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Arbovirus-mosquito Interactions: RNAi Pathway

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Abstract

Arthropod-borne (arbo) viruses infect hematophagous arthropods (vectors) to maintain virus transmission between vertebrate hosts. The mosquito vector actively controls arbovirus infection to minimize its fitness costs. The RNA interference (RNAi) pathway is the major antiviral response vectors use to restrict arbovirus infections. We know this because depleting RNAi gene products profoundly impacts arbovirus replication, the antiviral RNAi pathway genes undergo positive, diversifying selection and arboviruses have evolved strategies to evade the vector's RNAi responses. The vector's RNAi defense and arbovirus countermeasures lead to an arms race that prevents potential virus-induced fitness costs yet maintains arbovirus infections needed for transmission. This review will discuss the latest findings in RNAi-arbovirus interactions in the model insect (*Drosophila melanogaster*) and in specific mosquito vectors.

Introduction

Arboviruses require hematophagous arthropod vectors to transmit virus between vertebrate hosts. *Aedes aegypti* (*Aea*) and *Culex quinquefasciatus* (*Cxq*) are mosquito vectors that transmit dengue viruses (DENV; *Flavivirus; Flaviviridae*) and West Nile virus (WNV; *Flavivirus; Flaviviridae*), respectively. Arboviruses replicate in the mosquito prior to transmission causing minimal pathologic changes and fitness costs [1,2]. The mosquito becomes persistently infected for the remainder of its adult life and can transmit virus with each bite. Barring barriers to infection, DENVs and WNV replicate in the mosquito's midgut and then disseminate to salivary glands where the virus enters saliva and transmits to a new host with the next bite. The time interval between initial infection of the midgut and virus in saliva is termed the extrinsic incubation period (EIP). The EIP can take 5-14 days for DENVs and WNV depending on virus and mosquito genotypes and environmental conditions [3-6]. Mosquitoes are not passive during EIP and the persistent phase of infection and mount a number of innate antiviral responses that modulate arbovirus infections (reviewed in [7-9]). The RNAi pathway is the major innate antiviral response vectors have

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to restrict arbovirus infections [10]. Neither are arboviruses passive during infection and have evolved several strategies to counter the vector's antiviral responses, although these are not well-characterized. The vector's innate immune responses and arboviral countermeasures lead to a balanced infection that prevents potential vector fitness costs yet maintains arbovirus infections at levels needed for effective transmission. In this review, we will discuss recent advances in our understanding of the antiviral RNAi pathway that illustrate this concept.

Drosophila melanogaster and mosquitoes

Drosophila melanogaster (*Dm*) and mosquito vectors are flies from the insect order *Diptera*. *Dm* has served as a model dipteran insect mainly due to its tractability in the laboratory leading to a rich history of genetic, molecular, physiological and biochemical discovery. *Dm* research has been fundamental to our present understanding of vector-arbovirus interactions. For instance, the complete *Dm* genome sequence has been available since 2000 [11] providing a framework for comparisons with genomes of the medically important arboviral vectors *Aae* [12,13] and *Cxq* [14,15] as they became available. Transcriptome analyses of virus-infected *Dm*, *Aae* and *Cxq* have demonstrated complex gene expression profile changes when compared with uninfected insects [16-18]. For instance transcriptome studies have demonstrated that the fly's and vector's antimicrobial responses (Toll, Imd and JAK-STAT pathways) can be activated to control viral infections in insects [19-25]. *Dm* researchers initially demonstrated that RNAi responses have a significant role in the insect's antiviral defense [26]. However, *Dm* is not a perfect infection model in that it is not a vector of arboviruses and often requires injection of abnormally high arbovirus titers for efficient infection. Nevertheless, *Dm* research has generated tools and approaches that have been fundamental for the study and understanding of arbovirus-vector interactions.

