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A Dissertation Presented

By

Bradford Michael Stadler

Submitted to the Faculty of the  
University of Massachusetts Graduate School of Biomedical Sciences, Worcester  
In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

March 15, 2007

Department of Molecular Genetics and Microbiology  
Program in Immunology and Virology

# **Interaction Of A Mammalian Virus With Host RNA Silencing Pathways**

A Dissertation Presented

By

Bradford Michael Stadler

Approved as to style and content by:

Trudy Morrison, Ph.D., Chair of Committee

Raymond Welsh, Ph.D., Member of Committee

Rachel Gerstein, Ph.D., Member of Committee

Craig Mello, Ph.D., Member of Committee

John Rossi, Ph.D., Member of Committee

Timothy Kowalik, Ph.D., Thesis Advisor

Anthony Carruthers, Ph.D.,

Dean of the Graduate School of Biomedical Sciences

Department of Molecular Genetics and Microbiology

March 15, 2007

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## ABSTRACT

In the complex relationships of mammalian viruses with their hosts, it is currently unclear as to what role RNA silencing pathways play during the course of infection. RNA silencing-based immunity is the cornerstone of plant and invertebrate defense against viral pathogens, and examples of host defense mechanisms and numerous viral counter-defense mechanisms exist. Recent studies indicate that RNA silencing might also play an active role in the context of a mammalian virus infection. We show here that a mammalian virus, human adenovirus, interacts with RNA silencing pathways during infection, as the virus produces microRNAs (miRNAs) and regulates the expression of Dicer, a key component of RNA silencing mechanisms.

Our work demonstrates that adenovirus encodes two miRNAs within the loci of the virus-associated RNA I (VA RNA I). We find that one of these miRNAs, miR-VA “g”, enters into a functional, Argonaute-2 (Ago-2)-containing silencing complex during infection. Currently, the cellular or viral target genes for these miRNAs remain unidentified. Inhibition of the function of the miRNAs during infection did not affect viral growth in a highly cytopathic cell culture model. However, studies from other viruses implicate viral miRNAs in the establishment of latent or chronic infections.

Additionally, we find that adenovirus infection leads to the reduced expression of Dicer. This downregulation does not appear to be dependent on the presence of VA RNA or its associated miRNAs. Rather, Dicer levels appear to inversely correlate with the level of viral replication, indicating that another viral gene product is responsible for this

activity. Misregulation of Dicer expression does not appear to influence viral growth in a cell culture model of infection, and also does not lead to gross changes in the pool of cellular miRNAs. Taken together, our results demonstrate that RNA silencing pathways are active participants in the process of infection with human adenovirus. The production of viral miRNAs and the regulation of cellular Dicer levels during infection implicate RNA silencing mechanisms in both viral fitness as well as potential host defense strategies.

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**CHAPTER I:**

**INTRODUCTION**

## **A. Overview of host-virus relationship**

Infection of host organisms by viruses is an active and constantly evolving interaction. Organisms go to great lengths in their attempts to stay free of pathogens, and examples of host defense mechanisms reside in species as diverse as mammals, plants, flies, and fungi. Mammalian host cells employ a dizzying array of mechanisms to combat viral infection, even going so far as to direct the equivalent of cellular euthanasia, or apoptosis, of infected cells for the overall benefit of the organism. As obligate parasites, viruses are dependent upon infection of host organisms to maintain their survival. Therefore, the process of viral infection is a delicate balance involving the ability of the virus to replicate efficiently in the face of host defense mechanisms. The virus not only needs to evade host defense mechanisms, but must also subjugate host cell machinery for its own use to achieve viral replication or to maintain a stable, latent state within cells. The continual interplay of invading viruses with host cells drives co-evolution of these parties. Similarly, our understanding of the viral and host factors dictating this relationship continues to evolve.

## **B. Mammalian immune defense against viruses**

### *Kinetics of the host immune response to virus*

The immune response of mammals is composed of an early, non-specific innate response, followed by a specific, acquired response to the virus. While initially believed to be separate and distinct responses to pathogens, numerous studies now indicate that these responses are entwined to produce a robust, integrated immune response for the host (90, 124). When virus succeeds in entering the host and infecting cells, it triggers a variety of “danger” signals, which serve to broadcast the presence of an infection to the host (123). Typical primary responders in this process are dendritic cells (DCs) (69, 191). Viral recognition by DCs or other innate immune cells via direct infection or virus-induced death of neighboring cells leads to the local production of chemokines and cytokines, triggering an inflammatory and adaptive immune response (69, 76). Of these cytokines, interferons (IFNs) are the key mediators in the induction of the innate response as well as the subsequent adaptive response (45, 76, 90). Type I and II interferons function to elicit numerous cellular immune factors which give rise to a general antiviral state at both the cellular and organismal level. In addition to upregulating expression of manifold cellular effector molecules, interferons also induce DCs to mature as antigen presenting cells for recognition by lymphocytes, and induce the expression of chemokines which direct monocytes and lymphocytes to the site of infection (69, 76). Recognition of viral antigen by specific T or B cells leads to rapid expansion of this

subset of cells and the production of virus-specific, cytotoxic helper T cells and antibody producing B cells, the cellular hallmarks of the acquired response (69, 191). Typically, the innate response begins with viral recognition and maintains in various forms throughout the infection, while the virus-specific cytotoxic T lymphocytes (CTL) responses normally peak at 7-10 days after infection, followed by the appearance of antibody-producing B cells (192).

*Virus recognition by the innate response: dsRNA*

Activation of the innate response by virus typically occurs when cells involved in the innate response, such as DCs, are directly infected or exposed to viral components through the lysis of nearby infected cells (191). The most common molecular indicator of viral infection is the presence of double-stranded RNA (dsRNA). For most viruses, spurious dsRNA species arise as a consequence of viral replication (68). Mammalian cells express a variety of factors that recognize dsRNA as a foreign, pathogenic material. Until very recently, it was believed that the initial cellular responders to dsRNA were protein kinase R (PKR), which shuts off host translation in response to dsRNA, and 2'-5' oligoadenylate synthetase (2-5A), which activates the endoribonuclease RNase L. Expression of both of these molecules is greatly induced by type-1 interferons and they are both active participants in recognition of dsRNA (45, 132). However, several studies of mice with genetic deletion of the PKR or RNase L genes indicated that these molecules were important as IFN-induced effectors, but did not play a significant role in

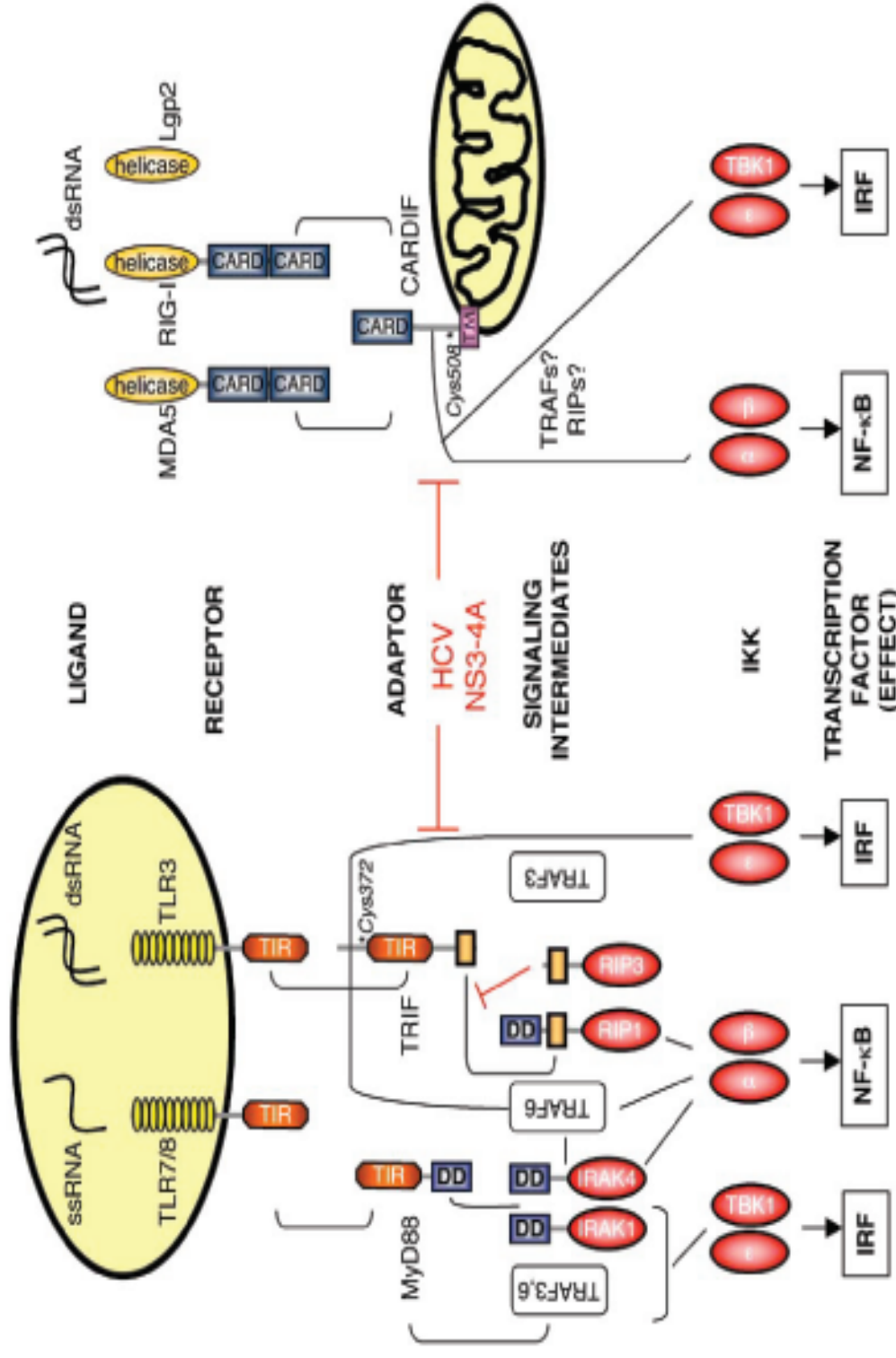
the initial induction of type-1 IFN (167, 195, 210). These experiments indicated that other dsRNA sensors were involved in the initial induction of IFN in virus-infected cells.

*Pattern recognition receptors as primary dsRNA sensors*

Recent studies have demonstrated that initial recognition of dsRNA occurs primarily through pattern recognition receptors (PRRs). These receptor molecules are expressed in a wide variety of cells involved in the innate immune response, and they function to detect specific conserved motifs or moieties common to pathogens, known as pathogen-associated molecular patterns (PAMPs) (76, 132, 148).

The best-studied PRRs are the Toll-like receptors (TLRs), a family of eleven receptor molecules that recognize PAMPs from a broad range of pathogens including viruses, bacteria, fungi, and protozoa (1, 76, 132). The various TLRs are expressed on different cell types as well as differing cellular compartments. Individual TLRs recognize specific PAMPs, such as bacterial lipopolysaccharide (TLR4), non-methylated CpG DNA (TLR9), single-strand RNA (TLR7, TLR8), and double-stranded RNA (TLR3) (1, 2). While most TLRs reside on the plasma membrane, the four family members which have been associated with viral recognition (TLR3, TLR7, TLR8, and TLR9) are located in intracellular compartments, such as endosomes. Activation of all of these TLRs leads to a signaling cascade culminating in the induction of cytokines, including type-1 IFN (Fig. 1.1) (76, 132).





**Fig. 1.1: Overview of TLR and RIG-I-dependent pathways.**

(Left) TLR7/8 and TLR3 or (right) RIG-I- and MDA5-dependent signaling pathways are represented according to the function of their respective constituents. The interactions and domains involved are shown. Abbreviations are as follows: CARD, caspase recruitment domain; DD, death domain; TIR, Toll-interleukin-1 receptor; and TM, transmembrane region. Reproduced from Meylan et. al., Mol. Cell, 22, pgs. 561-569.

In addition to TLRs, several other dsRNA-sensing PRRs have recently been identified. The cellular RNA helicases, retinoic acid inducible gene-I (RIG-I) and the melanoma differentiation-associated gene (MDA5/Helicard), also appear to act as intracellular PRRs. RIG-I contains two caspase recruitment domain (CARD)-like domains and a RNA helicase domain. The helicase domain recognizes viral dsRNA and the CARD domains are responsible for triggering a signaling cascade leading to interferon regulatory factor 3 (IRF3) and NK- $\kappa$ B (Fig.1.1) (132, 201, 207). Over-expression of RIG-I was able to block infection from several viruses, and studies using fibroblasts and DCs from RIG-I<sup>-/-</sup> mice demonstrated that these cells were almost completely deficient in their ability to produce type-1 IFN and inflammatory cytokines in response to viral infections (75, 201). MDA5 is closely related to RIG-I structurally and functionally. This molecule also contains an RNA helicase domain and two CARD-like domains that are critical for IRF and NK- $\kappa$ B activation. Over-expression and knockdown studies of this gene produced similar results as seen with RIG-I: over-expression of MDA5 induced a more robust type-1 IFN response to virus, and MDA5 knockdowns prevented appropriate IFN responses in the context of viral infection (5, 74, 200).

The study of PRRs is a relatively young field, and new candidate dsRNA-sensors are still being characterized (76, 132). The identification of PRRs helps shed light on the former immunological grey area of how a host can mount efficient innate responses to multiple and varied pathogens. TLR3, RIG-I, and MDA5 compose a network of innate dsRNA sensors that can cover a broad range of cells. It appears that these PRRs work in separate cell types as well as separate cellular compartments. Analysis of RIG-I<sup>-/-</sup> mice

suggests that TLRs are activated from endosomes of plasmacytoid dendritic cells (pDCs), while RIG-I appears to operate in the cytoplasm of DCs and fibroblasts (75). It is likely that these PRRs and potentially other yet to be identified dsRNA-sensing PRRs act as immune sentinels for identifying viral infection. Together with PKR and 2-5A, these molecules underscore the importance of dsRNA recognition in the early stages of the immune response to virus. Identification of dsRNA by these molecules leads to the production of an antiviral state, where high levels of type-1 IFN production sound the alarm for the host and kickstart the acquired phase of the immune response (76, 90).

### **C. Viral inhibition of cellular dsRNA-sensing molecules**

#### *Inhibitors of PRR signaling*

A general assumption in the study of virus/host interactions is that for every example of host-mediated immune defense against viruses, corresponding examples of viral inhibitors to this defense pathway exist. As initial responders to dsRNA introduced by viral infection, the PRRs are not an exception to this assumption. Several viral inhibitors which disrupt the signaling molecules downstream of PRRs have been identified (76, 132). The vaccinia virus A46R, hepatitis C virus (HCV) NS3-4A protein, paramyxovirus V proteins, and Kaposi's sarcoma-associated herpesvirus (KSHV) RTA protein all target components of the signaling cascade involved in induction of type-1 IFN production from dsRNA sensors (5, 43, 102, 170, 172, 202).

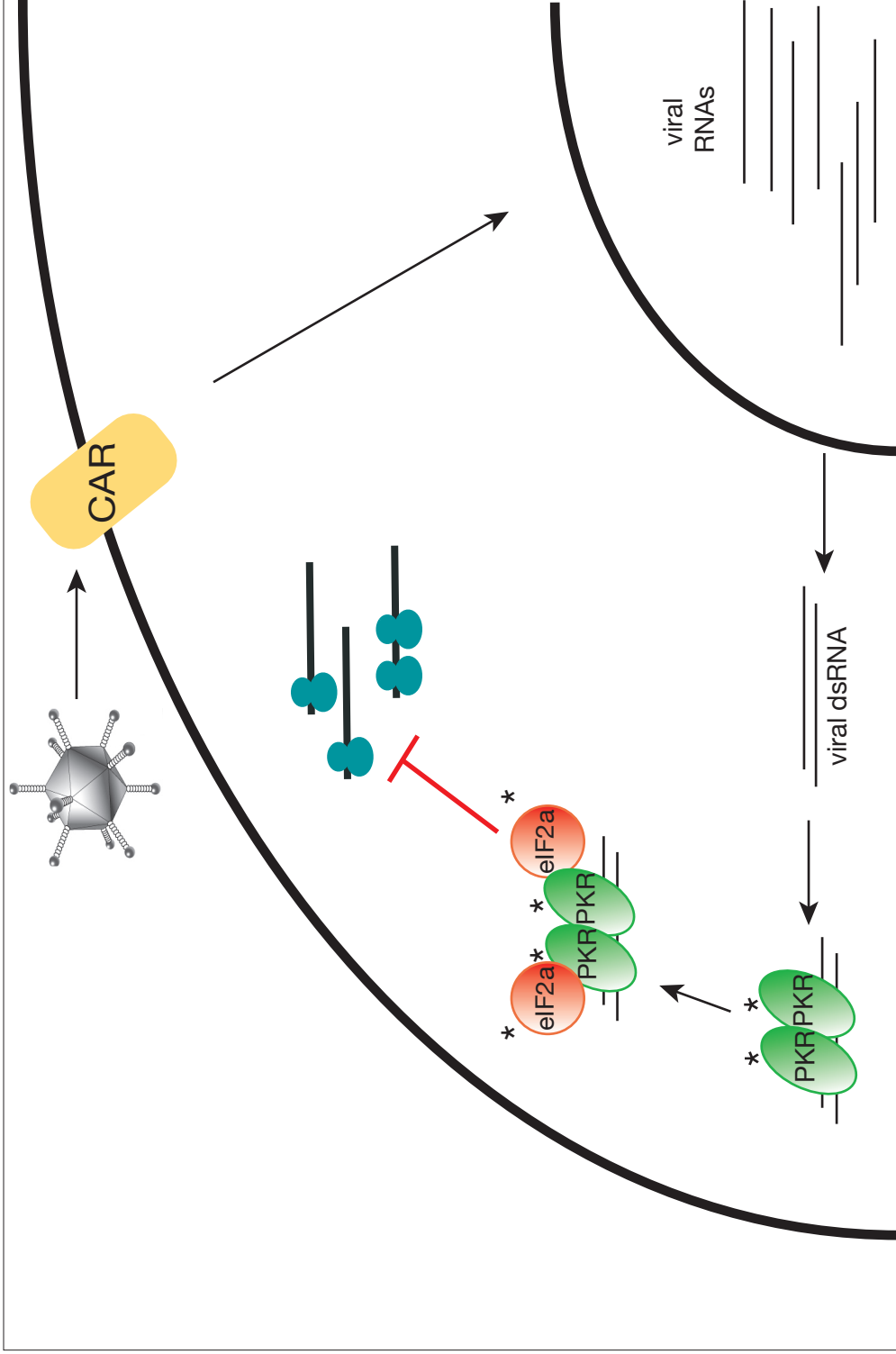
### *Inhibitors of 2-5A and PKR*

The IFN-induced dsRNA response factors 2-5A and PKR represent some of the best-studied examples of cellular targets for viral inhibition of the host immune response. A wide array of inhibitors encoded by numerous viruses focus on inactivation of these proteins, with a particular emphasis on PKR (45, 185). Inhibition of these proteins is essential for most infections, since downstream consequences of their activation include increased IFN production, destruction of mRNA transcripts, and host protein synthesis shutoff (185). For most viruses, transcription and translation of viral factors requires host cell components. The indiscriminate targeting of mRNAs, both viral and cellular, for degradation, and the ablation of global protein synthesis demonstrate a general anti-viral state in which the cell subjugates all activities in an effort to prevent dissemination of virus and to signal the presence of a viral pathogen in the host.

Activation of the 2-5A pathway occurs when 2-5A synthetase molecules come into contact with dsRNA. This interaction results in the formation of oligomers of ATP residues with a 2'-5' linkage (77, 209). These 2-5A molecules subsequently activate the cellular RNase L protein, which directs cleavage of mRNA at specific sequences (3' of UpNp) (41). The 2-5A pathway has been shown to influence replication of several viruses, including vaccinia virus, encephalomyocarditis virus (EMC), and reovirus (136, 151, 194). Correspondingly, several viral-encoded inhibitors of the 2-5A pathway have been identified. Not surprisingly, the highly immunomodulatory vaccinia virus interferes

with the pathway, as the E3L protein was shown to inhibit 2-5A synthetase function in *in vitro* assays (152). Additionally, herpes simplex viruses encode 2-5A derivatives which act as antagonists of 2-5A, and EMC virus and HIV have been demonstrated to block the 2-5A/RNase L pathway by inducing the expression of a cellular inhibitor of RNase L (119, 120).

The powerful and robust cellular PKR response results in the inhibition of cellular translation in response to dsRNA. Expressed at low levels in mammalian tissues, PKR becomes activated rapidly after binding dsRNA through its double-strand RNA binding domains (dsRBD) (10, 45, 51). Activation of the kinase function of PKR requires dimerization and subsequent intermolecular autophosphorylation events (141, 153). Activated PKR then phosphorylates the cellular translation factor, eukaryotic initiation factor 2 (eIF-2), blocking its normal role in translation initiation (28). The eIF-2 initiation factor is made up of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits. Under normal circumstances, the eIF-2 forms a ternary complex, along with GTP and the initiator methionyl tRNA. After initiation of translation is complete, eIF-2, now bound to GDP, is released from the ribosome. Another initiation factor, eIF-2b, acts as a guanosine exchange factor (GEF), catalyzing the exchange of a GTP molecule for the bound GDP on eIF-2, so that the complex can be recycled and start another round of initiation for polypeptide synthesis. However, PKR-induced phosphorylation of the  $\alpha$  subunit of eIF-2 prevents the ability of eIF-2b to recycle guanosines, resulting in the “trapping” of the eIF-2 initiation factor in an inactive conformation (Fig. 1.2). When sufficient eIF-2 becomes trapped, translation of both cellular and viral mRNAs is halted (45, 99, 122, 156).



**Fig. 1.2: PKR response to adenovirus dsRNA.**

Adenovirus infection results in bi-directional transcription of genome leading to production of dsRNA species. The dsRNA is bound by PKR, leading to dimerization and activation by autophosphorylation. Activated PKR phosphorylates the translation initiation factor, eIF2α, leading to a shut off of polypeptide synthesis.

While the 2-5A pathway has been formally demonstrated to regulate replication of only a select list of viruses, the PKR response appears to be of paramount importance to the replication of many viruses, based on the number of viral inhibitors directed at this pathway. Viral inhibition of the pathway can be found for every stage of the PKR response, from binding of dsRNA to the activation of eIF-2 $\alpha$  phosphatase (45, 185). Examples include viral proteins, as well as structured viral RNAs that bind and inhibit PKR, such as the adenovirus VA RNAs, Epstein-Barr Virus EBERs, and human immunodeficiency virus TAR RNA. Of these inhibitors, the VA RNAs are the best studied; in fact, the initial studies of VA RNA were the first to provide evidence of viral subversion of the PKR response.

#### **D. Adenoviruses and the VA RNA genes**

##### *Identification of adenovirus and associated diseases*

The initial identification of adenovirus occurred in 1953 when a previously unknown virus was isolated from primary cells derived from human adenoid tissue (155). Presently, the Adenoviridae family is composed of two genera: the Aviadenovirus, which infect birds, and the Mastadenovirus, which infect a variety of mammals, including humans, simians, pigs, and rodents (165). There are currently 49 distinct serotypes of adenovirus (divided into 6 subgroups (A-F)) that infect humans (Centers for Disease Control Website, 2/07). Adenovirus are classified as DNA tumor viruses based on the

ability of the virus to transform cultured cells, as well as the ability of particular strains of the virus to induce tumors in rodents (155, 175). Infection occurs primarily through the respiratory tract leading to respiratory disease, but the virus can also infect the conjunctiva, giving rise to conjunctivitis (“pink eye”), and adenovirus infection has also been associated with infantile gastroenteritis (165). While the virus is most commonly associated with acute infections, the virus is also able to establish a persistent infection, as virus has been isolated from lymphocytes in addition to adenoid and tonsillar tissue (48, 87, 181).

#### *General features of adenovirus*

Adenovirus virions are icosahedral, non-enveloped particles that infect cells through an interaction of the viral fiber protein and a membrane-bound coxsackievirus and adenovirus receptor (CAR) (13, 165). The genome of the virus is composed of linear, double-strand DNA (dsDNA) and the genome size is ~36 kilobases (kb) (165).

Adenovirus viral transcripts are produced from both strands of the genome and the virus uses a temporal mode of gene expression, with early gene products regulating expression of numerous host genes to establish an environment conducive to viral replication, and late gene products (L1-L5) encoding the structural components of the virus (165). The early gene products E1a and E1b were identified as the viral factors responsible for transformation of cultured cells. These proteins disrupt the activities of numerous cell cycle regulators, simultaneously activating transcription of viral genes,



while promoting cell cycle progression. Like the other DNA tumor viruses, adenovirus achieves cellular transformation by inactivating the cellular p53 and retinoblastoma (RB) proteins. The cultured human 293 cell line is the result of cellular immortalization and transformation by E1a and E1b. The other major early transcriptional units of adenovirus are the E2, E3, and E4 genes, and the virus-associated RNAs (VA RNAs) (165). The E2 gene encodes proteins involved in replication of the viral genome, while the E3 and E4 gene products, like the E1 gene products, regulate a wide range of cellular proteins to promote an environment for adenovirus replication. The activities of the E3- and E4-encoded proteins are broad, as E3 gene products have been reported to alter numerous immune and inflammatory mediators and the E4 gene products are reported to modulate transcription, the cell cycle, cell signaling, DNA repair, and immune mediators (39, 165, 190). Adenovirus vectors are now commonly used for transduction of transgenes into cultured cells and laboratory animals in laboratory settings and their clinical application for gene therapy is an active area of research. Importantly, these viral vectors contain deletions of the E1 and E3 genes to reduce the oncogenic and anti-immunogenic qualities of the vectors (47).

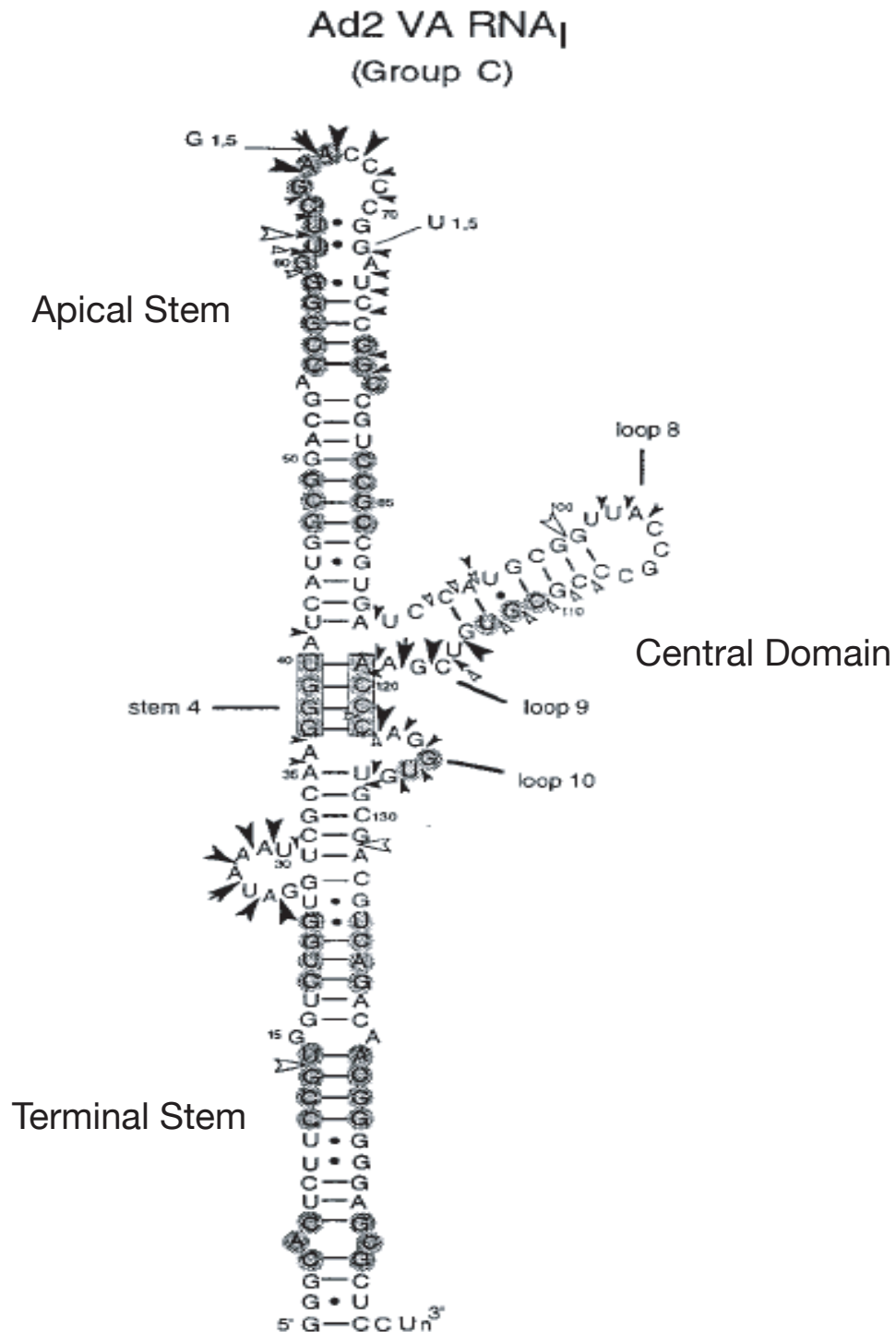
Historically, adenoviruses have been an extremely fruitful area of research, producing several groundbreaking studies that affected multiple biological fields. The most famous of these discoveries was the identification of the processing of mRNA molecules or “splicing” (9, 14). Additionally, investigation of the E1a function led to the discovery of the E2F family of transcription factors. The first E2F protein was so named because the first promoter it was found to bind was the adenovirus E2 promoter (84).

