Sinkins and Gould, 2006). Too many copies of an anti-pathogen gene could result in cosuppression of that gene, and thus loss of the anti-pathogen effect (Birchler *et al.*, 1999).

However, before homing endonucleases can be used as a gene drive mechanism, some questions need to be answered. First, the ability of a homing endonuclease to induce a dsDNA break in mosquitoes needs to be determined. Second, the mechanism of dsDNA break repair needs to be determined because gene drive relies on gene conversion events stimulated by homologous recombination.

Sindbis virus expression systems

Sindbis virus is an alphavirus in the family *Togaviridae* transmitted by mosquitoes (Rice *et al.*, 1987). The Sindbis virus genome is a nonsegmented positive sense single stranded RNA 11, 703 nucleotides long (Strauss and Strauss, 1994). The genomic RNA has a 5' cap and is polyadenylated at the 3' terminus (Rice *et al.*, 1987). The 5' two thirds of the genome encodes for the four nonstructural proteins (nsp1, nsp2, nsp3, and nsp4) while the 3' third of the genome encodes for five structural proteins, the capsid protein, the envelope proteins, and two small polypeptides E3 and 6K (Strauss and Strauss, 1994). As reviewed by Strauss and Strauss (1994),

alphaviruses replicate in both arthropods and vertebrate hosts. A lifelong infection is produced in the arthropod host while an infection in vertebrates is acute resulting in cell death.

Rice *et al.* (1987) constructed a full length cDNA infectious clone of the Sindbis virus genome downstream from an SP6 polymerase promoter. These cDNA clones are able to be transcribed *in vitro* producing infectious transcripts. Xiong *et al.* (1989) developed a replicon in which the structural proteins were replaced with a chloramphenicol acetyl transferase (CAT) gene yielding a self replicating RNA. They showed that 10^8 CAT particles were produced per infected cell in chicken embryo fibroblasts, *Aedes albopictus* C7-10, *Drosophila* Schneider 1, quail QT-6, hamster BHK-21, and human SW13 cell lines between 16 and 20 hours post-infection demonstrating the usefulness of Sindbis virus as a system to overexpress proteins.

Sindbis virus was seen as a potential vector for the expression of foreign genes because of 1) the broad host range, 2) gene expression is fast, efficient, and occurs in the cytoplasm, and 3) there are temperature sensitive mutants available that could be used to control expression based on environmental temperature (Xiong *et al.*, 1989). Where Xiong *et al.* (1989) used constructs without structural proteins which required the use of a helper system for packaging, Hahn *et al.* (1992) developed Sindbis virus vectors capable of self replication and packaging. In this system, a second subgenomic promoter was inserted in the Sindbis virus genome so that heterologous RNAs or proteins could be expressed with this promoter.

Sindbis virus applications

Sindbis virus vectors have been used extensively to express and study foreign proteins. Sindbis virus vectors have been used to express proteins or peptides to study antigen presentation for epitope mapping (Hahn *et al.*, 1992; London *et al.*, 1992; Lovett *et al.*, 1993), to study the function of cellular vesicle trafficking proteins such as Rab4 and Rab5 (Piper *et al.*, 1993a; Piper *et al.*, 1993b; Piper *et al.*, 1992), to study E1 and E2 envelope proteins from Hepatitis C (Dubuisson *et al.*, 1994), to study viral replication in Herpes simplex virus (Stabell and Olivo, 1993); to express functional RNAs (Huang and Summers, 1991) and antisense RNAs in mosquito cells to inhibit the growth of LaCrosse virus and dengue-2 virus (Olson *et al.*, 1994; Powers *et al.*, 1994); used to study vaccine (Pugachev *et al.*, 1995; Xiong *et al.*, 1993) and

antiviral development and use (Higgs *et al.*, 1995; Jiang *et al.*, 1995); and for nucleic acid immunization which relies on injection of nucleic acids into patients for *in vivo* expression of a desired protein to build immunity against a desired virus (Dubensky *et al.*, 1996). Sindbis virus has been examined as a potential gene transfer vector to target cancers *in vivo* in mice (Tseng *et al.*, 2002) and also to express epitopes to *Plasmodium yoelii* circumsporozoite protein or the nucleoprotein of influenza virus as a means for immunization against malaria or influenza A virus in mice. Sindbis virus has also been used for the long term expression of a heterologous protein using replicons in mammalian cells (Agapov *et al.*, 1998), in *Bombyx mori* for expression of antisense RNA to knock-down a transcription factor Broad-Complex (BR-C) (Uhlirova *et al.*, 2003), and to construct bivalent replicons that expresses one protein as a fusion protein and the second protein in native form from the first subgenomic promoter (Thomas *et al.*, 2003).

The research presented is aimed to determine whether recombinant homing endonucleases can induce dsDNA breaks at their recognition site in exogenously transfected DNA in cell culture and at recognition sites introduced in *Aedes aegypti* following germline transformation. Development of assays to determine if rHE can induce dsDNA breaks in *Aedes aegypti* will allow for the assessment of using homing endonucleases in targeted gene disruption, as a means to study dsDNA break repair, and as a possible mechanism for gene drive.

Chapter 2

DETERMINATION OF RECOMBINANT HOMING ENDONUCLEASE EXPRESSION, LOCALIZATION, AND ACTIVITY IN CELL CULTURE

INTRODUCTION

Vector-borne diseases are an emerging global public health issue. Yellow fever, dengue fever, and dengue hemorrhagic fever are some of diseases caused by viruses transmitted by mosquitoes. Approximately half of the world's population lives in areas at risk for contracting malaria and dengue fever, with an estimated 200 million cases of malaria diagnosed and approximately two million deaths each year (Gubler, 1998). Control of the vectors and prevention of these diseases is an issue being addressed by laboratories worldwide. New methods need to be developed to prevent the spread and transmission of these diseases. Genetic control strategies of mosquito vectors, in combination with current control strategies, offer a novel approach to prevention and possibly eradication of vector-borne diseases.

Homing endonucleases are DNA endonucleases that induce double-stranded DNA (dsDNA) breaks at a specific recognition site ranging from 14-40 base pairs long (Chen and Zhao, 2005). Homing is a site-specific process in which there is a transfer of an intron or intein sequence to the homologous chromosome which lacks the intron or intein sequence (Chevalier and Stoddard, 2001). Most described homing endonucleases belong to the LAGLIDADG family, but there are three other described families: H-N-H, GIY-YIG, and His-Cys families each of which is based on a conserved amino acid motif that evolved independently of one another (Jurica and Stoddard, 1999). Homing endonucleases may be used as part of a mechanism to drive refractory genes into a natural mosquito population with the goal to convert the natural population into a population refractory to a given pathogen. Homing endonucleases could also be used for targeted gene knock-down by modifying the homing endonuclease so that it recognizes a novel site.

Transposition assays have been used in cell culture to test the ability of transposons *piggyBac*, *Minos*, and *Hermes* to mobilize and integrate in cultured cells (Handler *et al.*, 1998; Klinakis *et al.*, 2000; Sarkar *et al.*, 1997; Wilson *et al.*, 2007). In these assays, a helper plasmid encoding a transposase, a donor plasmid encoding the transposon, and a target plasmid were used. Based on

successful use of these transposition assays, similar assays were developed for the evaluation of recombinant homing endonucleases (rHE) to induce dsDNA breaks at exogenously introduced recognition sites *in vivo*. Previous studies by Gruen *et al.* (2002) and Chen and Zhao (2005) have shown that it was possible to use a two plasmid *in vivo* endonuclease activity assay that linked rHE activity and cell survival (Chen and Zhao, 2005). Doyon *et al.* (2006) used a similar two plasmid system that selected for dsDNA breaks induced by a homing endonuclease resulting in the excision of the CcdB gene.

Sindbis virus expression systems have been used in insect and mammalian cells to express heterologous proteins. Sindbis virus is an alphavirus in the family *Togaviridae* which is transmitted by mosquitoes (Rice *et al.*, 1987). A double subgenomic Sindbis virus (dsSIN) system has been used to express chloramphenicol acetyltransferase in *Aedes albopictus* C6/36 cells (Olson *et al.*, 1994). LaCrosse virus envelope glycoprotein genes have been expressed using dsSIN in mammalian (BHK-21 cells) and mosquito cells (C6/36 cells) (Kamrud *et al.*, 1998). dsSIN has also been used to express the full-length premembrane coding region for dengue virus type 2 in C6/36 cells that resulted in resistant cells when challenged with dengue 2 virus (Gaines *et al.*, 1996).

The aim of these studies is to determine whether rHE (I-AniI, I-Cmoel, I-CreI, I-PpoI, and I-SceI) can catalyze dsDNA breaks at an exogenous recognition site in cell culture using C6/36 cells (*Aedes albopictus*) and S2 cells (*Drosophila melanogaster*).

MATERIALS AND METHODS

Plasmid construction

pSLfa/sacB. An *NheI* and *XbaI* fragment containing *sacB* from pDNR-EGFP (Jager *et al.*, 1992) was ligated into the *XbaI* site in pSLfa.

Cloning of HE sites flanking sacB. pSLfa/sacB w/2 HE sites- An *MluI* and *PstI* fragment containing an HE recognition site was ligated into the *MluI* and *PstI* sites 3' to *sacB*. A *BglII* and *SphI* fragment containing an additional HE recognition site was ligated into the *BglII* and *SphI* sites 5' to *sacB*. Oligonucleotide sequences used are in Table 2.1.

sacB/pSLfa w/ HE site in EcoRI. *sacB/pSLfa* was digested with *XbaI* followed by an *AvrII* digest to destroy one of two *EcoRI* sites located at the 3' end of the *sacB* gene. An *EcoRI* fragment containing an HE recognition site was ligated into the now unique *EcoRI* site in *sacB/pSLfa*. Oligonucleotide sequences used are in Table 2.2.

Cloning of synthesized rHE based on the Aedes aegypti codon bias. Recombinant homing endonuclease (rHE) genes were synthesized (Top Gene Technologies) with a nuclear localization signal (nls) and an S_{tag}, (Novagen) using the *Aedes aegypti* codon bias (<http://www.kazusa.or.jp/codon/>). *BamHI* codon optimized rHE fragments were ligated into the *BamHI* site in pKhsp82 to generate pKhsp82-Aa-nls-S_{tag}-I-*CmoE*I, pKhsp82-Aa-nls-S_{tag}-I-*Ani*I, and pKhsp82-Aa-nls-S_{tag}-I-*Cre*I.

Dmact5C-GFP/pSLfa. A fragment containing the *Drosophila melanogaster* actin5C promoter fragment was amplified using a proofreading DNA polymerase *Pfx* (Invitrogen) and primers 5'-ttttggateccTATTTCTAGTTTTCGATACCCCTCACCGCC-3' and 5'-ttttgtcgacTCTGGATTAGACGACTGCTGGCTGATGG-3' (94°C, 2 min; 94°C, 30 sec; 62°C, 1 min; 68°C, 3:30 min; 35 cycles; 68°C, 10 min) with *BamHI* and *SalI* compatible ends. Following restriction enzyme digestion, the amplicon was ligated into the *BamHI* and *SalI* sites in pSLfa/GFP SV40.

Table 2.1. Primers used to introduce HE sites flanking *sacB*

Primer Name	Sequence (5'-3') ¹
I-AniI F M+P² I-AniI R	<u>cgcg</u> TTATTTGAGGAGGTTTCTCTGTAATAATG <u>tgca</u> AATAAACTCCTCCAAAGAGACATTATTAC
I-AniI R S+B³ I-AniI F	<u>gatc</u> AATAAACTCCTCCAAAGAGACATTATTAC <u>catg</u> TTATTTGAGGAGGTTTCTCTGTAATAATG
I-CmoEI F M+P I-CmoEI R	<u>cgcg</u> ATCGTAGCAGCTCACGGTTA <u>tgca</u> TAGCATCGTCGAGTGCCAAT
I-CmoEI R S+B I-CmoEI F	<u>gatc</u> TAGCATCGTCGAGTGCCAAT <u>catg</u> ATCGTAGCAGCTCACGGTTA
I-CreI F M+P I-CreI R	<u>cgcg</u> CAAACGTCGTGAGACAGTTTGGT <u>tgca</u> GTTTTGCAGCACTCTGTCAAACCA
I-CreI R S+B I-CreI F	<u>gatc</u> GTTTTGCAGCACTCTGTCAAACCA <u>catg</u> CAAACGTCGTGAGACAGTTTGGT
I-PpoI F M+P I-PpoI R	<u>cgcg</u> ATGACTCTCTTAAGGTAGCCTAGAA <u>tgca</u> TAGTGAGAGAATTCCATCGGATCTT
I-PpoI R S+B I-PpoI F	<u>gatc</u> TAGTGAGAGAATTCCATCGGATCTT <u>catg</u> ATGACTCTCTTAAGGTAGCCTAGAA
I-SceI F M+P I-SceI R	<u>cgcg</u> TAGGGATAACAGGGTAATTGCA <u>tgca</u> ATTACCCTGTTATCCCTA
I-SceI R S+B I-SceI F	<u>gatc</u> ATTACCCTGTTATCCCTACATG <u>catg</u> TAGGGATAACAGGGTAAT

¹Lower case letters indicate restriction site compatible overhangs

²M+P for *MluI* and *PstI* compatible overhangs

³S+B for *SphI* and *BglII* compatible overhangs

Table 2.2. Primers used to introduce a HE site within *sacB*

Primer Name	Sequence (5'-3') ¹
I-<i>SceI</i> EcoRI F	<u>aatt</u> CATTACCCTGTTATCCCTAG
I-<i>SceI</i> EcoRI R	<u>aatt</u> CTAGGGATAACAGGGTAATG
I-<i>AniI</i> EcoRI F	<u>aatt</u> TTATTTGAGGAGGTTTCTCTGTAATAATG
I-<i>AniI</i> EcoRI R	<u>aatt</u> CATTATTACAGAGAAACCTCCTCAAATAA
I-<i>CmoI</i> EcoRI F	<u>aatt</u> ATCGTAGCAGCTCACGGTTA
I-<i>CmoI</i> EcoRI R	<u>aatt</u> TAACCGTGAGCTGCTACGAT
I-<i>CreI</i> EcoRI F	<u>aatt</u> CAAAACGTCGTGAGACAGTTTGGT
I-<i>CreI</i> EcoRI R	<u>aatt</u> ACCAAACGTCTCACGACGTTTTG
I-<i>PpoI</i> EcoRI F	<u>aatt</u> ATGACTCTCTTAAGGTAGCCTAGAA
I-<i>PpoI</i> EcoRI R	<u>aatt</u> TTCTAGGCTACCTTAAGAGAGTCAT
I-<i>SceI</i> EcoRI F	<u>aatt</u> TAGGGATAACAGGGTAAT
I-<i>SceI</i> EcoRI R	<u>aatt</u> ATTACCCTGTTATCCCTA

¹Lower case letters represent *EcoRI* compatible overhangs

Ub-nls-I-SceI/pSLfa. A fragment containing the nls-I-SceI fragment from pKhsp70-nls-I-SceI (Rong and Golic, 2000) was amplified using a proofreading DNA polymerase *Pfx* (Invitrogen) and primers 5'-ttttccatggATGGGATCATCATCAGACGACGAAGC-3' and 5'-ttttgcggccgcTTATTTTCAGGAAAGTTTTCGGAGGAGA-3' (94°C, 2 min; 94°C, 30 sec; 57°C, 1 min; 68°C, 1 min; 35 cycles; 68°C, 10 min) with *NcoI* and *NotI* compatible ends. Following restriction enzyme digestion, the amplicon was ligated into the *NcoI* and *NotI* sites in Ub-EGFP/pSLfa.

pTE/3'2J-rHE. A fragment containing Aa-nls-S_{tag}-I-*AniI*, Aa-nls-S_{tag}-I-*CmoEI*, Aa-nls-S_{tag}-I-*CreI*, nls-S_{tag}-I-*PpoI*, or nls-S_{tag}-I-*SceI* was amplified using a proofreading DNA polymerase *Pfx* (Invitrogen) and primers 5'-ttttggcgcgcccTTAAATTAACACGGATCCATGC-3' and 5'-tttttaattaaTGATCTTGATCTTCATGGTCGACGG-3' (94°C, 2 min; 94°C, 30 sec; 54°C, 1 min; 68°C, 2 min; 35 cycles; 68°C, 10 min) with *AscI* and *PacI* compatible ends. Following restriction enzyme digestion, the amplicon was ligated into the *AscI* and *PacI* sites in pTE/3'2J.

pTE/5'2J ME2-rHE. An *XbaI* fragment containing a multiple cloning site with *AscI* and *PacI* sites was ligated into the *XbaI* site in pTE/5'2J ME2 to generate pTE/5'2J ME2 MCS. A fragment containing Aa-nls-S_{tag}-I-*AniI*, Aa-nls-S_{tag}-I-*CmoEI*, Aa-nls-S_{tag}-I-*CreI*, nls-S_{tag}-I-*PpoI*, or nls-S_{tag}-I-*SceI* was amplified using a proofreading DNA polymerase *Pfx* (Invitrogen) and primers 5'-ttttggcgcgcccTTAAATTAACACGGATCCATGC-3' and 5'-tttttaattaaTGATCTTGATCTTCATGGTCGACGG-3' (94°C, 2 min; 94°C, 30 sec; 54°C, 1 min; 68°C, 2 min; 35 cycles; 68°C, 10 min) with *AscI* and *PacI* compatible ends. Following restriction enzyme digestion, the amplicon was ligated into the *AscI* and *PacI* sites in pTE/5'2J ME2 MCS.

pTE/5'2J ME2-EGFP. A fragment containing EGFP was amplified using a proofreading DNA polymerase *Pfx* (Invitrogen) and primers 5'-ttttggcgcgcccATGGTGAGCAAGGGCGAGGAGC-3' and 5'-tttttaattaaTACTTGTACAGCTCGTCCATGCC-3' (94°C, 2 min; 94°C, 30 sec; 57°C, 1 min; 68°C, 2 min; 35 cycles; 68°C, 10 min) with *AscI* and *PacI* compatible ends. Following restriction enzyme digestion, the amplicon was ligated into the *AscI* and *PacI* sites in pTE/5'2J ME2 MCS.

Cell culture

All cell lines were maintained in medium supplemented with 10% FBS (Innovative Research), 1% penicillin-streptomycin solution (Mediatech), and 1% L-glutamine (Mediatech). *Aedes albopictus* C6/36 cells were cultured in DMEM (Mediatech) in 5% CO₂ at 28°C. Cells were subcultured by scraping in medium and split at a ratio of 1:10. *Mesocricetus auratus* Syrian baby golden hamster kidney cells (BHK-21) and *Cercopithecus aethiops*, African green monkey kidney cells (Vero) were cultured in DMEM (Mediatech) in 5% CO₂ at 37°C. *Aedes aegypti* CCL-125 cells were cultured in MEM (Mediatech) in 5% CO₂ at 28°C. Cells were subcultured by removing medium, washing cells with phosphate buffered saline (PBS) and adding trypsin (Mediatech) until cells were completely detached from the flask. An equal volume of medium was added and cells were centrifuged at 1000 x g for 5 minutes at 4°C. Cells were split at a ratio of 1:10. Schneider's *Drosophila melanogaster* Line 2 cells and *Aedes aegypti* Aag2 cells were cultured in Schneider's *Drosophila* Medium (Lonza BioWhittaker) at 28°C. Cells were subcultured by scraping cells in medium and split at a ratio of 1:10.