RNAi is a primary antiviral response in *Dm* and mosquitoes

RNAi is a post-transcriptional response first described in the nematode *Caenorhabditis elegans* [27] with the observation that injection of long double stranded (ds)RNA depleted expression of targeted genes in a sequence-specific manner. Gene silencing triggered by dsRNA was soon described in *Dm* [28] and has since been a basic tool for functional analyses of genes in mosquitoes. With one exception, arboviruses are RNA viruses and many RNA viruses generate intracellular dsRNA intermediates during their replication suggesting that these might trigger RNAi to function as an antiviral response. Li, Li and Ding (2002) initially demonstrated that the RNAi response in *Dm* was triggered by dsRNA from a replicating pathogenic insect RNA virus, Flock House virus (FHV; *Alphanodavirus*; *Nodaviridae*) [29]. We (and others) reasoned that RNAi may restrict arbovirus infections in mosquitoes. Several years prior to the discovery of RNAi we inserted a 500 nt portion of the DENV2 genome into the genome of an unrelated arbovirus, Sindbis virus (SINV; *Alphavirus; Togaviridae*). We infected *Aea* with the chimeric SINV and found that the mosquitoes were refractory to subsequent DENV2 infection. Since the SINV/DENV2 genome generates dsRNA during its replication, we reasoned that RNAi may be the mechanism for the observed inhibition of DENV2 [30-32].

RNAi background

We now know that RNAi comprises three pathways, named for their effector RNAs: small interfering (si)RNA, micro (mi)RNA, and Piwi-interacting (pi)RNA pathways. Each has a distinct role in antiviral defense, regulation of development and host gene expression, or defense of the host genome against transposon mobilization and expression, respectively. The three pathways of RNAi have been discussed in some length in previous reviews [8,9]. In this review, we will concentrate on recent advances in understanding *Dm* and mosquito siRNA and piRNA pathway interactions with viruses. Furthermore the RNAi pathways appear to have interconnectivity within *Dm* [33] and mosquitoes [34]. The mosquito genes that encode major proteins in each pathway have been identified by homology to *Dm* genes [35].

Antiviral responses and the siRNA pathway in diptera

The RNAi pathway triggered by viral (exogenous) dsRNA is the siRNA pathway. Virus-generated dsRNA is recognized by Dicer 2 (Dcr2), a large protein with several functional domains that have been previously described [26,35]. Briefly, the Dcr2 RNase III domains cleave dsRNA into 19-22 bp siRNA duplexes with 2-nt overhangs at the 3′-OH-ends. The siRNA-Dcr2 complex associates with the dsRNA-binding protein, R2D2, facilitating siRNA loading into the multiprotein RNA-induced silencing complex (RISC) [26]. The effector protein of RISC is Argonaute 2 (Ago2), an RNase H-type endonuclease that also contains a PAZ domain, which binds and unwinds the siRNA, cleaves the “passenger” strand, and uses the bound siRNA “guide” strand to form a perfectly base-paired duplex with the complementary sequence on target viral mRNA [36]. The viral mRNA target is specifically cleaved at the center of the duplex by Ago2 endonuclease activity [37].

Dcr2's essential role in antiviral defense has been demonstrated in *Dm* and C6/36 (*Ae. albopictus*) mosquito cells lacking a functional Dcr2 [26,38,39]. Both mutant flies and C6/36 cells were highly susceptible to infection, resulting in increased viral loads in the absence of Dcr2 activity. Furthermore if Dcr2 (and the siRNA pathway) were depleted with Dcr2-dsRNA, *Aea* mosquitoes supported a 10-fold higher DENV2 load and a shorter EIP than control mosquitoes [40]. Ago2 also has an essential role in antiviral defense. Van Rij and colleagues (2006) showed that Ago-2-defective *Dm* were hypersensitive to infection with drosophila C virus (DCV; *Cripavirus*; *Dicistroviridae*) or cricket paralysis virus (CrPV; *Cripavirus; Dicistroviridae*) [41]. The *ago2* mutants had increased mortality and dramatic increases in viral RNA accumulation and virus titers. We have shown that Ago2 depletion in *Anopheles gambiae* and *Aea* mosquitoes also leads to increased viral RNA and virus titers after infection with o'nyong-nyong virus (*Alphavirus*; *Togaviridae*) and DENV2, respectively [40,42].