Finally, experiments performed to ascertain the function of the VA RNA genes of adenovirus demonstrated the importance of the cellular PKR response to viral infection (83, 173).

#### *Virus-associated RNAs (VA RNAs) of adenovirus*

VA RNAs are a class of small RNAs (~160 nucleotides) produced at very high levels during adenovirus infection (Fig. 1.3). These RNAs are transcribed by RNA polymerase III and contain neither a 5' cap structure nor a 3' poly-adenylate tail. Rather, these highly structured molecules accumulate to levels as high as  $10^8$  molecules (VA RNA I) and  $10^7$  molecules (VA RNA II) in infected cells (168). The RNAs contain internal Box A and Box B promoter sites, characteristic of RNA polymerase III transcripts (25, 42, 55). Mammalian adenoviruses of groups C, D, E, and most of B, encode two related VA RNA genes, VA RNA I and VA RNA II. Viruses of groups A, F, SA7, and several from group B, encode only a single VA species. Of the 49 known human serotypes, only 9 encode a single VA RNA gene, and in these instances, the single VA gene is of the VA RNA I family (114).

Extensive phylogenetic analysis of the VA RNA species from the various adenovirus groups has been performed. The genomic location of the VA RNA genes in relation to neighboring genes reflects the compact, highly efficient nature of viral genomes, as the VA RNA loci very closely abut protein-coding sequences. Only ~30 nucleotides separate the 5' end of VA RNA I from an upstream splice site for the pTP



**Fig. 1.3: Predicted structure of VA RNA I.**

Putative structure of VA RNA for Group C adenovirus, as determined from nuclease sensitivity assays, site directed mutagenesis, and MFOLD RNA structure prediction (Zuker analysis). Figure reproduced from Ma and Mathews, *J. Virol.*, 70, p. 5083-5099.

gene. Similarly, there are only ~20 nucleotides downstream from the 3' end of VA RNA II and a splice site of the 52,55K L1 protein (114). Essentially, only the ~100 nucleotides separating the VA RNA I gene from the VA RNA II gene are non-coding in this portion of the genome.

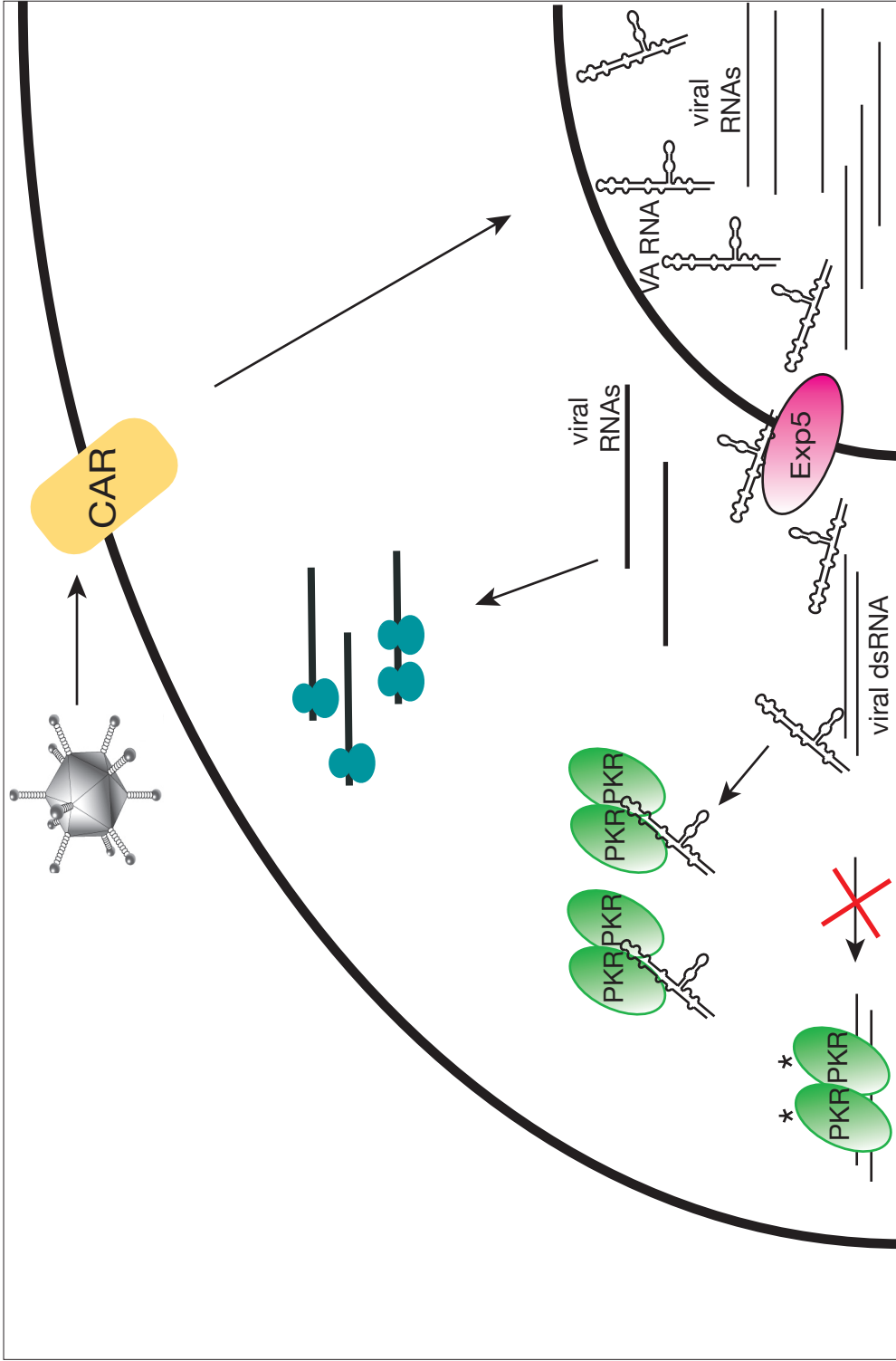
Genetic studies of the VA RNA molecules led to the seminal findings of the molecule's role in blocking the host PKR response. Viruses containing deletions of VA RNA II grew to the same level as wild type virus, while VA RNA I-deleted virus (dl331) were found to grow poorly in relation to wild type virus (~10 fold reduced virus production), and viruses containing deletions of both VA RNA genes grew even more poorly (~60 fold reduced). This result suggested that VA RNA II was able to partially substitute for VA RNA I in the dl331 single mutant virus (17, 173). Further studies of the dl331 provided evidence that the cell growth defect was caused by inefficient protein synthesis at late times of infection, and this defect was traced to defective initiation of translation (150, 173). Phosphorylated forms of the translation initiation factor eIF2 $\alpha$  were found in the dl331-infected cells, implying the activity of what was at the time known as eIF2 kinase, or dsRNA-activated inhibitor (DAI) (150, 159). This DAI kinase is now known as PKR. Additionally, it was shown that extracts from dl331-infected cells could be rescued for protein synthesis when eIF2 was added to the extract, and experiments in cells expressing low levels of PKR demonstrated that the dl331 virus grew similar to the wild type virus (139, 150, 159).

The implication of PKR as a target for VA RNA regulation led to the logical assumption that the structured VA RNA molecule likely acted as a competitive inhibitor

of PKR. This would prevent the recognition by PKR of virus-derived dsRNA that is produced during infection (116). A series of careful mutagenesis experiments have been performed on VA RNA I in order to determine the functional domains of the molecule and potential interactions with PKR (44, 113, 128). These studies identified the apical stem region of the VA RNA molecule as crucial to the binding of PKR, and the central domain as the region responsible for PKR inactivation (Fig. 1.3). Interestingly, phylogenetic analysis of the VA RNA molecules indicates that the apical stem and central domain are less conserved than the terminal stem of the molecule, which is not implicated in PKR interaction (114). However, the high conservation of sequence in the terminal stem may simply be attributed to the presence of the Box A internal promoter for RNA polymerase III (25, 42, 55).

Taken together, these results demonstrated that the high levels of VA RNA produced in infected cells act as competitive inhibitors of PKR, preventing its binding to virally-derived dsRNA and subsequent activation (Fig. 1.4). These studies were the first to define the important role for PKR in the virus/host relationship.

Recently, VA RNAs were shown to utilize the nuclear export factor, Exportin-5, to transit from the nucleus to the cytoplasm. Interestingly, this same export factor was found to export endogenous, structured dsRNA molecules called pre-microRNAs (pre-miRNAs) (59, 112, 206). These molecules are involved in an endogenous gene silencing pathway based on dsRNA substrates. Numerous examples of related gene silencing pathways have been described in a variety of organisms, and some of these have been shown to act as immune defense systems against pathogens (127).



**Fig. 1.4: Adenovirus VA RNA blocks PKR activation.**

Adenovirus infection results in bi-directional transcription of genome leading to production of dsRNA species. High levels of VA RNA bind and sequester PKR, preventing its activation. Viral and host mRNA are translated by cellular translational machinery.

## **E. RNA silencing**

### *The discovery of RNA silencing pathways*

In the early 1990s, plant researchers attempting to engineer more attractive flowers stumbled upon a puzzling result. By introducing a transgene to increase production of the chalcone synthetase gene responsible for pigment production in petunias, the researchers expected to generate flowers with greater purple hues. However, the plants that they generated displayed flowers with variegated coloring or sometimes completely lacking in pigment (135). Not long after these studies, a genetic screen devised to find mutants in developmental timing of worm larvae produced a loss-of-function gene that did not encode protein. Mysteriously, the gene encoded a short hairpin RNA molecule with antisense complementarity to a heterochronic gene (93). Just several years later, scientists attempting to use antisense technology to regulate gene expression in *C. elegans* were surprised to find that the controls for the experiments, sense RNA molecules, sometimes produced similar effects to the antisense RNAs (56, 131). Unbeknownst to them initially, all of these researchers had stumbled upon related mechanisms of RNA-based silencing.

The mechanisms and pathways of RNA silencing began to come into focus when Andrew Fire and Craig Mello solved the conundrum of the aforementioned antisense experiments. They proposed that both the sense and antisense RNA preparations in such

experiments were not completely pure populations of RNA, and that dsRNA contamination was likely present in small quantities in the RNA preparation (131). When intentionally-generated, specific dsRNA preparations were used in similar studies, highly efficient and specific silencing of the target gene was achieved. Additionally, silencing initiated in the worms could be passed through the germline to their progeny (40). This Nobel Prize-winning experiment describing RNA interference (RNAi) brought the study of dsRNA to the forefront of biology and led to the identification of a wide array of RNA silencing mechanisms that are still being uncovered at a rapid pace.

Examples of RNA silencing now include a diverse group of organisms that includes yeast, fungi, worms, flies, plants, and mammals. The common feature in all of these pathways is the recognition of a dsRNA substrate or “trigger”. Similar to the potent non-specific immune response to dsRNA in mammalian cells, a multitude of other organisms had now been found to rely on host mechanisms to detect and respond to long stretches of dsRNA as foreign, pathogenic material. Traditionally, the term RNAi is used to refer to the silencing pathways triggered by long dsRNA molecules, which leads to specific gene silencing. Examples of this occur in related, but distinct pathways in species such as worms, flies, and plants. Experiments in plants and fly extract demonstrated that in these organisms, the long dsRNA is processed into short, ~21-26 nucleotide dsRNAs, or short, interfering RNAs (siRNAs). These siRNAs are the functional intermediates of the pathway, which are responsible for performing site-specific, endonucleocytic cleavage of complementary mRNAs (62, 176, 205). The process is more complicated in worms, where an RNA-dependent RNA polymerase is



involved in the production of siRNAs for RNAi (142, 166). With these results in place, molecular biologists were off and running with this new technique to experimentally induce RNAi in their model system for the examination of a specific gene of interest.

Intuitively, it would seem that RNAi would not be a conserved mechanism used in mammals because of their aforementioned non-specific response to dsRNA. Previous studies had demonstrated that PKR is activated by dsRNA of 30 nucleotides or longer (115). However, as components of the RNAi response were identified, many of these factors were found to be conserved in mammals (15, 63). Indeed, mammalian cells were shown to retain RNAi capabilities, as studies in murine embryonic cell lines lacking a PKR response demonstrated efficient RNAi generated by long dsRNA (19). While this result from a specialized, seldom-studied cell line provided evidence that mammals maintained an RNAi response, it didn't translate into efficient use of experimentally-induced RNAi to study gene function, since most mammalian cell lines commonly used in laboratory research do maintain the non-specific dsRNA response. However, this limitation was circumvented when it was demonstrated that the introduction of siRNA directly into mammalian cells produced specific gene silencing without immune induction (35).

These initial studies of RNAi uncovered many of the key components conserved in RNA silencing pathways and provided a basic mechanistic framework for RNA silencing mechanisms. As the field expanded, so too did the number of identified RNA-based triggers, including several RNA species encoded by the host.

*miRNAs and endogenous small RNAs*

The aforementioned genetic screen in *C. elegans*, which uncovered a small hairpin regulatory RNA that regulates the expression of a heterochronic gene in a post-transcriptional fashion is now credited as the initial discovery of a microRNA (miRNA) (93). Soon after the initial discovery of RNAi, there were a series of studies describing large classes of phylogenetically-conserved, endogenously-expressed substrates for RNA silencing pathways (86, 88, 92). Studies of these genes have identified them as important regulators of gene expression, impacting a variety of cellular pathways including cell death, development, proliferation, and hematopoiesis. They are commonly described as regulators which “fine tune” gene expression (3, 11). These genes are initially processed from their hairpin precursors into siRNA-like molecules. However, unlike siRNAs, which specifically cleave their targets, miRNAs typically regulate their target genes by translational repression through conserved miRNA target sites in the 3'UTR of target mRNAs (3, 179). However, exceptions to this general rule exist, as examples of several human miRNAs and numerous plant miRNAs have been shown to perform direct cleavage of their targets (108, 197). To date, miRNA have been found in all metazoans examined. It has been demonstrated that the “seed” sequence of the miRNA (nucleotides 2-7/8) are the critical determinants for target recognition in the 3'UTR of mRNA. Amazingly, just 15 years ago, miRNAs had not been discovered, yet bioinformatic analysis now predicts that miRNAs regulate the expression of 30% of all human genes (100). More recently, global analysis of miRNA and mRNA expression patterns

indicates an intricate evolutionary relationship of miRNAs and mRNAs bearing target sites for these miRNAs. It was seen that mRNAs bearing conserved target sites for miRNAs were often expressed at early developmental stages prior to expression of miRNAs. Additionally, genes expressed at similar times in similar cells as miRNAs have evolved to selectively avoid the presence of potential non-conserved target sites in their 3'UTRs (38).

In addition to miRNA, several other classes of endogenously-expressed small RNAs have been described. Some of these endogenous small RNAs are similar to the exogenously-produced siRNAs of long dsRNA triggers, whereas other small RNAs have slightly larger sizes and enter into distinct silencing complexes (82). Naturally occurring siRNAs (Nat-sRNAs) of plants, or endogenous siRNAs (endo-siRNAs) of worms have been observed. These RNAs are believed to derive from the production of dsRNA created by annealing of endogenous sense and antisense transcripts (20, 94). The trans-acting siRNAs (tasiRNAs) of plants are derived from dsRNAs created by an RDRP using endogenous transcripts as its template. Interestingly, the siRNAs created by this process regulate different (trans) genes than the one from which the dsRNA was created (144). Studies in fruit flies uncovered a further class of small RNAs, called repeat-associated RNA (rasiRNAs), which are derived from repetitive sequence elements in the genome. These small RNAs are slightly larger in size than siRNAs and associate with different effector proteins (7, 8, 107, 178). And, very recently, a subset of small RNAs of ~26-31 nucleotides has been identified in mammalian testes. These so called piRNAs (PIWI-

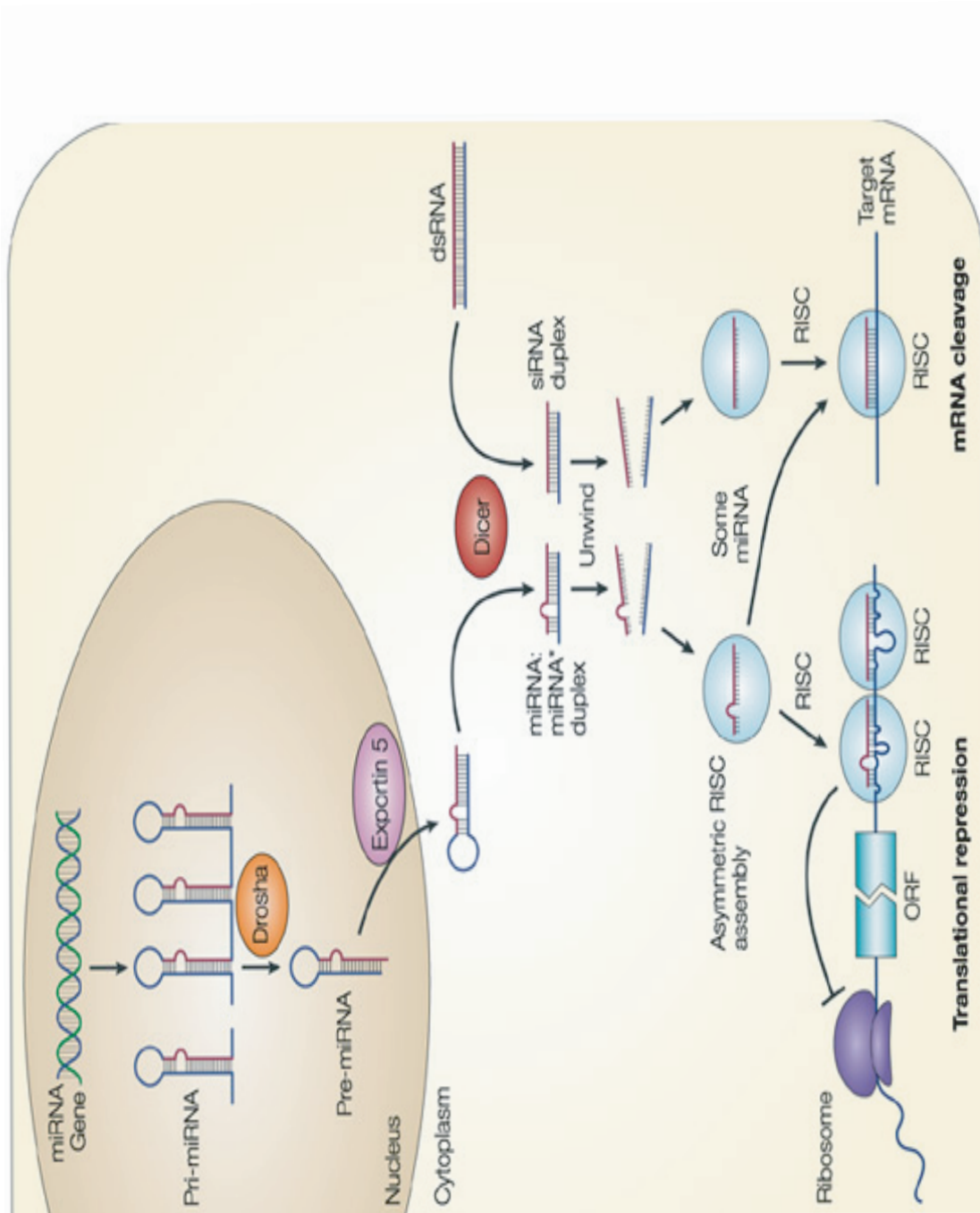
interacting small RNAs), like siRNAs, can perform cleavage of a target RNA *in vitro*, but their biological function is currently unknown (82, 89).

#### *Common components of the siRNA and miRNA silencing pathways*

The miRNA and siRNA pathways are mechanistically similar and share many common components (Fig. 1.5). A considerable amount of cross talk between the pathways exists, as it has been experimentally demonstrated that siRNAs can be forced to act like miRNAs, causing translational repression of a target; conversely, miRNAs have been demonstrated to act as cleaving siRNAs, both *in vitro* as well as *in vivo* (32, 67, 197). Extensive biochemical analysis of *in vitro* RNAi-competent extracts from fruit flies and mammals has greatly helped our understanding of how these pathways work. The following is a summary of the known factors involved in these pathways in humans, and the divergence of these pathways in different organisms where appropriate.

#### *Processing of dsRNA substrates into functional small RNAs*

Typically, triggers for RNAi/siRNA pathway are exogenously introduced sources of dsRNA, such as virally-produced dsRNA. The long dsRNA is recognized and processed into siRNAs by cellular RNase-III enzyme known as Dicer (15). In flies, there are two Dicer proteins, Dicer-1 and Dicer-2. The latter preferentially dices dsRNA triggers in the siRNA pathway, while the former is involved in the processing of miRNA



**Fig. 1.5: Overview of the siRNA and miRNA pathway.**  
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substrates. In humans, there is only one Dicer protein, which performs the processing of both dsRNA and pre-miRNA substrates (98, 127). Plants lead the way in number of Dicer proteins, as they encode four different Dicer molecules (184). Human Dicer performs dsRNA recognition and cleavage in conjunction with two cellular proteins, TAR RNA-binding protein (TRBP) and protein activator of protein kinase R (PACT) (26, 80, 96). Processing by Dicer results in the typical product of RNase III cleavage, leaving 5' phosphates and 2 nucleotide 3' overhangs (36). Importantly, these 3' overhangs appear to be key signatures for preventing recognition of the dsRNA by innate immune factors, such as PKR, RIG-I, and TLRs. Conversely, blunt-ended small siRNAs have been shown to trigger a RIG-I response in mammalian cells (80, 117, 118). The processed siRNAs are quickly assembled into the RNAi effector complexes, or RNA-induced silencing complex (RISC), following their production by Dicer.

The precursor molecules for the miRNA pathway are initially transcribed in the nucleus as long primary transcripts, called pri-miRNAs. These pri-miRNAs can contain one or several pre-miRNA precursors within the transcript. The RNase III enzyme Drosha, along with a dsRNA-binding protein called DGCR8 (PASHA in flies), form a "microprocessor complex" that cleaves the pre-miRNA hairpins from the pri-miRNA transcripts. The cleaved precursor molecule bears a 2 nucleotide 3' overhang at the site of Drosha cleavage, as is seen with Dicer cleavage (95, 97). The hairpin miRNAs are now able to exit the nucleus via the nuclear export factor, Exportin-5 (112, 206). Once in the cytoplasm, the pre-miRNA is then further processed by Dicer, to produce a mature miRNA/miRNA\* (microRNA/microRNA "star") duplex, which is similar in form to

siRNAs (53, 65, 98). The miRNA/miRNA\* duplex is extremely transient and is difficult to detect *in vitro*, as the mature miRNA is quickly loaded into an active RISC, while the miRNA\* strand is degraded.

#### *RISC complexes and the argonautes*

Processed siRNA and miRNA are loaded into the effector complexes of the pathway, as siRNAs associate into RISC complexes, and miRNAs into so-called miRNA ribonucleoprotein complex (miRNP). The transient double strand nature of the siRNA duplex or the miRNA/miRNA\* is quickly resolved upon incorporation into the RISC or miRNP. This incorporation of one strand into an active complex requires a strand selection method. Normally for siRNAs and miRNAs the mitigating factor involved in this strand selection is the thermodynamic stability at the ends of the small RNA molecule (79, 161). The strand incorporated preferentially into RISC is the one bearing less thermodynamic stability at its 5' end. For miRNAs, this is the mature miRNA as opposed to the miRNA\*, and for siRNAs, this is referred to as the “guide” strand of the siRNA, with the non-incorporated strand dubbed the “passenger”.

The RISC effector complexes are all characterized by the inclusion of a Argonaute protein family member. The Argonautes represent a large family of proteins that can be phylogenetically separated into five sub-families (143). It appears that differing Argonaute proteins are capable of performing different types of RNA silencing. The Argonaute-2 (Ago-2) protein was shown to be responsible for the site-directed

cleavage of target RNA in a RISC (105, 126). Cleavage of the target RNA strand occurs specifically at the phosphodiester bond on the target RNA located across from nucleotides 10 and 11 on the corresponding siRNA. In flies, miRNAs load into Argonaute-1 (Ago-1)-containing RISCs, and siRNAs load into Ago-2 RISCs (a.k.a “Slicer”) (127). Dauntingly, *C. elegans* express 27 different Argonaute proteins, which are involved in various aspects of RNA silencing (199). Many Argonaute proteins, such as *D. melanogaster* Ago-1, are capable of slicing if provided with the appropriate substrates. That Ago-1 miRNP complexes normally don’t slice their targets *in vivo* is largely due to the lack of perfect complementarity between the miRNA and target RNA (32, 61). The method by which miRNA-directed translational repression occurs is an area of active debate. Several studies demonstrated effects on mRNA stability, while others link translational repression to early termination of translation (179). Unlike the Argonautes of *Drosophila*, it has been formally demonstrated that of the four human Argonaute family members, only Ago-2 is capable of slicing (105, 126). Currently, very little is known about the other human Argonaute family, but some intriguing new data suggest that Ago-1 may be involved in a mechanism that specifically regulates transcription (70, 81, 154, 174). The wide variety of Argonaute proteins highlight the diversity of the RNA silencing pathways, as distinct Argonautes associate with differing triggers and elicit several different types of silencing mechanisms.



*The anti-viral role of RNA silencing pathways*

It is now becoming apparent that RNA-based silencing systems are the primary anti-viral defense system for several organisms. Evidence from *C. elegans* implicates RNAi in an anti-viral role, as well as a role in the defense against mobilization of transposons (78, 193). Evidence continues to accumulate in *Drosophila* that RNAi is the primary anti-viral response (182, 183, 186). It has been found that *ago-2* mutant flies are highly susceptible to viral infection, and additionally, several RNA-silencing suppressor proteins have been found in insect viruses (110, 183). Plants provide the clearest example of a fully functional anti-viral RNA response. Plants produce a virus-induced gene silencing (VIGS) response, producing virus-specific small RNAs that can target viral RNAs for destruction. Like worms, plants have the ability to spread their RNA silencing response from cell to cell, and it has been demonstrated that the plant VIGS response can lead to a systemic silencing (186, 189). Additionally, numerous plant viruses encode RNA silencing suppressors, which target a variety of steps in silencing pathways, from inhibitors that directly bind small RNAs, to inhibitors that prevent the silencing signal from spreading through the plant (160, 186, 203).

The relationship of anti-viral RNA silencing mechanisms and their viral counterparts show great similarity to the nature of the mammalian host/virus interactions. The silencing response of the host to virus displays the same characteristics of the mammalian adaptive immune response, in that it is specific, adaptive, and mobile (at least

in the instance of plants and worms). Additionally, similar to the numerous methods by which we observe mammalian viruses counteracting the host immune system, we see viral pathogens utilizing a wide array of silencing suppressors.

The idea that RNA silencing also plays a role in the host/virus response in mammals is gaining momentum. Recently, several virally-encoded miRNAs have been found in mammalian DNA viruses (54, 57, 146, 147, 171). Several of these miRNAs were found to regulate expression of viral genes bearing perfect complementarity to the miRNA. These activities generally appeared to aid in the ability of the virus to establish a chronic infection in the cells. In addition, several proteins encoded by mammalian viruses were shown to suppress silencing mechanisms in plants and insect cells (21, 30, 103). Finally, it was demonstrated that a cellular miRNA was able to restrict the growth of primate foamy virus (PFV) and that the virus produced an RNA silencing suppressor that inhibited silencing in mammalian cells and as well as plant cells (91). These results point to the possibility that RNA silencing mechanisms may play a role in the mammalian response to viruses.

**CHAPTER II:**

**MATERIALS AND METHODS**

## **A. Cell Culture**

HeLa cells (American Type Culture Collection (ATCC)) were maintained in Minimal Essential Media (Gibco/Invitrogen) with 10% (v/v) fetal bovine serum (HyClone); human 293 cells (ATCC) and HEL (human embryonic lung fibroblasts; ATCC) were maintained in Delbucco's Minimal Essential Media (Gibco) with 10% fetal bovine serum.