Transfection of plasmid DNA in cultured cells

Transfections of cultured insect cells were performed according to manufacturer instructions for the Qiagen Effectene Transfection Reagent. Cells were seeded in 12 well plates or 25 cm² flasks 24 hours prior to transfection. Adherent cells (C6/36 and CCL-125 cells) were transfected when cells were between 40-60% confluent. Semi-adherent/suspension cells (S2) were seeded at a density of 5x10⁵ cells/ml in 5 ml medium and transfected the following day. Photographs of cells were taken using a Zeiss Axiovert 200 microscope with a Cannon Powershot A260 camera.

Recombinant Sindbis virus production

Template DNA was generated by linearizing target vector with *Xho*I. Digested DNA was incubated with proteinase k, phenol:chloroform extracted, and ethanol precipitated. *In vitro* transcription reactions were performed using SP6 polymerase. BHK-21 cells were harvested using a trypsin treatment, washed three times with PBS, and seeded at a concentration of 1x10⁷ cells per 25 cm² flask and were electroporated with transcript RNA using a BTX Harvard Apparatus ECM 630 ElectroCell Manipulator with the following settings: 460V, 725Ω, and 0075 μF. Cells were pulsed twice and grown as previously described. Electrotransfected BHK-

21 cells were monitored for the presence of cytopathic effects (CPE). When cells demonstrated approximately 80% CPE, medium was centrifuged at 1000 x g for 5 minutes at 4°C to remove cellular debris. The supernatant containing virus was stored at -80°C. Viral titers were determined by plaque assays on Vero cell monolayers. Serial dilutions of virus (10^{-1} to 10^{-6}) were used to infect Vero cells for 1 hour at 32°C with gentle rocking every 10 minutes. A 0.4% agarose plug was overlaid following infection to prevent the spread of virus particles. Agarose plugs were pulled at 96 hours post-infection and plaques were stained with crystal violet (40% methanol, 0.25% crystal violet). Plaque forming units per ml (pfu/ml) were calculated based on an average from triplicate samples. C6/36 cells were infected with either 500 µl virus and 500 µl medium (TE/3'2J-rHE viruses) or a multiplicity of infection (MOI) of 1 (TE/5'2J ME2-rHE viruses) with gentle rocking for one hour at room temperature when cells were at a 40-60% confluency. Viral titers ranged from 1×10^4 to 2.5×10^6 pfu/ml for TE3'2J-rHE viruses. TE/5'2J ME2-rHE viruses had an average viral titer of 1×10^7 pfu/ml. Cells were maintained as previously described.

Recovery of DNA from transfected cells

Total DNA. Medium was removed and cells were washed three times with PBS. Cells were scraped in 400 µl PBS and transferred to an eppendorf tube and centrifuged at 1000 x g for 5 minutes at 4°C. Cell pellets were lysed in 200 µl Bender Buffer (0.1 M NaCl, 0.2 M Sucrose, 0.1 M Tris, pH 9.0, 0.05 M EDTA, 0.5 M SDS) and proteinase k treated at 50°C overnight. DNA was phenol:chloroform extracted, ethanol precipitated, and resuspended in DEPC water overnight.

Low molecular weight plasmid DNA. Medium was removed and cells were washed three times with PBS. Cells were scraped in 1 ml PBS and centrifuged at 1000 x g for 5 minutes at 4°C. Cell pellets were washed with PBS three times. A final 2 minute spin was done to ensure all PBS was removed. A Qiagen MINiprep was performed per the manufacturer's instructions with the following modifications: supernatant from the 10 minute spin was centrifuged for an additional 5 minutes to ensure all cell debris was removed before applying the supernatant to the column, and two additional PB wash steps.

Immunofluorescence assay (IFA)

Cells were seeded on glass coverslips in 12 well plates and either transfected or infected as described above. Cells were washed with PBS and fixed with ice cold acetone:PBS (75:25) for 2 minutes. Acetone was removed and cells were washed with 1 ml PBS and permeabilized in 0.3% Triton-X in PBS for 10 minutes at room temperature. Cells were washed with PBS and blocked in 2% bovine serum albumin (BSA) and 1% horse serum for 1 hour at room temperature. Coverslips were then incubated in a humidified 37°C chamber for 1 hour with 75 µl of primary antibody (Table 2.3) in 0.1% Triton-X and 0.2% BSA in PBS. Antibody was drained off and immersed in PBS with gentle agitation. Cells were washed with 0.1% Triton-X and 0.2% BSA and then incubated in a humidified 37°C chamber for 30 minutes with 75 µl of secondary antibody (Table 2.3) in 0.1% Triton-X and 0.2% BSA. Cells were washed with PBS and counterstained with a 0.025% Evan's Blue solution for 5 minutes followed by additional PBS washes. Coverslips were mounted on glass slides with ProLong Gold anti-fade reagent with DAPI (Molecular Probes) mounting solution. Coverslips were allowed to cure overnight at room temperature. Cells were examined under a Zeiss Axiovert 200 fluorescent microscope with a Cannon Powershot A260 camera and a Zeiss LSM META on an Axiovert 100 Inverted Microscope.

SDS-PAGE and western analysis

Cells were seeded in 25 cm² flasks and transfected or infected as described above. Cells were harvested as follows: cells were washed with PBS, scraped in 1 ml PBS, and centrifuged at 1000 x g for 5 minutes at 4⁰C. Cell pellets were washed three times with PBS and centrifuged. Cell pellets were lysed with 2X SDS Loading buffer (Novagen) and boiled at 100°C for 5 minutes. Sample lysates were centrifuged for 1 minute at 13,000 rpm, and were separated on a 4% stacking 10% resolving SDS polyacrylamide gel (0.75 mm). Gels were run at 90V for 90 minutes at room temperature. The gel, membrane, sponges, and filter paper were equilibrated in transfer buffer (39 mM glycine, 48 mM Tris, 1.3 mM SDS, 20% methanol) for 10 minutes. The gel was transferred to a nitrocellulose membrane (0.45-µm pore size; Biorad) at 100V for 1 hour. The membrane was blocked in 3% non-fat dry milk in Tris-buffered saline with 1% Tween-20 (TBST) for 30 minutes, incubated with primary antibody and rocked gently at room temperature followed by incubation with a secondary antibody. For antibody dilutions see Table 2.3. The

membrane was washed with TBST and incubated in the dark room with 500 µl of 2X luminol/enhancer and 500 µl stable peroxide solution (Novagen) for 1 minute. Proteins were detected using X-ray film (Kodak) and developed with a Konica SRX-101A processor.

Two plasmid assay to test for rHE ability to induce dsDNA breaks in cell culture

Cells were co-transfected with a target and an expression plasmid or infected and transfected with a target plasmid as previously described. Cells transfected with a heat inducible promoter were heat shocked at 37°C for an hour and allowed to recover for 1 hour before DNA was harvested. Either total DNA or low molecular weight plasmid DNA was harvested as described. DNA was quantitated using a NanoDrop® ND 1000 Spectrophotometer. DNA was RNase treated for 1 hour at 37°C, digested to linearize the target vector, and loaded on a 1% agarose gel for Southern analysis. Probes were either randomly primed with [α - P³²]dATP, specific activity 3000 Ci/mmol using the Amersham Megaprime DNA labeling System (GE Healthcare, Buckinghamshire, UK) and purified using illustra NICK columns (GE Healthcare) or labeled with biotinylated-dATPs using New England Biolabs NEBlot Phototope Kit. The membrane was visualized using Kodak BioMax maximum sensitivity film at -80°C.

Table 2.3. Antibodies used for protein detection

Antibodies for IFA	Dilution	Time
S-protein Antibody (Novagen)	1:400	1hr
Goat Anti-Mouse FITC Conjugate (Calbiochem)	1:400	30 min
Anti-I- <i>SceI</i> Goat Polyclonal Antibody (Santa Cruz)	1:200	1hr
Rabbit Anti-Goat IgG FITC Conjugate (Calbiochem)	1:100	30 min
Antibodies for Western	Dilution	Time
S-Tag monoclonal antibody mouse IgG (Novagen)	1:5000	30 min
Anti-E2 mouse monoclonal antibody (Mab92hs)	1:200	30 min
Anti-GPF mouse IgG mAb 11E5 (Molecular Probes)	1:200	1hr
Goat Anti-Mouse IgG Peroxidase Conjugate (Calbiochem)	1:10,000	30 min
Anti-I- <i>SceI</i> Goat Polyclonal Antibody (Santa Cruz)	1:200	1hr
Donkey Anti-Goat IgG HRP (Promega)	1:10,000	30 min

RESULTS

Transfections of rHE expression plasmids in C6/36 cells

Before assays using rHE could be performed, parameters were optimized to maximize transfection conditions for rHE expression plasmids. Optimization was performed using a plasmid expressing enhanced green fluorescent protein (EGFP) under the control of a mosquito ubiquitin (Ub) promoter (Ub-EGFP) or a *Drosophila melanogaster* ElaV promoter (Elav-EGFP). The amount of DNA and Effectene for 12 well plates and 25 cm² flasks was varied to determine the most efficient conditions for transfection. Transfection efficiencies were determined by counting the number of EGFP positive cells divided by the total number of cells (Table 2.4). Efficiency calculations were done in triplicate for each treatment and an average was taken. Optimal conditions for transfection for C6/36, S2, and CCL-125 cells can be seen in Table 2.4. For C6/36 cells, 0.5 µg DNA and 5 µl Effectene or 2.5 µg DNA and 17 µl Effectene were optimal conditions for 12 well plates and 25 cm² flasks, respectively, while 5 µg DNA and 50 µl Effectene was optimal for S2 cells in 25 cm² flasks. Three days post-transfection was observed as an optimal time point for maximized EGFP expression based on photographing transfected cells every day post-transfection for seven days (Figure 2.1.A). Based on this observation, western analysis at 3 days post-transfection was performed for detection of EGFP in C6/36 transfected with the Ub-EGFP (Figure 2.1.B).

IFA to detect for rHE nuclear localization

Once transfection conditions were optimized, an IFA was used to determine whether S_{tag}-rHE fusion proteins could be localized to mosquito cell nuclei. Five rHE constructs were made by fusing each homing endonuclease gene to an SV40 nuclear localization signal (nls) and an S_{tag} (Novagen). The S_{tag} was used for detection for both an IFA and western analysis.

Novagen's S_{tag} system is based on the interaction between a 15 amino acid S_{tag} peptide and ribonuclease S-protein (Kim and Raines, 1993). To test whether the nls-S_{tag}-rHE fusion protein was localized in mosquito cell nuclei, C6/36 cells were transfected with pKhsp82-rHE constructs. At 3 and 7 days post-transfection an indirect IFA was performed using an anti-S_{tag} primary antibody followed by a secondary antibody conjugated to fluorescein (Table 2.3).

Table 2.4. Transfection optimization in cell culture using Qiagen Effectene Transfection Reagent.

Cell Line	Culture Vessel	DNA (μg)	Enhancer (μl)	Effectene (μl)	GFP ⁺ cells/ Total #cells	Transfection Efficiency	Standard Deviation
C6/36 ¹	12 Well Plate	0.5	2.4	5	1330/3513	38%	±11.3
	25 cm ²	2.5	22.4	17	372/3650	10%	±2.4
CCL-125	12 Well Plate	0.5	2.4	5	405/6034	7%	±2.4
S2 ²	25 cm ²	2.5	40	50	1199/5531	22%	±1.3

¹ C6/36 and CCL-125 cell transfections were optimized with a Ub-EGFP construct

² S2 cell transfections were optimized with an ElaV-EGFP construct

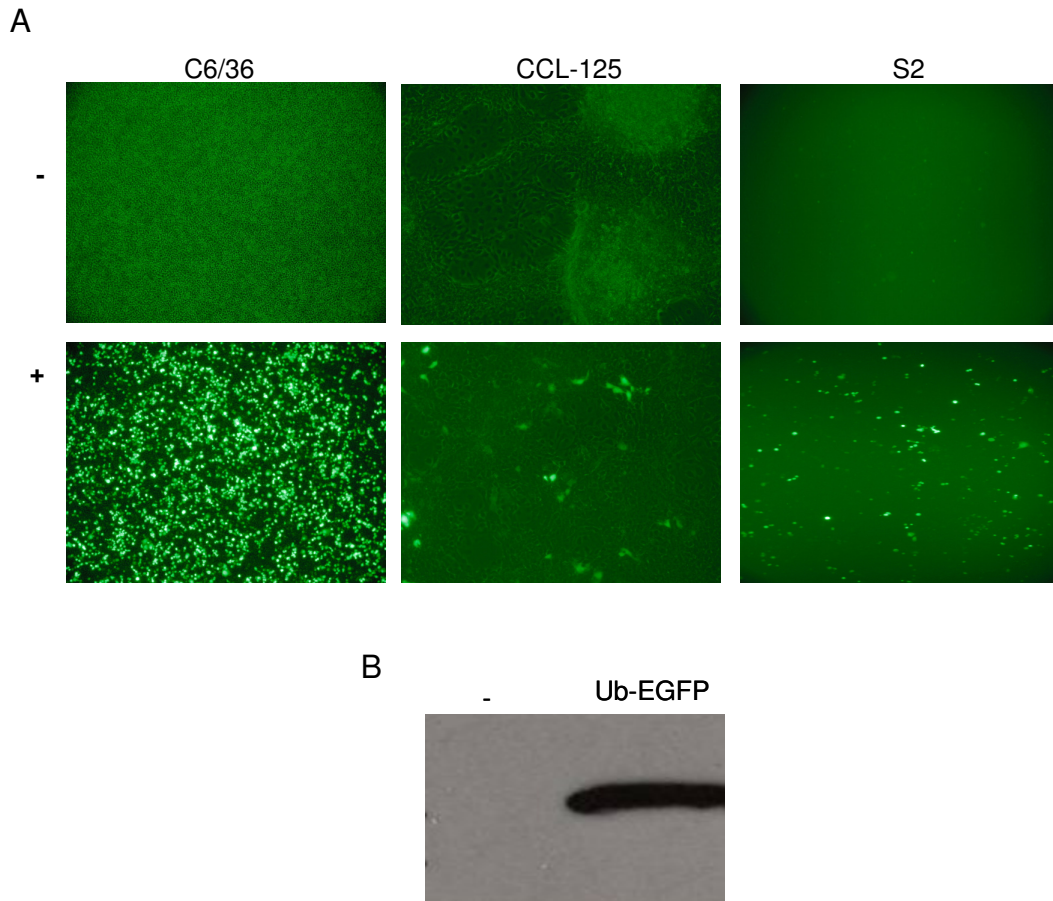


Figure 2.1. Monitoring of EGFP expression and detection in C6/36 cells. (A) Transfection optimization in C6/36 cells, CCL-125 cells, and S2 cells using a Qiagen MIDIprep of either Ub-EGFP/pSLfa for C6/36 and CCL-125 cells or ElaV-EGFP/pSLfa for S2 cells. Pictures were recorded at 3 days post-transfection from cells transfected in 12 well plates for C6/36 and CCL-125 cells and in 25 cm² flasks for S2 cells. (-) denotes untransfected cells and (+) denotes cells transfected with an EGFP expression plasmid. (B) Western analysis from C6/36 cells transfected with Ub-EGFP/pSLfa. Total protein extract was harvested 3 days post-transfection for SDS-PAGE and transferred to a nitrocellulose membrane for western analysis using an anti-GFP antibody.

Nuclear localization was observed for each S_{tag}-rHE fusion protein (Figure 2.2). IFA signals were scored as nuclear or cytoplasmic.

The number of cells positive for an IFA when transfected with pKhsp82-nls-S_{tag}-I-*AniI*, pKhsp82-nls-S_{tag}-I-*Cmoel*, and pKhsp82-nls-S_{tag}-I-*CreI* was lower compared to cells transfected with pKhsp82-nls-S_{tag}-I-*PpoI* and pKhsp82-nls-S_{tag}-I-*SceI* (data not shown). We reasoned that the low transfection rates observed could be due to codon preference. I-*AniI*, I-*Cmoel*, and I-*CreI* are encoded by introns from organelle DNA whereas I-*PpoI* is encoded by a nuclear intron and I-*SceI* had already been codon optimized for *Drosophila melanogaster* (Bellaiche *et al.*, 1999). rHE genes were synthesized de novo (Top Gene Technologies) with an nls and an S_{tag} (Novagen) using the *Aedes aegypti* codon preference. IFA results for C6/36 cells transfected with codon optimized pKhsp82 constructs (pKhsp82-Aa-nls-S_{tag}-I-*AniI*, pKhsp82-Aa-nls-S_{tag}-I-*Cmoel*, and pKhsp82-Aa-nls-S_{tag}-I-*CreI*) and non-codon optimized constructs (pKhsp82-nls-S_{tag}-I-*PpoI* and pKhsp82-nls-S_{tag}-I-*SceI*) can be seen in Figure 2.3. C6/36 cells observed to have an S_{tag}-rHE were counted after 3 and 7 days post-transfection. Codon optimized I-*AniI* and I-*Cmoel* resulted in a 3.5 to a 7.5 fold increase in the number of cells observed to have an S_{tag} signal. A Z-test for comparing two proportions was performed comparing the average percent of cells positive for an IFA in between non-codon optimized constructs and codon optimized constructs for I-*AniI* and I-*Cmoel* at 3 and 7 days post-transfection. There was a significant difference in the average percent of cells with an S_{tag} signal when the non-optimized and optimized results for I-*AniI* and I-*Cmoel* were compared within each time point (p<0.0001). The average number of cells demonstrating nuclear and cytoplasmic localization of S_{tag}-rHE was calculated, and IFA scores from codon optimized and non-codon optimized constructs were compared (Figure 2.4). S_{tag}-rHE fusion protein localization could not be determined in CCL-125 cells with any rHE construct (data not shown) despite varying fixation and permeabilization times.

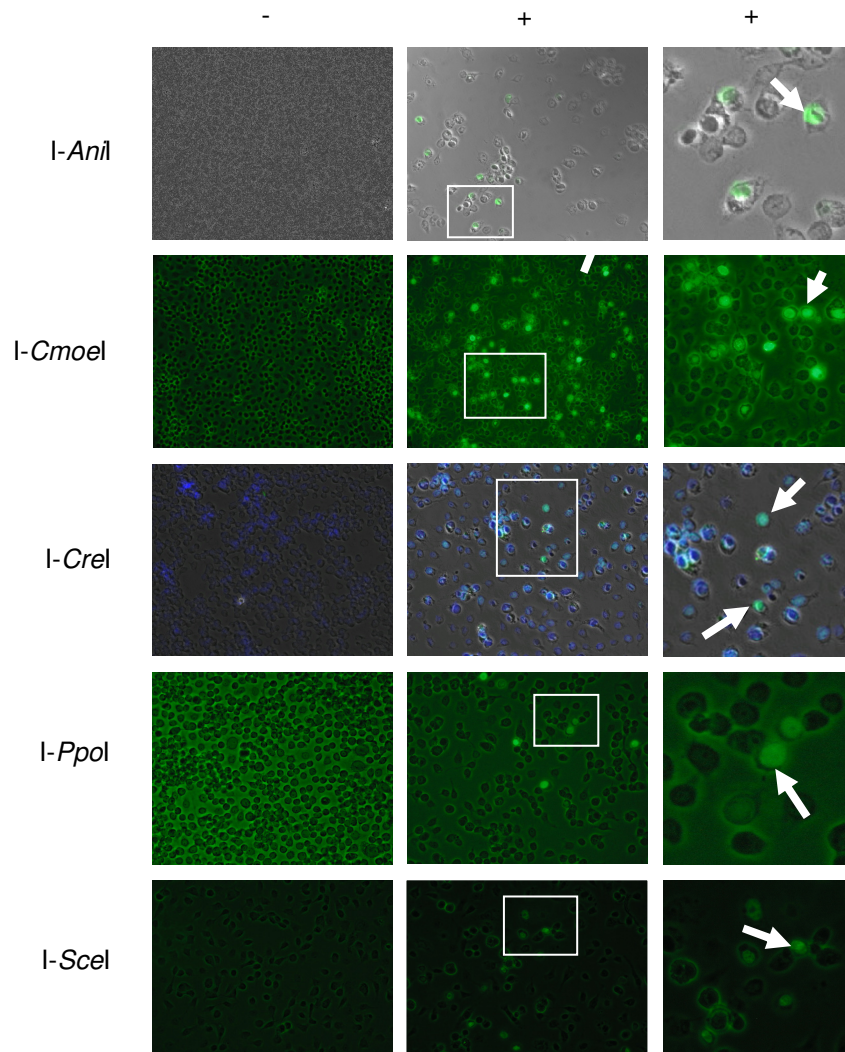


Figure 2.2. Immunofluorescence assay results for C6/36 cells transfected with pKhsp82 vectors expressing recombinant homing endonuclease (rHE). C6/36 cells were transfected with pKhsp82-rHE constructs which contain a nuclear localization signal (nls) and an S_{tag} (Novagen) used for detection. At 3 days post-transfection cells were fixed and subjected to an immunofluorescence assay using an anti-S_{tag} antibody. Arrows indicate nuclear localization of rHE. I-AniI, I-Cmoel, and I-CreI were codon optimized based on the *Aedes aegypti* codon preference. Images were taken at 200X.