The virus-insect arms race

Obbard and colleagues (2006) demonstrated in several drosophila species that siRNA pathway genes encoding Ago2 and Dcr2 are among the fastest evolving 3% of all genes, suggesting that infection with RNA viruses may drive diversifying selection in *Dm* [43]. To test this in mosquitoes, Bernhardt and colleagues (2012) analyzed the genetic diversity of

siRNA pathway genes (*ago2* and *dcr2*) in six distinct geographic populations of *Aea* [44]. Similar to the studies among drosophila species, analysis of nearly complete sequences of *Aea ago2* and *dcr2* revealed that these genes were undergoing rapid, positive, and diversifying selection among *Aea* populations. Interestingly, the *Aea* populations with lower vector competence for DENV2 significantly and positively correlated with the nucleotide diversity indices in *dcr2*. In a related study Lambrechts and colleagues (2013) surveyed allelic polymorphism in *dcr2* in different *Aea* populations experimentally exposed to genetically distinct isolates of DENVs [45]. They found that the *dcr2* genotype was associated with resistance to specific DENV2 genotypes. They further hypothesized that non-synonymous polymorphisms within *dcr2* generated differential dsRNA binding affinities for particular dsRNA sequences with genotype-by-genotype specificity. However, many arboviruses like DENVs provide only rare opportunities for selection at the vector population level since numerous field studies report that very few mosquitoes collected in areas endemic for DENV are actually infected [46,47]. The driver of rapid *dcr2* gene diversity in *Aea* remains a mystery but could include non-pathogenic, insect-specific viruses having no vertebrate host [48] or pathogenic insect viruses. A recent metagenomics study by Obbard and colleagues determined that in >2,000 individually collected wild adult *Dm* more than 30% had detectable virus, and more than 6% carried multiple viruses [49] In contrast to *Dm*, Bernhardt and colleagues found that *Aea* Dcr1 and Ago1, key proteins of the miRNA pathway, are also undergoing rapid, positive, and diversifying selection among *Aea* populations, suggesting inter-connectivity between siRNA and miRNA pathways [44].

Viral counterdefenses to the siRNA pathway

Many pathogenic insect viruses encode virulence factors that are potent suppressors of the siRNA pathway. Depending on the virus, suppressors of RNAi (VSRs) target different steps in the pathway [50,51]. VSRs would be advantageous for RNA viruses that do not require transmission by a vector and thus do not need to minimize fitness costs to the infected vector, but rather require high virus loads for efficient environmental transmission. As discussed earlier, arboviruses need to minimize fitness costs of vector infection or risk their elimination during EIP and prior to transmission. Complete suppression of the siRNA pathway, resulting in pathogenesis, is predictably detrimental to their transmission cycle [52,53]. Nevertheless, viral evasion of the mosquito immune response may be necessary in order to infect, disseminate and be transmitted. A candidate for limited suppression of RNAi is the subgenomic flavivirus RNA (sfRNA) [54]. A proposed mechanism of suppression was inhibition of Dcr protein because recombinant human Dcr had reduced activity when WNV-sfRNA was added to an *in vitro* assay. However, direct inhibition of mosquito cell Dcr2 was not demonstrated. The sfRNA also was observed to sequester mosquito 5′ exonuclease Xrn1, which might be a required downstream step to complete degradation following Ago2 cleavage of viral mRNA [55].

