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RNA activation in insects: The targeted activation of endogenous and exogenous genes

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ABSTRACT

RNA activation (RNAa) is a newly emerging area of research in which dsRNA targeting promoter regions can induce the expression of the target gene. Although still in its infancy, it is already having significant impacts in several research areas in particular as cancer therapeutics. So far, the scope of RNAa has been limited to mammals and *Caenorhabditis elegans* with no indication of its prevalence in insects. In this study, we aimed to demonstrate the presence of RNAa in the insect dengue vector *Aedes aegypti*. Furthermore, we looked to uncover some details surrounding the involvement of host factors in order to present this as a new technique for insect research. The outcomes of this study provide new opportunities to further research into arthropod-borne diseases and insect biology in the same way as RNA interference.

1. Introduction

In the last decade, the primary understanding of small RNAs such as microRNAs (miRNA) and short interfering RNAs (siRNA) has centered around the paradigm of down-regulating gene expression through the process of RNA interference (RNAi). This process has led to numerous benefits in modern science such as the use of miRNAs as therapeutic agents in human disease or use of siRNAs in transgenic crops for pest control (Kim and Rossi, 2008; Zotti et al., 2018). Recently, the role of non-coding RNAs (ncRNA) in biology has expanded from the concept of gene silencing to a new phenomenon known as RNA activation (RNAa) (Janowski et al., 2007; Li et al., 2006; Place et al., 2008). RNAa uses short activating RNAs (saRNA) to upregulate the expression of targeted genes.

RNAa shares a few similarities with RNAi, but differs in a few key ways. saRNAs are typically 21 nucleotides (nt) long and, like siRNAs, are loaded into an Argonaut (Ago) protein (Chu et al., 2010). Unlike RNAi however, RNAa takes place exclusively in the nucleus of cells and acts to recruit transcriptional elements in order to upregulate gene expression (Li et al., 2006). It is believed that the recruitment of co-factor proteins, together known as the RNA Induced Transcriptional Activation (RITA) complex, aid in chromatin remodeling and modification in order to increase the permissive state of the target gene allowing increased expression of the gene (Portnoy et al., 2011, 2016). Currently, much debate is centered around the nature of the interaction of saRNA duplexes with the target in the nucleus; some speculating the RNA-protein duplex binds directly to the DNA, and others stating it may bind to a nascent transcript in the region (Matsui et al., 2010; Meng et al., 2016; Schwartz et al., 2008). While a growing body of research has explored the RNAa phenomenon, many components of the mechanism and its regulation are poorly understood. Additionally, RNAa has only been described in *Caenorhabditis elegans* and in mammalian organisms, including mice and humans (Huang et al., 2010; Turner et al., 2014). Currently, no literature describing RNAa in other animal groups exists.

Aedes aegypti is a mosquito that primarily feeds on human blood and poses a very significant threat to human health by transmitting diseases, including dengue fever and Zika virus disease. Dengue virus (*Flaviviridae*) is currently one of the most important diseases affecting global human health (LaBeaud et al., 2011). There are an estimated two billion people at risk of dengue infection worldwide with 309 million cases annually, by the latest WHO estimates (WHO, 2009, 2016). The virus is primarily transmitted by the mosquito *Ae. aegypti,* which is also responsible for the spread of other arboviruses including Zika virus and Yellow fever virus (*Flaviviridae*) (Bhatt et al., 2013). There are currently no effective vaccines or treatments for dengue or Zika virus, which means the focus of disease prevention has primarily concentrated on vector control (Lee et al., 2016). Traditionally, this has been achieved through the use of pesticides to control mosquito populations, however, new technologies have emerged such as the use of the Sterile Insect Technique (SIT) and the release of *Wolbachia*-infected mosquitoes to reduce the spread of the viruses. There are a number of limitations to

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these methods which has prompted interest in the application of molecular techniques such as RNAi for the control of arboviral diseases.

In this study, we explored the potential for RNAa to occur in insects, particularly focusing on the mosquito *Ae. aegypti*. We explored induction of exogenous as well as endogenous genes. Furthermore, we examined the differences in approach through the use of longer dsRNA or saRNAs in order to upregulate gene expression. We aimed to tailor the approach to the upregulation of immune related genes in order to demonstrate its potential for infectious disease research.

2. Results

2.1. Induction of RNAa in mosquito cells using dsRNA

We examined the potential occurrence of the RNAa phenomenon in an *Ae. aegypti* insect cell line (Aag2) through co-transfection of both a commercially available insect expression vector (pIZ/V5-His) containing a Green Fluorescent protein (*GFP*) insert as a reporter and also a dsRNA fragment targeting the OpIE2 promoter. The OpIE2 promoter is derived from Orgyia pseudotsugata multiple nucleopolyhedrovirus immediate early 2 gene promoter, which is activated by host factors. Briefly, Aag2 cells were co-transfected with pIZ/GFP and dsRNA (500 bp) against the OpIE2 promoter (dsIE2pro) or an unrelated control dsRNA (dscrt) of the same length. RNA was extracted 72 h post-transfection. Northern blotting was performed using a *GFP*-specific radiolabelled probe, while rRNA was examined to confirm equal loading of samples. The results showed a considerable increase in the *GFP* transcript levels in cells transfected with dsIE2pro as compared to cells transfected with pIZ/GFP only or pIZ/GFP and control dsRNA (Fig. 1A). There were differences in the expression of *GFP* between the two replicates in the control samples that could be due to differences in the basal expression of *GFP* and efficiency of transfection in the two replicates as *GFP* expression is through a plasmid and transient. Nevertheless, in both instances there were substantial induction of GFP relative to the controls. Since RNAa is mainly a small-RNA mediated mechanism (Portnoy et al., 2011), we employed northern blot analysis using a pool of radioactively labelled small RNA probes designed to the OpIE2 promoter to check whether dsIE2pro is actually processed into small RNAs. The result clearly demonstrated that dsIE2pro is processed into small RNAs of 21 nt that could target the OpIE2 promoter to induce *GFP* expression (Fig. 1B).