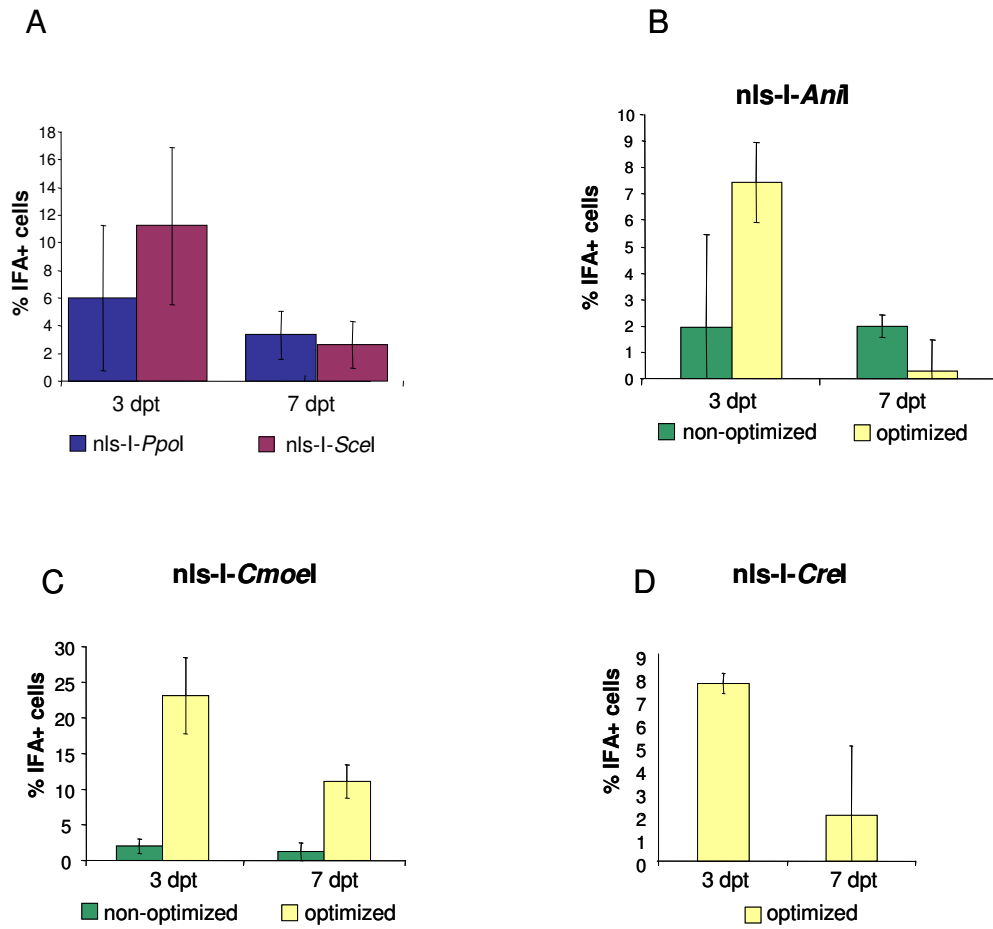


Figure 2.3. Average percent of cells with a fluorescent signal from an immunofluorescence assay in C6/36 cells transfected with pKhsp82-rHE. (A) IFA scores for C6/36 cells transfected with non-optimized nls-S_{tag}-I-*PpoI* or nls-S_{tag}-I-*SceI* constructs at 3 and 7 days post-transfection. (B-D) The average percent of cells that were positive for an IFA in C6/36 cells transfected with codon optimized versus non-codon optimized constructs at 3 and 7 days post-transfection for nls-S_{tag}-I-*AniI*, nls-S_{tag}-I-*Cmoel*, and nls-S_{tag}-I-*CreI*.

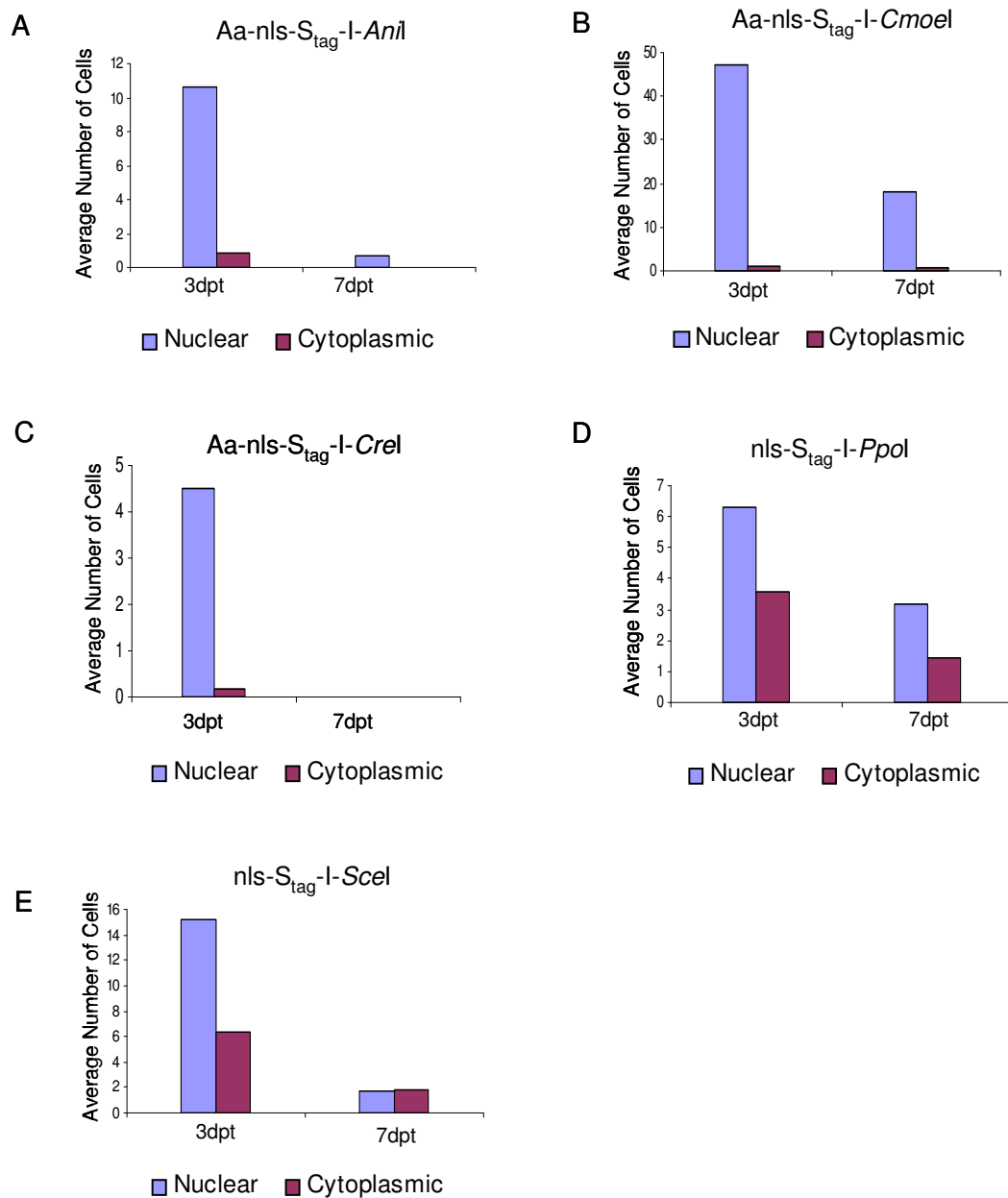


Figure 2.4. Nuclear versus cytoplasmic localization of rHE in C6/36 cells. Cells were transfected with each pKhsp82-rHE construct and scored for localization following an immunofluorescence assay at 3 and 7 days post-transfection. Represented here are the average number of cells that were positive for an IFA with either nuclear or cytoplasmic localization.

SDS-PAGE and western analysis

To determine if each S_{tag}-rHE was expressed as a unique and full length fusion protein, C6/36 cells were transfected with pKhsp82-rHE (Aa-nls-S_{tag}-I-AniI, Aa-nls-S_{tag}-I-CmoE, Aa-nls-S_{tag}-I-CreI, nls-S_{tag}-I-PpoI, and nls-S_{tag}-I-SceI), harvested, and subjected to western analysis using an anti-S_{tag} antibody (Table 2.3). Despite several attempts, rHE could not be detected (data not shown) from cells transfected with any of the pKhsp82 expression plasmids. This is likely not related to a low transfection efficiency since the conditions had been optimized and cells had been successfully transfected as seen by the IFA. Recombinant I-CmoE previously purified from bacteria was detected using an anti-S_{tag} antibody (data not shown) which confirmed the western transfer, reagents, and antibodies were functioning as expected. Expression of rHE following transfection may have been low due to use of the hsp82 *Drosophila pseudoobscura* promoter.

IFA of transfected C6/36 with Ub-nls-I-SceI/pSLfa

Since S_{tag}-rHE protein was not detected by western analysis in cells transfected using expression plasmids containing the *Drosophila pseudoobscura* hsp82 promoter, the nls-I-SceI gene was cloned under the control of the mosquito ubiquitin promoter to determine whether low protein expression was due to low transcript levels. Western analysis was performed 3 days post-transfection on C6/36 cell lysates transfected with Ub-nls-I-SceI/pSLfa. I-SceI recombinant protein was present (Figure 2.5.A) at levels similar to those observed in C6/36 cells transfected with Ub-EGFP (Figure 2.1.B). An IFA was performed on C6/36 cells transfected with Ub-nls-I-SceI using a primary anti-I-SceI (Santa Cruz) antibody and a secondary antibody conjugated to FITC (Calbiochem) [Table 2.3]. IFA results were inconclusive (Figure 2.5.B-D) similar to results seen using CCL-125 cells. The same primary anti-I-SceI antibody (Santa Cruz) was used for both an IFA and western analysis.

Although switching to a mosquito promoter yielded detectable expression of I-SceI, the expression was not robust. In order to further increase transcript levels and subsequent protein expression, we decided to switch to a viral expression system to express rHE in mosquito cells.

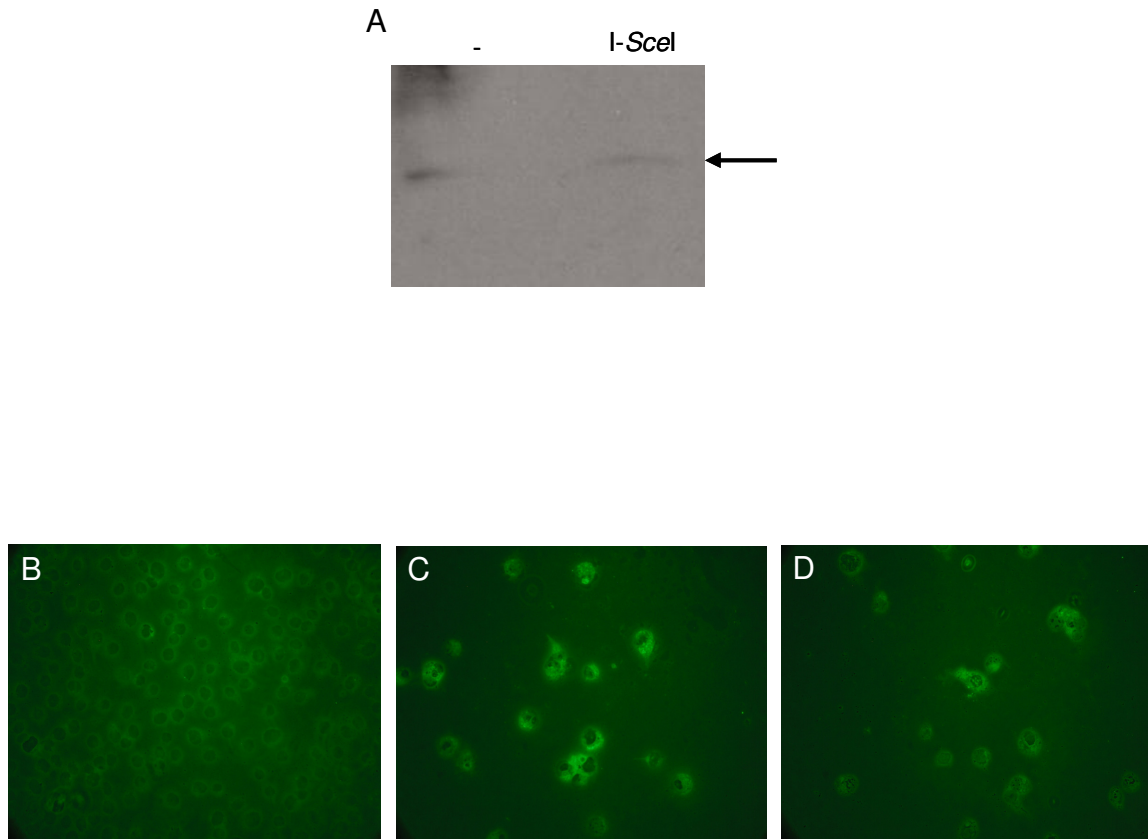


Figure 2.5. C6/36 cells transfected with Ub-nls-I-SceI/pSLfa. (A) Western analysis for detection of I-SceI. C6/36 cells transfected with Ub-nls-I-SceI/pSLfa were lysed in 2X SDS Loading Buffer 3 days post-transfection. Total protein extract was loaded for SDS-PAGE and transferred to a nitrocellulose membrane for western analysis using an anti-I-SceI antibody. The arrow indicates the expected size band. (B-D) Immunofluorescence assays from C6/36 cells transfected with rHE constructs. (B) Control uninfected cells. (C) pKhsp82-Aa-nls-S_{tag}-I-Cmoel transfected cells (anti-S_{tag}). (D) Ub-nls-I-SceI/pSLfa transfected cells (anti-I-SceI).

rHE expression using a recombinant Sindbis virus expression system

A recombinant double subgenomic Sindbis (dsSIN) virus vector, TE/3'2J, was used for expression of each rHE. Each nls-S_{tag}-rHE gene was inserted following the 3' subgenomic promoter in pTE/3'2J. Following rescue of each dsSIN-rHE virus, C6/36 cells were infected with each TE/3'2J-rHE virus (Aa-nls-S_{tag}-I-*AniI*, Aa-nls-S_{tag}-I-*CmoeI*, Aa-nls-S_{tag}-I-*CreI*, nls-S_{tag}-I-*PpoI*, and nls-S_{tag}-I-*SceI*) and harvested 24 hours post-infection. Western analysis at 24 hours post-infection from total cell protein extracts were used for detection of S_{tag} fusion rHE proteins (Figure 2.6). An expected single band of the correct molecular weight was detected for each rHE.

Since rHE were previously shown to exhibit nuclear localization (Figure 2.2) but were now expressed using the dsSIN virus system, an IFA was performed on C6/36 cells infected each TE/3'2J-rHE virus to re-confirm whether the expected localization was occurring. TE/3'2J-Aa-nls-S_{tag}-I-*AniI* virus-infected and TE/3'2J-Aa-nls-S_{tag}-I-*CmoeI* virus-infected C6/36 cells demonstrated S_{tag}-rHE nuclear localization (Figure 2.7.B and C). An IFA could not be performed on cells infected with TE/3'2J-Aa-nls-S_{tag}-I-*CreI* virus, TE/3'2J-nls-S_{tag}-I-*PpoI* virus, or TE/3'2J-nls-S_{tag}-I-*SceI* virus because the cells did not survive infection (data not shown). This was surprising, as both wild type and recombinant Sindbis viruses are known to establish a persistent infection in mosquito cells with no cytopathic effects while still expressing a heterologous gene (Higgs *et al.*, 1997). The cause of cell death was not determined but could be due to toxicity from overexpression and accumulation of rHE or due to rHE endonuclease activity within the cell.

In pTE/3'2J, heterologous genes are cloned under the second subgenomic promoter 3' to the structural genes. In pTE/5'2J ME2 the heterologous genes are cloned 5' to the structural proteins under the 5' subgenomic promoter (Hahn *et al.*, 1992). The 3' subgenomic is favored compared to the 5' subgenomic promoter based on the abundance of transcripts from the 3' subgenomic promoter. To reduce TE/3'2J-rHE virus toxicity in C6/36 cells, TE/5'2J ME2 virus was used to express each rHE. C6/36 cells were infected with each TE/5'2J ME2-rHE virus (Aa-nls-S_{tag}-I-*AniI*, Aa-nls-S_{tag}-I-*CmoeI*, nls-S_{tag}-I-*PpoI*, and nls-S_{tag}-I-*SceI*) and harvested 1, 2, 3, 4, 6, and 8 days post-infection. Using western analysis, rHE expression was not observed for 1, 2, or 3 days

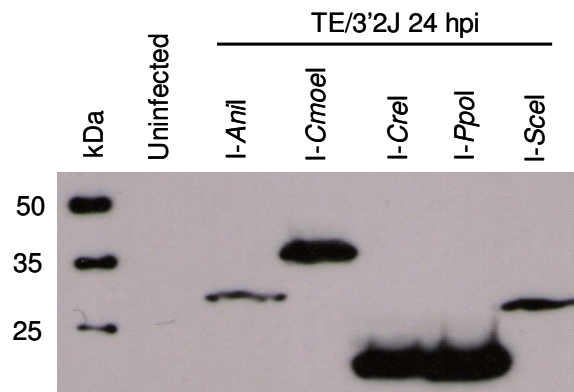


Figure 2.6. Western analysis for detection of rHE in C6/36 cells infected with TE/3'2J-rHE viruses. C6/36 cells were infected with each TE/3'2J-rHE virus, harvested, and lysed in 2X SDS Loading Buffer 24 hours post-infection. Total protein extract was loaded for SDS-PAGE and transferred to a nitrocellulose membrane for western analysis using an anti-S_{tag} antibody for detection.