The antiviral siRNA pathway response also may play an important role shaping the population structure of arboviruses in mosquitoes. Arthropod vectors are known to contribute to flavivirus quasispecies complexity [56,57]. Evidence suggests that viral genome diversity is critical for the adaptation of flaviviruses and other arboviruses to new and changing environments and hosts [58]. Brackney and colleagues (2015) then determined

that the siRNA pathway imposes diversifying selection on WNV genomes in infected *Dm* S2 cells [59]. S2 cells were used since Ago2 and Dicer2 depletion with cognate dsRNAs is well-established. In this study, the *Dm* siRNA pathway was left intact, depleted using dsRNAs targeting *dcr2* or *ago2* transcripts, or enhanced by addition of a dsRNA targeting a specific region of the WNV RNA genome. They then determined WNV quasispecies diversity in an amplicon spanning the 5′ untranslated region (UTR)-capsid-premembrane genes of WNV RNA from populations produced after a single round of infection or after five passages in *Dm* S2 cells. WNV diversity was significantly lower in the targeted sequence of WNV in RNAi-depleted cells but significantly greater in RNAi-stimulated cells relative to that of the intact controls [59]. They hypothesized that targeting viral genomes with siRNAs can lead to the rapid emergence of escape mutants containing mutations within intensely-targeted sequences leading to increased genome diversification of the virus. Similar experiments in mosquito cells and *Cxq* infected with WNV need to be performed to verify that increased genetic diversity observed in *Cxq*-derived WNV populations is siRNA pathway-driven.

Development of persistent infections in diptera

In a series of seminal discoveries, *Dm* researchers demonstrated that the siRNA pathway may play an important role in establishing persistent RNA virus infections in diptera. Goic and colleagues (2013) detected DNA forms of a non-retroviral RNA virus (FHV) after infecting *Dm* S2 cells [60]. The finding was surprising since FHV and other non-retroviral RNA viruses lack reverse transcriptase and machinery required to integrate their genomes into those of vector or host [61]. Their observation has been supported by an increasing number of studies showing partial DNA sequences from non-retroviral RNA virus genomes integrated into arthropod DNA genomes [62-65]. Goic and colleagues hypothesized that reverse transcriptase from endogenous retrotransposons most likely generated cDNA forms of FHV RNA as extrachromosomal or integrated DNAs in the insect genome [60]. To test this hypothesis they treated S2 cells with the nucleoside analogue reverse-transcriptase inhibitor azidothymidine (AZT) triphosphate prior to FHV infection and showed that AZT treatment inhibited production of the FHV DNA forms and increased the viral load up to 1,000-fold when compared with untreated FHV-infected S2 cells in which the DNA form was present. Furthermore, they observed that S2 cells without DNA forms of FHV failed to become persistently infected with FHV. They hypothesized that if the endogenized DNA sequence was transcribed, its transcript could generate dsRNAs for Dcr2 detection and processing by the siRNA pathway and may explain how the DNA form controls RNA virus infection and promotes persistence. The obvious next experiment, depletion of Dcr2 in cells persistently infected with FHV, resulted in a shift from a persistent to an acute infection and cell death. Deep sequencing analysis of the genome of persistently infected S2 cells showed that most viral DNA forms were fused to fragments corresponding to long-terminal repeat (LTR) retrotransposons. Goic and colleagues detected chimeric v-siRNAs derived from both retrotransposons and the FHV genome, confirming that FHV DNA templates were transcribed and then processed by the siRNA machinery [60]. Well over 50% of adult flies also generated DNA forms when infected with either FHV or SINV by 6 days post-injection [60]. Treatment of flies with AZT prior to infection led to significantly increased viral loads

and high mortality when compared to controls. They also suggested that defective interfering viral RNAs produced during infection may act as templates for the DNA forms as the DNA form nucleotide sequences are often highly rearranged similar to the nucleotide sequences of defective viral RNAs [60]. FHV expresses the potent VSR, B2, which suppresses RNAi by binding dsRNAs preventing the dicing action of Dcr2. So why doesn't the B2 VSR prevent Dcr2 surveillance and v-siRNAs from forming in persistently infected cells? Flynt and colleagues (2009) suggest that B2 preferentially binds to viral replicative intermediates and we suspect that B2 fails to bind to dsRNA products generated by transcription of DNA forms [66]. The extent to which reverse transcription drives arbovirus persistence in mosquitoes remains to be determined. However, it should be noted that 42% and 29% of the *Aea* and *Cxq* genomes, respectively, are composed of transposable element (TE) sequences and retrotransposons are the dominant type of TE in each genome [14]. (Refer to figure 1).