## **B. Viruses**

### *Adenovirus*

Wild-type Adenovirus type 5 was obtained from ATCC, and dl331 is a mutant Adenovirus strain kindly provided by Thomas Shenk (Princeton University, New Jersey), which contains a 29 base pair (bp) deletion in the VA RNA1 locus, preventing its expression (173). Viruses were propagated in HeLa or 293 cells and purified by centrifugation through cesium-chloride gradients. Virus titers were determined by immunohistochemical staining for the adenovirus hexon protein with an anti-adenovirus antibody (Bioscience International) and visualized by using a 3,3'-diaminobenzidine substrate kit from Vector Laboratories (24).

### *Adenovirus Infections*

Prior to infection, cells were washed with phosphate-buffered saline (PBS). Virus was added to cells in serum-free media containing 20nM Hepes, pH 8.0, and infections were performed at 37°C in 5% carbon dioxide (CO<sub>2</sub>) for one hour. All experiments in this work utilized a multiplicity of infection (MOI) of 5 or less (as noted in text). Following the one hour incubation, virus media were removed and replaced with serum-containing media and the cells were cultured as described above. For virus growth curve experiments, cells were resuspended in PBS containing 10% glycerol. The samples were then subjected to 3 freeze/thaw cycles in order to disrupt cells and release virus. Freezing was performed in a dry ice/EtOH bath, and thawing occurred in a 37°C water bath. Samples were then centrifuged for 20 minutes at 13,000 x g at 4°C to pellet cell debris. The virus-containing supernatant was subsequently used in immunohistochemical titering protocol described above.

### *Human Cytomegalovirus (HCMV)*

The Ad-169 cell culture adapted strain of HCMV (ATCC) was used in these studies. Virus stocks were titered using plaque assay on HELs. Briefly, cells were infected with a series of virus dilutions. At 3 hours post infection, virus media were removed, and cells were overlaid with DMEM containing 0.3% Seaplaque GTG agarose (FMC) and 0.2% FBS. Agarose overlays were allowed to set for 30 minutes at room

temperature prior to being placed at 37°C. Seven days after infection, monolayers were fixed by addition of 100% methyl alcohol and stained with Giemsa stain (Sigma-Aldrich). Triplicate samples were counted to determine the number of plaques.

### *HCMV Infections*

For the miRNA gene array, an MOI of 3 was used. Infections were performed as described for Adenovirus with the exception of a 2 hour infection period for HCMV.

## **C. In vitro RNAi Reactions**

### *Preparation of RNA Templates for Dicing Reactions*

Polymerase chain reaction (PCR) was used to generate dsRNA or structured RNA templates containing a T7 promoter upstream of the sequence of interest. The primers used in this study are listed below. VA RNA PCRs used the recombinant adenovirus vector, pAd-Easy (Stratagene), as template. EBERs PCRs used the pBR322-EBV BamC fragment (kind gift of Jae Jung, Harvard University) as template. Alu PCRs used genomic HeLa DNA as template. Purified PCR products were precipitated and resuspended in a volume equivalent to 1/10 of the original PCR reaction. This concentrated DNA template is then transcribed and radiolabeled in an in vitro

transcription reaction (60). Radiolabeled RNAs are gel purified, precipitated, and resuspended in water.

*Primers for svRNA Dicing Templates*

VA RNA I

**VARNA IVT5**- 5' GCGTAATAACGACTCACTATAGGGCACTCTTCCGTGGTCTGG 3'

**VARNA IVT3**- 5' AAAAAGGAGCACTCCCCCGTTG 3'

VA RNA II

**T7 + VA RNA2 5'** - 5' GCGTAATAACGACTCACTATAGGGCTCGCTCCCTGTAGCCG 3'

**VA RNA2 3'** - 5' AAAAGGGGCTCGTCCCTGTTTC 3'

ALU RNA

**Alu 5'+T7**- 5' GCGTAATAACGACTCACTATAGGGGCCGGGCGCGGTGGCTC 3'

**Alu 3'** - 5' TTTGAGACGGAGTCTCGCTCTGTCGCC 3'

EBER

**EBER 5'+ T7** - 5' GCGTAATAACGACTCACTATAGGGGGACCTACGCTGCCCTAGA 3'

**EBER 3'** - 5' AAAACATGCGGACCACCAGC 3'

*Dicing Assays*

In vitro dicing assays were performed using a final concentration of 5-10 nM radiolabeled substrate. A typical 10 µl reaction contained the following: 3 µl 40x reaction mix (60), 5 µl *D. melanogaster* embryo lysate (Haley 2003), 1 µl water, and 1 µl 50-100 nM uniformly labeled dsRNA (or structured RNA) substrate. Reactions were

incubated at 25°C for the indicated times. Reactions were quenched in 100 µl 2X PK Buffer (200 mM Tris-HCL, pH 7.5, 25 mM EDTA, 300 mM NaCl, 2% w/v sodium dodecyl sulfate) and were deproteinized by addition of 10 µl Proteinase K (E.M. Merck; 20 mg/ml dissolved in water) with 1 µl glycogen carrier (Roche). Proteinase K treatment was carried out at 65°C for 30-60 minutes. The reaction contents were extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and RNA was recovered by precipitation with 3 volumes of absolute ethanol. Precipitate is then washed with 80% ethanol and the pellet is dissolved in 20 µl formamide loading dye (98% w/v deionized formamide, 10 mM EDTA, pH 8.0, 0.025% w/v xylene cyanol, 0.025% w/v bromophenol blue). Samples were resolved on 15% denaturing polyacrylamide gels. Following electrophoresis, the gels were dried under vacuum, and detected by phosphorimagery. Dicing assays using recombinant human Dicer enzyme (Gene Therapy Systems) were performed according to manufacturer's protocol, and the samples were then Proteinase K-treated, phenol extracted, precipitated, and resuspended as described for the embryo extract dicing reactions.

#### *Gel Filtration of RNAi Reactions*

A preparative scale dicing reactions (100 µL) was prepared with 50nM radiolabeled VA RNA I template. Dicing reaction proceeded for 2 hours at 25°C, and the reaction was then centrifuged at 13,000 x g to remove cellular debris. Supernatant (~100 µl) was subjected to chromatography on a Superdex-200 HR 10/30 column (Pharmacia)



at 0.75 ml/min in lysis buffer using a BioCad Sprint (PerSeptive Biosystems). Following the passage of 7.5 ml through the column, sixty 200  $\mu$ l fractions were collected on a cooled stage (4°C). Odd numbered fractions were used for scintillation counting, and even numbered fractions were Proteinase K treated, phenol extracted and precipitated, and dissolved in 20  $\mu$ l formamide loading buffer as described above. Samples were electrophoresed on a 15% PAGE gel to analyze the distribution of the radiolabeled VA RNA-complexes in the column. Additionally, the Superdex-200 column was loaded with an additional 100  $\mu$ l of sample containing various size markers (100  $\mu$ l contained: 88  $\mu$ l Lysis Buffer (100 mM potassium acetate, 30 mM Hepes-KOH, pH 7.4, 2 mM magnesium acetate), 2  $\mu$ l of a 1  $\mu$ M radiolabeled siRNA, 5  $\mu$ l ferritin (15  $\mu$ g/ $\mu$ l), and 5  $\mu$ l catalase (15  $\mu$ g/ $\mu$ l)).

#### *Preparation of Cap-Radiolabeled RNA Templates for Target Cleavage Reactions*

RNA templates for target cleavage assays are prepared by PCR and transcribed as described for the dicing RNA substrates, with the exception that no radiolabeled NTPs are used for transcription. A list of PCR primers used to create the target RNA templates are included below. The PCR reaction uses the pGL2 luciferase plasmid (Promega) as a template, along with primers that amplify a portion of the luciferase sequence, as well as the T7 promoter site at the 5' end of the product and a perfectly complementary sequence to the miRNA of interest and to the lin41 sequence in the 3' of the product. Following transcription of the purified PCR template, the non-radioactive target RNA is

electrophoresed on a denaturing polyacrylamide gel, and the RNA band is detected and excised from the gel using UV shadowing. Following gel purification, the RNA is precipitated and resuspended in a small volume (10  $\mu$ l) of sterile water. The target RNA is then cap-radiolabeled in a reaction including: 10 pmol uncapped target RNA (in 1.75  $\mu$ l), 5  $\mu$ l of [ $\alpha$ - $^{32}$ P] GTP (3000 Ci/mmol, 40 mCi/ml, ICN Pharmaceuticals), 1  $\mu$ l 10x guanylyl transferase buffer (500 mM Tris-Cl, pH 8.0, 60 mM KCl, 12 mM MgCl<sub>2</sub>, 25 mM DTT), 0.5  $\mu$ l of 0.1 mM DTT, 0.25  $\mu$ l of 20U/ $\mu$ l RNasin (Promega), 1  $\mu$ l of 10 mM *S*-adenosyl methionine (SAM, Sigma-Aldrich) in SAM dilution buffer (5 mM H<sub>2</sub>SO<sub>4</sub>, 10% ethanol), and 0.5  $\mu$ l recombinant, heterodimeric vaccinia virus guanylyl transferase (Ambion). Reactions were carried out at 37°C for 1.5 hours. Free radiolabeled nucleotides were removed by passing the reaction contents over a G-50 spin column (Roche). Target RNA was then gel purified, phenol extracted, precipitated, and resuspended in a small volume of water.

*Primers used for preparation of target RNA templates*

miR-VA Target RNA

**T7 pGL3-ANR Prm-** 5' GCGTAATACGACTCACTATAGGGGATTACCAGGGATTTTCAGTC 3'  
**miR-VA1 Targ 3'Prm-** 5'CCCATTTAGGTGACACTATAGATTTACATCGCGTTGAGTGTA  
 GAGGTCGACGTCAGACAACGGGGGAGTGCTCCTTTTCCGAAGAGAGGAGTTCATGAT  
 CAGTG 3'

Dcr Target "A"

**T7 + DCR A 5'- 5'** GCGTAATACGACTCACTATAGGGGATAATTATCTGTGCATGGAGTG 3'  
**DCR A + Lin 3'- 5'** CGTTGAGTGTAGAACGGTTGTATAAAAGGGGCTGATTCTTCCCAA  
 TGCC 3'

Dcr Target "B"

**T7 + DCR B 5'- 5'** GCGTAATACGACTCACTATAGGGGAAGCTCTATCCTCCTGAA 3'  
**DCR B + Lin 3'- 5'** CGTTGAGTGTAGAACGGTTGTATAAAAGGGTCAATATCCAAAGT  
 GCTGG 3'

Dcr Target “C”**T7 + DCR C 5’- 5’** GCGTAATACGACTCACTATAGGGGAGCTAAGAGCCCAGACTG 3’**DCR C + Lin 3’- 5’** CGTTGAGTGTAGAACGGTTGTATAAAAGGCTTCAACGTGGAGCTT  
ACCAGG 3’Dcr Target “D”**T7 + DCR D 5’- 5’** GCGTAATACGACTCACTATAGGGGGTAAGCTCCACGTTGAAGTTT  
C 3’**DCR D + Lin 3’-5’**CGTTGAGTGTAGAACGGTTGTATAAAAGGTCTGCTCAGAATCCAT  
CCTGC 3’*Target Cleavage Assays (D. melanogaster embryo and human cell extracts) and Mapping of the Cleavage Product*

Reaction contents for a typical *D. melanogaster* target cleavage reaction include: 3  $\mu$ l 40x reaction mix (60), 5  $\mu$ l *D. melanogaster* embryo lysate, 1  $\mu$ l target radiolabeled RNA (1-10 nM final concentration), and 1  $\mu$ l synthetic siRNA (25-100 nM final concentration). Reactions are carried out at 25°C and were processed as described for dicing assays. For reactions using human cell extracts, no synthetic siRNA was added since extracts were prepared from siRNA-transfected or infected-cell extracts. Typical reactions for human cell extracts include: 3  $\mu$ l 40x reaction mix, 5  $\mu$ l human cell extract, 1  $\mu$ l target radiolabeled RNA (1-10 nM final concentration), and 1  $\mu$ l water. Reactions were carried out at 37°C, and were then processed and analyzed as described for *D. melanogaster* embryo lysate reactions. Experiments using 2’-O-methyl (2’oMe)-derived oligonucleotide inhibitors for calculating the half maximal inhibition of RISC (IC<sub>50</sub>) were performed as described (66). Base hydrolysis was performed by incubating labeled target RNA in a reaction containing 50 mM (NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub>, pH 9.5, at 95°C for varying lengths of

time (5, 10, and 15 minutes). RNase T1 (Sigma-Aldrich) digestion was carried out at varying concentrations of enzyme (0.1 U, 1 U, and 10U) for 10 minutes at 50°C in reactions containing 25 mM sodium citrate, pH 5.0 and 7M Urea. Base hydrolysis and RNase T1 reaction products were Proteinase K treated, phenol extracted, precipitated and resuspended in formamide loading buffer as described above prior to electrophoresis on denaturing polyacrylamide gels.

#### **D. Preparation of Human Extracts for in vitro RNAi Reactions**

Cells were infected or transfected as described and S10 extracts were prepared according to the protocol of Dignam and Roeder (31). Cells were scraped and collected in ice-cold PBS, pelleted by centrifugation at 1200 x g for 10 minutes at 4°C, and washed 2 more times in PBS. Cells were resuspended in 5 volumes hypotonic Buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM DTT) and swelled on ice for 5-10 minutes. The cell suspension was collected at 1500 x g for 10 minutes, resuspended in 2 volumes of Buffer A, then disrupted with ~50 strokes using a Dounce homogenizer (Kimble/Kontes, Vineland, NJ). Following homogenization, 1/10 volume of Buffer B (30 mM MgCl<sub>2</sub>, 1.4 M KCl, 300 mM HEPES, pH 7.9) was added and the suspension was centrifuged at 13,000 x g for 20 minutes at 4°C. The resulting supernatant was dialyzed 2 x 90 minutes against Buffer D (20 mM Tris, pH 7.9, 100 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, pH 8.0, and 20% Glycerol (w/v)) in a dialysis cassette (Pierce Slide-A-Lyzer, 10,000 MW cutoff). The extracts were centrifuged once more at 13,000 x g for 20 minutes at 4°C, then aliquoted, flash frozen in liquid nitrogen, and stored at -80 °C.

### **E. Northern Blot Analysis**

RNA was isolated using Trizol reagent (Gibco/Invitrogen) according to the manufacturer's protocol, then DNase treated (Promega) and was resuspended in water. RNA was quantitated by UV spectrophotometry and aliquots of appropriate amounts of RNA for gel loading were precipitated with 1/10 volume of 3M sodium acetate and 3 volumes absolute ethanol. The precipitated RNA was resuspended in formamide loading buffer, then resolved on a 15% denaturing polyacrylamide gel and transferred to Hybond membrane (Amersham-Pharmacia) in 0.5x TBE in a semi-dry transfer system (Transblot SD, Bio-Rad) at 20 V for 60 min. The RNA was UV cross-linked to the membrane using a Stratalinker (Stratagene). The full length VA RNA probe was prepared by in vitro transcription of a PCR template as described in for preparation of RNA templates above. The PCR primers used for construction of the template are listed below. For all other Northern blots, RNA (Dharmacon) or DNA (Integrated DNA Technologies, Coralville, IA) oligonucleotide probes (50 pmol per reaction) were 5'-radiolabeled with polynucleotide kinase (New England Biolabs) and  $\gamma$ -<sup>32</sup>P-ATP (NEN). After labeling, unincorporated radioactivity was separated from the labeled probe using a Sephadex G-25 spin column (Roche) and probes were hybridized to blots in Church's Buffer (0.5 M sodium phosphate, 7% SDS, and 1mM EDTA). Blots were washed at the same temperature used for hybridization using the following protocol: 1 X 20 minutes in 2X SSC/0.1% SDS, 3 X 20 minutes in 0.2X SSC/0.1% SDS, rinsed briefly in 5X SSC and exposed to phosphorimager screen (Fuji) and scanned and analyzed using an FLA-5000 phosphorimager (Fuji), Image Reader FLA-5000 version 1.0 (Fuji) and Image Gauge version 3.45 (Fuji).

*PCR primers for production of full length VA RNA probe*

**ANR IVT5-** 5'GCGTAATACGACTCACTATAGGGAAAGGAGCACTCCCCCGTTG 3'

**ANR IVT3-** 5' GGGCACTCTTCCGTGGTCTGG 3'

*DNA oligonucleotide primers used for Northern blot analysis*

**VA1 ProbeA-** 5' TTATCCACCAGACCACGGAAGAGTGCCC 3'

**VA1 ProbeB-** 5' CGGTCGTCCGCCATGATACCCTTGCGAAT 3'

**VA1 ProbeC-** 5' GGCCGGATCCGGGGTTCGAACCCCGG 3'

**VA1 ProbeD-** 5' ATGGATCACGGCGGACGGCCGG 3'

**VA1 ProbeE-** 5' TCGACACGCGGGCGGTAACCGCATGG 3'

**VA1 ProbeF-** 5' CGCTCCCCGTTGTCTGACGTCGCACACCTGGGT 3'

**tRNA-Ile-ATT Probe-** 5' TGGTGGCCCGTACGGGGATCGA 3' (64)

*RNA oligonucleotide probes used for Northern blot analysis*

miR-VA1 probe 5' - AAGGAGCACUCCCCGUUGUCU-3'

*Locked nucleic acid (LNA) probes used for Northern blot analysis*

miR-17-5p probe 5' - A<sup>+</sup>CTA<sup>+</sup>CCT<sup>+</sup>GCA<sup>+</sup>CTG<sup>+</sup>TAA<sup>+</sup>GCA<sup>+</sup>CTT<sup>+</sup>TG -3'

("+" denotes a locked nucleic acid modification)

**F. miRNA Gene Array Analysis**

Global miRNA gene analysis was performed on RNA from mock or Adenovirus (WT Ad-5)-infected HeLa cells (MOI= 5) and from mock or HCMV (Ad-169)-infected

HEL cells (MOI=3). RNA from adenovirus-infected cells was extracted using miRvana Total RNA Isolation (Ambion) at 24 hours post infection (hpi) and RNA from HCMV-infected cells was extracted at 48 hours hpi. Purification of the small RNA fraction and miRNA gene array was performed by LC Sciences (Houston, TX.). All known human miRNA and miRNA\* sequences known at the time of the experiment were probed on the micro array chips. Additionally, for the adenovirus array, a 1000 nt region of the adenovirus genome encompassing the VA RNA I and VA RNA II loci (10,320- 11,319, r-strand reading orientation) was probed using a single base tiling approach utilizing overlapping,  $T_M$ -normalized 22 nt probes. Similar single base tiling probes were used to analyze the 14 putative HCMV pre-miRNAs reported at the time of this work. Data was normalized according to the methods described by Lu et. al (109).

### **G. Primer Extension**

Primer extension using total RNA from HeLa cells infected with wild type Ad-5 was performed with the AMV Reverse Transcriptase Primer Extension System (Promega, Madison, WI). Briefly, 100 fmol of a 5'-radiolabeled DNA oligonucleotide (5' GGAGCACTCCCCCGTTGTCT 3', IDT) was annealed to 5  $\mu$ g total RNA at 55°C for 20 minutes, and then allowed to cool to room temperature (approximately 10 minutes). For the extension portion of the reaction, the indicated mixtures of dNTPs (2 mM) and reaction buffer were added to differentiate the extension product, and 30U of AMV reverse transcriptase was then added to the reactions, which were allowed to proceed for 1 minute at 42°C. Reactions were immediately placed on ice and 1 volume of formamide loading buffer was added to each. Control reactions containing either no template RNA or dNTPs for extension were also included. Additionally, synthetic RNA

oligonucleotides corresponding to the exact sequences of miR-VA “g” and miR-VA “A” (5′ GA CAA CGG GGG AGU GCU CCU UU 3′, and 5′ AGA CAA CGG GGG AGU GCU CCU U 3′, respectively, Dharmacon) were also used as RNA templates to act as markers for differentiation of the extension products. Products were resolved on a 20% denaturing PAGE gel and analyzed by FLA-5000 phosphorimager (Fuji).

#### **H. Transfections (siRNAs, 2′oMe Oligonucleotides, and Plasmids)**

Synthetic RNA and 2′-*O*-methyl oligonucleotides (2′oMe, Dharmacon) were deprotected and annealed according to manufacturer’s protocol. Sequences of siRNAs and 2′-*O*-methyl oligonucleotides used in these studies are listed below. The siRNAs used in this study were designed to bias the loading of the guide strand (g) of the siRNA into active RISC. This was accomplished by intentionally creating a mismatch at the proximal nt at the 5′ end of the guide strand/3′ passenger strand (p) pairing to produce a less thermodynamically stable (i.e. asymmetric) end of the siRNA, favoring incorporation of the guide strand into RISC (161). The human Dicer expression plasmid (pDcr) was the kind gift of Daryll Conte and Craig Mello (University of Massachusetts Medical School). This vector contains the full length human dicer cDNA cloned into a pCDNA backbone (Invitrogen). All transfections were optimized for each cell line (HeLa or 293) according to the manufacturer’s protocol. All siRNA and 2′oMe transfections were performed using Oligofectamine (Invitrogen) and used a final concentration of 25 nM of the transfected small RNAs. Plasmid transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.



*siRNAs*

siLuc (g) 5' UCGAAGUAUUCGCGUACGUG 3'  
 siLuc (p) 5' CGUACGCGGAAUACUUCGCUU 3'  
 siDcr (g) 5' UCCAGAGCUGCUUCAAGCAdTdT 3'  
 siDcr (p) 5' UGCUUGAAGCAGCUCUGGCdTdT 3'  
 siVA "g" (g) 5' GACAACGGGGGAGUGCUCCUU 3'  
 siVA "g" (p) 5' GGAGCACUCCCCCGUUGUCAG 3'  
 siVA "A" (g) 5' AGACAACGGGGGAGUGCUCCUU 3'  
 siVA "A" (p) 5' GAGCACUCCCCCGUUGUCAGG 3'  
 siDcr Trg A (g) 5' AGACAACUGCUGUGUAUCUdTdT 3'  
 siDcr Trg A (p) 5' AGAUACACAGCAGUUGUCCdTdT 3'  
 siDcr Trg B (g) 5' AGACAACAUGAAACCAGACdTdT 3'  
 siDcr Trg B (p) 5' GUCUGGUUUUCAUGUUGUCCdTdT 3'  
 siDcr Trg C (g) 5' AGACAACAUUGGCGCACUdTdT 3'  
 siDcr Trg C (p) 5' AAGUGCGCCAAUGUUGUCCdTdT 3'  
 siDcr Trg D (g) 5' AGACAACAAAAACACUGGdTdT 3'  
 siDcr Trg D (p) 5' CCAGUGUUUUUGUUGUCCdTdT 3'

*2'oMe Oligonucleotides*

2'-O- Luc 5' CAUCACGUACGCGGAAUACUUCGAAAUGUCC 3'  
 2'-O- let-7 5' UCUUCACUAUACAACCUACUACCUCAACCUU 3'  
 2'-O- miRVA1 5' UCUUCAAGGAGCACUCCCCCGUUGUCUAUUC 3'  
 2'-O- miRVA2 5' UUCAAGGGGCUCGUCCUGUUUCCGGAGAUUC 3'

## **I. Quantitative Reverse-Transcription Real-Time PCR (qRT-PCR)**

Total cellular RNA was prepared using Trizol reagent (Invitrogen) and 1.5 µg total RNA was reverse transcribed using Sensiscript III and oligo dT (12-18) primer (Invitrogen) according to manufacturer's instructions. qPCR analysis was performed using QuantiTech Sybr Green PCR Kit (Qiagen Inc., Valencia, CA) and analyzed using a DNA Engine Opticon 2 (MJ Research [Bio-Rad, Hercules, California]). Displayed results are the average of three separate experiments repeated in triplicate. Sequences for the real time primers are displayed below and primer set validation was performed for all three primer sets. Relative mRNA concentrations were calculated by the  $2^{-\Delta\Delta C_T}$  method (106).

### *qPCR Primers*

GAPDH (s) 5' GAAGGTGAAGGTCGGAGTC 3'

GAPDH (as) 5' GAAGATGGTGATGGGATTTC 3'

Dicer (s) 5' CAGAACGTTGCTCAGCGAGTC 3'

Dicer (as) 5' GGTTGCACGGGTATTCCTG 3'

Actin (s) 5' GCAAAGACCTGTACGCCAACA 3'

Actin (as) 5' ACACGGAGTACTTGCGCTCAG 3'

## **J. Protein Extraction and Western Blot Analysis**

Following indicated treatments, HeLa or 293 cells were harvested and centrifuged at 1500 x g for 10 minutes. Cell pellets were resuspended in ice cold PBS and centrifuged at 1500 x g for 5 min. The cells were then resuspended in WCE buffer (55

mM HEPES, pH 7.9, 275 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 10% Glycerol, 0.01 % NP-40, 1.1 mM DTT, + 1X Protease Inhibitors (Sigma-Aldrich)) and incubated on ice for 60 minutes. Following centrifugation at 13,000 x g for 10 minutes at 4°C, the supernatant was collected and used as whole-cell extract. Proteins were separated by SDS-polyacrylamide electrophoresis and transferred to a nitrocellulose membrane (Schleicher and Schuell). Western analysis was performed using a monoclonal antibody for Dicer (Clonogene) at a dilution of 1:150 and a secondary HRP-conjugated goat anti-mouse antibody (Amersham-Pharmacia) at a dilution of 1:10,000. Equal loading was confirmed using antibodies to detect  $\alpha$ -tubulin (Santa Cruz Biotechnology) at a dilution of 1:500 and a secondary HRP-conjugated goat anti-mouse antibody (Amersham-Pharmacia) at a dilution of 1:10,000. Blots were detected using ECL reagent (NEN) and quantified using an LAS-3000 phosphorimager (Fuji, Tokyo, Japan).

## **CHAPTER III:**

# **ADENOVIRUS VA RNA IS A SUBSTRATE FOR CELLULAR RNA SILENCING PATHWAYS**

## Introduction

The initial groundbreaking studies of RNA silencing phenomena in the 1990s has led to a massive amount of research directed at uncovering RNA silencing activities in a wide array of organisms (40, 135). These diverse organisms have evolved to use several related components of RNA silencing for a variety of applications, from host defense against pathogens to biogenesis of small RNAs that are critical regulators of gene expression. Not only do these pathways vary from organism to organism, but they also diverge in each species in relation to the substrate or trigger RNA being examined (204). Plants, for example, have related, but distinct pathways to handle dsRNA triggers from viruses, single strand triggers from transgenes, and several overlapping pathways to handle the wide array of endogenously produced small RNAs (184). It is becoming apparent that nearly any RNA sequence bearing a hairpin or extensive secondary structure may be a substrate for an RNA silencing pathway.

Along these lines, numerous viruses encode structured RNA molecules that have the potential to come into contact with host RNA silencing pathways during the virus life cycle. Examples of highly structured RNAs produced during viral infections include small RNA molecules transcribed during replication, such as the adenovirus VA RNAs, and Epstein-Barr virus EBERs, as well as large structured portions of viral genomes or transcripts, such as human immunodeficiency virus (HIV) TAR sequences and the internal ribosome entry sites (IRES) of RNA viruses. We theorized that since RNA silencing was shown to act as an immune defense system in plants and lower animals, it

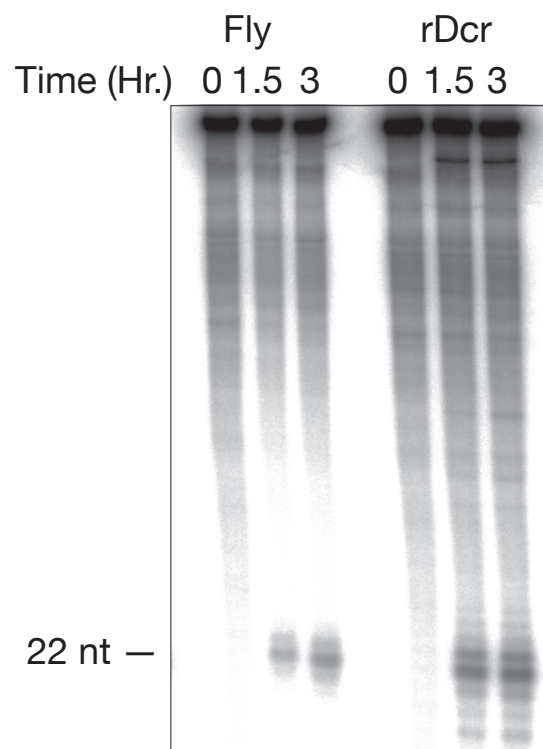
was likely that similar RNA silencing pathways may also be actively involved in the virus-host interaction for mammals. In taking a candidate-based approach to look for viral RNAs that enter into cellular RNA silencing pathways, we chose to study the VA RNA I molecule of adenovirus. This highly expressed, RNA polymerase III-transcribed RNA has been previously shown to play a crucial role in viral replication by blocking the robust interferon-inducing PKR response in mammals. Interestingly, VA RNA molecules display a gross resemblance to the predicted secondary structures of pre-miRNA molecules, known substrates for RNA silencing. Additionally, VA RNA utilizes the same nuclear export factor, Exportin-5, that pre-miRNAs use to traffic from the nucleus to the cytoplasm (59). These factors led us to hypothesize that adenovirus VA RNA molecules may act as substrates for host RNA silencing machinery in a similar fashion to dsRNA molecules or pre-miRNAs.