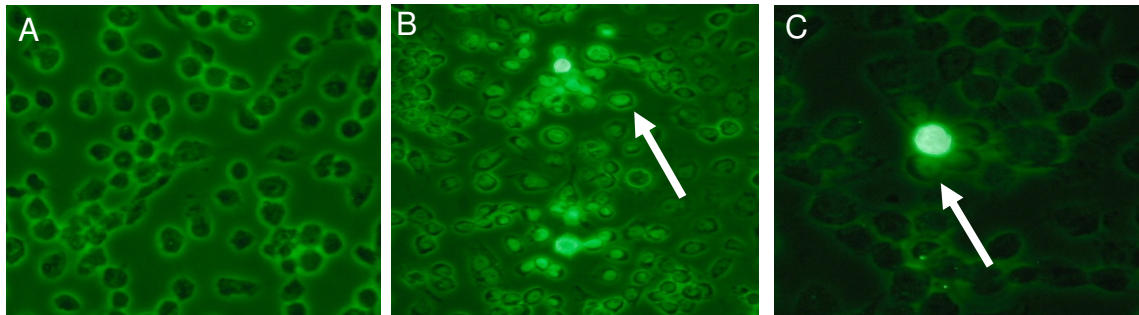


Figure 2.7. Immunofluorescence assay for C6/36 cells infected with TE/3'2J-rHE viruses. C6/36 cells were infected with TE/3'2J-rHE viruses. After 24 hours post-infection cells were fixed and subjected to an immunofluorescence assay. (A) Uninfected (B) TE/3'2J-Aa-nls-S_{tag}-I-AniI virus-infected (C) TE/3'2J-Aa-nls-S_{tag}-I-CmoEI virus-infected. Arrows indicate observed nuclear localization of rHE.

post-infection (data not shown). Cell lysates were used for detection of S_{tag} fusion rHE proteins (Figure 2.8.A, C and E.) or Sindbis E2 protein (Figure 2.8.B, D, and F.). rHE from TE/5'2J ME2-Aa-nls-S_{tag}-I-*Cmo*I virus and TE/5'2J ME2-nls-S_{tag}-I-*Ppo*I virus were detected at 4, 6, and 8 days post-infection. rHE from TE/5'2J ME2-Aa-nls-S_{tag}-I-*Ani*I virus and TE/5'2J ME2-Aa-nls-S_{tag}-I-*Cre*I virus were detected at 4 days post-infection while rHE from TE/5'2J ME2-nls-I-*Sce*I virus could not be detected at any time post-infection. E2, which is a Sindbis viral envelope protein, was detected at all time points post-infection for each recombinant virus. C6/36 cells infected with TE/5'2J ME2-EGFP virus (Figure 2.9) were used to visually monitor the expression of EGFP while western analysis was used for detection of EGFP or Sindbis E2 protein (Figure 2.8 G and H).

Since expression of each S_{tag}-rHE fusion protein was detected with the exception of I-*Sce*I (Figure 2.8), an IFA of TE/5'2J ME2-rHE virus-infected C6/36 was used to re-confirm previous observations demonstrating nuclear localization of rHE, but now in the context of the ME2 Sindbis virus genome. C6/36 cells were infected with TE/5'2J ME2 virus, TE/5'2J-Aa-nls-S_{tag}-I-*Cmo*I virus, or TE/5'2J ME2-nls-S_{tag}-I-*Ppo*I virus. As expected, nuclear localization of rHE was seen in C6/36 cells infected with TE/5'2J-Aa-nls-S_{tag}-I-*Cmo*I virus and TE/5'2J ME2-nls-S_{tag}-I-*Ppo*I virus (Figure 2.10.C. and D) and no fluorescent signal was observed in uninfected and TE/5'2J ME2 virus-infected cells (Figure 2.10.A and B). As we observed S_{tag}-rHE fusion proteins of the expected size were expressed and localized in mosquito cell nuclei, we were then able to perform activity assays using each rHE.

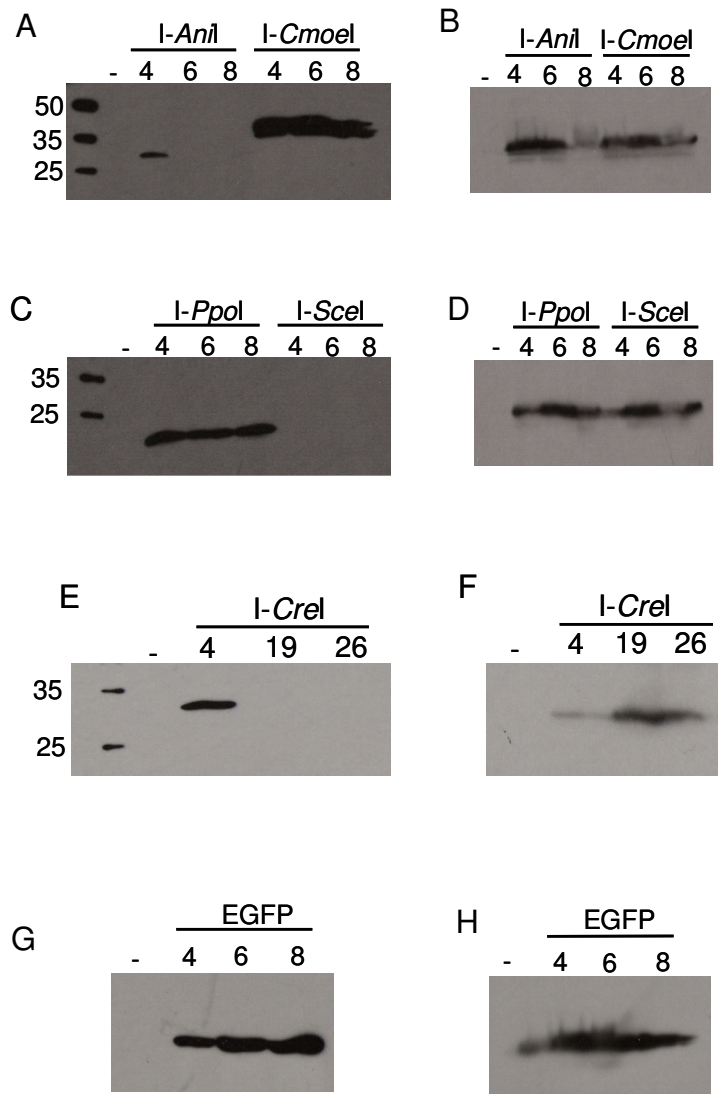


Figure 2.8. Western analysis for detection of rHE, Sindbis E2, or EGFP in C6/36 cells infected with TE/5'2J ME2-rHE viruses. Infected cells were harvested and lysed in 2X SDS Loading Buffer 4, 6, and 8 days post-infection. I-CreI infected cells were harvested and lysed 4, 19, and 26 days post-infection. Total protein extract was loaded for SDS-PAGE and transferred to a nitrocellulose membrane for western analysis using an anti-S_{tag} (A, C, and E), an anti-E2 (B, D, F, and H), or an anti-GFP (G) antibody. TE/5'2J ME2-EGFP virus infected cells were used to visually monitor viral infection.

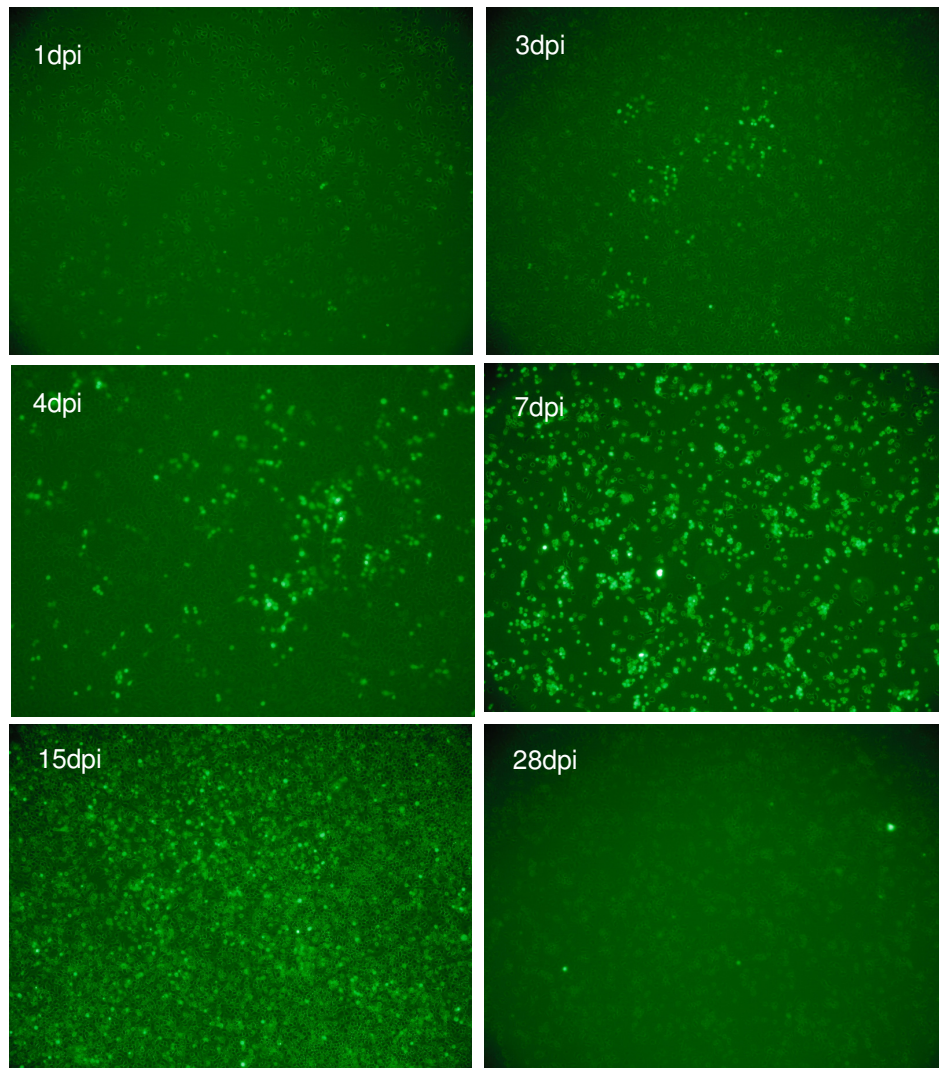


Figure 2.9. C6/36 cells infected with TE/5'2J ME2-EGFP virus. Pictures were taken every day post-infection. The time points post-infection represent the general trend of EGFP expression over time. The amount of EGFP observed was used to determine an optimal time for protein expression of rHE.

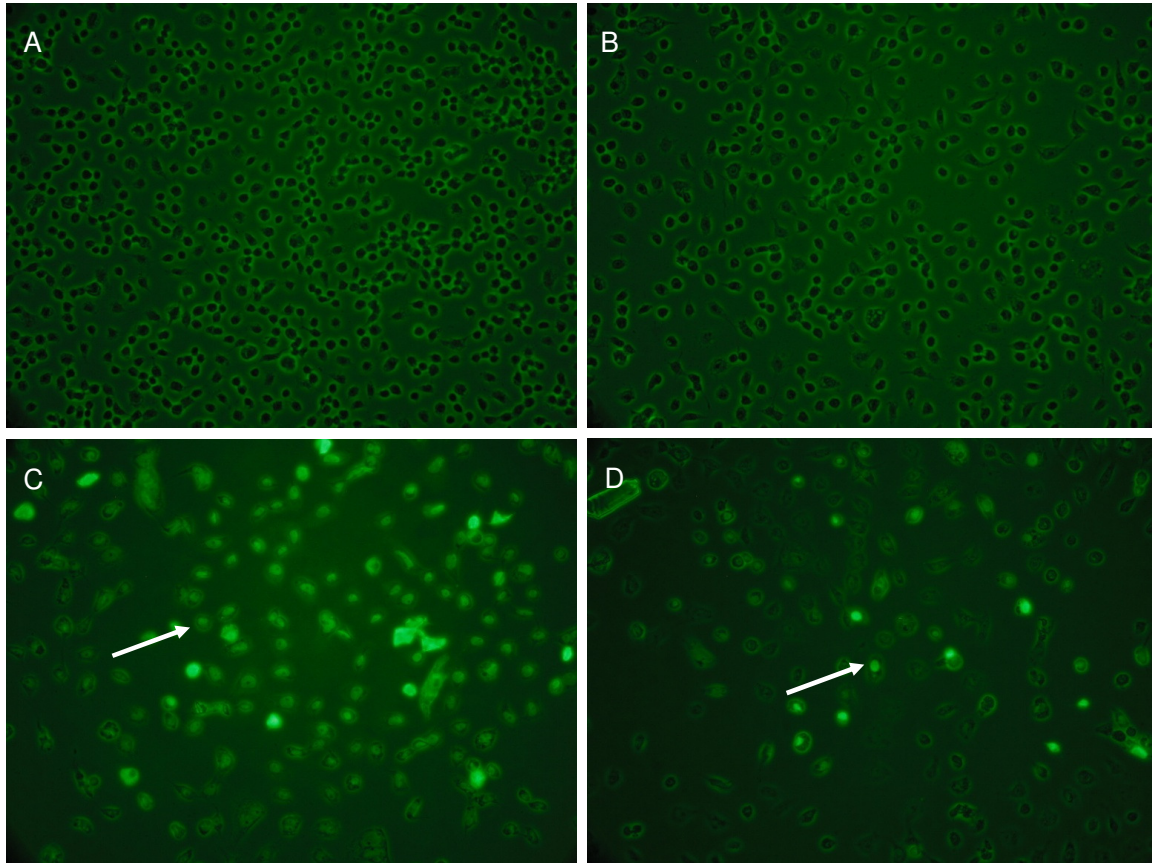


Figure 2.10. Immunofluorescence assay for C6/36 cells infected with TE/5'2J ME2-rHE viruses. C6/36 cells were infected with TE/5'2J ME2-Aa-nls-S_{tag}-I-CmoE1 virus or TE/5'2J ME2-nls-S_{tag}-I-PpoI virus (C and D, respectively) at an MOI of 1. Cells were fixed 5 days post-infection and subjected to an immunofluorescence assay. rHE appear to be localized in cell nuclei (indicated by arrows). No fluorescent signal was observed in uninfected cells or cells infected with TE5'2J ME2 virus (A and B, respectively).

Two plasmid assay to test for rHE ability to induce dsDNA breaks in cell culture

To assess whether rHEs were able to induce dsDNA breaks in *Aedes aegypti* cultured cells, a two plasmid assay was used. Two plasmids were co-transfected into C6/36 cells: a rHE expression plasmid and a target plasmid containing homing endonuclease sites flanking *sacB*, which is a lethal gene in the presence of sucrose (Gay *et al.*, 1985). C6/36 cells were co-transfected in 12 well plates with 0.25 µg of target plasmid pSLfa/*sacB* and 0.25 µg of either pKhsp82-nls-I-SceI or hsp70-nls-I-SceI expression plasmid. Cells transfected with hsp70-nls-I-SceI, with I-SceI under the control of the *Drosophila melanogaster* heat inducible promoter, were heat shocked 1 hour post-transfection at 37°C for an hour and allowed to recover at 28°C for 1 hour. Total DNA was harvested at 3 days post-transfection, RNase treated, digested with *XmnI* and loaded on a 1% agarose gel for Southern analysis. A biotinylated probe specific to *sacB* was used for detection of *sacB* fragments (Figure 2.11). If I-SceI was able to induce dsDNA breaks resulting in the excision of *sacB*, a 3 kb hybridization signal would be expected, but if I-SceI induced a single dsDNA break at one I-SceI recognition site, then a 1.5 kb hybridization signal would be expected. As seen in Figure 2.11, there was no evidence that *sacB* was excised or that there were unrepaired dsDNA breaks induced by I-SceI; only full length target plasmid was recovered.

Windbichler *et al.* (2007) developed an alternative two plasmid assay in *Anopheles gambiae* cells and embryos. Instead of homing endonuclease recognition sites flanking *sacB*, a single homing endonuclease recognition site was inserted within the *sacB* coding region downstream from the signal peptide required for secretion (Borchert and Nagarajan, 1991). Since I-SceI has been shown to induce dsDNA breaks in *Drosophila melanogaster* (Bellaiche *et al.*, 1999; Gong and Golic, 2003b; Rodin and Georgiev, 2005; Rong and Golic, 2000; Rong and Golic, 2001), we attempted to use S2 cells to repeat their assay. C6/36 cells and S2 cells were co-transfected with 1.25 µg of target plasmid (pSLfa/*sacB* w/SceI in *EcoRI*) and 1.25 µg of an expression plasmid hsp70-nls-I-SceI. Cells transfected with hsp70-nls-I-SceI were heat shocked, as described above, at 5, 11, and 23 hours post-transfection. Low molecular weight DNA was harvested 7, 13, and 25 hours post-transfection and digested with *MluI*. A biotinylated probe specific to *sacB* was used for detection. If there was evidence of I-SceI induced unrepaired dsDNA breaks, an 800 bp hybridization signal would be present, but there was no evidence of any unrepaired dsDNA breaks (Figure 2.12).

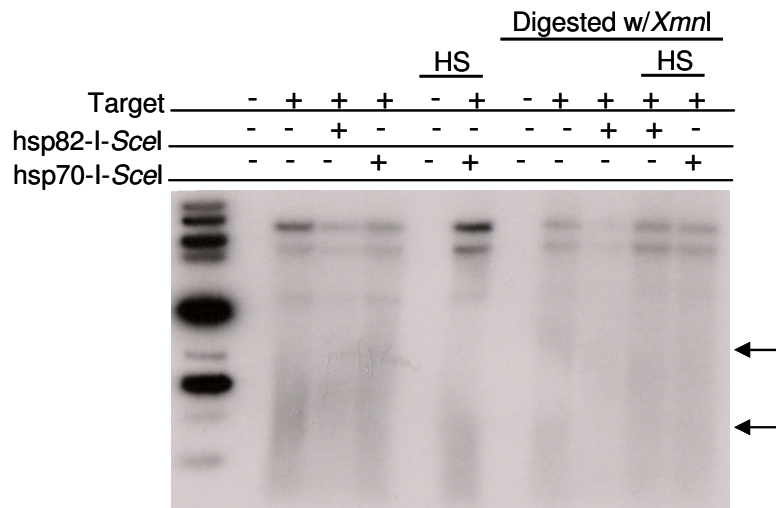


Figure 2.11. Southern analysis for detection of unrepaired dsDNA breaks induced by I-*Sce*I in C6/36 cells cotransfected with expression and target plasmids. Cotransfection of pSLfa/sacB with I-*Sce*I recognition sites flanking *sacB* and either pKhsp82-nls-S_{tag}-I-*Sce*I or hsp70-nls-I-*Sce*I in C6/36 cells. Total DNA was harvested at 3 days post-transfection, RNase treated, digested with *Xmn*I and loaded on a 1% agarose gel for Southern analysis. A biotinylated probe specific to *sacB* was used for detection. HS denotes cells heat shocked at 37°C for 1 hour followed by a 1 hour recovery at 28°C. Arrows indicate expected band sizes.

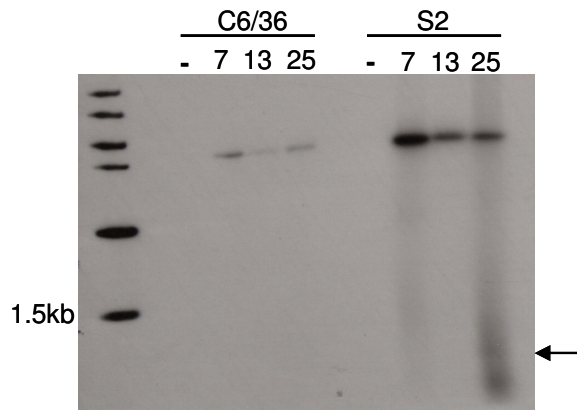


Figure 2.12. Southern analysis for detection of unrepaired dsDNA breaks induced by I-*SceI*. C6/36 cells and S2 cells were co-transfected with hsp70-nls-I-*SceI* and *sacB*/pSLfa w/I-*SceI* site in *EcoRI*. DNA was harvested 7, 13, and 25 hours post-transfection and digested with *MluI*. A biotinylated probe specific to *sacB* was used for detection of I-*SceI* induced dsDNA breaks. Arrow indicates the location of the expected band size if I-*SceI* induced a dsDNA break.