To find out if the induction of *GFP* is OpIE2 promoter-specific, we cloned *GFP* into another insect plasmid vector, pSLfa-PUb, under a polyubiquitin promoter and transfected it into Aag2 cells together with dsIE2pro. We used northern blot analysis to detect expression of *GFP* in cells transfected with either mock (Cellfectin transfection reagent only), dscrt (an unrelated control dsRNA) or dsIE2pro. Where previously dsIE2pro showed induction of *GFP* in pIZ/GFP, we found no increase in *GFP* expression when using a different promoter (Fig. 1C). When dsGFP was used, *GFP* transcripts were degraded, typical of an RNAi response (Fig. 1C; dsGFP). The result suggests that the *GFP* induction from pIZ/GFP in response to the targeting of the OpIE2 promoter is specific.

We were also interested to find out if activation of *GFP* through RNAa occurs in other insect cell lines. For this, we used Sf9 cells derived from *Spodoptera frugiperda*, which is used quite commonly for protein overexpression. Sf9 cells were co-transfected with pIZ/GFP and dsIE2pro. Protein lysates were collected 72 h post-transfection. Western blot was performed on protein lysates using a polyclonal anti-GFP antibody and anti-hsp70 as control. The results showed a considerable increase in the expression of the GFP protein in cells transfected with dsIE2pro compared to cells only transfected with pIZ/GFP (Fig. 1D). Conversely, dsGFP co-transfected with pIZ/GFP led to silencing of GFP. The results indicate that the RNAa phenomenon seen in mammals and nematodes indeed occurs in insects too. Further, RNAa can be used for boosting expression of exogenous proteins in insect cells utilizing expression vectors.

2.2. Targeting the promoter regions of endogenous Ae. aegypti genes with dsRNA induces gene expression

To explore the potential for dsRNA to induce gene transcription in *Ae. aegypti*, we chose three genes to predict their promoter regions and deliver dsRNAs to target their promoters. The upstream regions of *hsp70* and the immune genes, *Relish 1b* (*Rel1b*) and *Relish 2* (*Rel2*) were identified in the *Ae. aegypti* genome and analysed for potential promoter regions as described in Methods. 500 bp dsRNAs were designed and

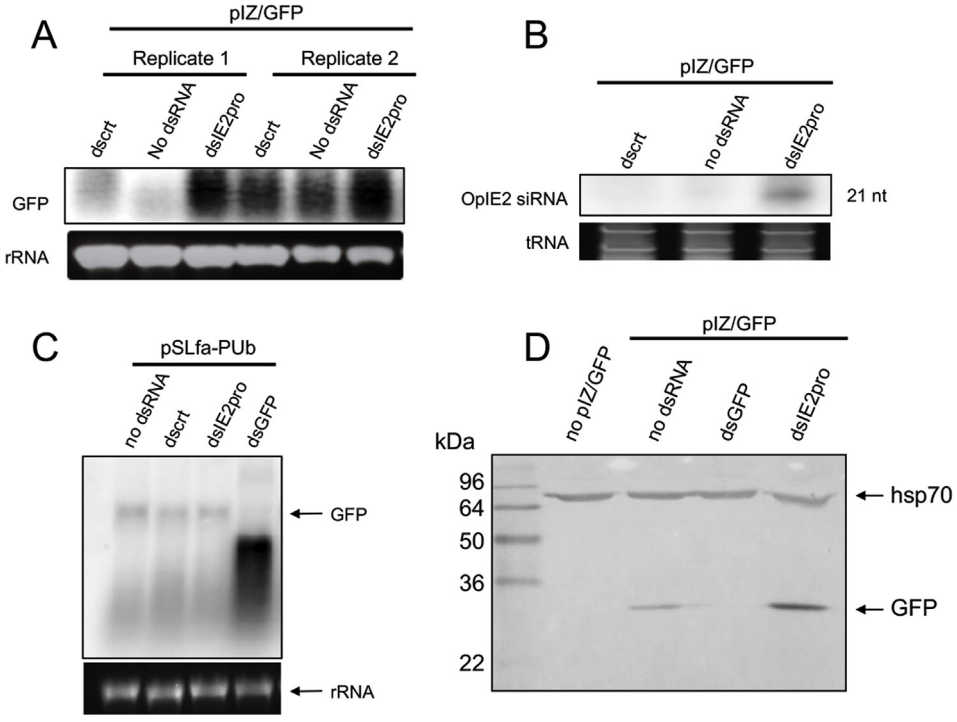

Fig. 1. Effect of dsRNA against the OpIE2 promoter on *GFP* expression in insect cells. A) Northern blot analysis of the *GFP* transcript levels after co-transfection of Aag2 cells with pIZ/GFP and an unrelated dsRNA control (dscrt) or dsIE2pro RNA along with mock transfection (no dsRNA). rRNA was used to check the equal loading of samples. B) Northern blot detection of small RNAs produced from dsIE2pro RNA processed by the Dicer enzyme. tRNA as a relatively small RNA molecule was used to confirm equal loading. C) Northern blot analysis of the *GFP* transcript levels after co-transfection of Aag2 cells with pSLfa-PUb/GFP and dscrt or dsIE2pro RNA along with mock transfection. rRNA was used as loading control. D) Western blot analysis of the GFP protein expression after co-transfection of Sf9 cells with pIZ/GFP and dsGFP RNA (dsGFP) or dsIE2pro RNA along with mock transfection which consisted of Cellfectin only (no pIZ/GFP). A polyclonal antibody to GFP was used. Hsp70 was used as an internal control, which showed equal loading of protein samples.