## Results

### *Adenovirus VA RNA I is diced to produce small RNAs*

Recent studies in plants, insects, and worms have established RNA silencing pathways as integral components of a host response to pathogens (186). In plants, dsRNA created during viral replication can lead to a virus-induced gene silencing (VIGS) response and the production of small RNAs that specifically target viral transcripts (188). Additionally, the Tombusvirus was shown to encode a suppressor of RNA silencing, p19, that binds and sequesters siRNAs (196). These studies indicated that Dicer family proteins were important members of the RNA silencing response to viruses in plants. As such, we decided to test whether a structured RNA from a mammalian virus may be a substrate for Dicer cleavage. For our initial analysis, we chose to test the adenovirus VA RNA I molecule, a highly structured, ~160 nucleotide RNA produced by RNA polymerase III transcription. *In vitro*-transcribed, radiolabeled VA RNA I was tested for its ability to be diced in *D. melanogaster* embryo lysate and in an *in vitro* reaction with recombinant human Dicer enzyme (Fig. 3.1). Our results demonstrate that both the RNAi-competent fly extract and the recombinant Dicer enzyme are able to efficiently process small RNAs characteristic of RNA silencing from the VA RNA molecule.

To more closely examine the production of VA-specific small RNAs in fly extract, we performed a large scale dicing reaction and fractionated the contents by gel filtration on a Superdex-200 column. Previous work had demonstrated specific peaks of



**Fig. 3.1: VA RNA I is diced to produce small RNAs.**

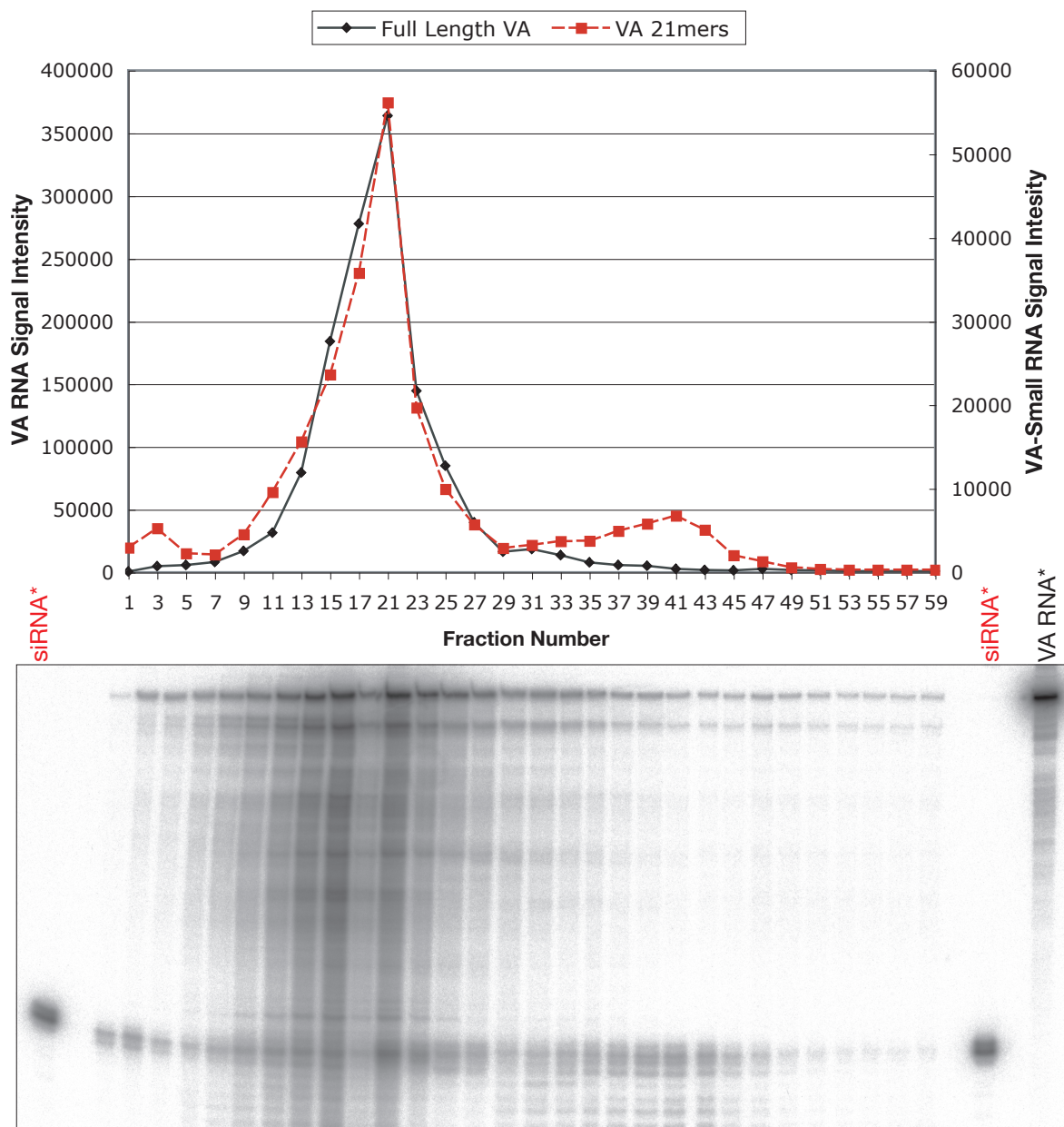
Radiolabeled VA RNA I was incubated for the indicated times in RNAi-competent *D. melanogaster* embryo lysate (Fly) or an *in vitro* reaction with recombinant human Dicer (rDcr) enzyme (Gene Therapy Systems). RNA was electrophoresed on 15% polyacrylamide gel to detect presence of processed small RNAs.



protein/siRNA complexes (siRNP) associated with RNAi activity in specific column fractions (137). When VA RNA was processed in an RNAi reaction and separated on the column, we observed a peak of both full-length VA RNA molecules and VA-specific small RNAs (~21 nt) at an apparent molecular weight of 220-230 kD (fraction 17) (Fig. 3.2). This correlates well with the predicted size of *D. melanogaster* Dicer-2 protein, and also coincides with the fractions previously identified for siRNP formation (137). When unprocessed, radiolabeled VA RNA or siRNA (not incubated in the fly extract) was loaded and fractionated on the column, the RNAs accumulated in lower molecular weight fractions, indicating that the VA RNA in our dicing reaction was bound, and presumably cleaved, in larger ribonucleoprotein complexes (data not shown). Additionally, a second peak of VA-specific small RNAs was observed in fractions 39-43, which were previously identified as fractions supporting siRNA-based target cleavage (137). Although not functionally demonstrated in our assay, we can speculate that this peak of small RNAs is reflective of VA-specific small RNAs loaded into an argonaute-containing RISC complex.

#### *VA RNA-specific small RNAs accumulate in infected cells*

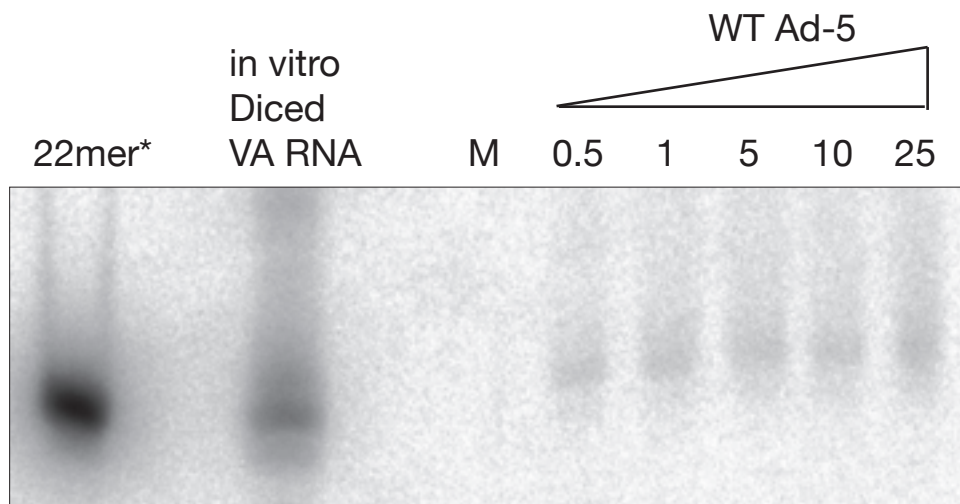
In order to determine whether the previously observed *in vitro* production of VA-small RNAs was relevant to adenovirus infection, we performed northern blot analysis to look for the presence of these small RNAs in infected cells. HeLa cells were infected with wild-type adenovirus (Ad-5) at varying multiplicities of infection (MOI), and RNA



**Fig. 3.2: VA-specific small RNAs appear to co-fractionate with RNAi components.** The contents of a large scale dicing reaction containing radiolabeled VA RNA was fractionated on a Superdex-200 gel filtration column. RNA was isolated from the odd numbered fractions and analyzed on a 15% polyacrylamide gel. The signal intensity of full length VA RNA and VA-specific small RNAs in each fraction are plotted above.

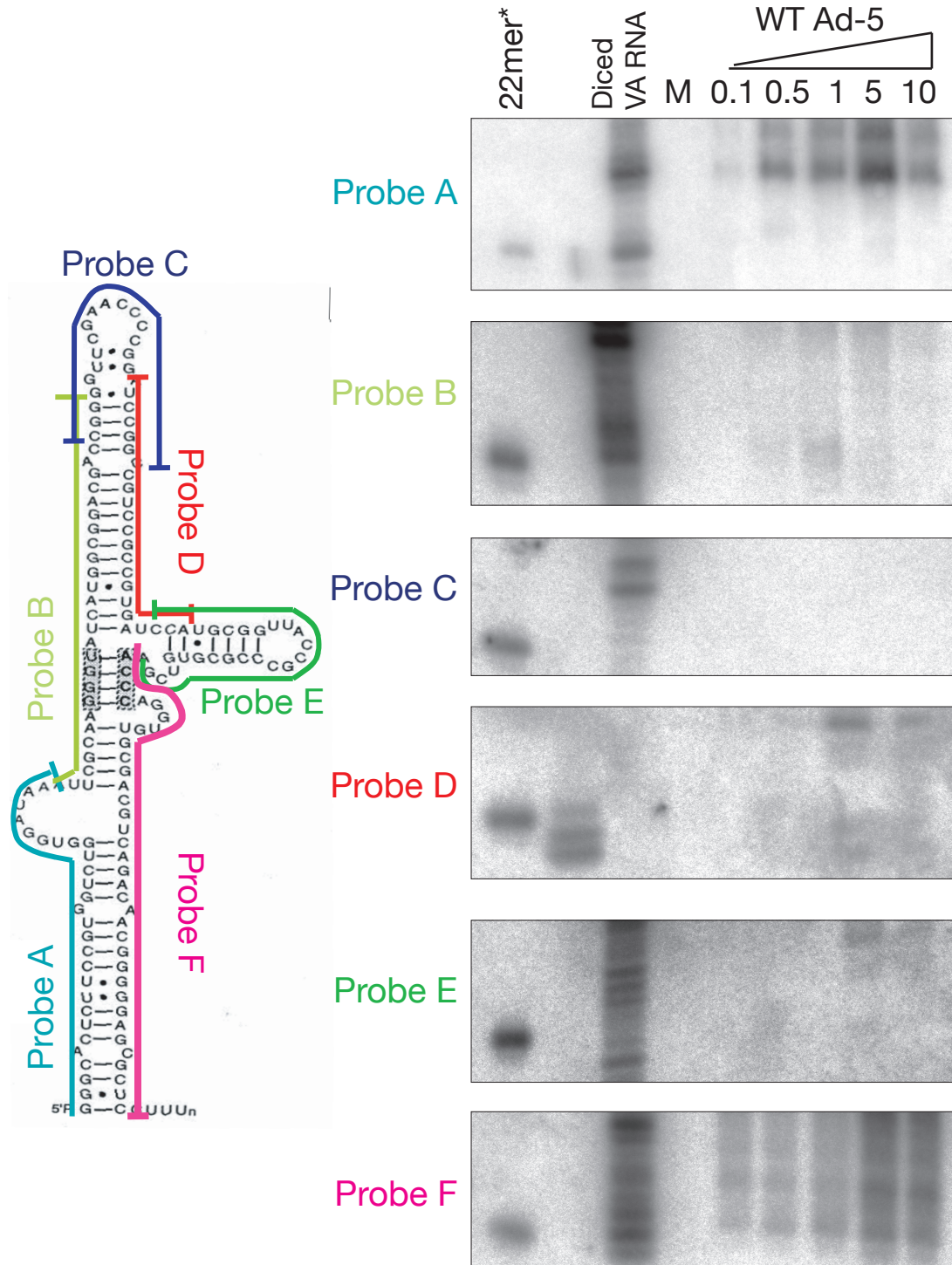
was examined for VA RNA-specific small RNAs, using a probe complementary to the full length VA RNA molecule. We observed the presence of VA-specific small RNAs of approximately 21-22 nucleotides in the infected cells (Fig. 3.3). This result indicated that VA RNA may indeed be a substrate for host RNA silencing pathways *in vivo* and that this interaction may be important to the process of infection, the host response to virus, or both.

To further delineate the region of the VA RNA I molecule producing the small RNAs, we performed northern blot analysis using a series of overlapping oligonucleotide probes, which span the entire VA RNA molecule (Fig. 3.4). The region of VA RNA I that gives rise to the 21-22 nucleotide small RNAs occurs in the 3' end of molecule, in the descending arm of the terminal stem (probe F, Fig. 3.4). These small RNAs are similar in size to those seen by the *in vitro* dicing assays (Figs. 3.1 & 3.2) and those seen in the northern blots using the full length VA probe (Fig. 3.3). We dubbed this VA-specific small RNA "miR-VA", due to its similarity in size to miRNAs, and because it appeared to accumulate asymmetrically, as has been reported for miRNAs (79, 161). That is, we saw the 21-22 nt miR-VA accumulate in infected cells with probe F, but did not see the corollary 21-22 nt miRNA\* strand when performing northern analysis with probe A. Interestingly, several of the other probes (A, B, D) also detected small RNA products of a size slightly larger than that characteristic of siRNAs and miRNAs. Whether or not these represent functional small RNAs or by-products of enzymatic cleavage or degradation remains to be determined. Recently, several other groups have similarly reported the production of small RNAs from the VA RNA locus (4, 6, 158).



**Fig 3.3: VA RNA-specific small RNAs are produced in infected cells.**

HeLa cells were mock-infected (M) or infected with increasing MOIs of wild type adenovirus. RNA was harvested at 24 hours post-infection and northern blots were probed with a radiolabeled full length, anti-sense VA RNA probe. Markers include a radiolabeled 22 nt. siRNA, and RNA from an *in vitro* VA RNA dicing reaction using non-radiolabeled VA RNA as substrate.

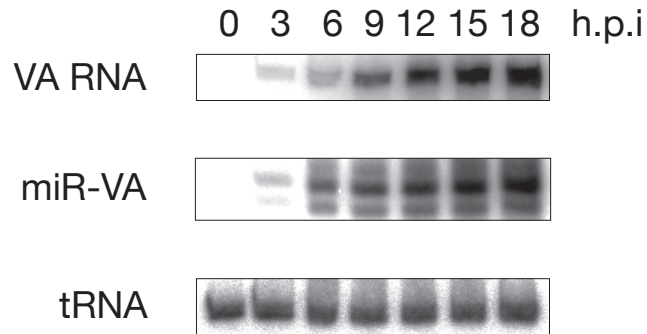
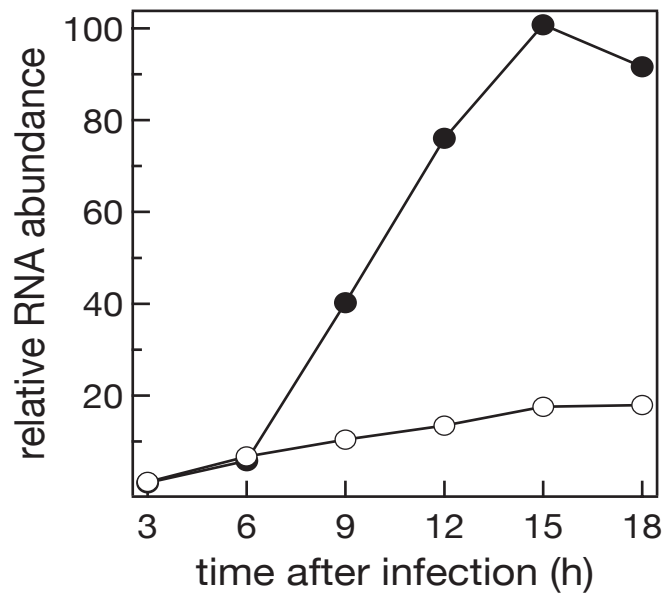


**Fig 3.4: VA RNA-specific small RNAs map to the 3' end of the VA RNA molecule.** HeLa cells were mock-infected or infected with increasing MOIs of wild type adenovirus. RNA was harvested at 24 hours post-infection and northern blots were probed with overlapping, radiolabeled, antisense oligonucleotide probes as indicated.

We further analyzed the production of the miR-VA in infected cells by following its accumulation over the early stages of an infection. Previous experiments indicated that in our experimental system, full length VA RNA reached its peak accumulation by 24-30 hours post-infection (hpi). In order to examine the relative levels of miR-VA and VA RNA accumulation, we performed northern blot analysis on RNA extracted during the first 18 hours of infection (Fig. 3.5). From our analysis, it appears that miR-VA accumulates early in infection and is detected at similar levels to full length VA RNA at 3 hours post-infection. While the level of VA RNA quickly rises to extraordinarily high levels in infected cells, the level of miR-VA appears to level off rather quickly. During this initial 18 hour analysis, the level of miR-VA does not appear to exceed 10% of the level of full length VA produced. However, since it has been reported that up to  $10^8$  molecules of VA RNA I can accumulate in infected cells, this small percentage of miR-VA could still represent an impressive and potentially significant amount of viral miRNA in the infected cells (121).

*Mapping of the miR-VA molecule reveals the presence of multiple miR-VA species*

Because miR-VA seemed to accumulate asymmetrically in infected cells, we sought to determine the precise identity of miR-VA with the goal of using this information to identify potential targets of the putative miRNA. We employed several different strategies in order to determine the exact sequence of miR-VA. In the first technique, the small RNA fraction from Ad-5 infected cells was subjected to single-

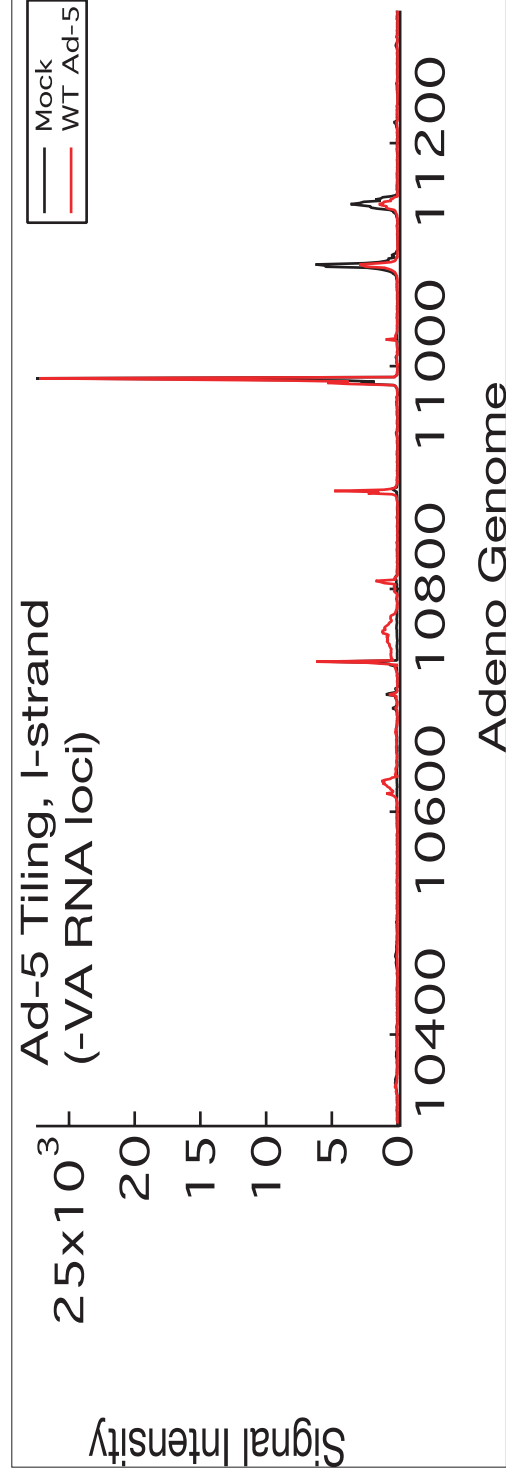
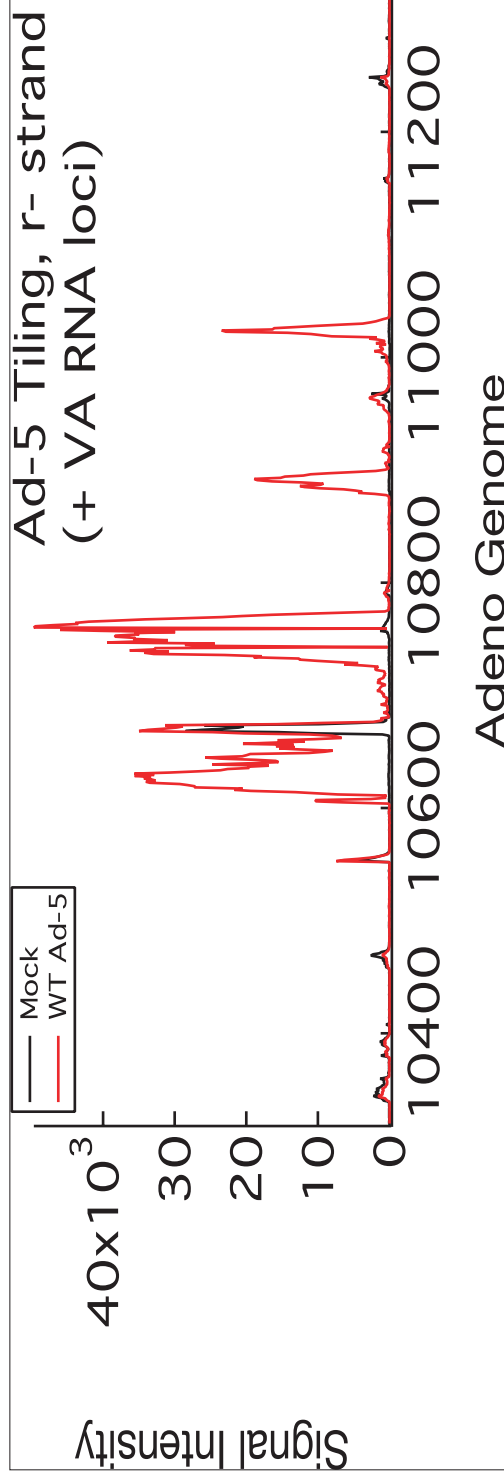
**A****B**

**Fig. 3.5: miR-VA1 accumulates during infection.**

HeLa cells were infected with WT Ad-5, total RNA was collected at the indicated times, and northern blots were probed with a miR-VA specific oligonucleotide RNA probe. Accumulation of full length VA RNA molecule (closed circles) was compared to that of miR-VA (open circles) (B). Each data point is internally normalized to the control tRNA level, and the data points were then normalized to the level of VA RNA at 3 h.

nucleotide, hybridization-based, tiling analysis with microarray detection (LC Sciences), using a series of 22 nucleotide  $T_M$ -normalized probes. The overlapping series of tiling probes differ by a single nucleotide, as each probe is “n+1” of the preceding probe. These  $T_M$ -normalized probes provide adequate sensitivity to differentiate between a probe bearing perfect complementarity to a miRNA and another probe bearing only a single mismatch with the miRNA of interest (LC Sciences, (178)). We performed this tiling analysis to examine all small RNAs potentially produced from nucleotides 10,320-11,319 on the right-reading strand of the Ad-5 genome, and the corresponding nucleotides on the left-reading strand (Fig. 3.6). The VA RNA I and II genes reside on the right-reading strand at nucleotides 10,620-10,779 and 10,876-11,038, respectively. As seen in the tiling analysis of the right-reading strand, the highest signal detection occurred between nucleotides 10,749-10,777, which corresponds to the region identified as being responsible for production of miR-VA by our northern blot analysis (Fig. 3.5). The exact sequence detected by this probe corresponds to nucleotides 10,756-10,777. The tiling analysis also revealed high levels of VA-specific small RNA detection in the 5' end of VA RNA I in the ascending arm of the terminal stem, as well as the ascending arm of the apical stem. This is also in accordance with the northern blot analysis using the overlapping oligonucleotide probes (Fig. 3.4), which detected small RNAs with probes A and B. The small RNAs from the ascending arm of the terminal stem detected in the tiling experiment and by probe A in northern blots (Fig. 3.4) is likely to result from dicer cleavage of a miR-VA/miR-VA\* duplex produced from the terminal stem. The tiling analysis additionally demonstrates that small RNAs are produced from the VA

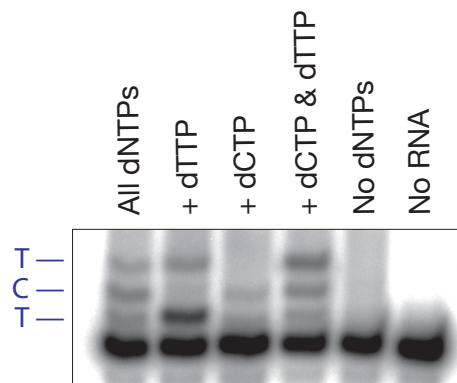




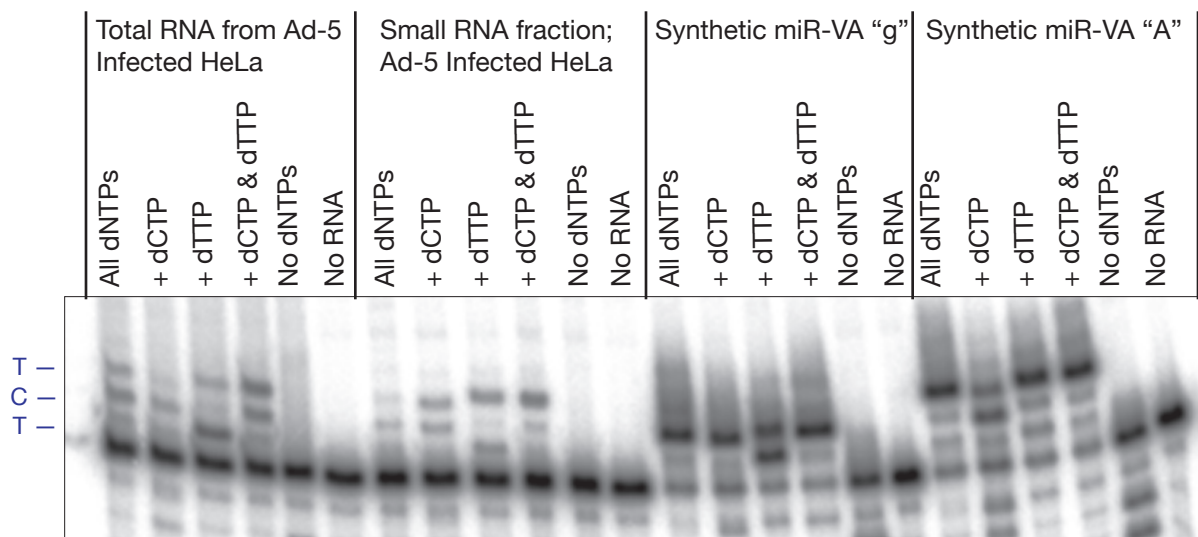
**Fig. 3.6: Identification of VA-specific small RNAs by oligonucleotide tiling analysis.** RNA from mock-infected or WT Ad-5-infected cells was subjected to single nucleotide tiling analysis using overlapping, T<sub>M</sub>-normalized, 22 nt oligonucleotide probes. RNA sequences corresponding to nucleotides 10,320- 11,319 of the Ad-5 genome (containing the VA RNA I and VA RNA II) loci was probed.