As described earlier (Figure 2.5), the recombinant Sindbis virus expression system TE/3'2J was used to express each rHE. C6/36 cells were seeded in 25 cm² flasks and infected when cells were 90% confluent with TE/3'2J-nls-S_{tag}-I-*PpoI* virus or TE/3'2J-nls-S_{tag}-I-*SceI* virus. At 24 hours post-infection, cells were transfected with 0.5 μg *sacB*/pSLfa with I-*SceI* sites flanking *sacB*. At 1, 2, and 4 hours post-transfection, low molecular weight DNA was harvested from cells and electrophoresed on an agarose gel for Southern analysis (Figure 2.13.A). Cells infected with TE/3'2J-nls-S_{tag}-I-*PpoI* virus were used to control for rHE recognition site specificity. If I-*SceI* induced a dsDNA break, a 3.5 kb hybridization signal would be expected. Results show an increase in the amount of target plasmid DNA recovered as a function of time, but there was no evidence of *sacB* excision as seen by the lack of 3.5 kb hybridization signal in all experimental treatments (Figure 2.13.A).

TE/5'2J ME2-rHE viruses, which were used because of the toxicity observed from TE/3'2J-rHE viruses, were used in assays to detect for induced dsDNA breaks following exposure to each rHE. C6/36 cells were infected with TE/5'2J ME2 virus, TE/5'2J ME2-EGFP virus, or TE/5'2J-rHE viruses (Aa-nls-S_{tag}-I-*AniI*, Aa-nls-S_{tag}-I-*CmoeI*, Aa-nls-S_{tag}-I-*CreI*, nls-S_{tag}-I-*PpoI*, and nls-S_{tag}-I-*SceI*). Cells were infected and split as needed and cell pellets were harvested for western analysis for S_{tag}, Sindbis E2, or EGFP detection (Figure 2.8). EGFP expression was monitored over the course of the infection (Figure 2.9) to determine when protein expression was optimal for transfection of plasmid DNA which was at 4 days post-infection.

C6/36 cells were infected with TE/5'2J ME2-Aa-nls-S_{tag}-I-*CmoeI* or TE/5'2J ME2-nls-S_{tag}-I-*PpoI* viruses, split 4 days post-infection, and transfected 24 hours later with target plasmid pMosDsRed-5HE-UbGFP. Low molecular weight DNA was harvested at 6 and 12 hours post-transfection and digested with *MluI*. A biotinylated probe specific to the 5HE region was used for detection of rHE induced dsDNA breaks (Figure 2.13.B). An 800 bp hybridization signal and would indicate dsDNA breaks induced following exposure to a rHE, but there was no evidence of unrepaired dsDNA breaks resulting from exposure to a rHE except in a positive control which was target plasmid recovered and digested with *MluI* and a commercial preparation of I-*SceI*.

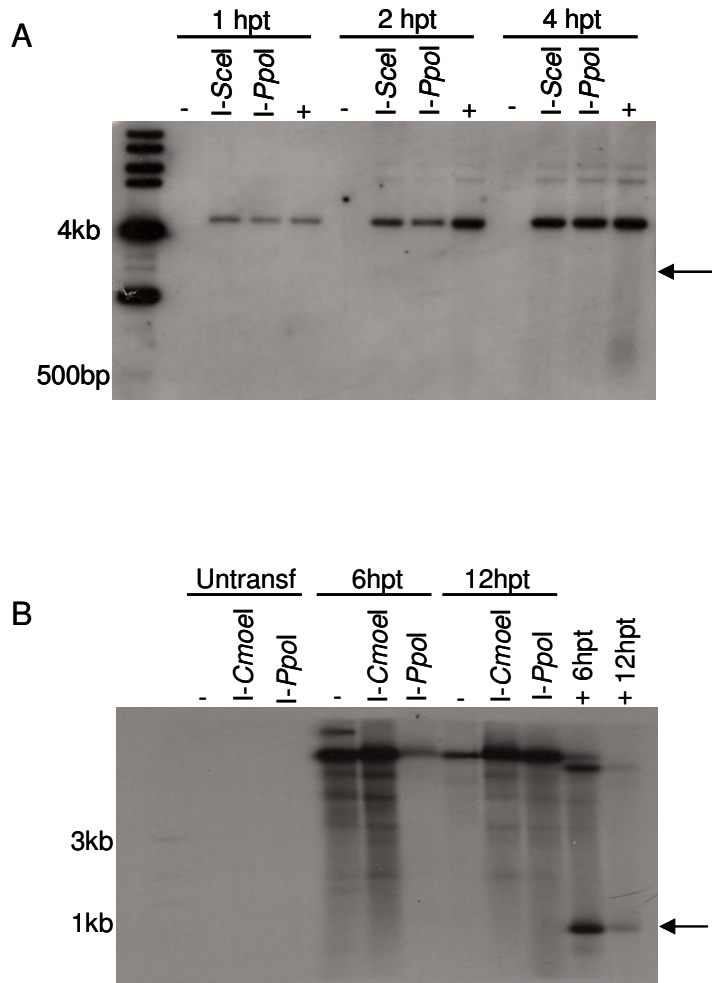


Figure 2.13. Southern analysis to detect unrepaired dsDNA breaks induced by rHE in C6/36 cells infected with TE/3'2J-rHE viruses or TE/5'2J ME2-rHE viruses. (A) C6/36 cells infected with TE/3'2J-nls-S_{tag}-I-PpoI virus or TE/3'2J-nls-S_{tag}-I-SceI virus for 24 hours followed by transfection with *sacB*/pSLfa with I-SceI sites flanking *sacB*. (+) denotes uninfected cells transfected with target plasmid. (-) denotes uninfected and untransfected cells. Arrow indicates expected band size. TE/3'2J-nls-S_{tag}-I-PpoI virus-infected cells were used to control for specificity at target homing endonuclease recognition site. A biotinylated probe specific to *sacB* was used for detection. (B) C6/36 cells infected with TE/5'2J ME2-nls-S_{tag}-I-Cmoel virus or TE/5'2J ME2-nls-S_{tag}-I-PpoI virus for 4 days, split and transfected at 5 days post-infection with a target plasmid construct with five homing endonuclease recognition sites, pMosDsRed 5HE UEGFP. DNA was harvested at 6 and 12 hours post-transfection and digested with *Mlu*I. A biotinylated probe specific to the 5HE region was used for detection. Arrow indicates expected band size.

DISCUSSION

While most described homing endonucleases belong to the LAGLIDADG family, homing endonucleases from multiple families were used to find one that was able to induce dsDNA breaks most efficiently in mosquitoes. I-*Cmo*I and I-*Cre*I are homing endonucleases encoded by a chloroplast intron of *Chlamydomonas moewusii* and *Chlamydomonas reinhardtii*, respectively (Durocher *et al.*, 1989; Rochaix *et al.*, 1985). I-*Ani*I is encoded by a mitochondrial intron from *Aspergillus nidulans* (Waring *et al.*, 1982). I-*Sce*I is also encoded by a mitochondrial intron from *Saccharomyces cerevisiae* (Colleaux *et al.*, 1986). Finally, I-*Ppo*I is encoded by an intron from nuclear DNA in *Physarum polycephalum* (Muscarella and Vogt, 1989). I-*Cmo*I is part of the HNH family, I-*Cre*I, I-*Ani*I, and I-*Sce*I are part of the LAGLIDADG family, and I-*Ppo*I is part of the His-Cys Box family (Jurica and Stoddard, 1999).

S_{tag}-rHE fusion proteins were successfully localized in mosquito cell nuclei when expressed from the *Drosophila pseudoobscura* hsp82 promoter and from both TE/3'2J virus and TE/5'2J ME2 virus expression systems, but nuclear localization of C6/36 cells transfected with I-*Sce*I under the control of the *Aedes aegypti* ubiquitin promoter were inconclusive. Based on detection of I-*Sce*I using western analysis, inconclusive IFA results may have resulted from an antibody not sensitive enough for an IFA. In an IFA, tagged proteins are detected in a native form while proteins detected on a western blot are denatured. Expression for all rHE was detected when expressed from TE/3'2J viruses; however, only I-*Cmo*I and I-*Ppo*I were detected in cells infected with TE/5'2J ME2-rHE viruses. Expression was not observed when cells were transfected with pKhs82-rHE expression vectors. rHE may have been expressed but at a level too low for detection.

Since nuclear localization and expression of rHE through the dsSIN virus expression system was confirmed, assays to test rHE ability to induce dsDNA breaks in cell culture were attempted. The two plasmid assay system and subsequent Southern analysis was based on detection of unrepaired dsDNA breaks. This assay was limiting because only open, unrepaired breaks were being examined and unrepaired dsDNA breaks were either at a level below the threshold for detection, the break had been repaired, or S_{tag}-rHE were unable to induce dsDNA breaks, none of which could be concluded based on this analysis. No evidence of unrepaired dsDNA breaks was

recovered using a cotransfection of target and expression plasmids or transfection of target plasmid followed by infection with a dsSIN virus expressing rHE.

Our results are inconsistent with previous findings using similar assays to evaluate the ability of rHE to induce dsDNA breaks *in vivo* (Chen and Zhao, 2005; Gruen *et al.*, 2002; Windbichler *et al.*, 2007). Since our two plasmid assay system was inconclusive in cell culture, it would be interesting to infect C6/36 cells expressing a rHE, like I-*PpoI*, and then transfect infected cells with a target plasmid containing a homing endonuclease recognition site within *sacB*. Instead of using a Southern analysis to detect for unrepaired cleavage events, harvested low molecular weight DNA could be transformed and cells could be selected by plating bacterial cells on medium containing sucrose. The *sacB* gene is a structural gene from *Bacillus subtilis* and encodes for levansucrase (sucrose:2,6- β -D-fructan; EC 2.4.1.10), which is a 50-kilodalton enzyme that is secreted by *B. subtilis* after induction by sucrose (Gay *et al.*, 1985). Gay *et al.* (1985) found that the production of levansucrase by *E. coli* and other bacteria is lethal in the presence of 5% sucrose. Windbichler *et al.* (2007) found that following exposure to I-*PpoI* or I-*SceI* imperfect gap repair via non-homologous end joining occurred with deletions at the homing endonuclease recognition site. By transforming recovered DNA and selecting for cells that could survive on sucrose, this assay could detect imperfectly repaired dsDNA breaks at the homing endonuclease recognition site. Only cells that had imperfect repair resulting in a disruption of *sacB* following exposure to a rHE would be able to grow on sucrose. Those colonies could be sequenced and analyzed for imperfect repair events within the *sacB* gene.

We were unable to demonstrate, with any of the five rHE tested, that any were able to induce dsDNA breaks in cultured cells. Optimization of transfection procedures and successful recovery of plasmid DNA following transfection was performed in order to test whether rHE were able to induce dsDNA breaks in *Aedes albopictus* C6/36 cells, but there was no evidence of unrepaired dsDNA breaks following exposure to a rHE.

Chapter 3

DEVELOPMENT OF A SOMATIC ASSAY FOR TESTING HOMING ENDONUCLEASE ACTIVITY IN THE YELLOW FEVER MOSQUITO, *Aedes aegypti*

INTRODUCTION

Vector-borne diseases are an emerging global public health issue. Yellow fever, dengue fever, and dengue hemorrhagic fever are diseases caused by viruses transmitted by mosquitoes. Dengue fever is the most common arthropod-borne virus causing an estimated 12,000 deaths per year (Kay and Nam, 2005). *Aedes aegypti*, the primary vector responsible for the spread and transmission of these diseases, is geographically found world-wide with approximately half of the world's population at risk from infection (Halstead, 1988). *Ae. aegypti* are highly anthropophilic but only females are hematophagous (Halstead, 2007). *Ae. aegypti* are container breeders and their distribution and abundance has increased due to the lack of urban infrastructure which results in an inadequate and unreliable water supply requiring people to store water in containers which are ideal habitats for larvae (Kay and Nam, 2005). Prevention of vector-borne diseases and control of vector populations is an important global issue and one proposed method for prevention of vector-borne disease is using a genetic control strategy in which transgenic mosquitoes refractory to a target pathogen could be used to replace the wild-type mosquito population.

Homing endonucleases can be used as a tool to study dsDNA break repair mechanisms in mosquitoes. In *Ae. aegypti* it is currently unknown which repair pathway is used or favored following a dsDNA break. Potential applications of HEGs could be used for decreasing vector fitness to decrease population densities, to disrupt a gene or genes necessary for pathogen transmission, to bias the sex ratio towards males, and as a mechanism to drive an anti-pathogen gene into a vector population as part of a population replacement strategy (Deredec *et al.*, 2008; Sinkins and Gould, 2006). The hypothesis behind using homing endonucleases as a means for genetic control of mosquito populations is that if there is a reduction in vector competence, then there should be a corresponding reduction in the prevalence of diseases transmitted by the target vector.

Sindbis virus expression vectors have been used to express heterologous proteins in a variety of organisms. Transient expression of anti-sense RNA against dengue type 2 virus resulting in the inhibition of virus replication in the salivary glands of *Ae. aegypti* and against transgenic expression of luciferase in *Ae. aegypti* (Adelman *et al.*, 2001; Johnson *et al.*, 1999; Olson *et al.*, 1996); to express green fluorescent protein in *Ae. aegypti* midguts (Olson *et al.*, 2000); and to express the scorpion Scotox gene in mosquitoes (*Ae. aegypti*, *Ae. triseriatus*, *Culex pipiens*), houseflies (*Musca domestica*), and ticks (*Dermacentor andersoni*) for the evaluation of this gene as a biocontrol agent (Higgs *et al.*, 1995).

Identification of mutations leading to mismatch base pairing between DNA strands is an important tool (Kulinski *et al.*, 2000). Mismatch specific DNA endonucleases are found commonly in plants in the roots, stems, leaves, flowers, and fruits (Yang *et al.*, 2000). CEL I, isolated from celery, is a nuclease that recognizes distortions resulting from base-substitution mismatches in DNA and subsequently cleaves the 3'-side of a mismatch in one of the two DNA strands (Oleykowski *et al.*, 1998). CEL I has been used to identify mutations, mismatch repair, and polymorphisms in the human BRCA1 gene (Kulinski *et al.*, 2000; Oleykowski *et al.*, 1998; Pimkin *et al.*, 2007; Yang *et al.*, 2000) and human mitochondrial DNA mutations associated with respiratory chain defects (Bannwarth *et al.*, 2005). It has also been used in screening induced point mutations in *Arabidopsis* (Colbert *et al.*, 2001; Till *et al.*, 2003) and in *Lotus* (Perry *et al.*, 2003). Furthermore, CEL I has been used to determine whether zinc finger nucleases induce mutations by non-homologous end joining in endogenous human genes *VEGF-A* and *HoxB13* (Maeder *et al.*, 2008). A CEL I ortholog, CEL II, is able to cleave both strands of DNA on the 3'-side of a mismatch nonspecifically recognizing base substitutions, insertions or deletions, multiple mutations in DNA even when there is a small proportion of mismatched DNA present (Qiu *et al.*, 2004). CEL II treatment of DNA is a simple and reliable mutation detection assay. In this assay, PCR is used to amplify target DNA from mutants and wild-type samples. A hybridization step between mutant and wild-type DNA is performed to form heteroduplexes. The DNA from both samples is then treated with nuclease and DNA analysis is performed, most commonly with gel electrophoresis (Qiu *et al.*, 2004).

This objective seeks to address whether recombinant homing endonucleases (rHE) can induce dsDNA breaks in chromosomal DNA in *Ae .aegypti*. We hypothesize that if rHE are able to induce dsDNA breaks at exogenously introduced homing endonuclease sites, then there should be evidence of repair at the homing endonuclease recognition site confirmed through sequence analysis.

MATERIALS AND METHODS

Rearing mosquitoes

Aedes aegypti (*kh^w* and Liverpool strains) were maintained at 28°C in an environmental chamber with a relative humidity of ~80% with a 15 hour light and 9 hour dark cycle. *Aedes aegypti* transgenic lines UUGFP #18 and UUGFP #P17A were also maintained at 28°C in environmental chambers with a relative humidity of ~80%. Transgenic lines were screened using a fluorescent Leica MZ16F microscope as either larvae or pupae for DsRed⁺ eyes/EGFP⁺ bodies.

Injections of double subgenomic Sindbis virus expressing rHE in *Ae. aegypti*

Approximately 2 day old adult female transgenic mosquitoes were intrathoracically injected with 0.4-0.5 µl of recombinant Sindbis virus ranging from 2250 to 11305 plaque forming units (pfu) for TE/5'2J ME2-rHE viruses. Whole bodies and head/thorax were harvested at various times post-infection, snap frozen, and stored at -80°C.

SDS-PAGE and western analysis

Cells were seeded in 25 cm² flasks and infected with a multiplicity of infection of one. Cells were harvested as follows: cells were washed with PBS, scraped in 1 ml PBS, and centrifuged at 1000 x g for 5 minutes at 4°C. Cell pellets were washed three times with PBS and centrifuged. Cell pellets were lysed with 2X SDS Loading buffer (Novagen) and boiled at 100°C for 5 minutes. Sample lysates were centrifuged for 1 minute at 13,000 rpm, and were separated on a 4% stacking 10% resolving SDS polyacrylamide gel (0.75 mm). Gels were run at 90V for 90 minutes at room temperature. The gel, membrane, sponges, and filter paper were equilibrated in transfer buffer (39 mM glycine, 48 mM Tris, 1.3 mM SDS, 20% methanol) for 10 minutes. The gel was transferred to a nitrocellulose membrane (0.45-µm pore size; Biorad) at 100V for 1 hour. The membrane was blocked in 3% non-fat dry milk in Tris buffered saline with 1% Tween-20 (TBST) for 30 minutes, incubated with primary antibody and rocked gently at room temperature followed by incubation with a secondary antibody. For antibody dilutions see Table 3.1. The membrane was washed with TBST and incubated in the dark room with 500 µl of 2X luminol/enhancer and 500 µl stable peroxide solution (Novagen) for 1 minute. Proteins were detected using X-ray film (Kodak) and developed with a Konica SRX-101A processor.

Four mosquitoes per experimental treatment were crushed with a pestle in 2X SDS Loading buffer (Novagen) and boiled at 100°C for 5 minutes. Sample lysates were centrifuged for 5 minutes at 13,000 rpm. The supernatant was removed and centrifuged again for 5 minutes to remove all debris. The supernatant was decanted and separated on a 4% stacking 10% resolving SDS polyacrylamide gel (0.75 mm) as described above.

Genomic DNA extraction and Southern analysis

Six female mosquitoes per experimental treatment per time point were crushed with a pestle in Bender Buffer (0.1 M NaCl, 0.2 M Sucrose, 0.1 M Tris, pH 9.0, 0.05 M EDTA, 0.5 M SDS). Lysates were incubated with 20 µl proteinase K at 50°C overnight. DNA was phenol:chloroform extracted twice with gentle rocking followed by an isopropanol precipitation. DNA was resuspended in DEPC-treated water overnight. Genomic DNA isolated from TE/5'2J ME2-rHE virus-infected mosquitoes was digested overnight, ethanol precipitated, and loaded on an agarose gel for Southern analysis. The probe was randomly primed and labeled with [α -³²P] dATP, specific activity 3000 Ci/mmol using the Amersham Megaprime DNA labeling System (GE Healthcare, Buckinghamshire, UK) and purified using illustra NICK columns (GE Healthcare). The membrane was exposed to Kodak BioMax maximum sensitivity film at -80°C.