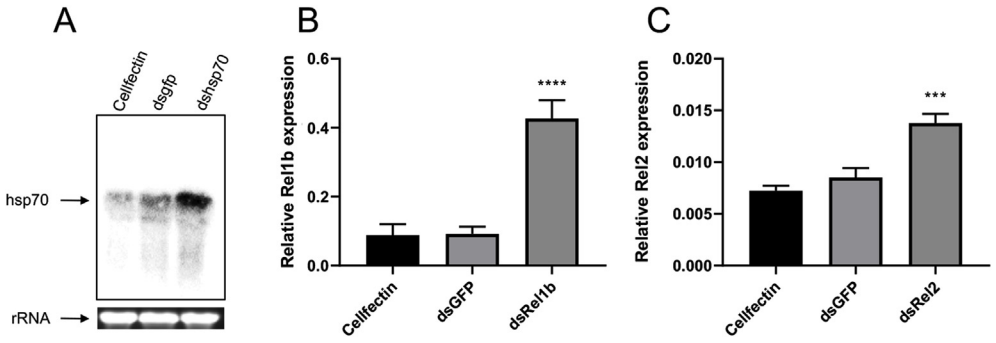

Fig. 2. Activation of endogenous genes *hsp70*, *Rel1b* and *Rel2* by dsRNA to their promoter regions in *Ae. aegypti* Aag2 cell line. A) *hsp70* transcripts identified by northern blot using a *hsp70*-specific probe with rRNA shown as loading control. Transcript levels of (B) *Rel1b* and (C) *Rel2* measured by RT-qPCR as compared with Cellfectin-only transfected cells and dsGFP-transfected cells as controls. Error bars represent standard error of the mean of four and six biological replicates (***, $p < 0.001$; ****, $p < 0.0001$).

synthesized targeting regions up to 100 bp before the Transcriptional Start Site (TSS) and transfected into Aag2 cells alongside controls. Total RNA was collected from the cells 48 h after transfection and analysed on a northern blot using a *hsp70*-specific probe. Treatment with dsRNA led to an increase in *hsp70* transcript levels when compared to Cellfectin only or control dsRNA (dsGFP) (Fig. 2A). Similarly, dsRNAs of the same length targeting regions in the *Rel1b* and *Rel2* promoters were transfected into *Ae. aegypti* Aag2 cells alongside controls. Total RNA was collected from cells following 72 h of incubation and analysed using RT-qPCR, which showed a four-fold and two-fold increase in gene expression of *Rel1b* and *Rel2*, respectively (Fig. 2B and C).

To find out if induction of *Rel1b* and *Rel2* has any effect on the downstream genes they regulate, RNA from dsRelb and dsRel2 transfected Aag2 cells were analysed with RT-qPCR. The effector molecules *Gambicin* (*Gam1*) and *Defensin D* (*DefD*) were upregulated in samples transfected with *Rel1b* targeting dsRNA (Fig. 3A and B). Similarly, the induction of *Rel2* by dsRel2 translated into the induction of the genes *Defensin E* (*DefE*) and *Cecropin A* (*CecA*) (Fig. 3C and D). These results indicate that the functional product of genes induced by RNAa is upheld through the cascade of signals (Kumar et al., 2018).

2.3. Targeted dsRNA design to highly optimized binding regions more effectively induces RNAa

Following the induction of the immune genes, based on TSS-based localization predictions of the promoter, we attempted to improve the efficiency through new identification techniques. Promoter regions of *Dome* (AAEL012471) and *Hop* (AAEL012553) were scanned for saRNA binding sites through identification of optimal binding conditions by the use of an algorithm developed by (Harris et al., 2017). This program produces a list of 21 nt regions with an associated score based on its sequence makeup. The output was scanned for 500 bp regions that contained a large number of higher scoring saRNA targets, and dsRNA was generated for the corresponding region. The 500 bp dsRNAs were transfected into Aag2 cells along with controls, and total RNA was collected 72 h post-transfection. Gene expression was analysed by RT-qPCR and modest increases in gene expression were detected (Fig. 4A and 4B). No induction was observed when randomly generated dsRNA targeting promoter regions of these regions were transfected into the cells (data not shown). This may indicate that not all regions of the promoter are equal in their ability to induce gene expression and it may