We have generated a *mariner* (*Mos1*)-transformed *Aea* line (Carb109M) containing an inverted repeat (IR) DENV2-specific cDNA sequence in chromosome 3 [67]. The effector DNA was designed to be transcribed in midgut epithelial cells soon after acquisition of a bloodmeal [67,68]. The transcribed IR RNA formed dsRNA that was processed by the siRNA pathway machinery. Carb109 mosquitoes were refractory to DENV2 challenge [8,67]. The refractory phenotype may be due to nucleus-derived dsRNA being processed more efficiently by the siRNA machinery than dsRNAs from viral replicative intermediates formed during infection, although unlike persistent infections the dsRNA sequence was not derived from DNA forms of viral interfering RNAs.

9. Arboviruses and the piRNA pathway in vectors

The Piwi-interacting RNA (piRNA) pathway is a dicer-independent RNAi response. In *Dm*, antisense primary piRNAs are transcribed in the nucleus from transposons and/or genomic loci termed piRNA clusters [67]. These are processed to 24-27 nucleotide piRNAs with 5′U (U1) and 2′-O-methylated 3′ ends by unknown mechanisms and are loaded onto one of the PIWI family proteins Aubergine (Aub) or Piwi in or near the nucleus. The antisense RNA-loaded (Aub) proteins act as piRNA-induced silencing complexes (piRISCs) to initiate the cytoplasmic “ping-pong” amplification loop. The Aub-RISC hybridizes to and cleaves sense strand secondary piRNA precursors complementary to its bound antisense piRNA, and this “slicer” reaction determines and forms the 5′ end of piRNAs that have an A10 nucleotide and are loaded onto Ago3. Ago3 with bound sense piRNA continues the “ping-pong” amplification loop by cleaving complementary antisense piRNA precursors, generating the 5′U ends of antisense piRNAs that subsequently are loaded onto Aub. The 3′ end of piRNA is formed by an unknown nuclease (or nucleases), followed by 2′ O-methylation mediated by HEN1 [69].

This complex and poorly understood pathway is primarily utilized to silence transposon activity in the *Dm* germline, but surprisingly, in mosquitoes virus-specific piRNAs have been detected in somatic cells and recent studies have implicated piRNAs in mosquito antiviral defense [70-72]. DENV2 infected C6/36 cells lacking Dcr2 activity show no vsiRNAs but do show vpiRNAs, indicating that the piRNA pathway is detecting and

processing arboviral RNAs, and vpiRNAs are clearly detected in infected mosquitoes [38,39]. The trigger for a piRNA antiviral response to cytoplasmic arboviruses like DENV remains a mystery but the presence of DNA forms of DENV2-infected vector cells may suggest an answer. Antisense primary piRNAs may be transcribed from the DNA forms in the nucleus of the infected cell and interact with Piwi family proteins to initiate the "ping-pong" amplification loop. The Piwi/Argonaute gene family of mosquitoes has undergone expansion by comparison to the *Dm* genome [35]. The genome of *Culex pipiens* encodes 7 Piwi-family genes and *Aea* mosquitoes have 8 Piwi-family genes. We have speculated that this expansion reflects an antiviral role for the piRNA pathway in mosquito vectors. Sequence analyses examining transcriptional changes in response to blood feeding reveal Piwi4 expression is up-regulated under these conditions in multiple strains of *Aea* [73-75]. It was recently reported that cytoplasmic Piwi4 and Ago3 or Piwi5 and Ago3 proteins are involved in piRNA biogenesis from Semliki Forest virus or SINV RNA, respectively in infected *Aea* cells [76,77]. Most organisms utilize several piwi proteins in concert, at least one that is cytoplasmic and one that shuttles between the nucleus and the cytoplasm. Piwi4 is a protein that potentially can bridge between nucleus and cytoplasm (Andino and Kunitomi, personal communication, 2015). While a major function the piRNA pathway is to restrict transposon activity, the antiviral response may rely on a different set of PIWI proteins for viral piRNA biogenesis. Future research activity should focus on whether vpiRNA biogenesis is dependent upon reverse-transcription of viral RNA and identify core components of the piRNA pathway required for an antiviral response (Figure 2).