RNA II locus (Fig. 3.6). The peak signals again corresponded with the 5' and 3' termini of the molecule, raising the possibility that a miR-VA2/miR-VA2\* duplex is produced by the VA RNA II locus. All microarray tiling was also performed on mock- infected RNA samples to ensure that the peaks identified in the infected-cell RNA were specific to the virus. The tiling analysis of the left-reading strand revealed very few peaks specific to virus and those peaks were of much lower intensity than those seen in the right-reading strand. The very high level of signal detected throughout the VA RNA loci may result from the detection of full length and/or truncated or cleaved VA RNA molecules, since the small RNA fraction can contain double stranded nucleotides of ~125 nucleotides or less.

To distinguish the precise 5' end of miR-VA, we performed primer extension analysis on RNA from infected cells. We analyzed several primers to define the approximate end of miR-VA (data not shown), and then performed an extensive analysis with a primer that produced a 2-3 nucleotide extension product. The results indicated that miR-VA appears to have two major products in infected cells, with those miRNAs differing by a single nucleotide at the 5' end of the molecule (Fig. 3.7 A). The 5' end of these miRNAs map to nucleotides 10,756 and 10,757 of the Ad-5 genome. We referred to these miRNAs as miR-VA "A" and miR-VA "g" based on the identity of the proximal 5' nucleotide of each miRNA. Extension reactions using only the two nucleotides involved in the extension (dTTP, dCTP, or both) confirmed the identity of the extension product seen in the reaction when all dNTPs are present. Control reactions lacking either total RNA template or dNTPs confirmed the specificity of the extension (Fig. 3.7 A).

**A**

3' TCT GTT GCC CCC TCA CGA GG 5' \* Primer  
 miVA 5' AGA CAA CGG GGG AGU GCU CCU U 3'

**B**

**Fig. 3.7: Primer Extension analysis reveals two VA-specific small RNAs.**

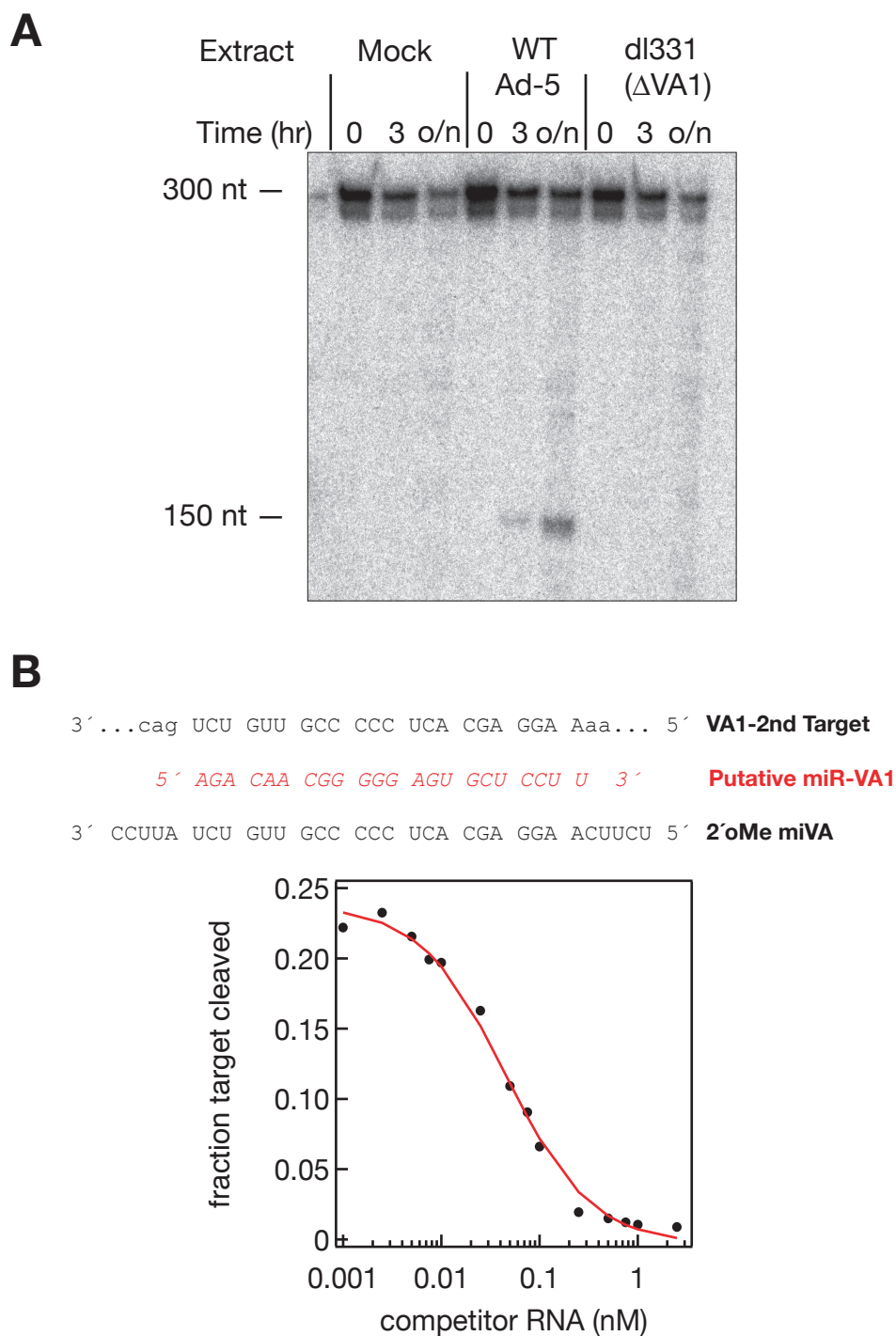
RNA from WT Ad-5-infected HeLa cells was analyzed by primer extension using the 5' radiolabeled DNA oligo shown above (A). Synthetic RNAs corresponding to miR-VA "g" and miR-VA "A" were used as size markers to show appropriate extension lengths (B).

Further confirmation of the precise extension products was seen in reactions using synthetic RNA templates comprising the exact miR-VA “g” and miR-VA “A” sequences (Fig. 3.7 B). These control reactions indicated that our primer extension protocol was revealing authentic extension products and that the presence of multiple products in the total RNA samples was not the result of a non-templated nucleotide addition by the enzyme. These primer extension reactions indicated that there are two major miRNAs (miR-VAs) produced from the 3' end of VA RNA I. This is in accordance with our dicing assays and northern blot analysis, which both displayed several products in the small RNA size range. Interestingly, two other groups identified miR-VAs by different techniques, with one of these groups identifying miR-VA “g” as the authentic miR-VA and the other group characterizing miR-VA "A" as the predominant miR-VA produced (6, 158).

*miR-VA is an authentic miRNA that enters RISC*

We further explored the interaction of VA RNA with the RNA silencing pathway by determining whether miR-VA proceeded from Dicer cleavage into the next stage of the RNA interference pathway, assembly into a functional RISC complex. In *D. melanogaster*, as a general rule, siRNAs segregate into slicing competent Ago2-RISC complexes, while miRNAs enter non-slicing Ago1-RISC complexes (140). There are four members in the human argonaute subfamily (Ago1-4), and it has been shown that siRNAs and miRNAs can associate with all four of these argonaute proteins; however,

only Ago2 (Slicer) has been shown to be capable of performing target cleavage (105, 126). Therefore, we established an *in vitro* cleavage assay to determine if miR-VA resided in an Ago2-containing RISC complex, which could slice a perfectly complementary target. We constructed a cap-labeled target RNA molecule containing a sequence that bore perfect complementarity to miR-VA. This target RNA was then incubated in an *in vitro* RNAi reaction containing extract from mock-infected or adenovirus-infected HeLa cells (Fig. 3.8 A). In this setting, specific target cleavage would only result from miR-VA loaded into Ago2-RISC complexes *in vivo*, prior to preparation of the extract. We found that in the WT Ad-5-infected extracts, specific cleavage of the target RNA occurs in the region corresponding to miR-VA. This cleavage product does not accumulate in mock-infected extracts, and more importantly, in the dl331 ( $\Delta$ VA1)-infected extracts. The dl331 adenovirus is a mutant virus containing a deletion of the VA RNA I gene (173). Therefore, the observed cleavage in the WT Ad-5 extracts is specific to VA RNA I. To further establish the specificity of the miR-VA region of the molecule as being responsible for the slicing of the target, we performed related cleavage experiments using a specific inhibitor of miR-VA. It has been shown that antisense oligonucleotides composed of nucleotides with 2'-*O*-methyl (2'oMe) base modifications are highly efficient, stable, and specific inhibitors of small RNA function (66). We repeated our target cleavage analysis in WT Ad-5-infected extracts using increasing concentrations of a specific 2'oMe inhibitor of miR-VA (Fig. 3.8 B). As the concentration of the inhibitor is increased, the level of target cleavage (measured as



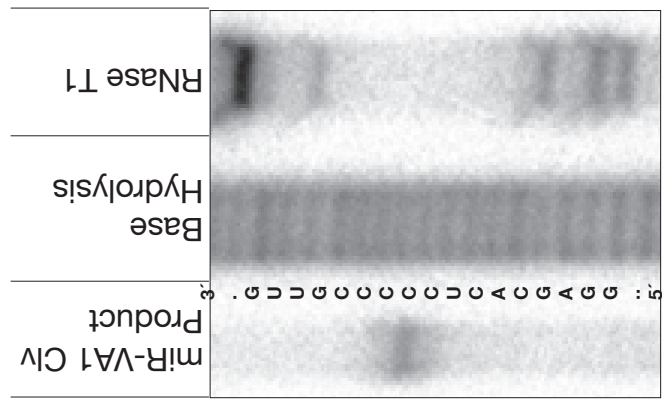
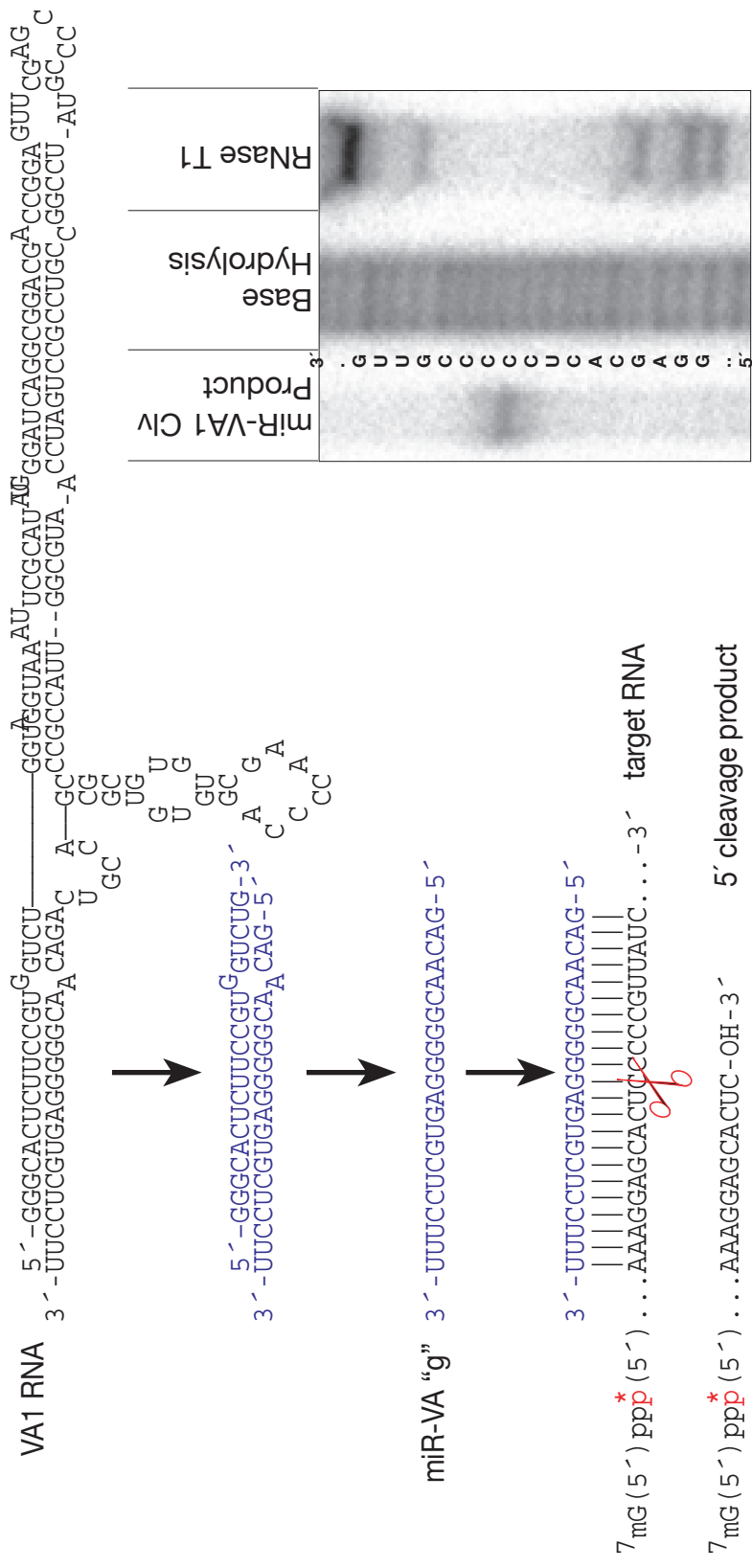
**Fig. 3.8: miR-VA enters a functional Ago-2 RISC**

Cytoplasmic extracts prepared from mock-infected or adenovirus-infected HeLa cells were incubated in an *in vitro* RNAi reaction with a 5' radiolabeled target RNA bearing perfect complementarity to miR-VA (A). A 2'oMe RNA molecule corresponding to miR-VA competes with the VA-specific target cleavage demonstrated in the *in vitro* reactions (B).

(product/substrate+product)) decreased, indicating that the cleavage was specific to miR-VA.

*miR-VA "g" resides in Ago2 RISC*

The *in vitro* cleavage assay indicated that only a single cleavage product accumulates, rather than two distinct products resulting from miR-VA "g" and miR-VA "A"-generated slicing. In order to distinguish which miR-VA was responsible for Ago2-mediated target cleavage, we mapped the cleavage product using a long polyacrylamide sequencing gel. Marker lanes included the target RNA subjected to RNase T1, which cleaves RNA at guanosine residues, as well as a lane containing target RNA subjected to base hydrolysis in alkaline conditions. Resolution of the precise scissile phosphate at which target cleavage occurs allows for the determination of the small RNA responsible for the cleavage, since it has been demonstrated that target cleavage occurs at the phosphate opposite of nucleotides 10 and 11 of the miRNA guide strand (65, 105, 126, 169)). Therefore, by identifying the precise nucleotide at which cleavage of our target RNA occurs, we were able to extrapolate the 5' end of the cleaving miR-VA, which maps to miR-VA "g" (Fig. 3.9). To confirm this mapping, we prepared extracts from HeLa cells transfected with siRNAs corresponding to the exact guide strand sequences of miR-VA "g" and miR-VA "A". These extracts were used to perform similar *in vitro* cleavage assays, and they generated specific cleavage products of the expected sizes (data not shown). When the products of these cleavage reactions were electrophoresed on a long



**Fig. 3.9: miR-VA "g" is loaded into Ago-2 RISC.**

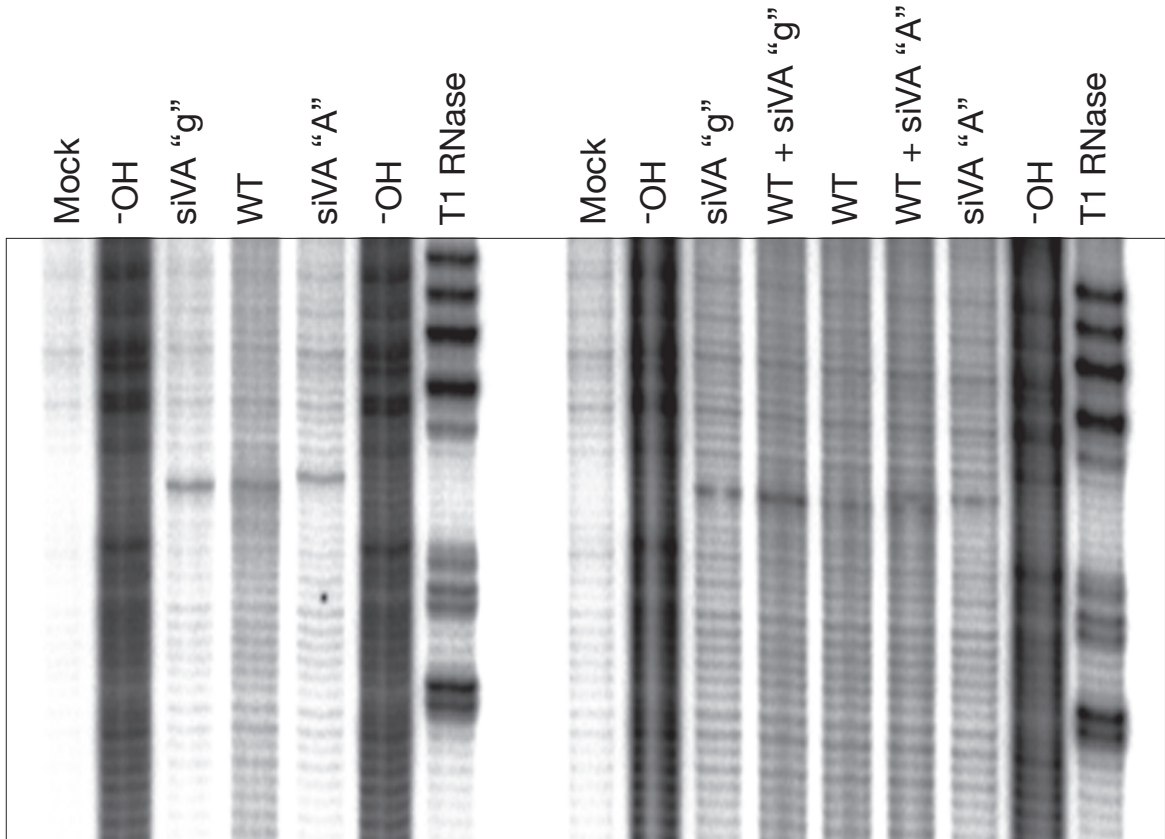
The miR-VA-specific cleavage product was electrophoresed on a polyacrylamide sequencing gel to provide single nucleotide resolution. Target RNA treated with RNase T1 and alkaline hydrolysis were used for precise mapping of the cleavage product. The resulting cleavage product can be used to infer the miR-VA sequence responsible for the cleavage. (Portions of this figure provided by P. Zamore).



polyacrylamide sequencing gel alongside the products of cleavage assays performed in the WT Ad-5-infected extracts, we saw that the infected cell cleavage product migrated to the same position on the gel as the product from the siVA “g” transfected extracts (Fig. 3.10). This product was discernibly one nucleotide shorter than the product generated in the siVA “A” transfected extracts. When portions of these reactions were mixed, the siVA “g”/WT mixture produced only a single cleavage product, while the siVA “A”/WT mixture produced two distinct products (Fig. 3.10). These results indicate that miR-VA "g" produced in infected cells forms an Ago2-associated RISC complex capable of cleaving a target RNA, while miR-VA "A" does not appear to enter a slicing-competent complex. Furthermore, several other groups have reported that miR-VAs can regulate RNAs containing miR-VA target sites in cell culture using luciferase-based reporters (4, 6, 158).

*Structured small RNAs related to VA RNA I are processed to small RNAs*

Since we determined that VA RNA I was a substrate for Dicer and that it enters into the RNA silencing pathway, we chose to look at several other structured RNAs, including the VA RNA II molecule of adenovirus, the Epstein-Barr Virus (EBV) EBERs, and Alu RNA, a human genetic short, interspersed element (SINE). The EBV EBERs are small RNAs transcribed by RNA polymerase III. They have been shown to perform similar PKR-inactivating functions to VA RNA, and they can functionally substitute for adenovirus lacking VA RNA (18). Alu SINES make up roughly 10% of the mass of the

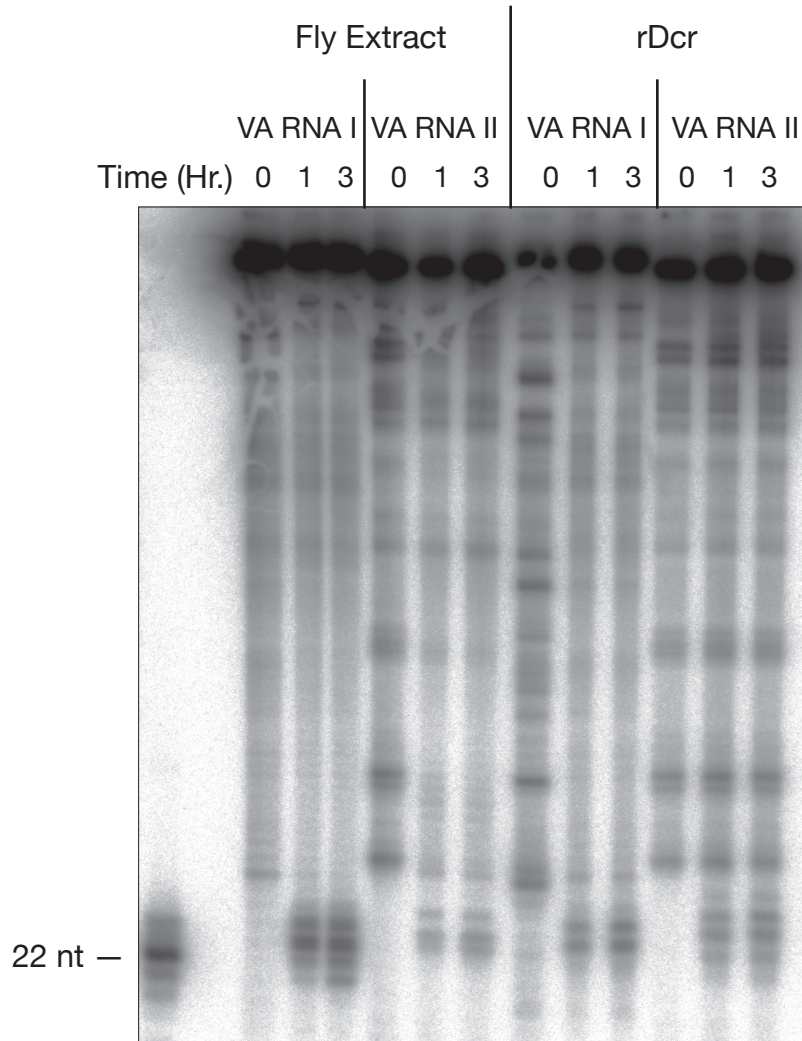


**Fig. 3.10: miR-VA "g" resides in cleaving RISC.**

Cytoplasmic extracts were prepared from mock-infected, WT Ad-5-infected, or siRNA (siVA "g" or siVA "A")-transfected HeLa cells. Standard *in vitro* RNAi reactions containing a 5' radiolabeled miR-VA-specific target was incubated in the extracts. RNA was electrophoresed on a polyacrylamide sequencing gel.

human genome and when transcribed, they produce structured RNAs of ~300 nucleotide and Alu sequences (12). They are derivatives of 7SL RNA genes and are believed to have originated in primates 65 million years ago (134, 177). It is believed that retrotransposition of these elements can occur following RNA polymerase III transcription (12). Interestingly, like VA RNA, Alu RNAs have been shown to stimulate translation of co-expressed plasmids in cell culture transfections (157). We constructed radiolabeled transcripts for VA RNA II, EBERs RNA, and Alu RNA to test in our *in vitro* dicing assays.

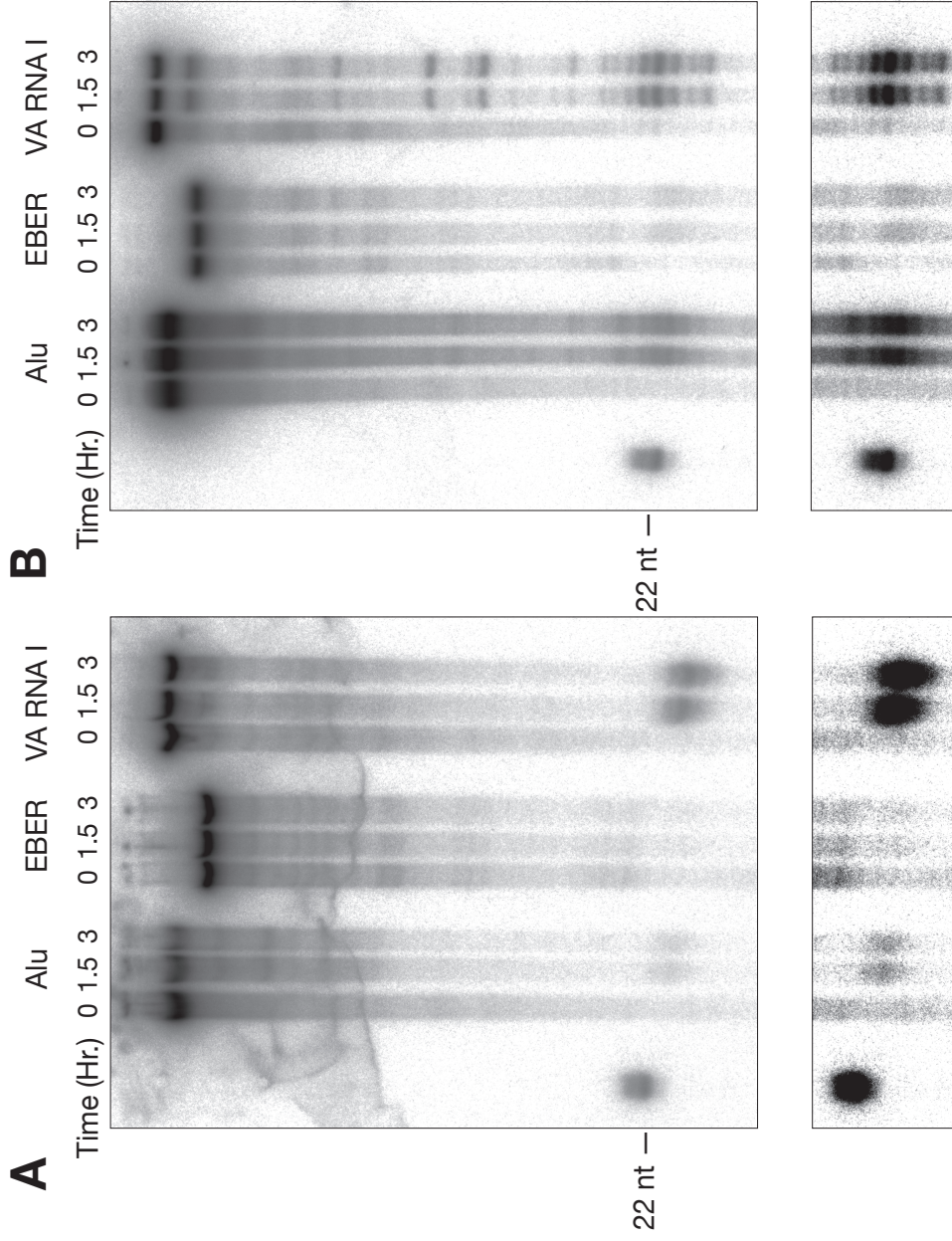
Our previous microarray tiling experiment and northern blots provided indications that VA RNA II produced small RNAs during infection. When we incubated VA RNA II in our dicing assays, we observed the production of small RNAs characteristic of RNA silencing phenomena (Fig. 3.11). The production of these small RNAs appears to be less efficient than those produced from the VA RNA I substrate. Similarly, northern blot analysis revealed very weak expression of VA RNA II-specific small RNAs (data not shown). The production of these small RNAs was increased slightly in dl331 ( $\Delta$ VA1) infected cells, which produce higher levels of VA RNA II to compensate for the absence of VA RNA I (42). However, our repeated attempts to identify miR-VA2 specific cleavage in our *in vitro* cleavage assay were unsuccessful (data not shown). Nevertheless, our dicing assays, northern blot analysis, and microarray tiling data suggest that VA RNA II may produce functional miRNAs. Indeed, recent data suggests that small RNAs derived from VA RNA II can cleave *in vitro* targets and regulate luciferase-based VA RNA II targets in cell culture (4).



**Fig 3.11: VA RNA I and VA RNA II are processed to small RNAs indicative of RNA silencing.**

Radiolabeled VA RNA I and VA RNA II was incubated in *in vitro* reactions with either RNAi-competent *D. melanogaster* embryo extract or recombinant human dicer enzyme as in Fig. 3.1.