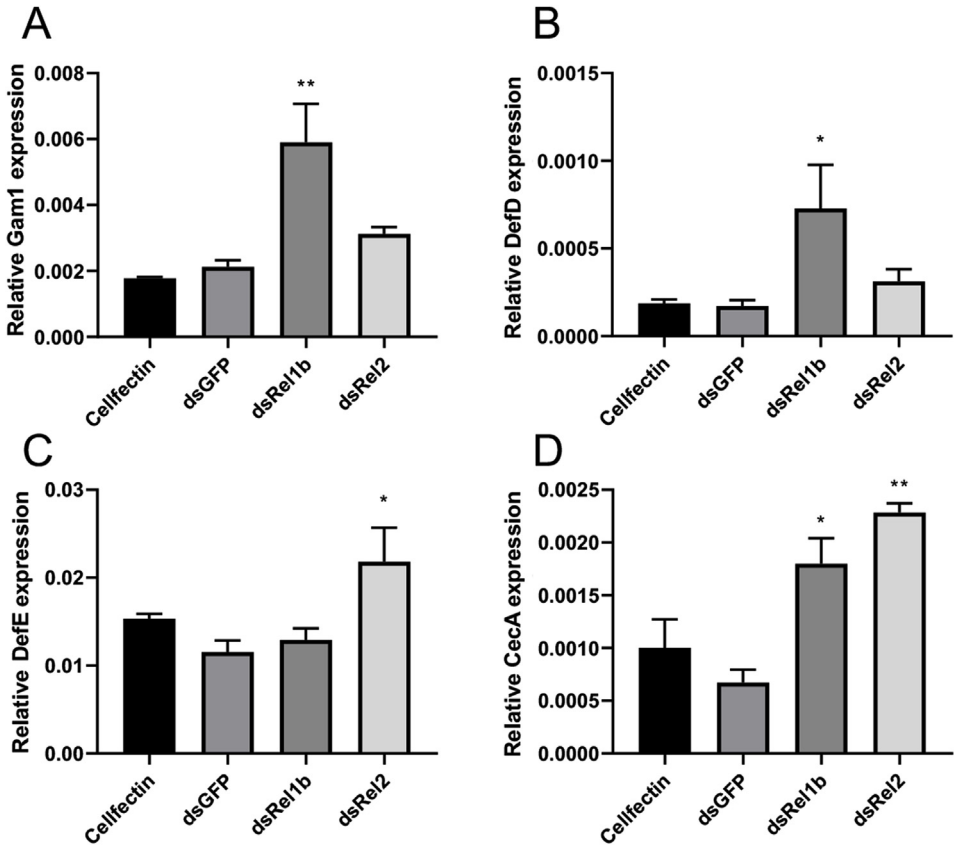

Fig. 3. RNAa of *Rel1b* and *Rel2* leads to the induction of the downstream genes *Gam1, DefD, DefE* and *CecA*. The relative expression of downstream effectors genes (A) *Gam1,* (B) *DefD* (*Rel1b*), (C) *DefE* (*Rel2*), and (D) *CecA* (*Rel1b* and *Rel2*) were measured by RT-qPCR. Error bars represent standard error of the mean of three biological replicates (*, $p < 0.05$; **, $p < 0.01$).

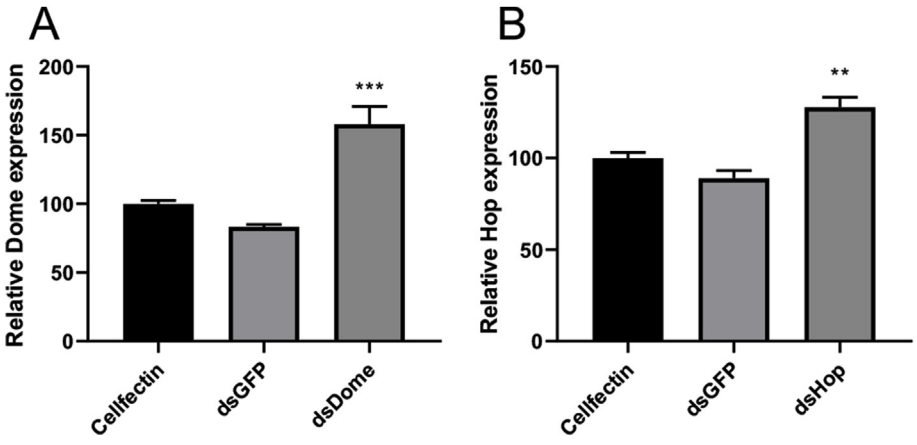

Fig. 4. Activation of endogenous immune genes *Dome* and *Hop* by dsRNA in Aag2 cells. Relative transcript levels of (A) *Dome* and (B) *Hop* measured by RT-qPCR 72 h after transfection of 500 bp dsRNA targeting regions in their respective promoters. Error bars represent standard error of the mean of three and six biological replicates (**, $p < 0.01$; ***, $p < 0.001$).

prove to be more effective to increase the general binding favorability of the dsRNA through targeted design.

2.4. Small saRNAs can induce RNAa in insects

Short RNAs of 21 nt were also tested for the ability to activate gene expression, due to this length being the size of the Ago loaded RNA. Firstly, three short saRNAs were designed to the OpIE2 promoter region with Pro-sa1 targeting the TSS, Pro-sa2 targeting the TATA-box and Pro-sa3 further upstream of the *GFP* reporter construct (Fig. 5A). The expression of *GFP* was measured by RT-qPCR in response to treatment of the three saRNAs and compared to the longer dsRNA as tested above (Fig. 5B). The results showed that both Pro-sa2 and Pro-sa3 were effective in inducing *GFP* expression at a level at least as effective as the dsRNA. Pro-sa1, which targeted the TSS, was not able to induce the expression of *GFP*, which is congruent with the literature describing the design of saRNAs outside the TSS (Wang et al., 2015).

We also tested the specificity of saRNAs by changing the seed region (nucleotides 2–8 from the 5' end) of the Pro-sa2 saRNA (Pro-sa4). When comparing the two saRNAs, we observed the inability of Pro-sa4 to induce expression of *GFP* beyond that of the controls (Fig. 5C). This not only indicates that Pro-sa2 was target specific to the OpIE2 promoter but also indicates that the seed region of the saRNA is essential for target recognition. This correlates to the seed dependent nature of saRNAs identified in mammals (Meng et al., 2016).

To further explore the use of saRNAs in RNAa, two saRNAs were designed to the promoter region of the *Dome* gene by using a target prediction tool as mentioned previously. This tool generated two targets with high scores, which were synthesized as saRNAs (Fig. 6A). These were transfected into Aag2 cells with controls on two occasions with 72 h between each administration. Following a further 72 h, total RNA was extracted, and the transcript levels of *Dome* were analysed using RT-qPCR. The results indicated an increase in the expression of *Dome* between 40 and 50%, which is comparable to that observed in the above results with the 500 bp dsRNA (Fig. 6B). This may indicate that short saRNAs are equally effective as longer dsRNA at inducing gene expression in the mosquito cell line.

To find out whether RNAa occurs in mosquitoes, three saRNAs were designed to target the promoter region of *Gam1* and injected into female *Ae. aegypti* mosquitoes. Three days after injection, the expression levels of *Gam1* were assessed by RT-qPCR. The saRNA targeting the TATA-box of the *Gam1* gene induced *Gam1* expression, whereas the control saRNAs, which target the transcription factor binding domains of Rel2 and STAT, did not (Fig. 7). The results showed that RNAa can also occur in mosquitoes.