10. Conclusions/Future directions

The RNAi pathway is the major innate antiviral response vectors use to restrict arbovirus infections. As described in this review, RNAi, particularly the siRNA pathway, may have an additional role in establishing persistent infections in mosquitoes and changing the population structure of the infecting arbovirus in ways that evade the vector's RNAi responses. The importance of the RNAi pathway in virus-insect interactions is demonstrated by siRNA pathway genes undergoing positive, diversifying selection in flies and vectors and the association of siRNA gene diversity with vector competence. The vector's innate immune responses and arboviral countermeasures are in an arms race to minimize virus-induced fitness costs but maintain arbovirus infections for transmission to a vertebrate host. Although evidence has been presented for restriction of arbovirus infections of mosquitoes by canonical innate immune pathways, knowledge that the siRNA pathway is the unique and most potent mechanism of antiviral innate immunity in mosquitoes provides opportunities to develop novel strategies for controlling arbovirus transmission [8,67] and incentives to more completely understand the mechanism. A few questions on which future research might be focused are the following: What viral RNA structures in addition to long dsRNA are required for Dcr2 recognition? What is driving diversifying selection of *dcr2* and *ago2*? Can we routinely find DNA forms of arboviruses in infected vectors and what role do DNA forms have in establishing persistence? What activities are required downstream from Ago2 cleavage of viral RNA to complete the RNAi pathway? What is the role of piRNAs in anti-viral immunity and which Piwi-clade proteins are involved? What are arboviral strategies for RNAi evasion?

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Highlights

1. RNAi is a post-transcriptional antiviral response comprising three pathways, named for their effector RNAs: small interfering (si)RNA, micro (mi)RNA, and Piwi-interacting (pi)RNA pathways. The siRNA pathway is the major innate antiviral response vectors have to restrict arbovirus infections but the piRNA pathway also has antiviral activity.
2. RNAi component genes in the siRNA pathway encoding Ago2 and Dcr2 are among the fastest evolving genes in *Drosophila melanogaster* and *Aedes aegypti*. RNA viruses may drive diversifying selection for these genes leading to an arms race between arboviruses and vectors to maintain vector fitness yet allow sufficient arbovirus replication for effective virus transmission.
3. No potent arboviral suppressor of RNAi has been found since the absence of RNAi may well eliminate the infected vector prior to virus transmission. The siRNA response may shape the structure of arboviral populations in the vector by the rapid emergence of viral escape mutants containing mutations within sequences intensely-targeted by siRNAs. However, arboviral diversity has only been studied in insect cells and needs to be further investigated in arbovirus-infected vectors.
4. Reverse transcriptase from endogenous retrotransposons in drosophila and vectors likely generates cDNA forms of RNA virus genomes that can be detected as extrachromosomal DNA or integrated DNA in the insect genome. Transcription of the viral DNA forms generates RNAs that can be recognized by dicer-2 and the siRNA pathway. Persistent infections of RNA viruses in insects can be terminated by treatment with drugs (AZT) that blocks reverse transcription.
5. The piRNA pathway is a complex and poorly understood pathway primarily utilized in germline cells to silence transposon activity. Surprisingly, in mosquitos virus-specific piRNAs have been detected in somatic cells and recent studies have implicated piRNAs in mosquito antiviral defense. Future research activity should focus on whether viral piRNA biogenesis is also dependent upon reverse-transcription of viral RNA and identify core components of the piRNA pathway required for an antiviral response.