Genomic DNA footprint analysis

Cloning of homing endonuclease site. Genomic DNA from UUGFP #18 uninfected and TE/5'2J ME2-nls-S_{tag}-I-PpoI virus-infected mosquitoes was amplified using a proofreading DNA polymerase Pfx (Invitrogen) and primers 5'-CGAAACGGTGAATACGGCACGCTA-3' and 5'-CGCCACCACCTGTTTCCTGTA-3' (94°C, 2 min; 94°C, 30 sec; 58°C, 1:30 min; 68°C, 1min; 35 cycles; 68°C, 10 min). The amplified 1 kb region contained recognition sites for five homing endonucleases. This PCR amplicon was cloned with a Zero Blunt TOPO PCR Cloning kit (Invitrogen) and sequenced. Genomic DNA from UUGFP #18 uninfected and TE/5'2J ME2-nls-S_{tag}-I-SceI virus-infected mosquitoes was amplified using Phusion polymerase (New England Biolabs) and the same primers listed above (98°C, 1 min; 98°C, 15 sec; 58°C, 1 min; 72°C, 1min; 35 cycles; 72°C, 10 min). This PCR amplicon was digested with a commercial preparation of I-SceI (New England Biolabs), electrophoresed, and the undigested area was gel extracted and reamplified as described above. The enriched PCR amplicon was digested again with I-SceI and

electrophoresed. The resulting PCR amplicon was cloned as described above. Genomic DNA from UUGFP #P17A uninfected and TE/5'2J ME2-nls-S_{tag}-I-*PpoI* virus-infected mosquitoes was amplified using a proofreading DNA polymerase *Pfx* (Invitrogen) and primers 5'-CGCCACCACCTGTTTCCTGTA-3' and 5'-AACGTGTGAACGGTGGTTTCAACGCTTC-3' (98°C, 1 min; 98°C, 15 sec; 58°C, 30 sec; 72°C, 1:30 min; 35 cycles; 72°C, 10 min). The amplified 2.1 kb region contained recognition sites for five homing endonucleases flanking EGFP. This PCR amplicon was cloned with a Zero Blunt TOPO PCR Cloning kit (Invitrogen) and sequenced.

Surveyor Nuclease assay. Additionally, PCR amplicons from genomic DNA generated from UUGFP #18 uninfected and TE/5'2J ME2-nls-S_{tag}-I-*PpoI* virus-infected mosquitoes were digested with Surveyor Nuclease according to the manufacturer's protocol (Transgenomic). For digests with Surveyor Nuclease, reactions were vortexed and incubated at 42°C for 20 minutes and stopped by the addition of 2 µl (1/10th volume) stop reagent. Digested products were loaded on a 2% agarose gel prior to Southern analysis. Detection was performed using a biotinylated probe following instructions in the NEBlot Phototope Kit (New England Biolabs).

Commercial homing endonuclease assay. PCR amplicons from genomic DNA generated from UUGFP #18 uninfected and TE/5'2J ME2-nls-S_{tag}-I-*PpoI* virus-infected mosquitoes were digested with commercial preparations of available homing endonuclease enzymes (*I-PpoI*, Promega; and *I-SceI*, New England BioLabs). Digested products were electrophoresed on a 1% agarose gel.

Table 3.1. Antibodies used for protein detection

Antibodies for Western	Dilution	Time
S-Tag monoclonal antibody mouse IgG (Novagen)	1:5000	30 min
Anti-E2 mouse monoclonal antibody (Mab92hs)	1:200	30 min
Anti-GPF mouse IgG mAb 11E5 (Molecular Probes)	1:200	1hr
Goat Anti-Mouse IgG Peroxidase Conjugate (Calbiochem)	1:10,000	30 min

RESULTS

SDS-PAGE and western analysis

To determine if S_{tag}-rHE could be expressed in mosquitoes, adult female *Aedes aegypti* (line UUGFP #18) were injected with recombinant Sindbis virus TE/5'2J ME2-rHE (Aa-nls-S_{tag}-I-AniI, Aa-nls-S_{tag}-I-CmoEI, nls-S_{tag}-I-PpoI, or nls-S_{tag}-I-SceI). Following injection, whole bodies were harvested and analyzed for S_{tag}-rHE expression at 24, 48, 96 hours post-infection and 10 days post-infection using western analysis. S_{tag}-rHE were detected using an anti-S_{tag} (Figure 3.1.A, C, and E) and E2 viral protein was detected with an anti-E2 antibody (Figure 3.1.B, D, and F). S_{tag}-rHE expression was not observed at 24, 48, or 96 hours post-infection. However, after 10 days post-infection, both S_{tag} fusion-rHE and E2 proteins were detected in mosquitoes infected with TE/5'2J ME2-Aa-nls-S_{tag}-I-CmoEI virus or TE/5'2J ME2-nls-S_{tag}-I-PpoI virus (Figure 3.1.E and F). Mosquitoes infected with TE/5'2J ME2-EGFP virus were used as a control since EGFP was observed in infected mosquitoes using fluorescence microscopy. Both EGFP (Figure 3.1.G) and E2 were detected (Figure 3.1.H) at all time points post-infection.

Western analysis was performed on head/thorax samples from UUGFP #18 female mosquitoes injected with TE/5'2J ME2-Aa-nls-S_{tag}-I-CmoEI virus or TE/5'2J ME2-nls-S_{tag}-I-PpoI virus. Mosquitoes were infected for 4, 7, 10, and 14 days after which rHEs were detected using an anti-S_{tag} or an anti-E2 antibody. E2 was detected in mosquitoes infected with either virus at all time points, but no rHE was detected (data not shown). A 14 day post-infection time point was not available for TE/5'2J ME2-nls-S_{tag}-I-PpoI virus-infected mosquitoes because infected mosquitoes did not survive beyond 10 days post-infection. High mortality rates in mosquitoes infected with TE/5'2J ME2-nls-S_{tag}-I-PpoI virus may be a consequence of an I-PpoI recognition site located on the 28S rRNA (Monnat *et al.*, 1999).

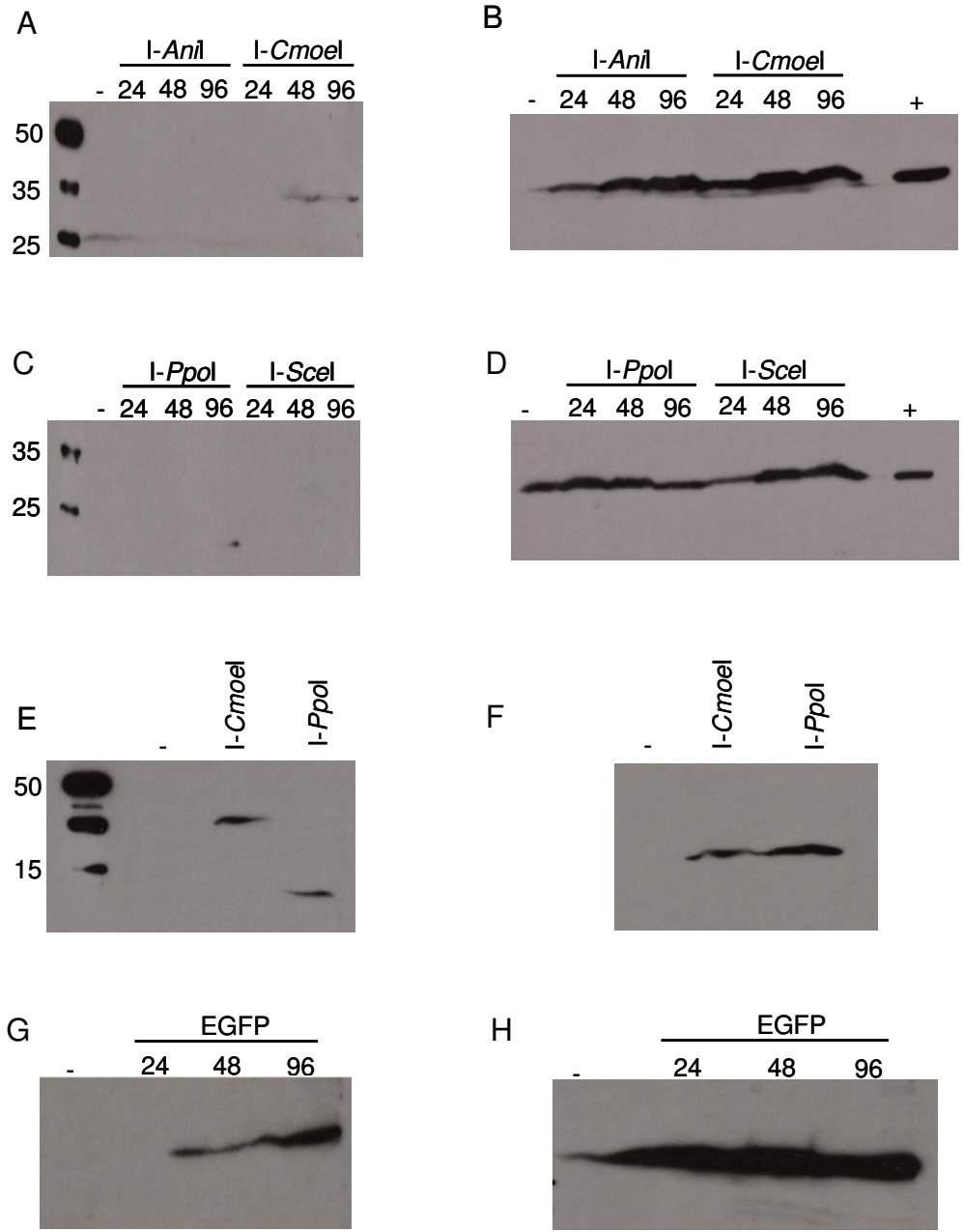


Figure 3.1. Western analysis for detection of rHE, Sindbis E2, or EGFP in UUGFP #18 mosquitoes infected with TE/5'2J ME2-rHE viruses. UUGFP #18 female mosquitoes injected TE/5'2J ME2-rHE viruses were lysed in 2X SDS Loading Buffer 24, 48, and 96 hours post-infection (A-D, G, H) and 10 days post-infection (E and F). Total protein extract was loaded for SDS-PAGE and transferred to a nitrocellulose membrane for western analysis using an anti-S_{tag} (A, C, and E), an anti-E2 (B, D, F, and H), or an anti-GFP (G) antibody.

Southern analysis for rHE activity

In order to determine if rHE were able to induce dsDNA breaks in mosquito chromosomal DNA, adult female mosquitoes were intrathoracically injected with TE/5'2J ME2-EGFP, TE/5'2J ME2-Aa-nls-S_{tag}-I-*AniI*, TE/5'2J ME2-Aa-nls-S_{tag}-I-*CmoI*, TE/5'2J ME2-nls-S_{tag}-I-*PpoI*, or TE/5'2J ME2-nls-S_{tag}-I-*SceI* viruses. rHE viruses were injected into transgenic *Ae. aegypti* line (UUGFP #18) which contains an exogenous DsRed/EGFP dual transgene with homing endonuclease sites between the two marker genes was used (Figure 3.2). Southern analysis was performed 96 hours post-infection following exposure of rHE to detect for unrepaired dsDNA breaks induced by rHE (Figure 3.3). If there was evidence of dsDNA breaks induced by a rHE, then there would be a shift from a common hybridization signal at 4 kb to a 2.7 kb hybridization signal, but no shift in signal was observed (Figure 3.3). No unrepaired breaks were detected. We hypothesized that either dsDNA breaks were not induced by rHE, breaks were induced at a level below the threshold for detection, induced dsDNA breaks were repaired too rapidly to be observed with this assay.

Genomic DNA footprint analysis

As we could not detect open, unrepaired dsDNA breaks using Southern analysis, a genomic footprint analysis was performed to detect evidence of imperfectly repaired dsDNA breaks induced by rHE at each homing endonuclease recognition site. Two different assays were used as part of this genomic DNA footprint analysis: a Surveyor Nuclease assay and a digest using commercial preparations of available homing endonucleases.

Surveyor Nuclease assay. A CEL II-based Surveyor Nuclease assay was used to detect evidence of mismatch base repair. PCR amplicons (125 ng) generated from UUGFP #18 uninfected mosquitoes or mosquitoes infected with TE/5'2J ME2-nls-S_{tag}-I-*PpoI* virus were digested with 1 µl of various dilutions (1:2, 1:5, 1:10, 1:15, 1:20, or 1:50) of both Surveyor Nuclease and enhancer (Transgenomic) in 20 µl total volume. We hypothesized that if rHE induced dsDNA breaks followed by imperfect repair, then we would expect to see 400 and 600 bp restriction fragments. It appeared as though there was imperfect repair at the I-*PpoI* recognition site as seen by the presence of the expected 400 and 600 bp restriction fragments which were not seen in uninfected mosquitoes (Figure 3.4.A). No evidence of imperfect repair was seen for TE/5'2J

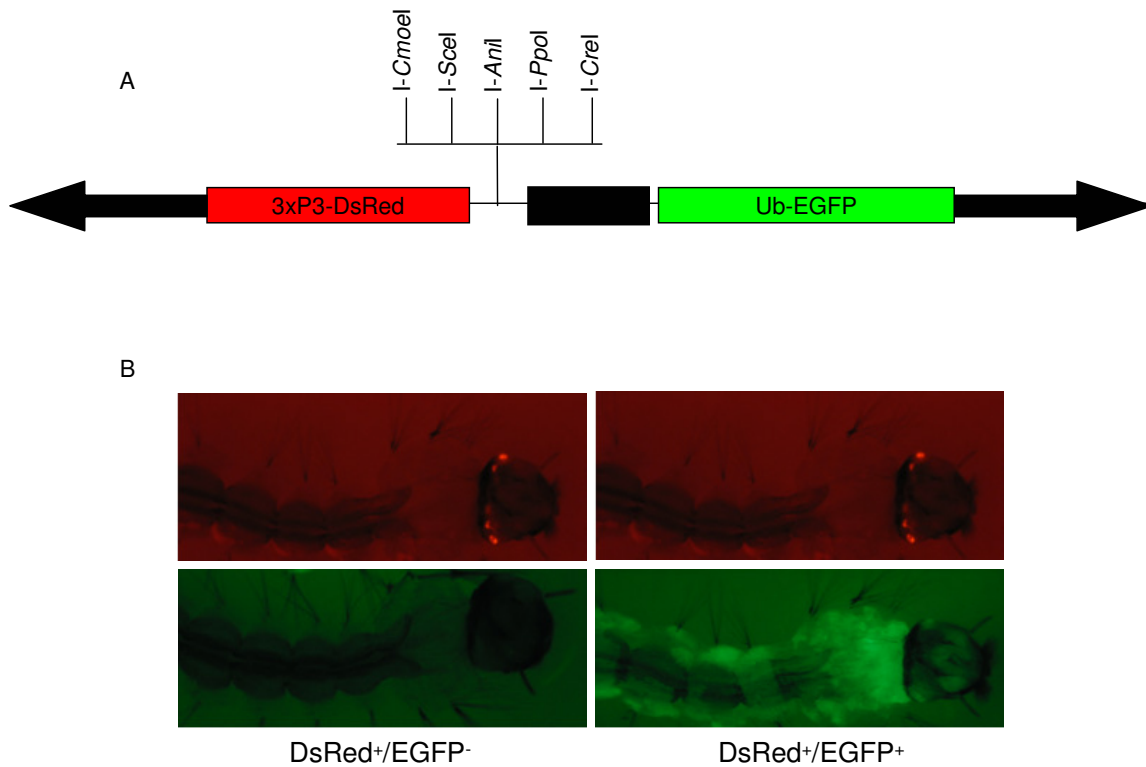


Figure 3.2. Schematic depiction of *Aedes aegypti* transgenic UUGFP #18. (A) DsRed is under the control of the eye specific 3xP3 promoter while EGFP is under the control of the mosquito ubiquitin promoter which is expressed throughout the body during larval development. The dual transgene construct contains five homing endonuclease recognition sites between the two marker genes. (B) Pictures of DsRed⁺/EGFP⁺ and DsRed⁺/EGFP⁻ larvae.

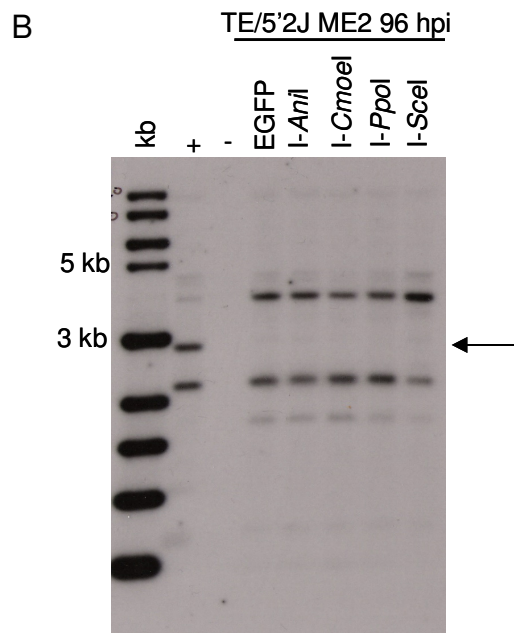
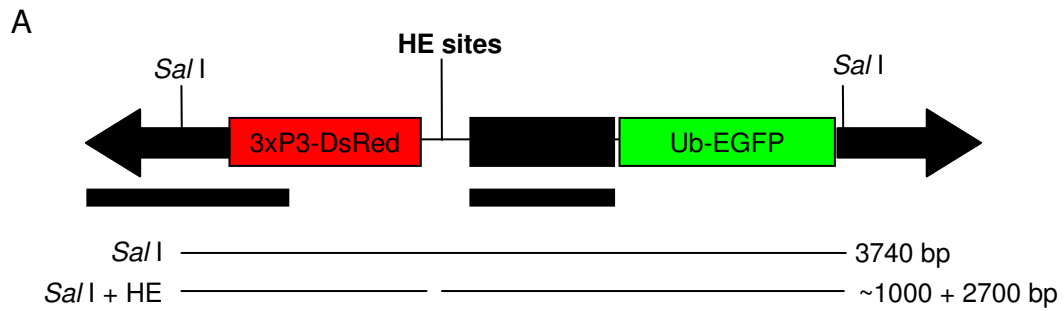


Figure 3.3. Southern analysis for unrepaired dsDNA breaks induced by a rHE. (A) Schematic for line UUGFP #18 with location of restriction and homing endonuclease sites with expected restriction fragment sizes. The black lines below the construct represent where the probe hybridizes. (B) Genomic DNA from UUGFP #18 female mosquitoes injected with TE/5'2J ME2-EGFP, TE/5'2J ME2-Aa-nls-S_{tag}-I-AniI, TE/5'2J ME2-Aa-nls-S_{tag}-I-Cmoel, TE/5'2J ME2-nls-S_{tag}-I-PpoI, or TE/5'2J ME2-nls-S_{tag}-I-SceI viruses was extracted 96 hours post-infection and digested with *SalI*. Unrepaired breaks were detected using a ³²P labeled probe from plasmid pMosDsRed-5HE-RH-AeUbEGFP-5HE digested with *HindIII*. Arrow demonstrates expected hybridization signal indicative of rHE induced dsDNA breaks. (+) denotes uninfected UUGFP #18 genomic DNA digested with *SalI* and a commercial preparation of I-SceI (New England Biolabs) (-) denotes genomic DNA from *kh^w* mosquitoes.