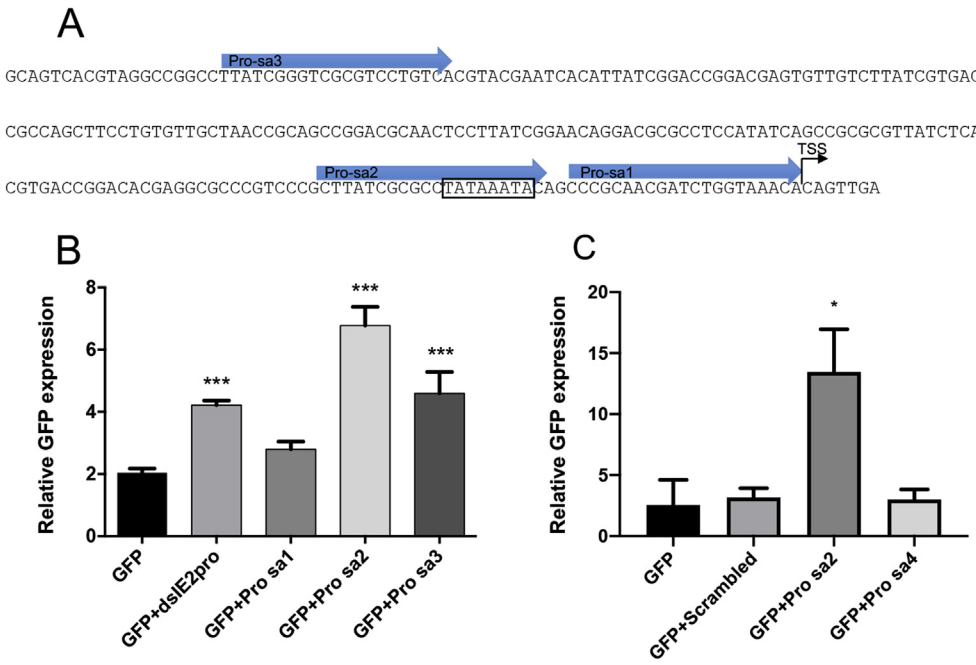

Fig. 5. Effect of saRNAs targeting the OpIE2 promoter in the pIZ/V5 vector. A) Schematic representation of saRNAs designed against different positions in the OpIE2 promoter. The TATA-box is boxed. B) Relative transcript levels of *GFP* analysed by RT-qPCR in Aag2 cells co-transfected with pIZ/GFP and dsIE2pro or saRNAs Pro-sa1, Pro-sa2 or Pro-sa3 designed to different regions in the OpIE2 promoter. C) Relative transcript levels of *GFP* analysed by RT-qPCR in Aag2 cells co-transfected with pIZ/GFP and saRNAs Pro-sa2 and Pro-sa4, which had a six nucleotide change to the sequence of Pro-sa2 in positions 2–8 of the saRNA. A scrambled sequence short RNA was used as control. Error bars represent standard error of the mean of three biological replicates (*, $p < 0.05$; ***, $p < 0.001$).