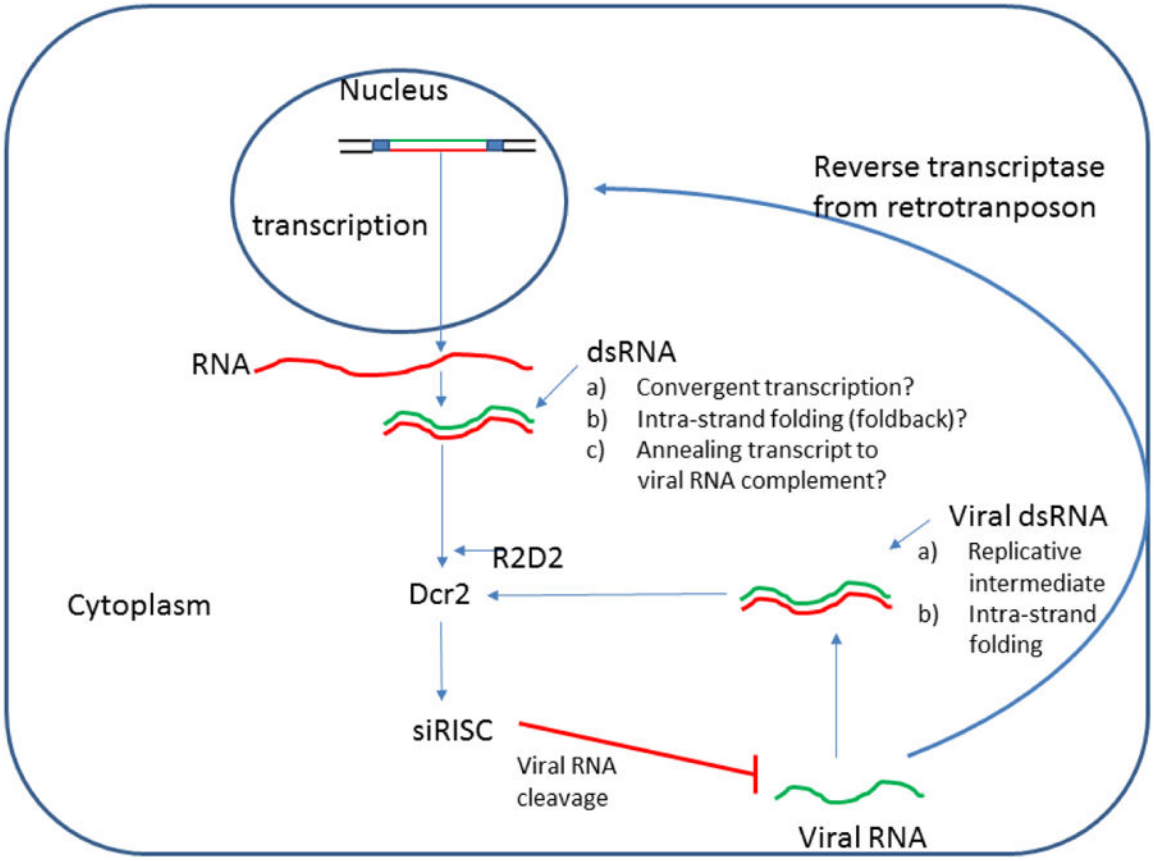

Figure 1.

Antiviral siRNA response with proposed vector-virus interactions. Diagram showing components of the siRNA pathway in vectors during an antiviral response to a positive sense RNA virus: genomic viral positive sense RNA; long dsRNA generated by virus replication, dicer2 (Dcr2) which cleaves dsRNA, R2D2 a dsRNA binding protein, the RNA interference silencing complex (siRISC) that includes the guide RNA and argonaute 2 (Ago2), the effector in cleaving target viral RNA. In this diagram a model for persistent infections has been included using reverse transcription activity from endogenous retrotransposons of viral RNA to generate DNA forms as extrachromosomal DNAs (not shown) or the vector genome- integrated forms (shown). In this model, when the integrated DNA is transcribed, the RNA forms dsRNA in a manner not yet determined, although three possible ways of generating dsRNA are described. Once virus-specific cellular dsRNA is formed it can enter the siRNA pathway to control virus replication during the persistent state. Figure was modified from Figure 1 [60]