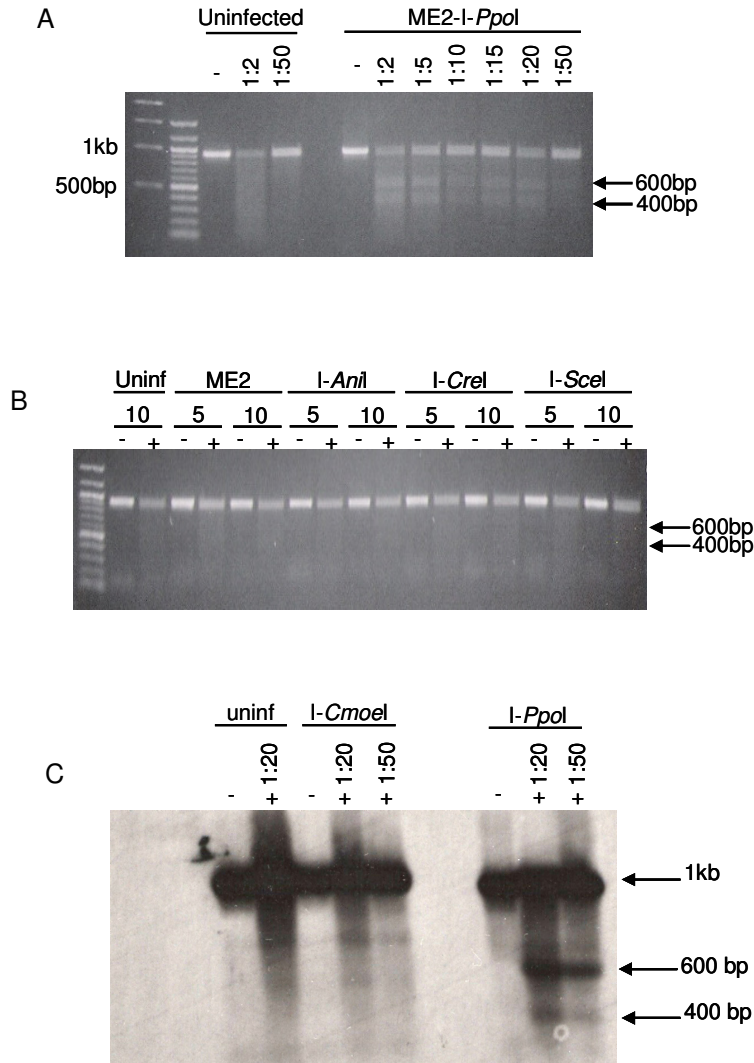


Figure 3.4. Detection of mismatch base pairing in TE/5'2J ME2-rHE virus-infected UUGFP #18 mosquitoes using Surveyor Nuclease. (A) Detection of mismatch base pairing using PCR amplicons from uninfected or TE/5'2J ME2-nls-S_{tag}-I-PpoI virus-infected mosquitoes. Amplicons were digested with varying dilutions of Surveyor Nuclease. (B) PCR amplicons from uninfected, TE/5'2J ME2, TE/5'2J ME2-Aa-nls-S_{tag}-I-AniI, TE/5'2J ME2-Aa-nls-S_{tag}-I-CreI, or TE/5'2J ME2-Aa-nls-S_{tag}-I-SceI virus-infected mosquitoes digested with Surveyor Nuclease at a 1:20 dilution. (-) denotes undigested and (+) denoted digested with Surveyor Nuclease. (C) PCR amplicons from mosquitoes infected with TE/5'2J ME2-nls-S_{tag}-I-PpoI virus or TE/5'2J ME2-nls-S_{tag}-I-Cmoel virus were digested with varying dilutions of Surveyor Nuclease. Southern analysis was used to detect mismatch repair in mosquitoes infected with TE/5'2J ME2-nls-S_{tag}-I-Cmoel virus or TE/5'2J ME2-nls-S_{tag}-I-PpoI virus. The PCR amplicon from uninfected mosquitoes was used as a biotinylated probe for detection of hybridization signals indicative of rHE induced dsDNA breaks. Arrows indicate hybridization signal sizes expected if rHE induced dsDNA breaks.

ME2 virus-infected mosquitoes, as expected, but also for TE/5'2J ME2-Aa-nls-S_{tag}-I-AniI, TE/5'2J ME2-Aa-nls-S_{tag}-I-CreI, or TE/5'2J ME2-nls-S_{tag}-I-SceI virus-infected mosquitoes (Figure 3.4.B). Southern analysis was used for increased sensitivity in TE/5'2J ME2-Aa-nls-S_{tag}-I-CmoE virus-infected mosquitoes, but no evidence of mismatch repair was observed as seen by the lack of hybridization signals of the expected size (Figure 3.4.C). If mismatch repair occurred in TE/5'2J ME2-Aa-nls-S_{tag}-I-CmoE virus-infected mosquitoes, it was below the level of detection for this assay.

Commercial homing endonuclease assay. Whereas the Surveyor Nuclease assay was non-specific and used to detect mismatch repair, commercial homing endonuclease digests were used to detect evidence of imperfect repair of dsDNA breaks in an rHE-specific manner. Genomic DNA was extracted from uninfected or TE/5'2J ME2-nls-S_{tag}-I-PpoI virus-infected female UUGFP #18 mosquitoes at 7 and 10 days post-infection. PCR was used to amplify a 1 kb fragment containing the I-PpoI recognition site (Figure 3.5.A). This PCR amplicon (100 ng) was digested with a commercial preparation of I-PpoI (Promega) at a dilution of 1:10. If I-PpoI induced a dsDNA break which was repaired imperfectly, then a commercial preparation of I-PpoI would not be able to recognize the site. We hypothesize that the PCR amplicon from uninfected mosquitoes would be digested completely resulting in 400 and 600 bp restriction fragments while PCR amplicons from TE5'2J ME2-nls-I-PpoI virus-infected mosquitoes would have a mixture of digested and undigested DNA (1 kb, 600, and 400 bp restriction fragments).

Digest analysis demonstrated that the PCR amplicon from uninfected mosquitoes was digested completely (Figure 3.5.B) while the PCR amplicon from TE5'2J ME2-nls-S_{tag}-I-PpoI virus-infected mosquitoes was incompletely digested (Figure 3.5.B). This, in combination with the Surveyor Nuclease assay, suggested that I-PpoI was able to induce dsDNA breaks at its recognition site which were repaired imperfectly through non-homologous end joining.

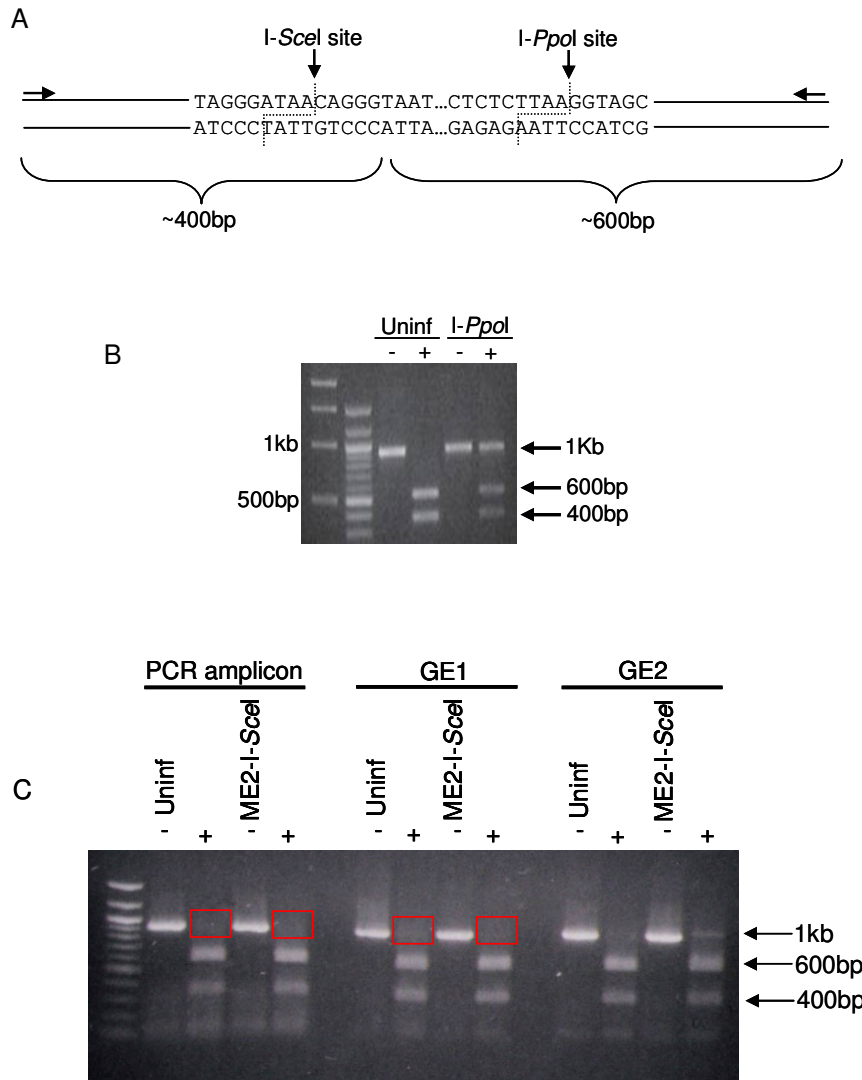


Figure 3.5. Commercial homing endonuclease digests for detection of imperfect repair at the homing endonuclease recognition site in UUGFP #18 mosquitoes exposed to a rHE. (A) Schematic depiction for the generation of PCR amplicons from uninfected or TE/5'2J ME2-rHE virus-infected mosquitoes. Brackets represent predicted band size following a commercial homing endonuclease digest. (B) PCR amplicon from uninfected or TE/5'2J ME2-nls-S_{tag}-I-PpoI virus-infected mosquitoes digested with I-PpoI (Promega). (C) PCR enrichment for rare imperfect repair events following exposure to I-SceI (New England Biolabs). PCR amplicons were generated from genomic DNA extracted from uninfected or TE/5'2J ME2-nls-S_{tag}-I-SceI virus-infected mosquitoes. Gel extracted 1 (GE1) was the 1 kb undigested area from the original PCR amplicon digested with I-SceI which was gel extracted and re-amplified followed by an I-SceI digest. GE2 was the 1 kb undigested area from GE1 which was re-amplified followed by an I-SceI digest. PCR enriched amplicons from GE2 were cloned and used for sequence analysis. Red boxes represent the 1 kb undigested template used for PCR amplification and enrichment. 400 and 600 bp restriction fragments represent digested PCR amplicon. (-) denotes undigested (+) denotes digested with I-SceI.

Additionally, genomic DNA was extracted from TE/5'2J ME2-nls-S_{tag}-I-SceI virus-infected female UUGFP #18 mosquitoes at 7 and 10 days post-infection. PCR was used to amplify a 1 kb fragment containing the I-SceI recognition site. This PCR amplicon (100 ng) was digested with a commercial preparation of I-SceI (New England Biolabs). Complete digestion was observed based on the presence of 400 and 600 bp restriction fragments (Figure 3.5.C). The undigested 1 kb region was gel extracted, and used as a template for re-amplification followed by an I-SceI digest to enrich for rare events in which there was incomplete digestion, possibly due to imperfect repair at the I-SceI recognition site (Figure 3.5.C). After two rounds of enrichment, an undigested fragment was observed. This suggested that there were rare events when dsDNA breaks were induced following exposure to I-SceI.

Confirmation of imperfect repair using DNA sequence analysis. To confirm that rHE-induced dsDNA breaks were occurring at exogenously introduced homing endonuclease recognition sites, PCR amplicons from uninfected and TE/5'2J ME2-nls-S_{tag}-I-PpoI virus-infected UUGFP #18 mosquitoes were cloned and sequenced. Based on observations from commercial homing endonuclease digests and Surveyor Nuclease assays, we hypothesized that in DNA exposed to I-PpoI there would be evidence of imperfect repair at the I-PpoI recognition site. Sequence results from 23 out of 23 clones (100%) from uninfected mosquitoes revealed no alteration at the exogenous I-PpoI site (Figure 3.6). In contrast, 8 out of 20 clones (40%) exposed to I-PpoI had evidence of imperfect gap repair overlapping the I-PpoI recognition site. In 5 out of 8 clones exclusively at the recognition site, 2 out of 8 overlapping both the I-PpoI and I-CmoEI, I-SceI, and I-AniI sites, and 1 out of 8 overlapping both the I-PpoI and I-CreI sites (Figure 3.6). Deletion of base pairs was observed ranging from a 1 to a 65 bp deletion with no other base pair changes observed in the sequenced PCR product. The average deletion size observed was 20 bp while the median deletion size was between 4 and 6 bp.

I-Ppol ↓

```

#18      ... I-CreI ... I-Scel ... I-Aml ... I-CreI ...
...TCGTAGCAGCTCACGGTTAGGGATAACAGGGTAATTATTTGAGGAGGTTTCTCTGTAATAATGACTCTCTTAAAGGTAGCCTAGAACAAAAACGTCGTGAGACAGTTTGGT...
uninfected ...TCGTAGCAGCTCACGGTTAGGGATAACAGGGTAATTATTTGAGGAGGTTTCTCTGTAATAATGACTCTCTTAAAGGTAGCCTAGAACAAAAACGTCGTGAGACAGTTTGGT... 23/23
ME2-I-Ppol ...TCGTAGCAGCTCACGGTTAGGGATAACAGGGTAATTATTTGAGGAGGTTTCTCTGTAATAATGACTCTCTTAAAGGTAGCCTAGAACAAAAACGTCGTGAGACAGTTTGGT... 12/20
...TCGTA-----TTAAGGTAGCCTAGAACAAAAACGTCGTGAGACAGTTTGGT... 2/20
...TCGTAGCAGCTCACGGTTAGGGATAACAGGGTAATTATTTGAGGAGGTTTCTCTGTAATAATGACTCTC-----GGTAGCCTAGAACAAAAACGTCGTGAGACAGTTTGGT... 2/20
...TCGTAGCAGCTCACGGTTAGGGATAACAGGGTAATTATTTGAGGAGGTTTCTCTGTAATAATGACTCTCTTA-GGTAGCCTAGAACAAAAACGTCGTGAGACAGTTTGGT... 2/20
...TCGTAGCAGCTCACGGTTAGGGATAACAGGGTAATTATTTGAGGAGGTTTCTCTGTAATAATGACTCTCT-----AGCCTAGAACAAAAACGTCGTGAGACAGTTTGGT...
...TCGTAGCAGCTCACGGTTAGGGATAACAGGGTAATTATTTGAGGAGGTTTCTCTGTAATAATGACTCTCTTAA-----AAACGTCGTGAGACAGTTTGGT...

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↓

Figure 3.6. Sequence analysis of UUGFP #18 mosquitoes exposed to I-Ppol. PCR amplicons from UUGFP #18 uninfected or TE/5'2J ME2-nls-S_{tag}-I-Ppol virus-infected mosquitoes were cloned and sequenced to determine if there was evidence of imperfect repair. Imperfect gap repair was observed in 8/20 clones (40%) from mosquitoes exposed to I-Ppol. There was no evidence of repair in uninfected mosquitoes.

Similarly, PCR enriched amplicons from uninfected or TE/5'2J ME2-nls-S_{tag}-I-SceI virus-infected UUGFP #18 mosquitoes were cloned and sequenced. Sequence results from 13 out of 13 clones (100%) from uninfected mosquitoes revealed no alteration at the I-SceI site (Figure 3.7); however, 3 out of 14 clones (21%) exposed to I-SceI had evidence of imperfect gap repair at the I-SceI recognition site. Like mosquitoes exposed to I-PpoI, deletions only occurred at the I-SceI site and between 1 to 14 bp. The average deletion size observed was 6 bp with the median size being 3 bp.

The commercial I-SceI digest results from TE/5'2J ME2-nls-S_{tag}-I-SceI virus-infected mosquitoes suggested that the Surveyor Nuclease assay was not as sensitive compared to specific commercial digests. PCR amplicons from unaltered homing endonuclease sites and rare events where there was manipulation at the recognition site were of equivalent sizes. An alternative for enriching for rare events is to use an additional transgenic line so that there would be a size difference between PCR amplicons that had no dsDNA breaks and those with rHE-induced dsDNA breaks. Based on the PCR amplicon size difference, detection of rHE-induced dsDNA breaks could be selected. Line UUGFP #P17A contains a dual DsRed/EGFP transgene and homing endonuclease sites flanking the EGFP gene (Figure 3.8). Evidence of rHE-induced dsDNA breaks and subsequent repair could be detected based on the excision of the EGFP gene. The homing endonuclease recognition sites are arranged asymmetrically so that each rHE will leave a unique and identifiable pattern and number of sites following the excision of EGFP which will allow for differentiating between perfect repair and no induced dsDNA breaks at a homing endonuclease site. Also, this second transgenic line was used to verify that rHE activity was independent of transgene position in the chromosome. Since I-PpoI had been shown to induce dsDNA breaks in line UUGFP #18, I-PpoI was used as a positive control for line UUGFP #P17A.


```

#18 ... TACGAAGTTATAATCGTAGCAGCTCACGGTTAGGGATAACAGGGTAAATTATTTGAGGAGGTTTCTCTGTAATAAT...
Uninfected ... TACGAAGTTATAATCGTAGCAGCTCACGGTTAGGGATAACAGGGTAAATTATTTGAGGAGGTTTCTCTGTAATAAT... .13/13
ME2-I-SceI ... TACGAAGTTATAATCGTAGCAGCTCACGGTTAGGGATAACAGGGTAAATTATTTGAGGAGGTTTCTCTGTAATAAT... .1/14
... TACGAAGTTATAATCGTAGCAGCTCACGGTTAGGG-----ATTTGAGGAGGTTTCTCTGTAATAAT... 1/14
... TACGAAGTTATAATCGTAGCAGCTCACGGTTAGGG---ACAGGGTAAATTATTTGAGGAGGTTTCTCTGTAATAAT... 1/14
... TACGAAGTTATAATCGTAGCAGCTCACGGTTAGGG-TAACAGGGTAAATTATTTGAGGAGGTTTCTCTGTAATAAT... 1/14

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I-Cmoel
I-SceI
I-Anil

Figure 3.7. Sequence analysis of UUGFP #18 mosquitoes exposed to I-SceI. PCR enriched amplicons from UUGFP #18 uninfected or TE/5'2J ME2-nls-S_{tag}-I-SceI virus-infected mosquitoes were cloned and sequenced to determine if there was evidence of imperfect repair. Imperfect gap repair was observed in 3/11 (27%) clones in mosquitoes exposed to I-SceI. There was no evidence of repair in uninfected mosquitoes.

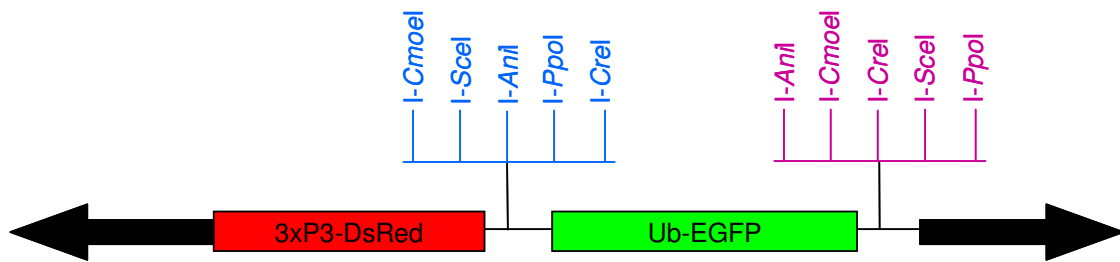


Figure 3.8. Schematic depiction of *Aedes aegypti* transgenic UUGFP #P17A. DsRed is under the control of the eye specific 3xP3 promoter while EGFP is under the control of the mosquito ubiquitin promoter which is expressed throughout the body during larval development. The dual transgene construct contains five homing endonuclease recognition sites flanking the ubiquitin and EGFP transgene.