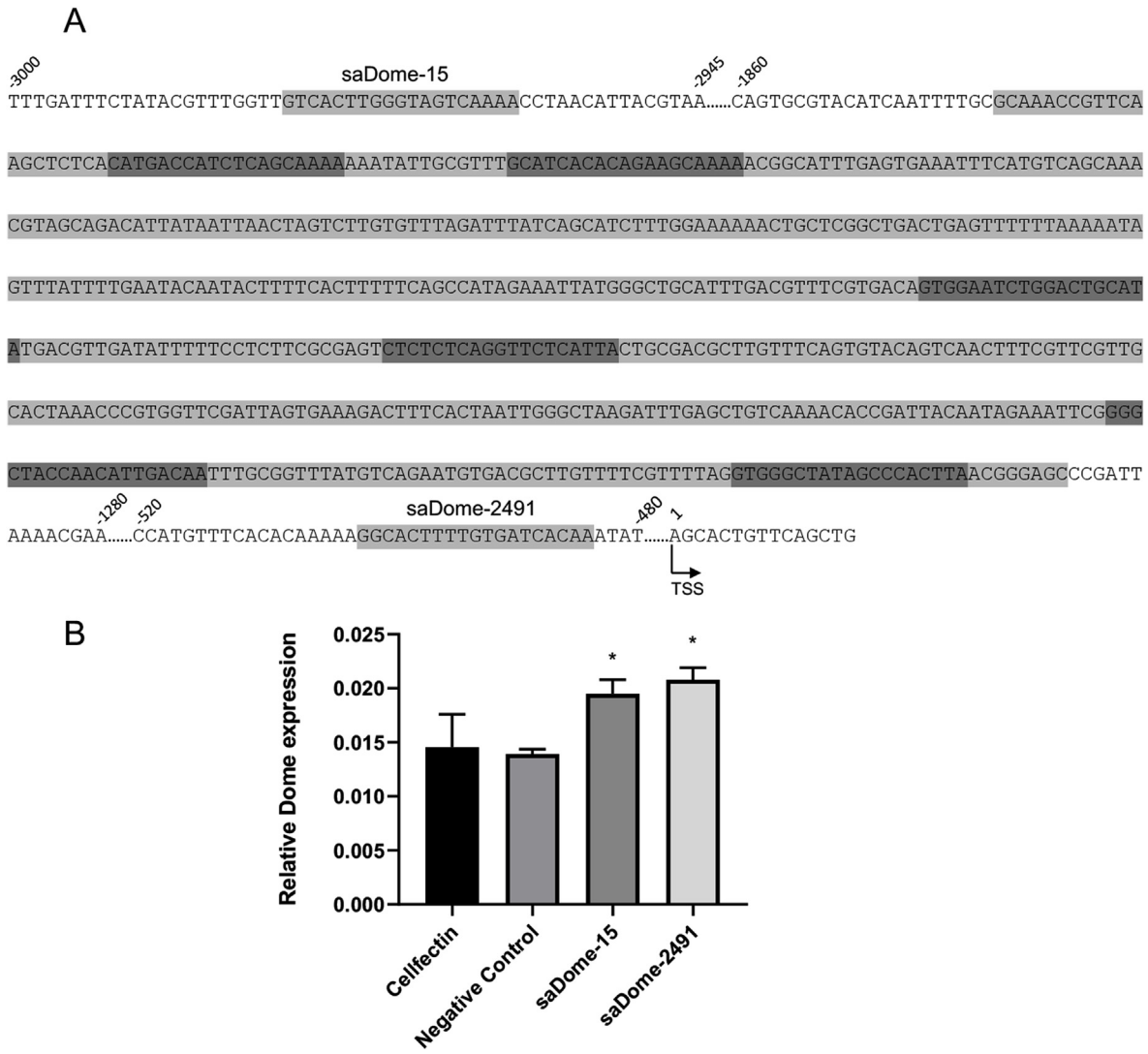

Fig. 6. Effect of saRNAs targeting the promoter region of *Dome*. A) Schematic representation of saRNAs designed against different positions in the *Dome* promoter. Light grey indicates the region for dsRNA to the promoter, and saRNA binding sites are highlighted in dark grey. Two saRNAs saDome-15 and saDome-2491 were selected for their higher score. B) Relative transcript levels of *Dome* analysed by RT-qPCR in Aag2 cells transfected with saRNA targeting the promoter regions as designed by the algorithm reported. Error bars represent standard error of the mean of three biological replicates (*, $p < 0.05$).

2.5. Single stranded RNA in both sense and antisense orientations mediates RNAa

saRNAs administered in mammalian cells targeting either the sense or antisense strand have proven to be effective (Meng et al., 2016). To investigate the dynamics of strand bias in our mosquito cells, single stranded RNAs (ssRNA) to the entire OpIE2 promoter were synthesized against both the sense and the antisense strands and were transfected separately into Aag2 cells along with the plasmid pIZ/GFP. The expression of *GFP* was analysed by RT-qPCR and northern blot following 72 h incubation. The results showed that RNAa of *GFP* with either of the ssRNAs was comparable to that of the dsRNA (Fig. 8A and B). There was approximately a 30% increase in *GFP* expression when the sense ssRNA was applied, which may elude to a more favourable binding interaction on sense strand that is driven by the increased abundance in the ssRNA relative to the dsRNA.

2.6. The Argonaute 2 protein is a major determinant of RNAa in mosquito cells

In insects, two major Argonaute proteins (Ago1 and Ago2) are involved in different aspects of RNAi and miRNA pathways. To determine which one is primarily involved in our observed RNAa, we tested the ability of our previously described dsIE2pro to activate the *GFP* reporter gene, while silencing either *Ago1* or *Ago2*. dsRNAs targeting the mRNAs of *Ago1* and *Ago2* were transfected into Aag2 cells previously transfected with the pIZ/GFP vector, in order to knockdown gene expression. Following 72 h of incubation, cells were again transfected with *Ago* dsRNAs before also being transfected with dsIE2pro. Following a further 72 h incubation, the expressions of *GFP, Ago1*, and *Ago2* were measured using RT-qPCR. Silencing of the expression of *Ago1* (84%) and *Ago2* (70%) was confirmed in cells transfected with pIZ/GFP or pIZ/GFP + dsIE2pro transfected cells (Fig. 9A and B). Results from this experiment indicated that while silencing of both *Ago1* and *Ago2* suppressed RNAa, *GFP* was not significantly induced in *Ago2* silenced cells with dsIE2pro (Fig. 9C).

3. Discussion

RNA activation is still an emerging field/technique, and so far, its use has been limited to only a few major mammalian models. This has not prevented it from already having far reaching impacts with roles for

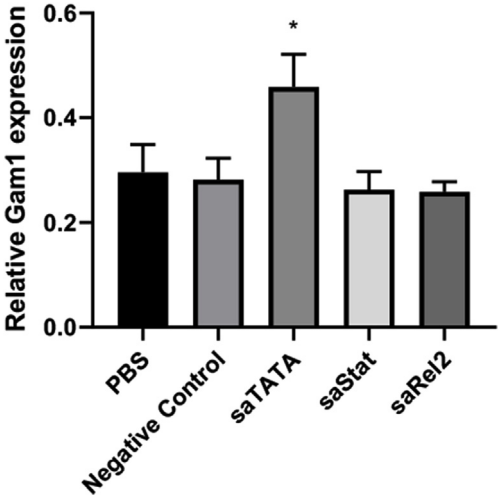

Fig. 7. RNAa occurs in mosquitoes. saRNAs targeting three regions in the *Gam1* promoter region, including the controls (saStat and saRel2), were injected into female adult mosquitoes. Negative control is a scrambled small RNA. RNA was collected and relative expression of *Gam1* was measure by RT-qPCR. Error bars represent standard error of the mean of three biological replicates each a pooling of three mosquitoes (*, $p < 0.05$).

saRNAs now being appreciated as therapeutics in cancer research (Setten et al., 2018; Wang et al., 2018). While major components of the phenomenon are slowly coming to light, still much is left to be understood about the mechanism of RNAa. At this stage, RNAa has yet to be utilized in any model outside of mammals and the use of this technique has the potential to expand research in important models such as flies and mosquitoes in much the same way as RNAi.

To begin to test whether RNAa occurs in insects, we utilized the *Ae. aegypti* Aag2 mosquito cell line together with a *GFP* construct with a well-known promoter region as the basis for dsRNA design. The result of targeting the OpIE2 promoter with dsRNA was significant upregulation of *GFP*. In addition to mosquito cells, GFP expression was also boosted in the lepidopteran Sf9 cells by targeting the OpIE2 promoter with dsIE2pro. These observations in turn led us to explore whether the phenomenon could be used to upregulate the expression of endogenous genes related to immunity in the mosquito. We targeted regions including and proximal to the 5' UTR of the *Rel1b* and *Rel2* genes with dsRNA in Aag2 cells, which demonstrated a significant increase in gene expression. We were further able to demonstrate the ability of promoter-targeting dsRNA to activate endogenous genes by completing a more repeatable design of target regions. This approach was able to generate a modest increase in gene expression in the *Hop* and *Dome* immune genes. Further, we found that when *Rel1b* and *Rel2* were induced by RNAa, the downstream effector antimicrobial genes (*CecA*, *DefD*, *DefE* and *Gam1*) were also induced.