We also performed our *in vitro* dicing assay using the EBV EBERs and Alu RNA as substrates. We again saw that small RNAs of ~21-22 nucleotides could be produced from these substrates (Fig. 3.12). The efficiency of dicing for both molecules was again much lower than that seen with VA RNA I. Follow up experiments using northern blot analysis were unable to detect any EBER-specific RNA in EBV infected cell lines. Similarly, attempts to detect Alu-specific small RNAs by northern blot analysis in several cell lines were unsuccessful (data not shown). Studies indicate that Alu RNAs are expressed when cells are stressed, so it would be prudent to repeat our analysis under such conditions (157). While it is tempting to speculate that these substrates can produce functional small RNAs, it is also likely that a wide variety of structured RNAs may be cleaved by Dicer or Dicer-like enzymes in *Drosophila* extract or that the recombinant Dicer enzyme may cleave structured RNAs in an indiscriminant fashion. Further experiments are needed to address whether these and other viral and cellular structured RNAs enter into RNA silencing pathways.



**Fig 3.12: Small RNAs are produced from other structured, viral RNA precursors and genetic elements.** Radiolabeled RNA corresponding to the Epstein-Barr virus EBERs, denovirus VA RNA I, or the Alu genetic element were incubated for the indicated times in RNAi-competent *Drosophila* embryo extract (A) or with recombinant human Dicer enzyme (Gene Therapy Systems, Inc.) (B). A darker exposure of the bottom portion of the gel is included below the respective gels.

## Discussion

### *Adenovirus VA RNA enters the RNA silencing pathway*

Our studies show that VA RNA I enters into the mammalian RNA silencing pathway, as it can be processed to small RNAs by Dicer, and enters into a functional RISC *in vivo*. Additionally, it had been shown that VA RNA and pre-miRNAs utilize the same nuclear export factor, Exportin 5, to transit to the cytoplasm (59, 111, 112, 206). These studies, combined with our data, indicate that VA RNA interacts with at least three protein complexes involved in RNA silencing during infection.

The initial dicing assays we performed demonstrated that VA RNA I is a structured RNA substrate that is efficiently processed to small RNA species by Dicer (Fig. 3.1). The chromatography of the *in vitro* dicing reaction further revealed that VA RNA appears to be processed by a Dicer-associated complex based upon the fractions with which the VA-specific small RNAs co-fractionated (Fig. 3.2, (137)). Additionally, this experiment provided an indication that these small RNAs may proceed further into the RNAi pathway, since an additional peak of small RNAs occurred in fractions previously identified to contain RISC activity (137).

Importantly, we demonstrated that these small RNAs were not artifacts of *in vitro* processing assays, as we utilized several nucleotide-based detection methods to identify the presence of the small RNAs in infected cells. Using overlapping probes for northern blot analysis, we identified the 3' end of the molecule as the progenitor of the 21-22

nucleotide small RNAs, miR-VAs, seen in infected cells (Fig. 3.4). When analyzing the accumulation of the miR-VA in infected cells, we saw that it was detectable very early after infection (3 hours), but that the accumulation of miR-VA quickly leveled off, while the production of full length VA RNA continued to steadily increase (Fig. 3.5). This may reflect saturation of components required for miRNA biogenesis in the cell. Indeed, although less than 10% of VA RNA appears to be converted to miR-VA, this may still represent a very large pool of miR-VA, since such massive quantities of VA RNA are produced in infected cells. Alternatively, full length VA RNA production may occur at the expense of miR-VA at later times in infection, when VA RNA-based inactivation of PKR becomes necessary, as dsRNA intermediates are being produced during virus replication (116).

We additionally performed microarray-based single nucleotide tiling analysis of the portion of the Ad-5 genome encoding the VA RNA I and II genes (Fig. 3.6). This analysis revealed distinct peaks of small RNA detection that coincided with the regions we had previously mapped by northern analysis (Fig. 3.4 & 3.5). Peaks corresponding to the terminal stem of both VA RNA I & II were identified, with the highest signal occurring at the region we had previously identified as miR-VA. This data indicated that the terminal stems of VA RNA molecules may act as a pre-miRNA which gives rise to miR-VA/miR-VA\* duplexes.



*VA RNA I encodes a miRNA*

The demonstration that infected cell extracts could perform miR-VA specific target cleavage provided evidence that miR-VA acts as an authentic miRNA, which enters into an Ago2-RISC complex (Fig. 3.8). Concurrent studies by another group speculated that VA RNA acted as a competitive inhibitor of Dicer, with its goal being to prevent Dicer-mediated cleavage of viral RNA or cellular pre-miRNAs (111). However, our work demonstrated that the interaction of Dicer with VA RNA produced a functional miRNA molecule that was capable of regulating target RNAs. Additional target cleavage studies indicate that the putative miR-VA\* was also capable of cleaving a complementary target *in vitro* (data not shown). Related studies by other groups provided evidence that small RNAs produced from VA RNA could regulate target RNAs both *in vitro* and in cell culture (4, 6, 158).

We further extended our analysis of miR-VA by deciphering the precise 5' terminus of the miRNA. Elucidation of the mature miRNA sequence could aid in the identification of cellular or viral targets for the miRNA. Our primer extension analysis revealed the presence of two distinct miR-VA species, miR-VA "g" and miR-VA "A", which differed by a single nucleotide at the 5' of the miRNA, with miR-VA "A" being n+1 of the miR-VA "g" sequence. These miRNAs were described independently in two other studies, with one group claiming miR-VA "g" as the miRNA product, and the other group describing miR-VA "A" as the major product (6, 158)). It is possible that miR-VA "g" inhabits an Ago2-RISC, while miR-VA "A" is loaded into a different argonaute

family member. To this point, very little is known about the function of the other human argonaute proteins. The identification of two miRNA species is consistent with the presence of multiple VA RNA dicing products and multiple miR-VA specific products being detected by northern blot analysis (Figs. 3.1 and 3.4). Additional heterogeneity of miR-VA sizes may be introduced at the 3' end of the molecule, since the miR-VA sequence extends into the poly-uridine (poly-U) tract of nucleotides at the 3' terminus of VA RNA. This poly-U stretch is the result of the RNA polymerase III termination signal (a run of adenosine residues in the template), and VA RNAs with variable length poly-U sequences at their 3' termini have been described (25).

Intriguingly, phylogenetic analysis of a wide cohort of VA RNA genes from adenovirus subfamilies indicates that the region of the molecule containing the miR-VAs, the terminal stem, is the most conserved at the sequence level (114). The main function ascribed to VA RNA is its inhibition of PKR activation, and further studies demonstrated that it was the apical stem and central domain of VA RNA that were responsible for the binding and inactivation of PKR, respectively (27, 44, 46, 128-130, 173). It is somewhat surprising that these regions are not the most conserved portion of the VA RNA molecule, since they are responsible for such a crucial activity during infection. Additionally, mutational analysis of the miR-VA region of VA RNA demonstrated that viruses deleted for the region encoding miR-VA or containing mutated sequences in the miR-VA region grew very poorly in comparison to wild type virus and other viruses containing mutations in the apical stem and central domain (16, 44). It therefore seems likely that binding and inactivation of PKR is dependent upon the secondary structure of

VA RNA, and is not solely reliant on the primary sequence of the molecule. It is tempting to speculate that the terminal stem is the most highly conserved region of the molecule because it contains the miR-VA sequence. Also, the fact that virus mutants bearing interrupted or mutated miR-VA sequences have dramatic effects on virus growth gives credence to the idea that the miR-VA may be of crucial importance for the ability of the virus to replicate (16, 44). However, it must also be considered that the terminal stem of the molecule contains elements responsible for transcription initiation and termination. The ascending arm of the terminal stem contains the Box A internal promoter for RNA polymerase III, and the descending arm of the terminal stem contains the transcription termination signal (25, 42, 55). There is likely a high degree of selective pressure to maintain these sequences to allow for appropriate transcription of the VA RNA molecule. Regardless, the fact remains that the miR-VA region of the VA RNA I molecule is highly conserved and may therefore serve a related function in multiple strains of adenovirus.

Our results demonstrate that adenovirus produces miRNAs, miR-VAs, which enter into host RNA silencing pathways during the course of infection. This provocative finding suggests that these miRNAs may play important roles in viral pathogenesis. Due to their compact genome sizes, viruses typically only encode factors required for efficient infection and replication. Numerous examples of viral factors performing multiple, disparate functions exist. VA RNA may well represent another of these multivalent factors, as it now appears to play a role in the RNA silencing pathway in addition to its previously established role in blocking the PKR-induced interferon response.

Finally, we additionally demonstrated that other structured viral or cellular RNAs may be substrates for RNA silencing pathways (Fig. 3.11 & 3.12). Our results indicating that VA RNA II is diced *in vitro* correlates with our observation that small VA RNA II-specific RNAs are detectable in infected cells by northern blot and microarray tiling analysis. While our attempts to identify a functional miR-VA2 capable of producing RISC-mediated cleavage were unsuccessful, another study did demonstrate that VA RNA II-specific small RNAs could cleave a target *in vitro* and regulate a target reporter gene in cell culture (4). Further experiments to improve the sensitivity of our cleavage assay should be performed, as well as studies to examine the potential function of VA RNA II-specific small RNAs in infected cells. While our dicing assay results from the other structured viral and cellular RNAs (EBERs, Alu) are only preliminary, *in vitro* studies, they do raise the possibility that production of small RNAs by mammalian viruses is a common theme in virus infections. To this end, numerous miRNAs produced from other animal viruses have recently been described (22, 29, 54, 57, 146, 147, 171).

## **CHAPTER IV:**

# **ADENOVIRUS INFECTION DISRUPTS MULTIPLE COMPONENTS OF HOST RNA SILENCING PATHWAYS**

## **Introduction**

The co-evolution of viruses with their hosts has led to a molecular arms race as viruses seek to elude the host pathogen defense pathways and successfully infect and propagate in the host. There is a seemingly endless array of viral mechanisms deployed for counteracting host defense measures. There are also numerous examples of viruses co-opting the use of a cellular pathway to aid in their viral replication. Prior to the discovery of RNA silencing pathways in the 1990s, the mammalian host defense against viruses was believed to be comprised solely of a rapidly responding non-specific innate immune defense system, followed by a specific protein-based adapted immune response. However, recent discoveries of RNA-based forms of immunity in plants and insects raised the possibility that similar mechanisms may exist in the mammalian host response to viruses.

Our work, along with others, has demonstrated that during adenovirus infection, the viral RNAs, VA RNA I & II, enter the host RNA silencing pathway and are processed into authentic miRNAs (4, 6, 158). This definition of “authenticity” is based on the ability of the miRNA to regulate experimentally introduced target RNAs in a sequence specific fashion. However, this does little to define the importance of the miR-VAs to the process of viral infection or the host response to infection. We undertook experiments to refine our understanding of miR-VA’s role in virus infection, as well as the importance of host RNA silencing mechanisms in the context of a viral infection.

## Results

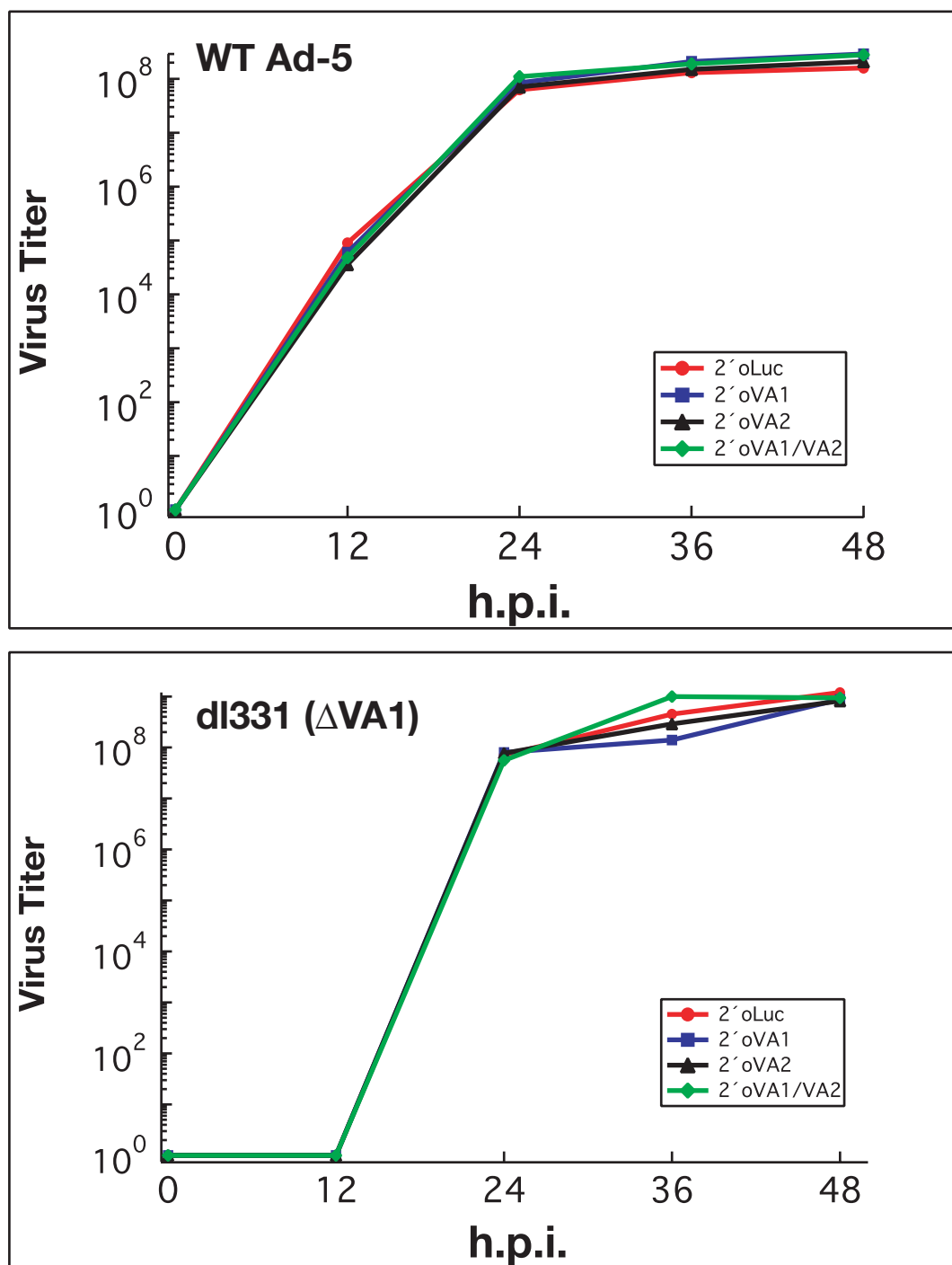
### *Inhibition of miR-VA does not affect viral growth*

The demonstration that miRNAs are produced by adenovirus raises the obvious question as to what their function is in the context of infection. There have been a plethora of recent studies, both computational and experimental, demonstrating the presence of miRNAs in animal viruses (22, 29, 54, 57, 146, 147, 171). For those miRNAs that have been experimentally tested, a variety of different functions have been found. A miRNA produced from the Epstein-Barr virus is perfectly complementary to the 3'UTR of the DNA polymerase gene transcribed on the opposite strand. The miRNA is able to perform site-specific cleavage of the polymerase transcript, which may contribute to the establishment of a latency stage for the virus (147). Similarly, a miRNA produced by the simian virus, SV40, slices early gene RNAs which bear perfect complementarity to the miRNA, thereby reducing expressing of viral T antigens, making the infected cells less susceptible to immune control (171). Since there are no viral transcripts containing perfect target sites for miR-VA, we performed experiments to try to ascertain the importance of miR-VA to the process of virus replication.

In order to test whether miR-VA affected replication of adenovirus, we utilized a sequence-specific 2' oMe oligonucleotide inhibitor of miR-VA to block its function during infection. The use of 2' oMe oligonucleotides as potent inhibitors of siRNA and miRNA-loaded RISC has been studied in variety of settings, including *in vitro* reactions,

*D. melanogaster* embryos, cultured human cells, and *C. elegans* larvae (66, 104, 125). We previously verified that a miR-VA specific 2' oMe inhibitor was able to block target cleavage in *in vitro* reactions (Fig. 3.8). For this experiment, we additionally tested a 2' oMe inhibitor specific for the region of VA RNA II that microarray tiling and northern blot analysis indicated also give rise to a viral miRNA. Accordingly, we also performed infections using the dl331 ( $\Delta$ VA I) virus, under the assumption that if there is a functional miR-VA2, it would be easier to test for its function in the absence of VA RNA I. We further reasoned that since miR-VA was produced so quickly after infection (Fig. 3.5), we should provide the 2' oMe inhibitors a priori in order to effectively block its function. Therefore, we transfected HeLa cells with the 2' oMe inhibitors 12 hours prior to infection with WT of dl331 virus. The supernatant and cell pellet were removed every 12 hours post infection for a total 48 hours, and titering assays were performed to determine virus levels. In our experiments, neither the 2' oMe specific for VA I or VA 2, or a combination of both was able to affect the growth of the virus in relation to a control inhibitor (2' oLuc) (Fig. 4.1). In experiments using both the WT and dl331 virus, the virus growth curves looked remarkably similar for all of the 2' oMe inhibitors used, indicating that their presence did not influence virus replication. While our experiment produced negative results, we did have a measure for efficacy of the 2' oMe treatment. Extra wells in the experiment were transfected with a 2' oMe inhibitor specific for the cellular miRNA, let-7. Bioinformatic predictions and *in vitro* experiments have shown that let-7 regulates the human Dicer mRNA (71, 163). We performed western blot analysis on these samples and saw that Dicer protein levels were elevated in the 2'-o-let-





**Fig 4.1: Inhibition of miR-VA does not affect viral replication.**

HeLa cells were transfected with 20 nM of the specified 2' oMe oligonucleotide inhibitors 12 hrs prior to infection with WT Ad-5 or dl331. Lysates were collected and viral titer was determined at the indicated timepoints.

7-treated cells relative to 2'-o-Luc-treated cells, indicating that our transfection protocol worked (data not shown). From these studies, it does not appear that miR-VA impacts virus propagation in this cell culture setting.

*miR-VA "A" is predicted to target Dicer mRNA*

To date, two examples of miRNA produced from animal viruses have been found to regulate viral transcripts bearing perfect complementarity (147, 171). In the case of miR-VA, this is unlikely, since there is no transcript produced antisense to the miR-VA locus in the adenovirus genome and additionally, there are no other cis sequences within the virus bearing perfect complementarity to the miR-VA seed sequence. However, it is still formally possible that miR-VA regulates expression of cellular genes. Bioinformatic algorithms have been developed to aid in the prediction of mRNA targets for miRNAs of interest (37, 101). We provided the sequences of miR-VA "g" and miR-VA "A" to the developer of the TargetScan prediction tool and were provided with a series of putative targets for the miRNAs. However, a serious limitation of this approach was imposed by the species-restricted tropism of adenovirus. A key component of the target prediction algorithm is conservation of the miRNA sequence and its putative target sequence across several species (101). Conservation of these sequences implies selective pressure to maintain those sites, underscoring their importance. Because human adenoviruses only infect humans, the algorithm cannot use the same cohort of species normally used in its analysis for targets of a conserved miRNA. Additionally, the algorithm did not provide a

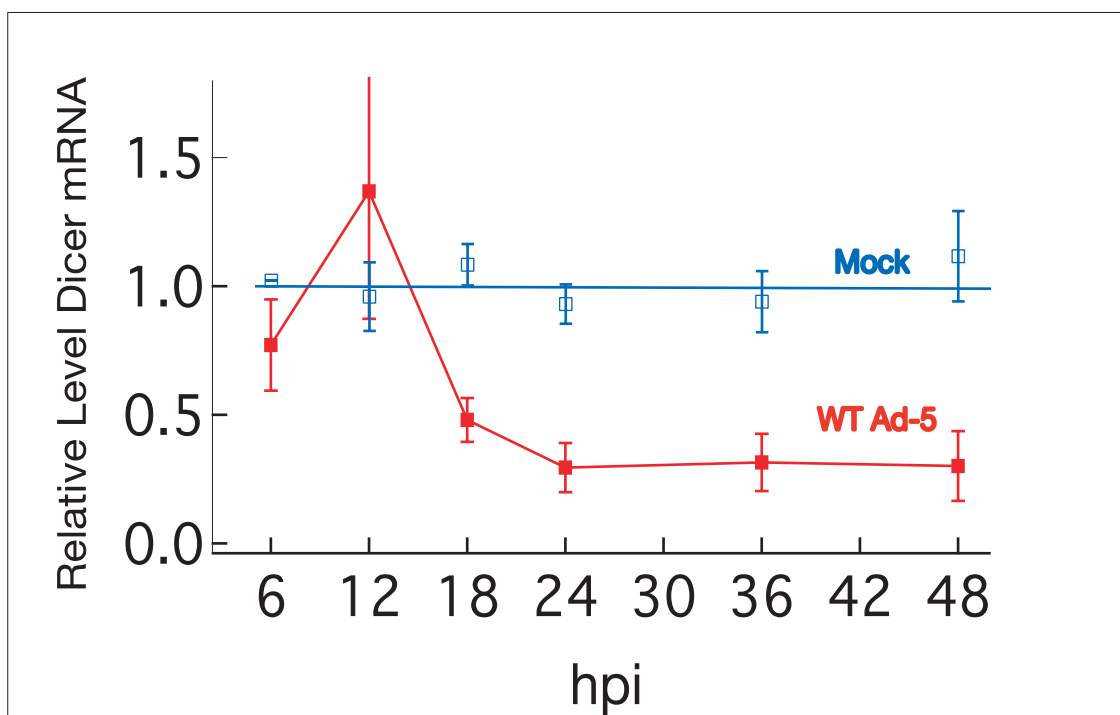
sufficient list of high scoring targets for miR-VA "g". At the time this analysis was performed, there had not been a single human miRNA identified that encoded a guanosine at the 5' end. Therefore, a bias against miRNAs with a 5' guanosine and respective target sequences was built into the algorithm. Since that time, there have been 49 human miRNAs found to encode a 5' guanosine from a listing of 474 total human miRNA (~ 10%). While the algorithm provided few targets for miR-VA "g", it generated a large list of targets for miR-VA "A", including several high scoring putative targets bearing multiple predicted sites of regulation. Of the list, one of the top scoring potential targets was the mRNA for human dicer. The TargetScan algorithm predicted 4 potential miR-VA "A" sites of regulation, with two sites residing in the coding region of the gene and the other two in the 3' UTR (Fig. 4.2). All four of these sites maintain a high level of pairing between the seed sequence of miR-VA and the target mRNA.

#### *Dicer mRNA and protein levels decrease during adenovirus infection*

To examine the potential effect of miR-VA on cellular Dicer levels, we first investigated the levels of Dicer mRNA during adenovirus infection. This was performed using quantitative real-time PCR on reverse-transcribed cDNA derived from total RNA in infected cells. We found that infection of HeLa cells reduced by ~3-fold the steady state abundance of Dicer mRNA (Fig. 4.3). Interestingly, and reproducibly, we saw an initial increase in Dicer mRNA levels after infection, peaking around 12 hours after infection, prior to the levels of mRNA dropping dramatically.



**Fig. 4.2: Computational algorithms predict miR-VA to target human dicer mRNA.** The sequence for miR-VA “A” is predicted by TargetScan computer algorithm to target dicer mRNA. The four putative target sites are shown above. Two sites (A & B) lie in the coding region and two sites (C & D) lie in the 3’UTR of dicer mRNA. The miR-VA sequence is displayed above the Dicer mRNA sequence. (figure courtesy of Ben Lewis, Whitehead Institute, Cambridge, MA)



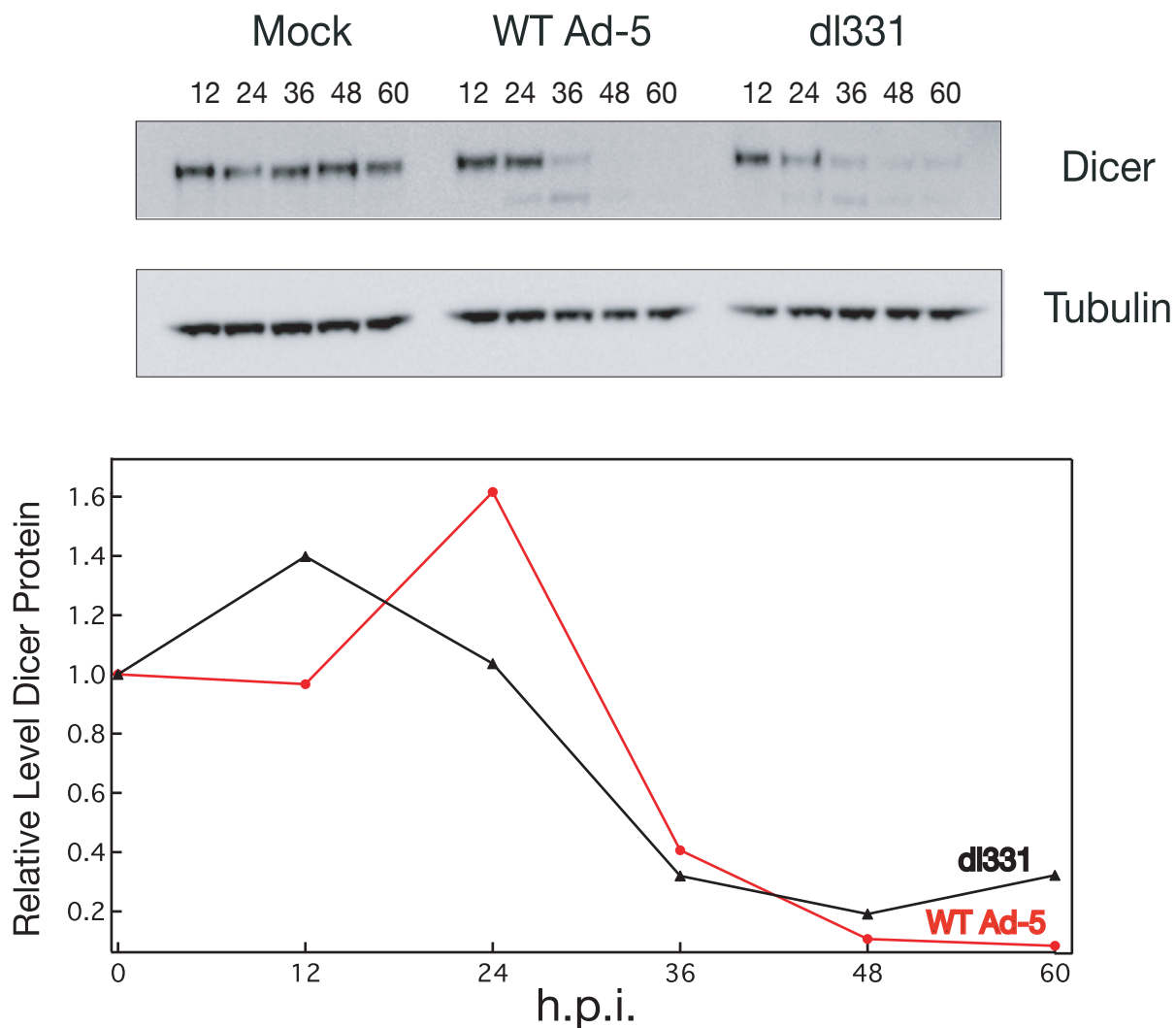
**Fig. 4.3: Dicer mRNA levels are reduced during adenovirus infection.**

Total RNA was collected from mock-infected or WT Ad-5-infected HeLa cells at the indicated times, was reverse transcribed, and was used for quantitative real-time PCR using primers to amplify Dicer or a housekeeping gene (GAPDH). All data points for relative dicer mRNA levels are internally normalized to GAPDH mRNA levels.

Additionally, we found that Dicer protein levels also decreased over the course of the infection (Fig. 4.4). Again, we see Dicer protein levels initially rise, before rapidly decreasing. However, in this experiment we also observed that the level of Dicer protein is decreasing in cells infected with the dl331 virus (Fig. 4.4). Because this mutant does not express VA RNA I, it appears that the downregulation is not directly linked to the presence of VA RNA, and therefore, miR-VA "A".