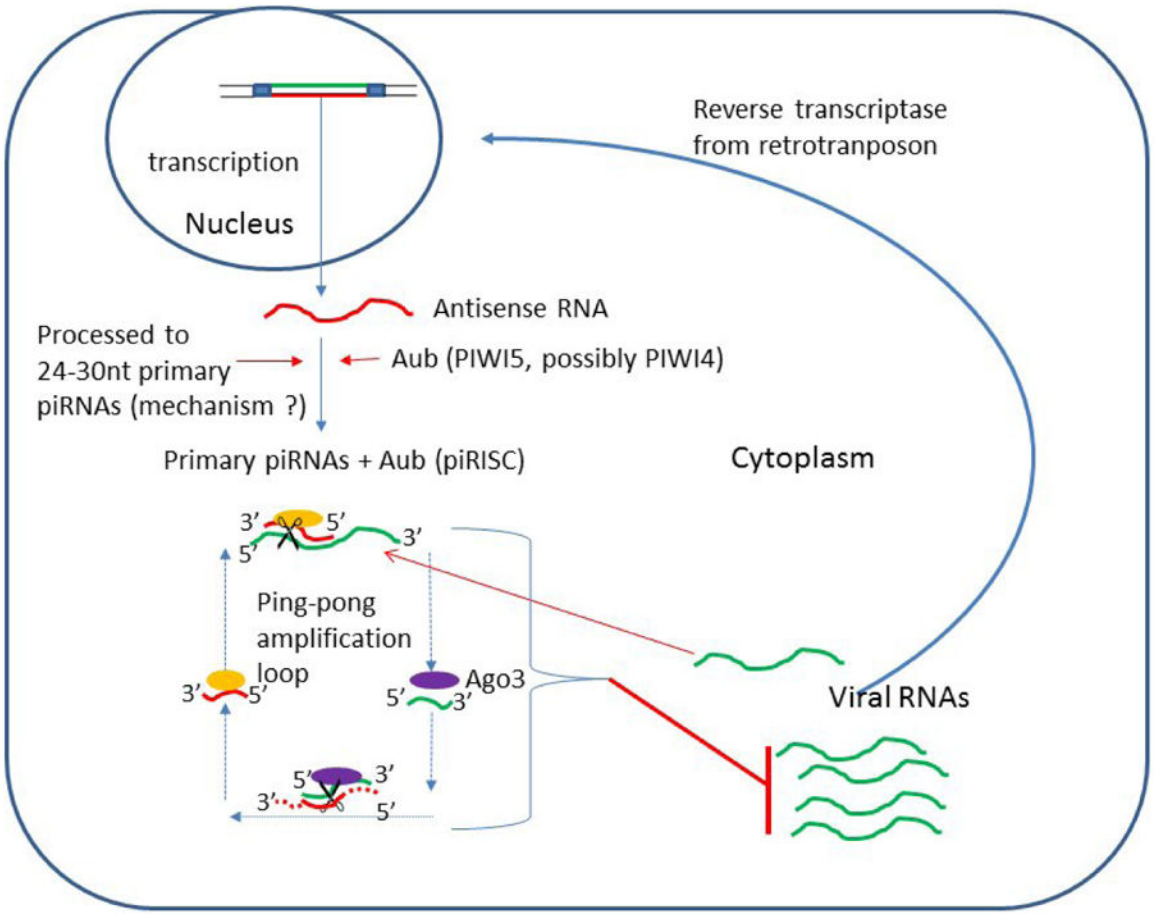

Figure 2.

Antiviral piRNA response with proposed vector-virus interactions. Diagram showing components of the piRNA pathway in vectors during an antiviral response to a positive sense RNA virus: genomic viral positive sense RNA; virus-derived antisense RNA transcribed in nucleus, 24-27 nucleotide piRNAs loaded onto PIWI family proteins (Aub in Dm) in or near the nucleus and entry of primary piRNAs into the “ping-pong” amplification loop. In this model we have included reverse transcription activity from endogenous retrotransposons of viral RNA as a means of generating DNA forms as extrachromosomal DNAs (not shown) or the vector genome-integrated forms (shown) that may be transcribed to form the initial primary antisense RNA.