When we compare insects to mammalian systems, there is a clear difference in the innate immune system of the two. Insects lack the interferon gamma (IFNγ) response that is present in mammals and is triggered by longer dsRNAs (Gantier and Williams, 2007). Due to this response in mammals, only short small RNAs can be used in RNAi and RNAa (Elbashir et al., 2001). Unlike mammals, the absence of the IFN pathway in insects has allowed the use of longer dsRNAs in RNAi (Yu et al., 2013) as we have demonstrated here for RNAa. Despite this, we were interested in demonstrating the ability of saRNAs to modulate gene expression. Several saRNAs were designed for select regions of the OpIE2 promoter and the *Gam1* promoter. The results from these experiments indicated that targeting the TATA-box proved to be a strong inducer of gene expression. One complication of the higher abundance of A and T nucleotides in the *Ae. aegypti* genome is that TATA-boxes are difficult to accurately predict *in silico*. We were also able to identify that saRNAs in close proximity to the TSS were not effective in inducing gene expression, which has previously been discussed and shown to lead to unfavorable outcomes for RNAa (Wang et al., 2015). With this in mind, we utilized an saRNA prediction tool through Python, which identifies regions in the promoter of selected genes based on optimized binding interactions. Using this, we were able to demonstrate that two saRNAs were also able to induce the expression of *Dome*. Interestingly, when we compared the efficacy of the saRNAs for *Dome* versus the longer dsRNA, we observed similar levels of increase indicating that the longer and more directed dsRNA is as effective as the saRNAs. This may provide a significant benefit to experimentation as longer dsRNA is easily synthesized in most laboratory settings as compared to what can be an expensive purchase to produce saRNAs from commercial entities.

The reliance of strand selection for gene activation was explored through the use of ssRNA targeting the OpIE2 promoter. This assay indicated that when comparing the dsRNA to either the positive or negative single strand, the activation was at least as effective when using ssRNA. This corresponds to findings in mammalian cell lines, which indicate that RNAa can be achieved through targeting either strand (Meng et al., 2016). Final exploration focused on examining the involvement of Ago proteins in RNAa as these proteins have been implicated in RNAa in mammalian cells (Chu et al., 2010; Portnoy et al., 2016). This was performed by silencing *Ago1* or *Ago2* and monitoring the effect these have on RNAa. The results demonstrated that while silencing of both led to reductions in RNAa, *Ago2* is apparently more

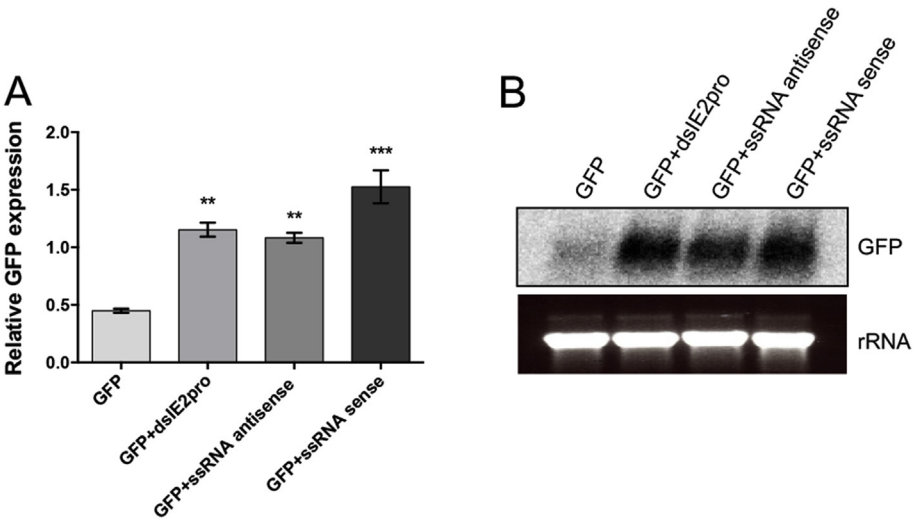

Fig. 8. Role of sense and antisense strand of OpIE2 promoter in RNAa. Relative *GFP* expression analysed by RT-qPCR in Aag2 cells co-transfected with pIZ/GFP along with dsIE2pro or antisense ssRNA or sense ssRNA to dsIE2pro. Error bars represent standard error of the mean of three biological replicates (**, $p < 0.01$; ***, $p < 0.001$). B) Northern blot analysis of RNA samples probed with *GFP* described in (A). Lower bands of rRNA show equal loading of all samples.

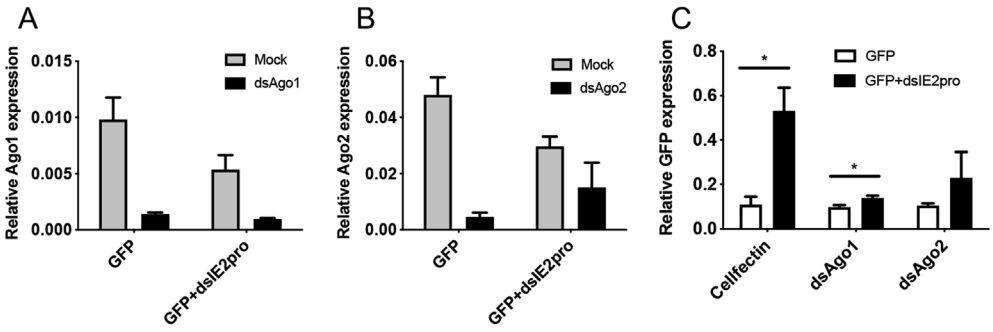

Fig. 9. Role of Ago1 and Ago2 in RNAa. A) Relative expression of *Ago1* transcripts in Aag2 cells transfected with *Ago1* dsRNA and pIZ/GFP vector with or without dsIE2pro. Mock, Cellfectin only. B) Relative expression of *Ago2* transcripts in Aag2 cells transfected with *Ago2* dsRNA and pIZ/GFP vector with or without dsIE2pro. C) Relative expression of *GFP* transcripts in Aag2 cells transfected with pIZ/GFP vector and *Ago1* or *Ago2* dsRNA for RNAi with or without dsRNA targeting the promoter region of OpIE2.

involved in the process of RNAa, despite less efficient silencing of *Ago2*.