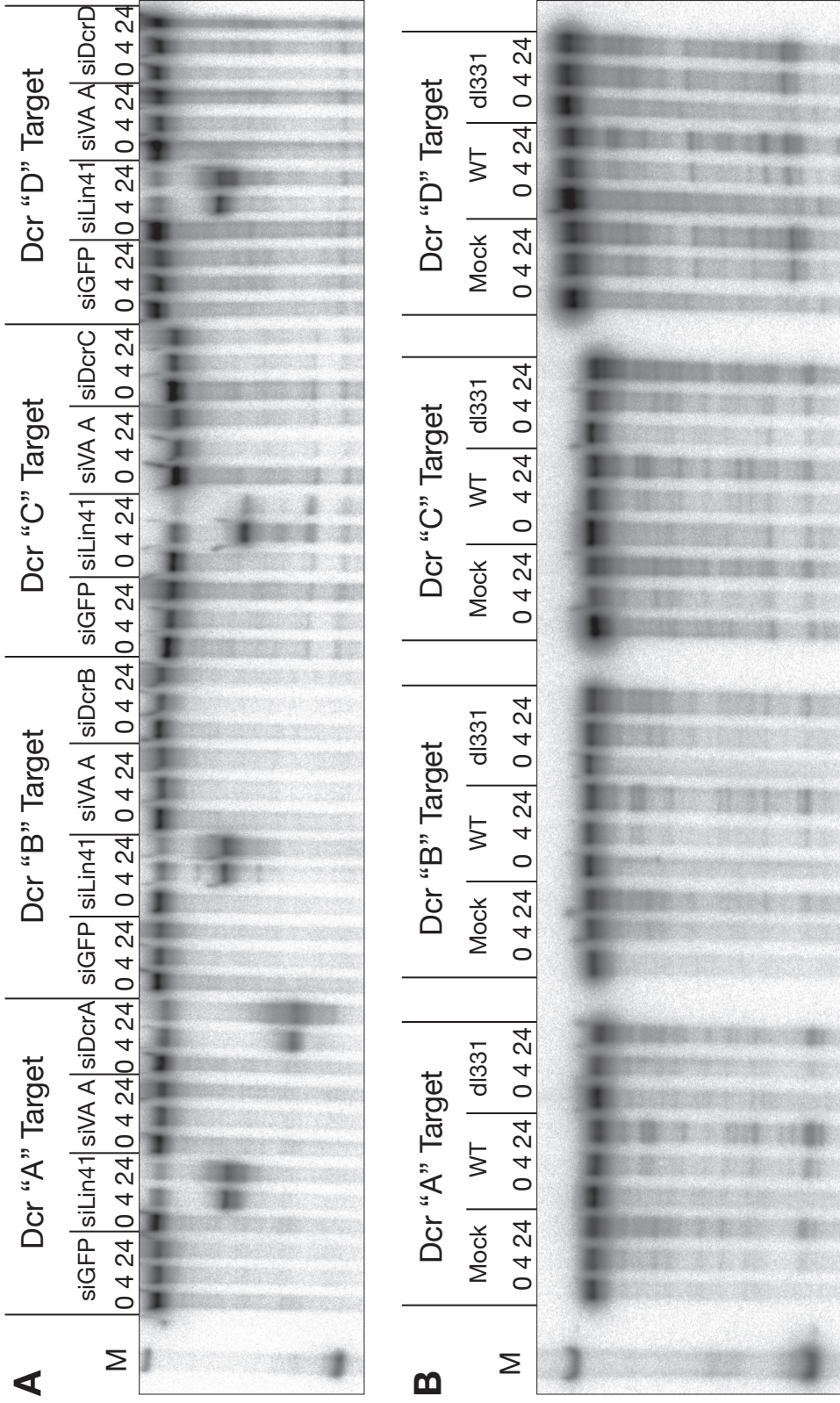
*miR-VA "A" does not directly regulate Dicer*

We performed several additional assays which verified that miR-VA "A" does not directly impact Dicer levels. First, we set up an *in vitro* cleavage assay to determine if the predicted miR-VA "A" target sites for Dicer mRNA could be cleaved by an siRNA corresponding to the guide strand of miR-VA "A" in *D. melanogaster* extract. For this assay, we developed four new target RNAs similar to the perfectly complementary target created for miR-VA "A" (Fig. 3.8). Each of these targets contained the perfect complement to one of the predicted miR-VA "A" target sites in Dicer mRNA (sites A-D, Fig. 4.2). These targets were incubated in fly extracts pre-loaded with siRNA corresponding to miR-VA "A", a control siRNA (siLuc), a positive control siRNA (siLin41), which cleaves a perfect-pairing site engineered into all targets, and an siRNA corresponding to the predicted Dicer target site. (siDcrA-D) (Fig. 4.5 A). As expected, the siLin41 siRNA cleaved each target, while the siLuc did not cleave the target. Importantly, none of the targets was cleaved by miR-VA "A" over the timecourse.



**Fig. 4.4: Dicer protein levels are reduced in adenovirus-infected cells.**

Whole cell extracts were prepared from infected HeLa cells at the indicated timepoints. Western blot analysis was performed using a monoclonal antibody for human Dicer (Clontech) and  $\alpha$ -tubulin (Santa Cruz). Data from the Western was quantitated (internally normalized to tubulin) and plotted.



**Fig. 4.5: Predicted Dicer mRNA target sites are not cleaved by siVA "A".**

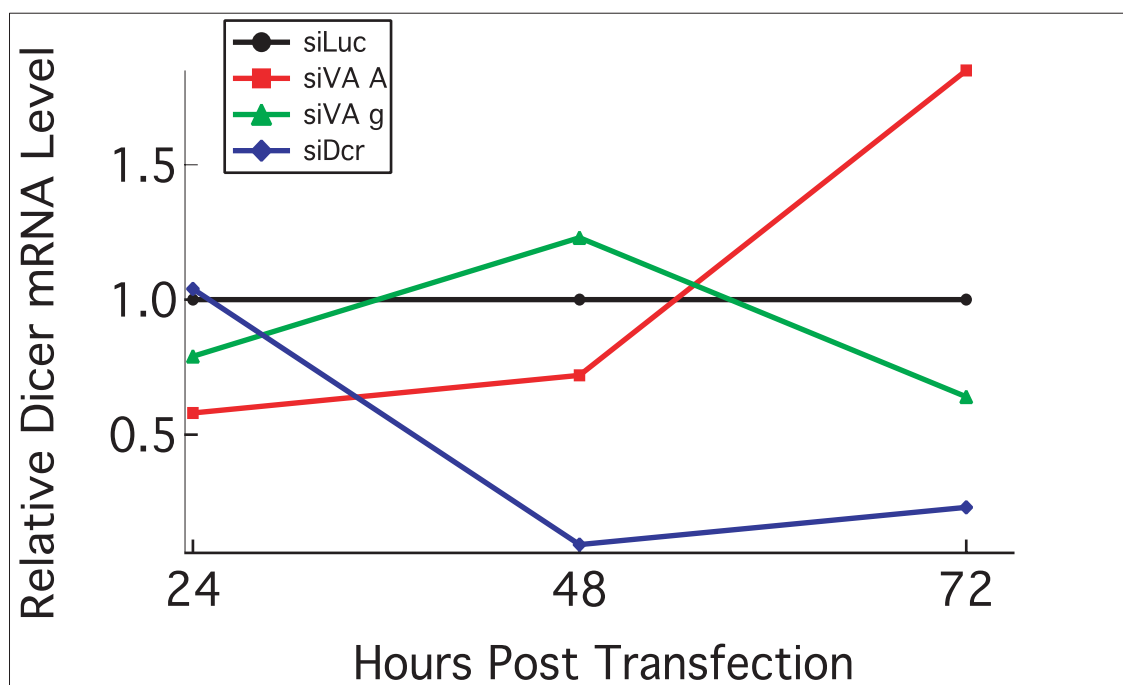
Four separate RNA targets containing complementary sequences to the predicted dicer target sites (Fig. 4.2) were constructed and used in *in vitro* RNAi reactions. The radiolabeled targets were incubated in *D. melanogaster* embryo lysate with the indicated siRNAs (A). The siDcrA-D siRNAs correspond to synthetic siRNAs bearing perfect complementary to the putative miR-VA target sites in each target. The four targets were also incubated in RNAi reactions in mock-infected or adenovirus-infected HeLa cell extracts (B). M- 100 bp ladder size markers.



However, only one of the perfectly matched target site siRNAs (siDcrA) was able to cleave its target. Further studies are needed to verify that these siRNAs and targets contain the appropriate sequences. These targets were also incubated in infected HeLa cell extracts, and no specific cleavage products for any of the targets were detected (Fig. 4.5 B).

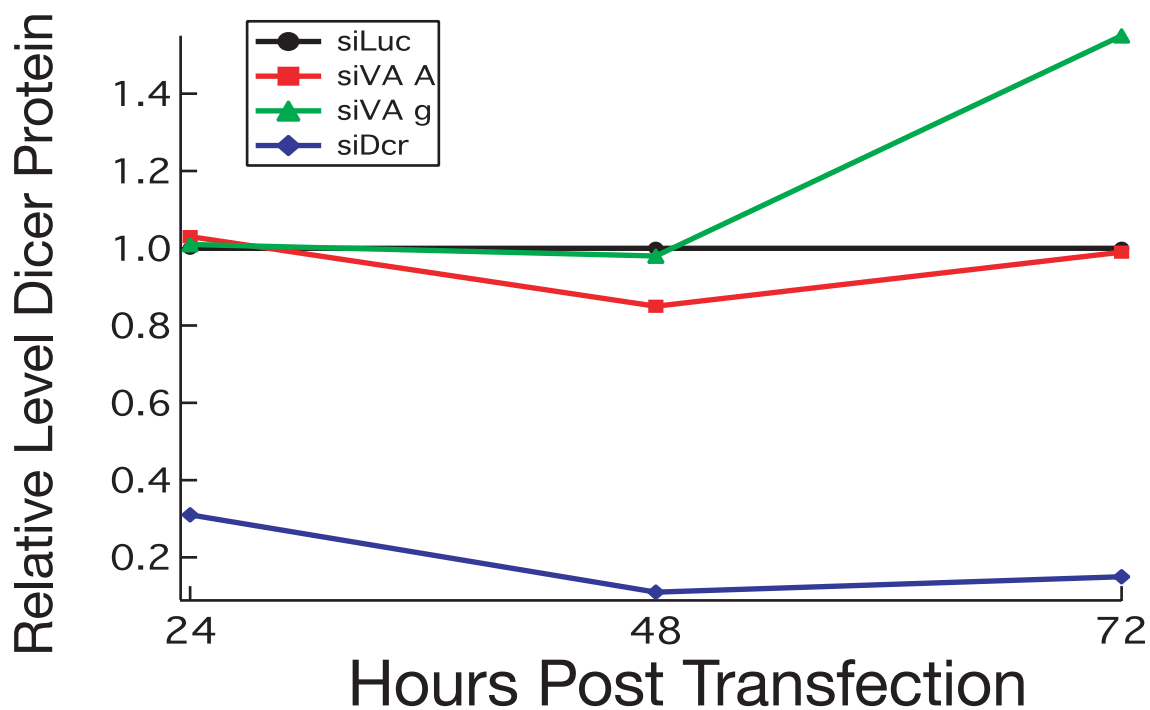
Finally, in order to further examine if miR-VA has any role in regulating Dicer mRNA or protein, we used siRNAs corresponding to the guide strand of miR-VA "g" or miR-VA "A" to perform transfections in cultured human cells. HeLa cells were transfected with the siRNA and total RNA or protein was collected every 24 hours for 3 days. The RNA samples were reverse transcribed and used for quantitative real-time PCR analysis. We saw that transfection of siVA "A" alone was unable to reduce the level of Dicer mRNA (Fig. 4.6). As expected, siVA "g" also did not regulate Dicer mRNA levels, whereas the siRNA specific for Dicer (siDcr) was able to greatly knockdown Dicer expression. Similarly, when we looked at protein levels from another set of transfections, we saw that the miR-VA-siRNAs were unable to downregulate Dicer protein levels, while the siDcr did provide a significant reduction in Dicer protein (Fig. 4.7).

Therefore, despite the *in silico* prediction that miR-VA "A" targets Dicer mRNA, we find that miR-VA "A" is not solely responsible for the observed decrease in Dicer mRNA and protein levels in infected cells. It has been demonstrated that typical mRNAs bear multiple miRNA target sites for a heterogeneous population of miRNAs (101). Indeed, detection of miRNA regulation by a single miRNA in cell culture typically requires the



**Fig. 4.6: miR-VA alone does not regulate Dicer mRNA.**

HeLa cells were transfected with the indicated siRNAs and total RNA was extracted every 24 hrs. for three days. Reverse transcription and quantitative real-time PCR was performed to determine the level of dicer mRNA at each timepoint. Each sample is internally normalized to GAPDH mRNA then further normalized to the siLuc sample at each timepoint.



**Fig. 4.7: miR-VA alone does not regulate Dicer protein.**

HeLa cells were transfected with the indicated siRNAs and whole cell extracts were prepared every 24 hrs. for three days. Western blot analysis was performed using a monoclonal antibody for human Dicer (Clontech) and  $\alpha$ -tubulin (Santa Cruz). Data from the Western was quantitated (internally normalized to tubulin) and plotted.

presence of 4 or more target sites to be generated in the 3'UTR of the gene of interest (32). Therefore, it is still formally possible that miR-VA "A" plays a synergistic role with other cellular miRNAs in the downregulation of Dicer mRNA/protein levels in infected cells. Perhaps there are cellular miRNAs expressed in response to adenovirus infection which perform this collaborative action. However, the demonstration that the dl331 virus was able to induce downregulation of Dicer protein suggests that miR-VA "A" is not essential for this downregulation (Fig. 4.4).

*Dicer protein levels inversely correlate with the level of replication in infected cells*

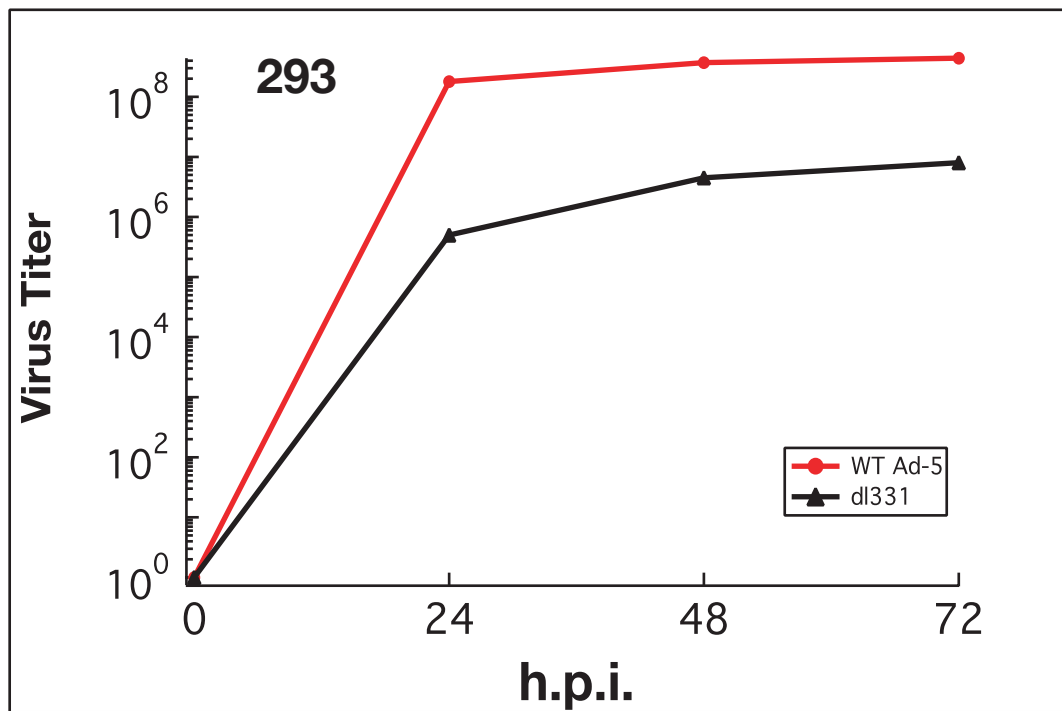
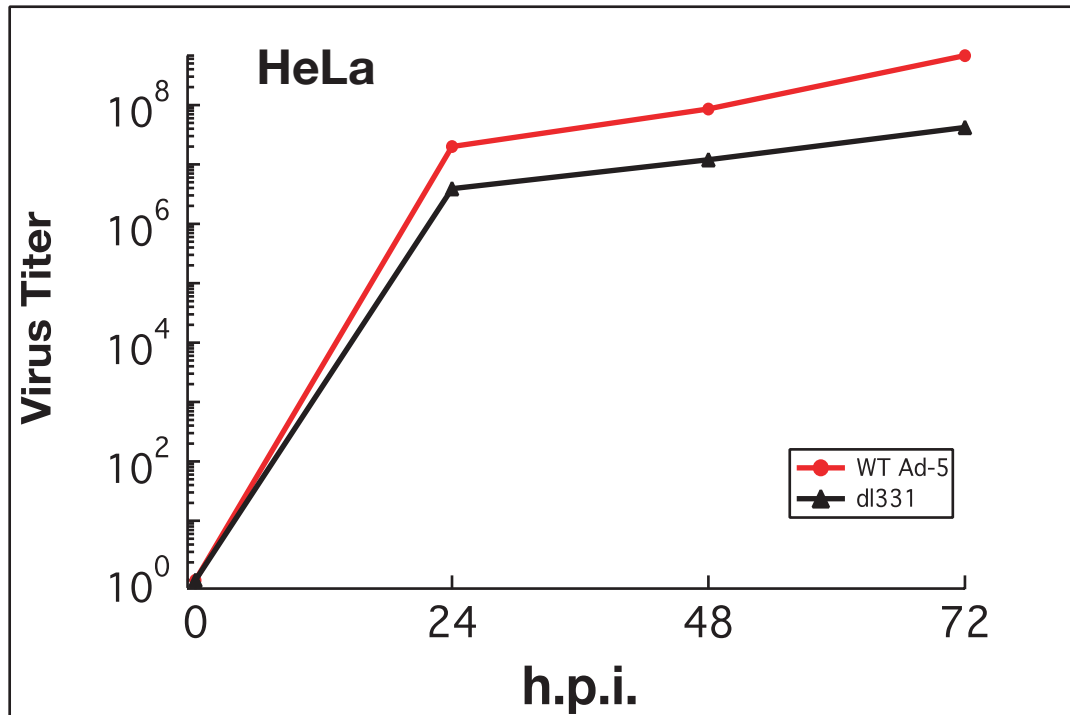
Since miR-VA "A" does not appear to specifically cause the observed reduction in Dicer levels, there is likely another viral factor mediating this effect. In the course of our studies, we observed differential effects on Dicer protein levels depending upon the particular virus and cell line used. Specifically, we found that the dl331 ( $\Delta$ VA I) virus did not cause a dramatic loss of Dicer protein levels in 293 cells. A search of the VA RNA literature reveals studies performed in the 1980s discussing this mutant virus growth defect. Inhibition of PKR is the main activity described for VA RNA I and to a lesser extent, VA RNA II (83, 159). The growth defect of dl331 in 293 cells has been attributed to the inability of the virus to block PKR activation and the subsequent shutdown of host translation. This growth defect appears to be proportional to the level of PKR in the infected cell, as the virus has a greater than 1 log growth defect in 293 cells, which express high levels of PKR, but grows similar to WT virus in cells with low

levels of PKR, such as GM2767A cells (159). We performed a growth curve analysis for WT and dl331 viruses in 293 and HeLa cells (Fig. 4.8). As predicted, dl331 grew poorly in relation to the WT virus, as there was nearly a 2 log difference in titer at 24 hours post infection. Contrastingly, there was only a modest difference in titer for the viruses when the infections were performed in HeLa cells.

When we performed western blot analysis to examine Dicer in infected 293 and HeLa cells, we saw that Dicer protein levels during infection also inversely correlate with the replication of virus (Fig. 4.9). In HeLa cells, where the dl331 virus grows at nearly the same level as the WT virus, Dicer protein levels were decreased for both viruses. In contrast, when the same analysis is performed in 293 cells, we observed that the poorly growing dl331 virus does not cause the same diminution of Dicer as WT Ad-5. Therefore, there is likely another viral factor produced during active infection that causes the observed effect. This downregulation of Dicer is unlikely to be part of a large scale downregulation of host mRNA and protein expression, since several microarray studies have indicated that surprisingly few cellular genes (~100) are significantly regulated by adenovirus at 24 hours post infection (34, 50, 208).

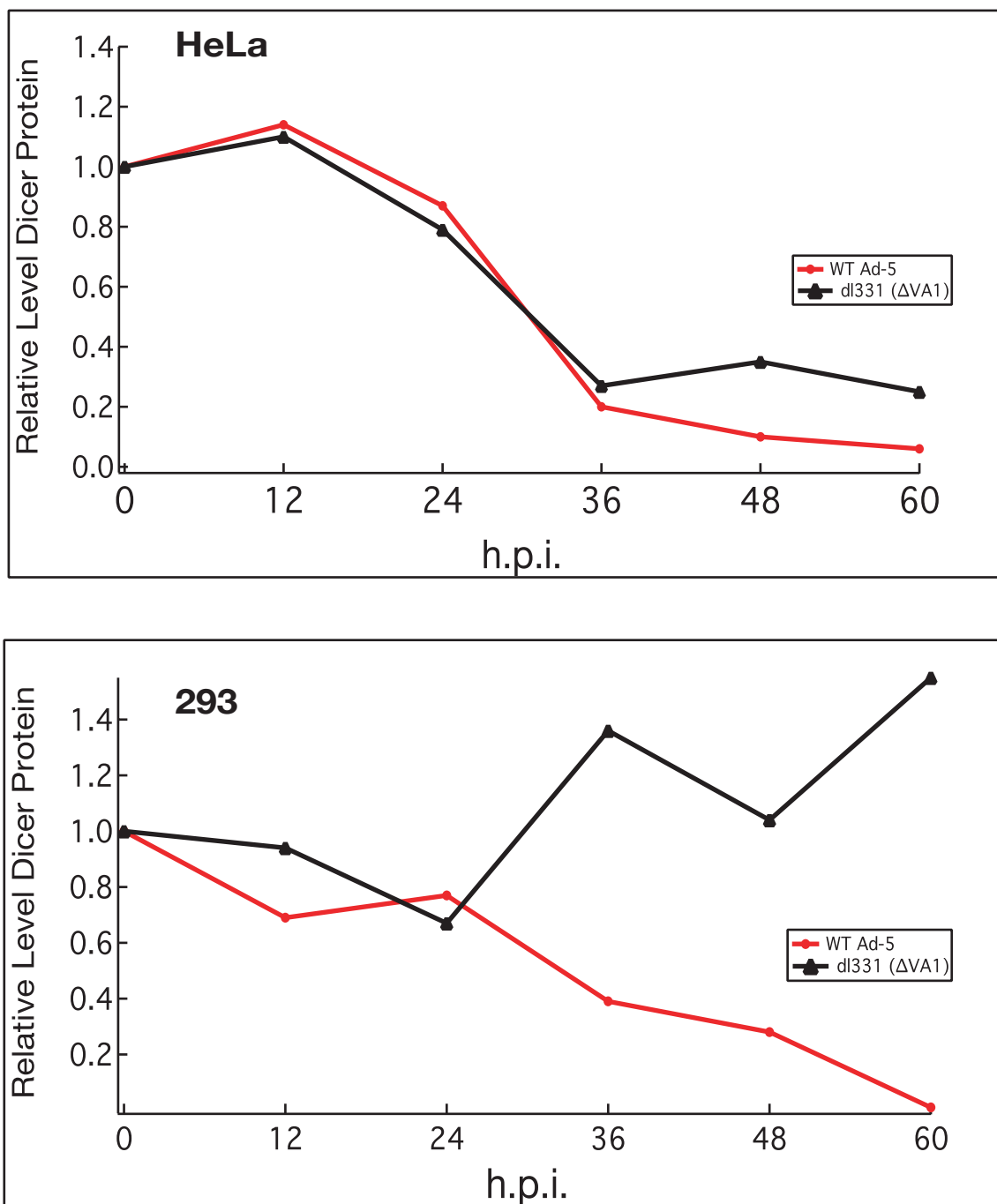
#### *Dicer protein levels do not affect virus replication*

Since we reproducibly observed that dicer levels were not reduced in 293 cells infected with dl331 virus, we asked whether Dicer was partially responsible for the observed attenuation of virus replication. The described growth defect of the dl331 virus



**Fig 4.8: dl331 ( $\Delta$ VA1) virus has a growth defect in 293 cells.**

HeLa or 293 cells were infected with WT Ad-5 or dl331. Lysates were collected and virus titers were determined at the indicated timepoints.



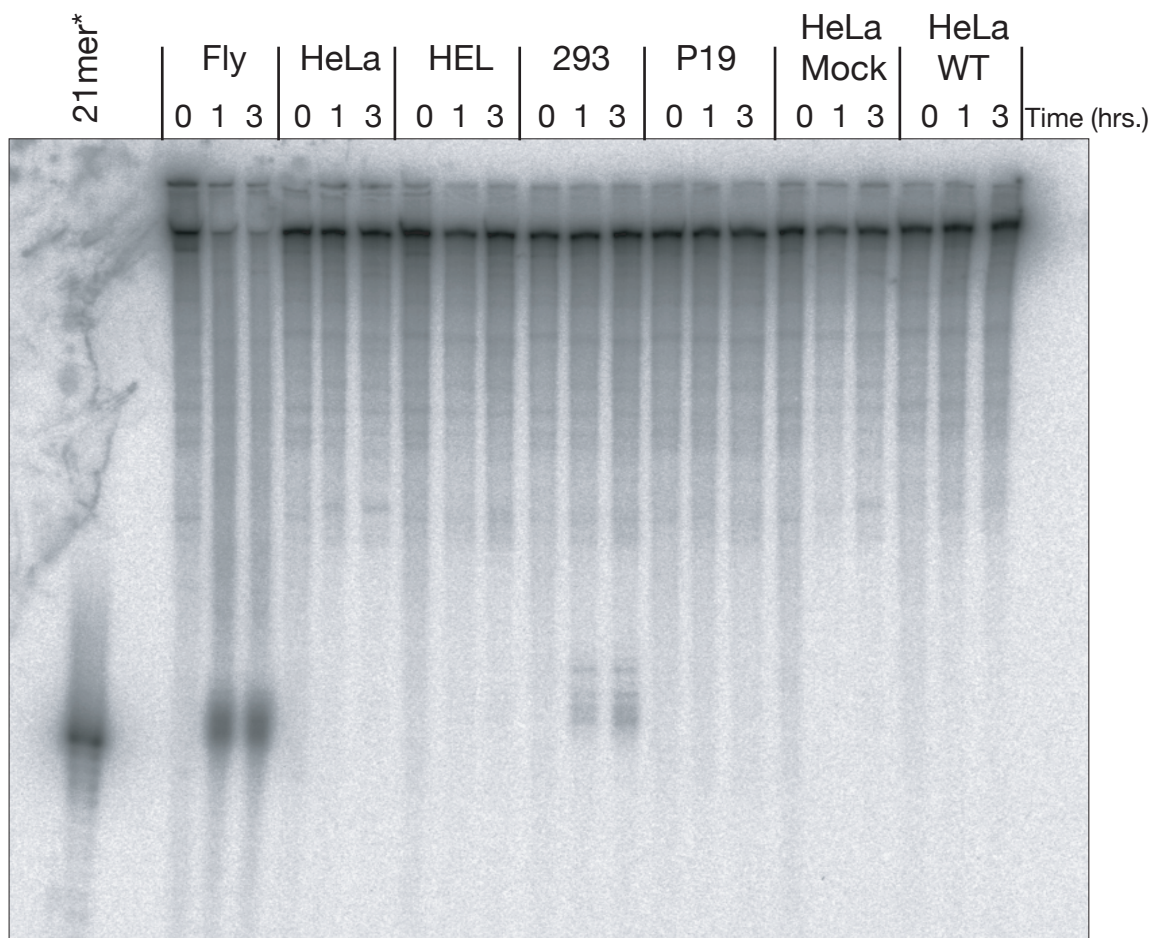
**Fig. 4.9: Adenovirus-induced downregulation of Dicer inversely correlates with replication.**

Whole cell extracts were prepared from mock-infected or adenovirus-infected HeLa or 293 cells at the indicated timepoints. Western blot analysis for Dicer protein levels was performed. Each sample is internally normalized to  $\alpha$ -tubulin, and samples were then normalized to mock infected sample at each timepoint.

in 293 cells has been solely attributed to the high levels of PKR in these cells (159). However, we have also observed that 293 cells express higher levels of Dicer protein and have more dicing activity than other human cell lines examined (Fig. 4.10). Unfortunately, we were unable to derive a quantitative measure of Dicer activity in 293 cells in relation to other human cell lines because we were unable to detect dicing in any cell line other than 293 cells. We reasoned that perhaps the compromised ability of the dl331 virus to grow in 293 cells was also partially due to the higher level of Dicer protein in these cells. To test this, we employed two related approaches. The first was to reduce levels of Dicer protein in cells prior to infection via siRNA knockdown. In the second approach, we increased levels of Dicer prior to infection by expression of a plasmid encoding the human Dicer cDNA. If Dicer plays a role in limiting adenovirus replication, then we would expect that reduction of Dicer levels would make 293 cells more permissive to infection. Conversely, overexpression of Dicer in a cell line containing lower levels of endogenous Dicer expression (HeLa cells) would be expected to limit replication of adenovirus.