PCR amplicons from uninfected or TE/5'2J ME2-nls-S_{tag}-I-PpoI virus-infected #P17A mosquitoes were cloned and sequenced. Sequence results from 16 out of 16 clones (100%) from uninfected mosquitoes revealed no alteration at either I-PpoI site (Figure 3.9.A), consistent with results from UUGFP line #18 uninfected mosquitoes. In contrast, 19 out of 21 clones (90%) exposed to I-PpoI had evidence of imperfect gap repair overlapping the I-PpoI recognition site with 18 out of 21 clones exclusively at the I-PpoI recognition site and 1 out of 21 spanning downstream from the recognition site (Figure 3.9.B). Interestingly, in 2 out of 21 clones (9.5%), the I-PpoI recognition site was intact, which is evidence that following the excision of EGFP, perfect repair occurred restoring the I-PpoI site. Consistent with results seen in UUGFP #18, deletions were observed at the I-PpoI recognition site ranging from 1 to 122 bp. The average and median deletion size for line #P17A was calculated only when deletions were observed, not when there was a perfect restoration of the homing endonuclease recognition site. The average deletion size observed was 8 bp while the median was 1 bp. Since I-PpoI was shown to induce dsDNA breaks in both transgenic lines used, additional homing endonucleases that have not been previously used in mosquitoes were tested in line UUGFP #P17A.

I-CreI has been shown to induce dsDNA breaks in human cells (Monnat *et al.*, 1999) but has never been used in mosquitoes. To test whether I-CreI could induce dsDNA breaks in *Ae. aegypti*, mosquitoes were infected with TE/5'2J ME2-Aa-nls-S_{tag}-I-CreI virus. I-CreI was able to induce dsDNA breaks and there was evidence of imperfect gap repair with deletions ranging from 2-146 bp in 16 out of 20 (80%) of clones sequenced with four clones containing a perfect restoration of the I-CreI site (Figure 3.10). Also, in one sequence there was the addition of 4 bases which may be from the repair event. In 3 out of 20 clones there were deletions exclusively at the I-CreI site while in 5 out of 20 clones the deletions span the I-CreI and I-SceI sites. In 7 out of 20 clones the deletion also included the I-CreI, I-PpoI, and the I-SceI sites. In 1 out of 20 clones, the deletion included the I-CreI and I-PpoI sites. The average size deletion observed was 60 bp with a median size of 65 bp.

A

```

#P17A ... TCGTAGCAGCTCACGGTTAGGGATAACAGGTAATTAATTGAGGAGGTTTCTCTGTAATAATGACTCTCTTAAGGTAGCCTAGAACAAAAACGTCGTGAGACAGTTTGGT...
uninfected ... TCGTAGCAGCTCACGGTTAGGGATAACAGGTAATTAATTGAGGAGGTTTCTCTGTAATAATGACTCTCTTAAGGTAGCCTAGAACAAAAACGTCGTGAGACAGTTTGGT... 16/16

#P17A ... TCGTAGCAGCTCACGGTTAGGGATAACAGGTAATTAATTGAGGAGGTTTCTCTGTAATAATGACTCTCTTAAGGTAGCCTAGAACAAAAACGTCGTGAGACAGTTTGGT...
uninfected ... TCGTAGCAGCTCACGGTTAGGGATAACAGGTAATTAATTGAGGAGGTTTCTCTGTAATAATGACTCTCTTAAGGTAGCCTAGAACAAAAACGTCGTGAGACAGTTTGGT... 16/16

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B

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#P17A (no EGFP) ... TTGAGGAGGTTTCTCTGTAATAATGACTCTCTCTTAAGGTAGCTAGAAA... CGACGTCAAAA... 2/21
ME2-I-Ppol ... TTGAGGAGGTTTCTCTGTAATAATGACTCTCTTAAGGTAGCCTAGAAA... CGACGTCAAAA... 2/21
... TTGAGGAGGTTTCTCTGTAATAATGACTCTCTTAAGGTAGCCTAGAAA... (106) --- CGACGTCAAAA... 1/21
... TTGAGGAGGTTTCTCTGTAATAATGACTCTC---GTAGCCTAGAAA... CGACGTCAAAA... 1/21
... TTGAGGAGGTTTCTCTGTAATAATGACTCTC---GGTAGCCTAGAAA... CGACGTCAAAA... 2/21
... TTGAGGAGGTTTCTCTGTAATAATGACTCTC---AGGTAGCCTAGAAA... CGACGTCAAAA... 5/21
... TTGAGGAGGTTTCTCTGTAATAATGACTCTC---AAGGTAGCCTAGAAA... CGACGTCAAAA... 2/21
... TTGAGGAGGTTTCTCTGTAATAATGACTCTCTT---GGTAGCCTAGAAA... CGACGTCAAAA... 2/21
... TTGAGGAGGTTTCTCTGTAATAATGACTCTCTTAA---TAGCCTAGAAA... CGACGTCAAAA... 1/21

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I-Ppol

↓

M6s LH

Figure 3.9. Sequence analysis of UUGFP #P17A mosquitoes exposed to I-PpolI. PCR amplicons from both (A) uninfected or (B) TE/S'2J ME2-nls-S_u^g-I-PpolI virus-infected mosquitoes were cloned and sequenced to determine if there was evidence of repair as seen by the excision of EGFP. Excision of EGFP was observed in 100% of clones sequenced with imperfect gap repair observed in 19/21 (90.5%) and a perfect restoration of the I-PpolI recognition site in 2/21 (9.5%) clones from mosquitoes exposed to I-PpolI. There was no evidence of repair in uninfected mosquitoes.

I-*AniI* has not been previously used to induce dsDNA breaks in any organism. To test whether I-*AniI* could induce dsDNA breaks in *Ae. aegypti*, mosquitoes were infected with TE/5'2J ME2-Aa-nls-S_{tag}-I-*AniI* virus. I-*AniI* induced dsDNA breaks with evidence of imperfect gap repair in both clones sequenced with deletions occurring at the I-*AniI* recognition site (Figure 3.11). Deletions ranged from 3 to 13 bp with an average size deletion of 8 bp and a median size between 3 and 12 bp. For both clones the deletions occurred exclusively at the I-*AniI* site.

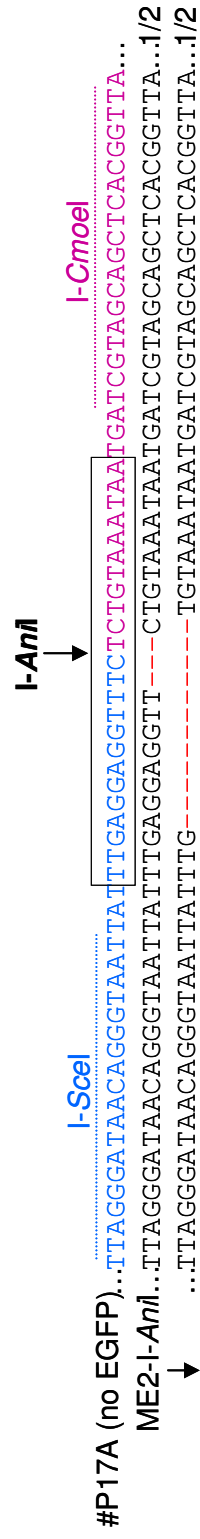


Figure 3.11. Sequence analysis of UUGFP #P17A mosquitoes exposed to I-AniI. PCR amplicons from TE/5'2J ME2-Aa-nls-S_{tag}-I-AniI virus-infected mosquitoes were cloned and sequenced to determine if there was evidence of imperfect repair as seen by the excision of EGFP. In 100% of clones sequenced there was excision of EGFP with imperfect gap repair observed in both clones. There was no evidence of repair in uninfected mosquitoes (Figure 3.9.A).

DISCUSSION

Our results demonstrate that four different homing endonucleases, *I-PpoI*, *I-SceI*, *I-CreI*, and *I-AniI* are able to recognize and induce a dsDNA break at exogenously introduced recognition sites in *Ae. aegypti*. This is the first report of *I-CreI* and *I-AniI* inducing dsDNA breaks in mosquitoes. Sequence analysis at each homing endonuclease recognition site demonstrated the use of imperfect gap repair through non-homologous end joining after exposure to a rHE. In 40% of clones derived from UUGFP line #18 mosquitoes infected with TE/5'2J ME2-nls-S_{tag}-*I-PpoI* virus, there was evidence of imperfect gap repair with most deletions between 1 and 6 bp. This is most likely an underestimation of the frequency of *I-PpoI* induced dsDNA breaks because perfect repair events at the recognition site cannot be determined with this method. Also, 90% of clones from UUGFP line #P17A demonstrated imperfect gap repair with most deletions between 1 and 5 bp. Interestingly, in 2 clones there was evidence that EGFP was excised followed by a perfect restoration at the *I-PpoI* recognition site. Perfect restoration of a homing endonuclease site may be due to direct ligation of two DNA strands cleaved by a homing endonuclease. The Surveyor Nuclease assay, in combination with the *I-PpoI* digest in TE/5'2J ME2-nls-S_{tag}-*I-PpoI* virus-infected mosquitoes, provided evidence that rHE can catalyze dsDNA breaks in *Ae. aegypti*.

In addition, there was evidence that *I-SceI*, *I-CreI*, and *I-AniI* were also able to successfully induce dsDNA breaks in mosquitoes. In mosquitoes infected with TE/5'2J ME2-nls-S_{tag}-*I-SceI* virus, imperfect gap repair was observed in 21% of clones sequenced. In mosquitoes infected with TE/5'2J ME2-Aa-nls-S_{tag}-*I-CreI* virus, imperfect gap repair was observed in 83% of clones sequenced. Finally, in mosquitoes infected with TE/5'2J ME2-Aa-nls-S_{tag}-*I-AniI* virus, imperfect gap repair was also observed in 8% of clones sequenced. For mosquitoes infected with TE/5'2J ME2-nls-S_{tag}-*I-SceI* virus, the percentage of clones is not a direct estimate of the frequency of imperfect repair events recovered because multiple rounds of PCR enrichment were used. PCR enrichment has been used before in determination of sequence degeneracy allowed at each recognition site for both *I-PpoI* and *I-CreI* (Argast *et al.*, 1998). Since *I-PpoI* is derived from a nuclear encoded intron, and has evolved to induce dsDNA breaks in nuclear DNA, it may be that *I-PpoI* is more efficient in locating and inducing a dsDNA break at its recognition site as opposed to *I-SceI*, *I-AniI*, and *I-CreI* which are encoded in mitochondrial or chloroplast introns.

Following exposure to I-*SceI*, no extensive deletions at the break site were observed which was similar to what has been previously observed in *Drosophila* (Bellaiche *et al.*, 1999).

Our results from I-*PpoI* exposed mosquitoes resulted in recognition sites that contained deletions which is consistent with other findings (Windbichler *et al.*, 2007), but unlike reports that there are insertions of bases following I-*PpoI* exposure (Monnat *et al.*, 1999). Additionally, mosquitoes exposed to I-*PpoI* had a high mortality rate, which has also been seen in *Anopheles gambiae* embryos (Windbichler *et al.*, 2007) and in human cells (Monnat *et al.*, 1999). Similar to findings by Windbichler *et al.* (2007), there is an I-*PpoI* recognition site in the 28S rDNA in *Ae. aegypti* (not shown) which could be used to bias the sex ratio towards males causing a population crash which would result in less disease transmission.

A limitation of our genomic DNA footprint analysis is that this is a qualitative assay, not quantitative therefore we cannot directly compare frequencies or efficiencies for each rHE used. We wanted to determine whether rHE could be expressed and induce dsDNA breaks in mosquitoes. While viral titers were similar for each TE/5'2J-rHE virus, the size of the inserts differed. I-*CmoE* was the largest gene inserted into the dsSIN virus system while I-*CreI* and I-*PpoI* were the smallest genes inserted. No evidence of dsDNA breaks induced by I-*CmoE* was observed. Larger genes inserted are more likely to contain deletion mutants which may be why there were no observed dsDNA breaks induced by I-*CmoE*; however, this was not determined experimentally.

Using western analysis, S_{tag}-rHE fusion proteins were not consistently detected, yet evidence of dsDNA breaks induced by rHE was observed when the I-*PpoI* digest and Surveyor Nuclease assays were repeated on head/thorax genomic DNA extracted from UUGFP #18 female mosquitoes infected with TE/5'2J ME2-nls-S_{tag}-I-*PpoI* virus at 4, 7, 10, and 14 days post-infection (data not shown). Inability to detect rHE expression may be due to S_{tag}-rHE fusion protein levels below the threshold for detection.

For the genomic DNA analysis, the Surveyor Nuclease assay was determined to be the least sensitive assay compared to the commercial homing endonuclease digest, which allowed for

multiple rounds of amplification. In PCR amplicons from TE/5'2J ME2-nls-S_{tag}-I-*PpoI* virus-infected mosquitoes, evidence of imperfect repair was detected using both the Surveyor Nuclease assay and the commercial I-*PpoI* digest. Using the Surveyor Nuclease assay, we were unable to detect any evidence of mismatch repair in TE/5'2J ME2-nls-S_{tag}-I-*SceI* virus-infected mosquitoes, yet we were able to detect undigested PCR amplicon in the commercial I-*SceI* digest. Due to the sensitivity and ability to perform multiple rounds of amplification for rare events, purification of I-*AniI* and I-*CmoE* would allow for homing endonuclease specific digests to be performed, similar to the I-*SceI* digests used to enrich for rare events repaired imperfectly.

Two different transgenic *Ae. aegypti* lines were used to test the ability of rHE to induce dsDNA breaks in mosquitoes. Multiple lines were used to determine whether the insertion position of the transgene containing homing endonuclease recognition sites was independent of rHE ability to induce dsDNA breaks. The location of transgene insertion and accessibility depends on the chromatin structure and how tightly the DNA is bound to histones and whether the insertion occurred in an actively transcribed region. While we have observed that transgene position does not seem to have an affect on the ability of rHE to induce dsDNA breaks, analysis of more mosquito lines should be performed to further confirm this finding.

Homing endonucleases have been engineered to have new target sequence specificity (Arnould *et al.*, 2006; Ashworth *et al.*, 2006; Doyon *et al.*, 2006; Rosen *et al.*, 2006; Seligman *et al.*, 2002; Smith *et al.*, 2006; Volna *et al.*, 2007). If the sequence specificity for a homing endonuclease could be designed to knock-down a gene suspected to be involved in virus transmission, then there could be a reduction in disease transmission. Such a tool could also be used for reverse genetics studies in mosquitoes.

Homing endonuclease-induced dsDNA breaks offer an alternative system for site-specific recombination. Random integration of transgenes using transposons can lead to positional effects and reduced integration efficiency with larger transgenes, and there can also be remobilization of the transposon. Site-specific recombination systems such as the FLP/FRT from the plasmid of *Saccharomyces cerevisiae* (O'Gorman *et al.*, 1991), cre-loxP from bacteriophage P1 (Sauer and Henderson, 1988), and phi C31 from *Steptomyces* bacteriophage

(Thorpe and Smith, 1998) have been used for the integration of transgenes. The cre-loxP system has been used in *Ae. aegypti* for the excision of a marker gene (Jasinskiene *et al.*, 2003). The FLP/FRT system has also been used in mosquito embryos resulting in the excision of specific DNA sequences (Morris *et al.*, 1991). Using a site-specific recombination system in combination with rHE-induced dsDNA breaks is a powerful combination for creating transgenic organisms and for the manipulation of genomes.

Determination of repair mechanisms used after dsDNA breaks occur in mosquitoes is important for a basic understanding of mosquito biology but also to evaluate the use of rHE in a gene drive mechanism. The predominant form of repair that we observed was imperfect gap repair through non-homologous end joining, although perfect repair resulting in the restoration of the homing endonuclease site following rHE induced excision of EGFP was also observed. Utilization of homing endonucleases to drive a gene into a wild-type mosquito population is contingent on homologous recombination being the preferred method of repair so that a gene conversion occurs. If homologous recombination occurs at a low frequency then homing endonucleases may not be an ideal choice for a gene drive system. Our assay did not aim to determine the frequency of dsDNA break repair but to demonstrate whether rHE could induce a dsDNA break at exogenously introduced homing endonuclease recognition sites in *Ae. aegypti*. Further studies into repair mechanisms needs to be performed to evaluate the frequency of homologous versus non-homologous recombination.

The assay we developed allows for the evaluation of whether a particular rHE can induce dsDNA breaks in mosquito somatic cells. For rHE to be used in a gene drive system or for inherited targeted gene disruptions, rHE activity in germline cells needs to be determined. Also, a quantitative analysis of the frequency of repair events needs to be evaluated. In summary, we were able to develop a genomic footprint assay to test rHE ability to induce site-specific dsDNA breaks at exogenously introduced homing endonuclease recognition sites in *Aedes aegypti*.

Chapter 4

SUMMARY

GENERAL OVERVIEW

Homing endonucleases are meganucleases capable of self-prorogation. Their ability to induce site-specific dsDNA breaks makes them a valuable tool for the study of dsDNA break repair mechanisms, as a means for targeted gene disruption, and as a potential mechanism for gene drive of an anti-pathogen gene into a mosquito population. For this study, rHE were expressed in *Aedes aegypti* to determine whether they could induce dsDNA breaks *in vivo*.

CHAPTER 2

Transfection parameters and conditions were optimized for *Aedes albopictus* C6/36 cells, *Aedes aegypti* CCL-125 cells, and *Drosophila melanogaster* S2 cells. I-AniI, I-Cmoel, I-CreI, I-PpoI, and I-SceI were shown to localize in mosquito cell nuclei and full length protein was expressed using a double subgenomic Sindbis viral expression system. Determination of rHE ability to induce dsDNA breaks using a two plasmid assay for detection of unrepaired dsDNA breaks were unsuccessful. One limitation of our assay used was the detection for open, unrepaired dsDNA breaks instead of detecting for imperfectly repaired dsDNA breaks.

CHAPTER 3

We were able to develop a genomic footprint assay to test the ability of a rHE to induce dsDNA breaks at exogenously introduced recognition sites in *Ae. aegypti*. The assay consisted of three components. First, a mismatch specific digest to screen for imperfect repair events. Second, a specific digest using commercially available homing endonucleases to detect for imperfect repair events. Finally, sequencing of genomic DNA from mosquitoes exposed to different rHE for confirmation of imperfect repair at a recognition site. We found that I-PpoI, I-SceI, I-CreI, and I-AniI were able to induce dsDNA breaks at their respective recognition sites. I-PpoI and I-CreI were observed to be the most active in *Ae. aegypti*. For each rHE, imperfect gap repair using non-homologous end joining at each recognition site was observed with varying lengths of deletions depending on the rHE.

FUTURE RESEARCH

In this study, rHE was injected into mosquitoes. dsDNA breaks induced by chromosomally expressed rHE would be a further expansion of this work and would re-confirm the ability of rHE to induce dsDNA breaks at their recognition sites. In addition, the effect of rHE expression on the fitness of a mosquito needs to be evaluated. I-*PpoI* is active and capable of inducing dsDNA breaks in *Ae. aegypti*, but due to the presence of an I-*PpoI* recognition site in the 28S rDNA which is highly conserved in all eukaryotes, expression of I-*PpoI* would need to be highly regulated so that there was no associated toxicity. Non-homologous end joining was the only form of repair observed; however, there was no homologous sequence to use as a template for repair to determine whether gene conversion occurred. The frequency of homologous recombination following a rHE induced dsDNA break would need to be evaluated for the use of a rHE as part of a gene drive mechanism and for gene therapy. rHE induced-dsDNA breaks resulted in different size deletions due to the use of imperfect gap repair. Homing endonucleases have been engineered so that they recognize desired recognition sequences. By verifying rHE activity in mosquitoes and other organisms, it may be feasible to determine whether rHE could be used for targeted gene disruption. A rHE that induces dsDNA breaks followed by large deletions at the recognition site, like I-*CreI*, may be the most useful in targeted gene disruption.

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