When examining the results together, we noted that there are varying levels of induction between different targeted genes, which is more obvious when comparing exogenous reporter genes with the endogenous genes. We speculate that reasons contributing to these differences may relate to epigenetic modifiers, such as methylation state or chromatin density. Similarly, tissue specificity might impact the ability for induction by dsRNA-mediated RNAa. It might be possible to overcome some of these effects by targeting different regions of the promoter and comparing expression.

Overall, our results show that RNAa can occur in an animal other than mammals or nematodes. We showed for the first time that RNAa occurs in insects. RNAa may prove to be a highly useful tool for future research in insect biology. Further, by showing that RNAa occurs in the major arbovirus vector *Ae. aegypti*, we have demonstrated the potential for RNAa to be used for research into arbovirus-mosquito interactions. The use of RNAa may have far-reaching potential in modulating gene responses in order to restrict viral transmission and reduce the global burden of disease. In addition, RNAa may be used to boost ectopic expression of exogenous proteins in cell lines used for overexpression of recombinant proteins.

4. Methods

4.1. Cell culture and mosquitoes

Ae. aegypti (Aag2) cells were maintained at 27 °C in a medium with a 1:1 mixture of Mitsuhashi-Maramorosch and Schneider's insect media (Invitrogen) supplemented with 10% FBS and 1% penicillin-streptomycin mix. *Spodoptera frugiperda* (Sf9) cells were maintained at 28 °C in Sf-900II medium (Invitrogen).

Mosquitoes were reared from eggs in dechlorinated tap water and fed a constant diet of ground TetraColour® fish flakes daily. Pupae were transferred to emerging cups placed inside cages. Adults were given access to 10% sucrose and collected for injection 72 h post-emergence. Females were injected through the thorax with dsRNA or saRNA and allowed to recover for 72 h prior to RNA isolation.

4.2. Gene cloning and transfection of nucleic acids

The *GFP* gene was cloned into pIZ/V5-His insect expression vector (Invitrogen) and pSLfa-PUb (Addgene). Nucleic acids were transfected into mosquito cells using Cellfectin II (Invitrogen) as per the product's protocol.

4.3. RNA extraction and RT-qPCR

RNA was extracted from cells and mosquitoes using the Qiazol (Qiagen) reagent as per the instruction included with the product. RNA was treated with DNaseI (New England Biolabs) or TURBO DNase (Life Techonoliges) as per the manufacturer's instructions. cDNA was synthesized using mMuLV reverse transcriptase (New England Biolabs) as per the included protocol. qPCR was completed using specific primers to genes of interest (Table S1) and the Quantifast SYBR system (Qiagen) as per the instruction of the manufacturer.

4.4. Northern blot

5 μg of total RNA was run on a 1% (w/v) formaldehyde agarose gel, transferred to nylon membrane and then subjected to UV for cross-linkage. The probes were generated by labeling PCR products with [α-32P]-dCTP by using the Random primers labeling kit (Invitrogen). Probe hybridization and washing was done at 65 °C. In order to detect dsIE2pro-derived siRNAs (21 nt), 60 μg of total RNA from transfected cells with dsIE2pro, control dsRNA and no dsRNA was separated on 15% polyacrylamide–7 M urea gel in 0.5 × TBE buffer, stained with ethidium bromide to visualize tRNA and transferred onto Hybond N + membrane (Amersham) by electroblotting for 1h at 20V. The membrane was cross-linked at 65 °C for 2h using a chemical method (Pall and Hamilton, 2008). saRNAs designed to the OpIE2 promoter (Fig. 5A) were labelled with (α-32P)-ATP using PNK kit (Thermoscientific) according to the manufacturer's instructions. Hybridization was carried out by using Express Hybridization buffer (Clontech) after three washes (15 min each with 2 × SSC, 0.1%SDS) at 55 °C. Radioactive signalling was detected by phosphorimaging using a Typhoon scanner (GE Healthcare).

4.5. Western blot

Total proteins were isolated from Sf9 cells transfected with pIZ/GFP, dsRNA against the OpIE2 promoter and dsRNA against the *GFP* mRNA. Proteins were run on a 12% (w/v) polyacrylamide gel. Proteins were then transferred to a nitrocellulose membrane and probed with polyclonal anti-GFP (Abcam) and anti-hsp70 (Abcam) antibodies. The GFP and hsp70 protein bands were detected using an anti-rabbit alkaline phosphatase conjugated antibody (Sigma).

4.6. dsRNA design

The OpIE2 promoter sequence was obtained from the pIZ/V5-His sequence (Life technologies) for design of dsRNA. Upstream regions of the immune genes, *Rel1b* (AAEL006930) and *Rel2* (AAEL007624), and also *hsp70* (AAEL017975) were extracted from the previous *Ae. aegypti* genome assembly (AaeL3.4) (available from VectorBase; https://www.vectorbase.org) and were analysed for potential promoter regions by predicting the Transcription Start Site using Promoter 2.0 prediction software (http://www.cbs.dtu.dk/services/Promoter/). Regions of interest for the other three genes were narrowed down by using the Python script outlined in (Harris et al., 2017) and selected to encompass the largest number of matches from the output. Regions were amplified by targeted PCR with the T7 recognition site included in the forward and reverse primers. Tagged DNA amplicons were used as template to synthesize dsRNA using the T7 MEGAScript kit (Ambion) as per the manufacturer's instructions.

4.7. saRNA design

saRNAs were designed to target three different regions in the OpIE2 promoter region encompassing the TSS, TATA-box and a farther upstream region (Fig. 5A). Three saRNAs were also designed to target the TATA-box, and STAT and Rel2 transcription factor binding sites in the *Gambicin* promoter as outlined by (Harris et al., 2017). Two saRNAs were also designed to the *Dome* promoter region based on the output from the prediction tool outlined in (Harris et al., 2017). The saRNAs were synthesized by Genepharma.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2020.103325.

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