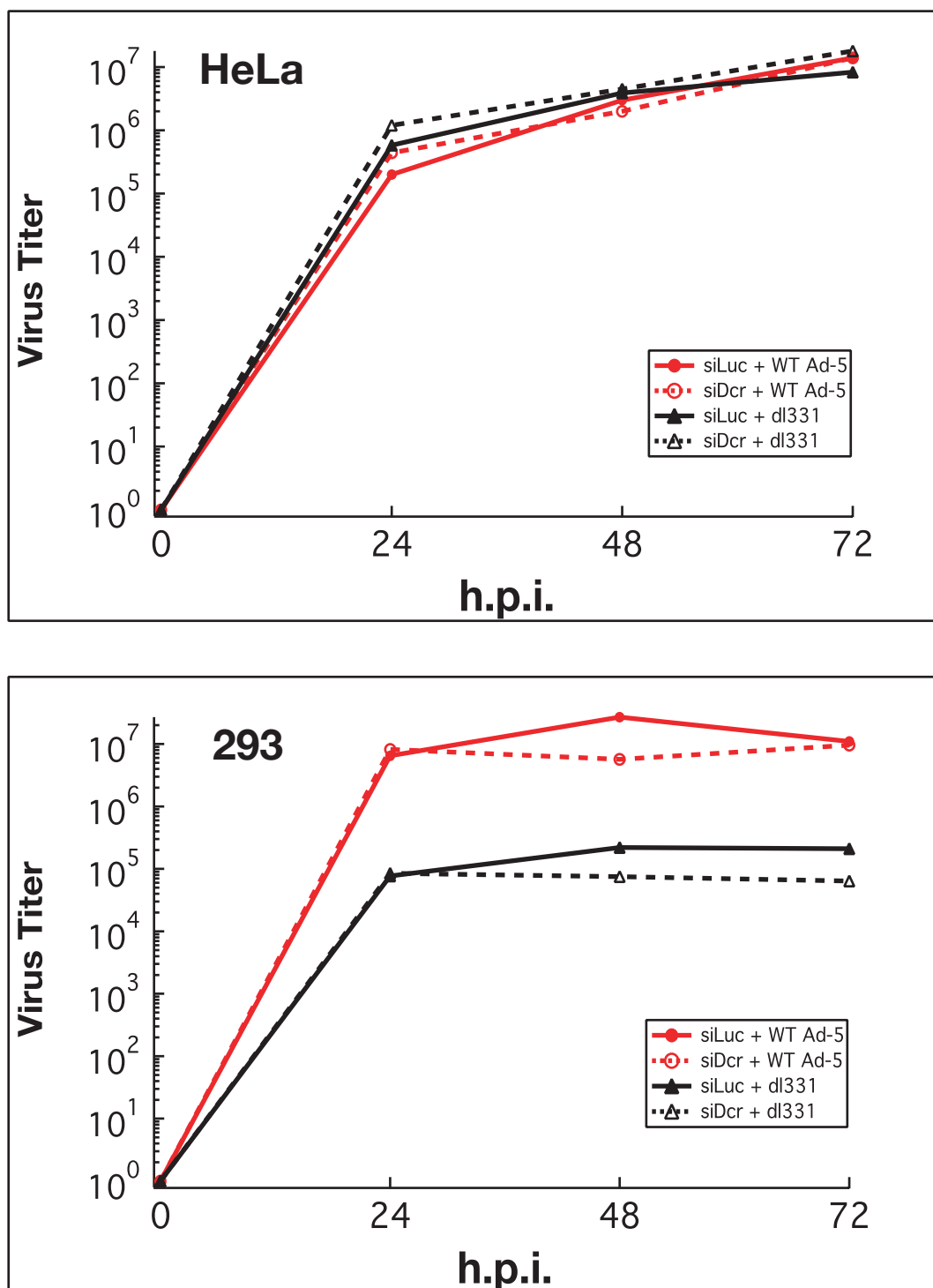
We saw no indication that the level of Dicer protein was of crucial importance to the efficiency of viral infection and replication. When siRNAs were used to knockdown Dicer levels prior to infection, we saw no difference in the growth of either the wild type or dl331 virus in HeLa or, more importantly, 293 cells (Fig. 4.11). Similarly, we saw no effect on virus growth when the converse experiment was performed. When Dicer was expressed exogenously in cells prior to infection, we did not see any impact on the growth of either the wild type or mutant virus in 293 or, more importantly, HeLa cells





**Fig. 4.10: 293 cells contains higher levels of dicing activity than other mammalian cell lines.**

RNAi-competent extracts were prepared from the indicated mammalian cell lines. Standard *in vitro* dicing reactions were performed using a 500 bp dsRNA target. RNA was electrophoresed on a 15% polyacrylamide gel.



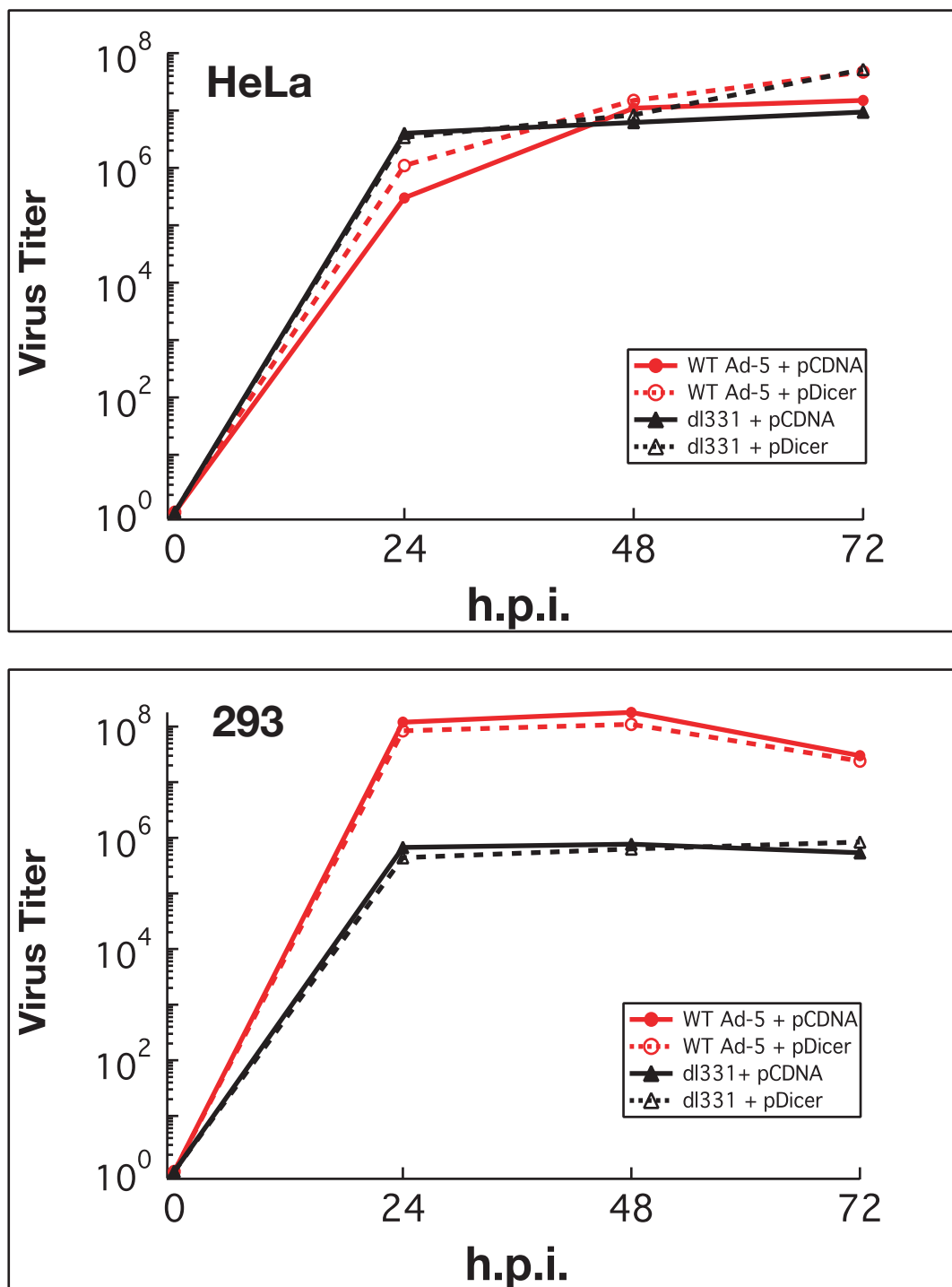
**Fig 4.11: Knockdown of cellular Dicer protein does not alter virus replication.**

HeLa or 293 cells were transfected with a control siRNA (siLuc) or an siRNA specific for human Dicer (siDcr). Twelve hours later, cells were infected with WT Ad-5 or dl331 virus. Lysates were collected and virus titers were determined at the indicated timepoints.

(Fig. 4.12). We confirmed the knockdown and overexpression of Dicer protein in these respective studies by performing western blot analysis for Dicer protein (data not shown). These experiments indicate that Dicer protein does not appear to regulate growth of adenovirus in these settings.

#### *Examining the impact of adenovirus infection on cellular miRNA biogenesis*

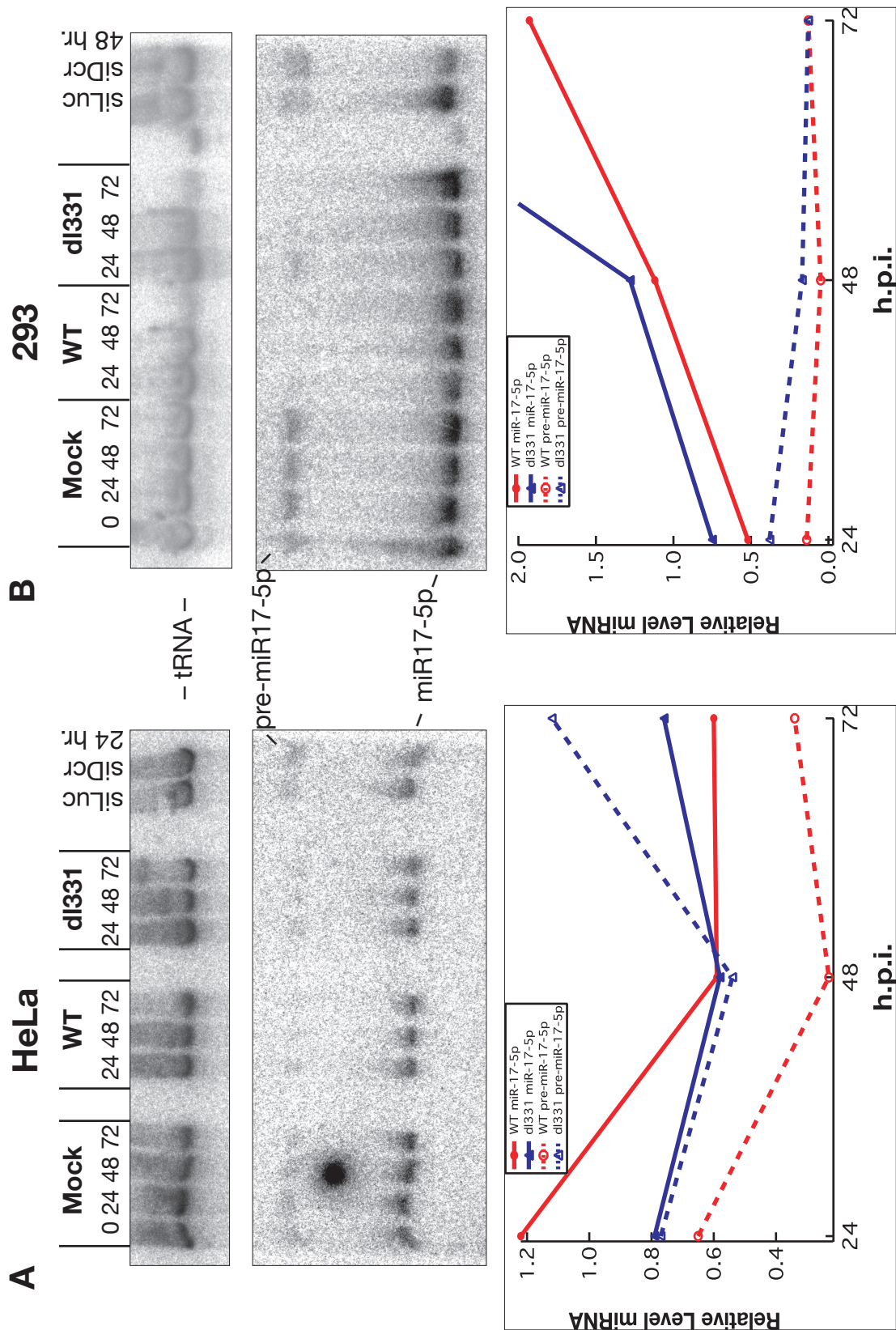
We and others have provided data that adenovirus utilizes several mechanisms to counteract Dicer function, including the reduction in cellular Dicer levels as well as the production of RNA molecules, VA RNA, that can compete with endogenous pre-miRNA for processing by Dicer and export by Exportin-5 (4, 111). These viral activities have the potential to greatly impact the endogenous miRNA pathway in infected cells. Because Dicer levels and function are being impaired, a prediction would be that cellular pre-miRNAs are not processed efficiently to mature miRNAs during adenovirus infection. To test this, we performed northern blot analysis to compare the levels of specific pre-miRNAs in mock-infected cells and adenovirus-infected cells. If adenovirus is affecting the ability of Dicer to process cellular miRNAs, then pre-miRNA levels would be expected to rise during infection. After several attempts using DNA and RNA-based probes for several different miRNAs, we were finally able to detect pre-miRNA species using an antisense probe containing locked nucleic acid nucleotides (LNA), which was specific for miR-17-5p (85, 180). These modified nucleotide probes have very high thermal stability when hybridized with DNA or RNA sequences (85, 145). The northern



**Fig 4.12: Dicer overexpression does not alter viral replication.**

HeLa or 293 cells were transfected with a control plasmid (pCDNA) or a plasmid overexpressing human Dicer protein (pDcr). Cells were infected with WT Ad-5 or dl331 virus 12 hours later. Lysates were collected and virus titers were determined at the indicated timepoints.

blot data for miR-17-5p indicated that there was not an increase in pre-miR-17-5p in either HeLa or 293 cells (Fig. 4.13). In fact, the level of the precursor appears to drop to nearly undetectable levels in infected cells. In analyzing the mature miRNA, we observe that after an initial modest increase in miR-17-5p levels in WT-infected HeLa cells, there was a marked decrease in the level of mature miRNA (Fig. 4.13). However, the same does not hold true in 293 cells, as there was no initial increase in mature miRNA; rather, there appears to be an increase of the miR-17-5p at later points of the infection, although analysis of the tRNA loading control indicates that there may have been unequal loading in both the WT and dl331 72 hour samples. This analysis only includes a single pre-miRNA, so major conclusions cannot be drawn from this experiment. Additional probes to other miRNAs, designed with the LNA modifications will be crucial in helping to accurately detect pre-miRNAs in future experiments. Also, the control samples on this blot indicate that downregulation of Dicer appears to produce only subtle differences in pre-miRNA levels at early timepoints. These control wells contain RNA from HeLa cells transfected with siRNAs specific for Dicer mRNA (siDcr) or a non-specific control (siLuc). Efficient knockdown of Dicer levels was obtained with these transfections (confirmed by western blot for Dicer protein), yet we only observe a modest effect on the level of pre-miRNA and miRNA at 24 hours post-transfection (HeLa blot). There is a 7% increase in pre-miR-17-5p and a 40% decrease in mature miRNA at 24 hours after this efficient transfection. In contrast, samples taken from 48 hours post transfection lead to a 40% increase in the level of pre-miRNA and a 70% decrease in the mature miRNA (Fig. 4.13, 293 blot). It is possible that in our experimental setting, where virus infection



**Fig. 4. 13 Adenovirus infection does not affect the accumulation of an endogenous miRNA precursor.** HeLa (A) or 293 (B) cells were infected with the indicated virus and RNA was harvested every 24 hours for 3 days. Northern blot analysis was performed using an antisense LNA probe to miR-17-5p. Each data point was internally normalized to the tRNA control, and pre-miRNA and miRNA levels were quantitated and plotted.

kills the cells rapidly (within 48-72 hours), there is not adequate time to observe potent effects on cellular miRNA biogenesis caused by disruption of cellular Dicer.

To further test for cellular miRNA function during infection, we performed *in vitro* target cleavage assays to examine the levels of a functional miRNA in the infected cells. We prepared a target RNA bearing a region of perfect complementarity for the cellular miRNA, let-7a (61). RISC-mediated target cleavage reactions were performed in extracts prepared from mock-infected or adenovirus-infected cells from various timepoints post infection. Therefore, this assay examines the levels of endogenous, RISC-associated let-7a in the infected cells. We also performed cell counts prior to preparation of the extracts, since adenovirus infection will influence the number of cells due to viral pathogenesis. A method for determining the concentration of a RISC-associated miRNA has been described for this *in vitro* target cleavage assay (66). In the assay, increasing amounts of a specific 2' oMe inhibitor for the miRNA are included in target cleavage reactions to inhibit target cleavage (as performed in Fig. 3.8). Since 2' oMe inhibitors act as stoichiometric inhibitors of RISC cleavage, the amount of 2' oMe required for half maximal inhibition of target cleavage ( $IC_{50}$ ) is directly proportional to the concentration of RISC (66). Using this analysis, we determined the [let-7-RISC] in each extract and found that the level of functional, RISC-associated let-7 was slightly increased during infection (Table 4.1). These results indicate that the viral manipulation of Dicer levels and function in infected cells does not lead to dramatic decreases in the level of RISC-associated miRNA.

<u>Extract</u>	<u>IC50</u>	<u>[RISC](nM)</u>	<u>Cell Conc. of extract</u>
Mock 12	0.34	0.69	1.016 x10 <sup>5</sup> cells/ $\mu$ l
WT 12	0.57	1.13	5.602 x10 <sup>4</sup> cells/ $\mu$ l
WT 24	0.97	1.94	5.463 x10 <sup>4</sup> cells/ $\mu$ l
WT 48	1.42	2.85	2.412 x10 <sup>4</sup> cells/ $\mu$ l

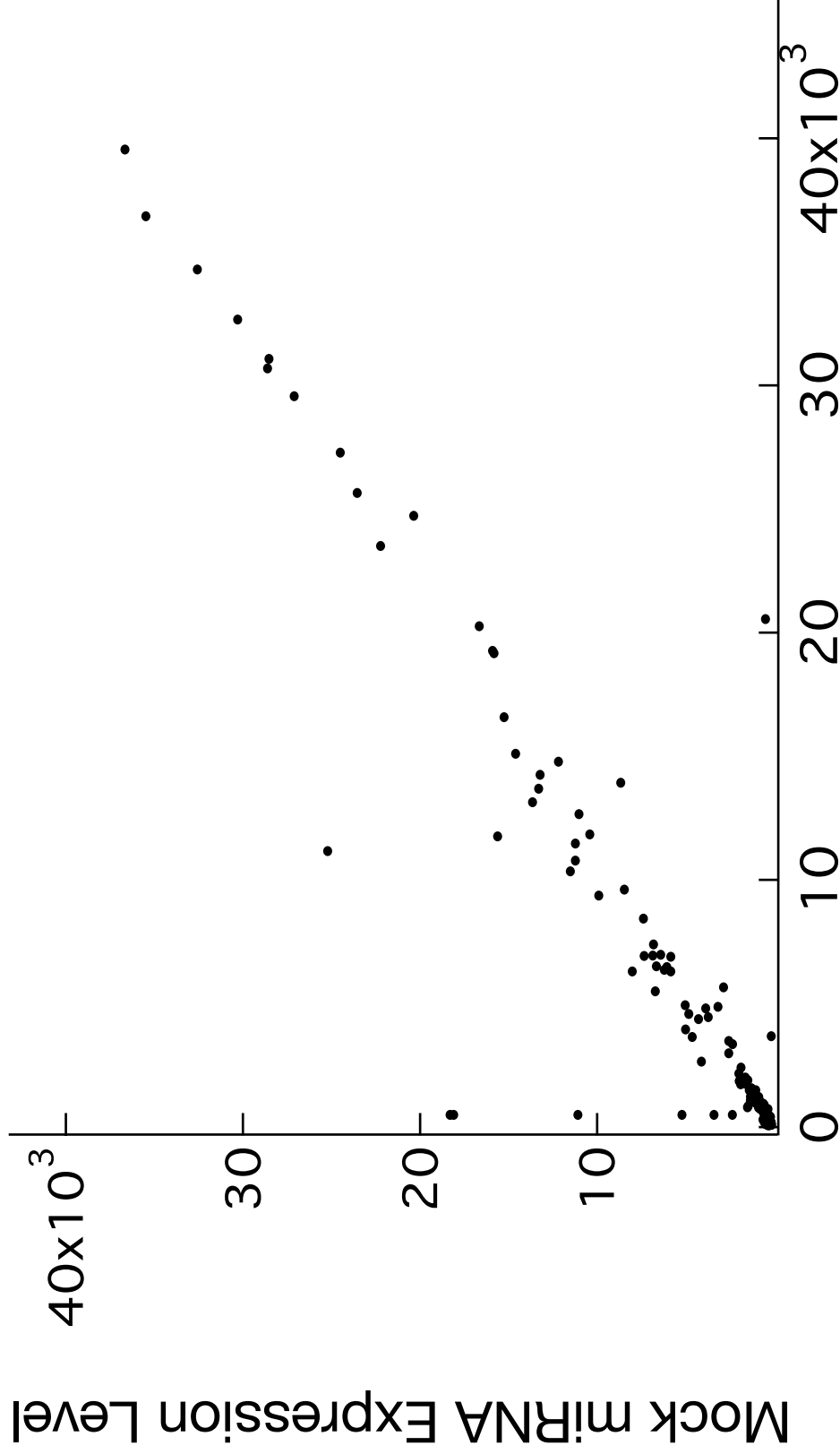
**Table 4.1: Adenovirus infection does not dramatically affect the level of a cellular RISC-associated miRNA.** RNAi-competent extracts were prepared from mock-infected or adenovirus-infected HeLa cells. Target cleavage assays were performed using a radiolabeled target containing a sequence bearing perfect complementarity to the endogenous miRNA, let-7a. Increasing concentrations of a specific 2'OMe oligonucleotide inhibitor for let-7 were used to determine the IC<sub>50</sub> for target cleavage, which was then extrapolated to infer [RISC].



*Adenovirus infection leads to altered expression of a small subset of miRNAs*

The previous experiments aimed at determining the effect adenovirus-induced perturbations of Dicer levels and functions has on infected cells examined only two cellular miRNAs for their expression and activity (Fig. 4.13 & Table 4.1). We also desired to gain a global perspective of how miRNA levels were changing in response to adenovirus. To examine the cellular pool of miRNAs during infection, we performed a miRNA microarray experiment. In this experiment, RNA from mock-infected and WT Ad-5-infected cells was hybridized to a microarray containing all known human miRNA and miRNA\* sequences identified at that time (LC Sciences, Houston, TX). We chose to use RNA from 24 hours post infection, a time at which infected cells are showing obvious signs of cytopathology, but prior to dramatic changes in cell morphology and detachment from the tissue culture plate. To date, there are no published studies examining the expression of all miRNAs in the context of infection. A literature search produced only one similar study, where an infectious clone of HIV was used to transfect cells prior to array detection of miRNAs (198).

Our miRNA analysis indicated that at 24 hours post infection, there are relatively few changes in miRNA gene expression between the mock-infected and adenovirus-infected samples (Fig. 4.14). Most miRNAs in this analysis had comparable expression in the samples, as indicated by the linear trend of the data points. We considered all miRNA genes whose expression changed (increased or decreased) by at least 1.5 fold as significant. By these criteria, only 19 miRNAs or miRNA\*s were differentially



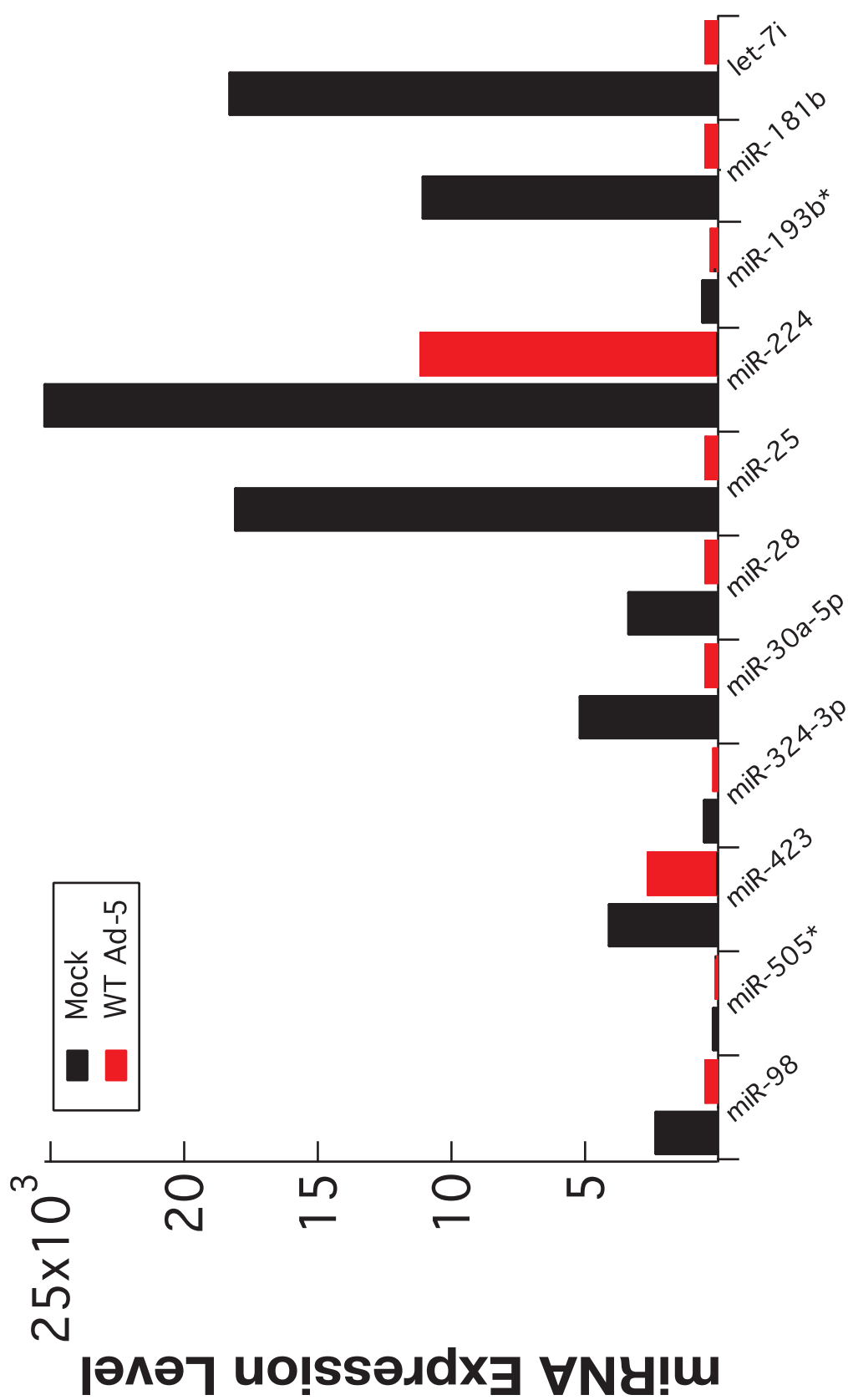
## WT Ad-5 Infected miRNA Expression Level

**Fig 4.14: Global Analysis of miRNAs expression during Adenovirus infection.** RNA from mock infected or WT Ad-5 infected HeLa was analyzed by miRNA microarray (LC Sciences). A space plot displaying mock and virus infected expression levels for each miRNA detected is shown.

regulated. This result is in accordance with our previous limited analysis of miRNAs in infected cells, where we did not detect appreciable changes in miRNA level or activity (Fig. 4.13 and Table 4.1). On a whole, it appears that the accumulation of miRNAs is not grossly affected 24 hours after infection. However, there are 19 miRNA or miRNA\* sequences that are significantly altered in infected cells (Fig. 4.15 & Fig. 4.16). Perhaps these miRNAs and miRNA\*s represent a subset of the miRNA population that helps establish an environment that is permissive for infection and replication. Alternatively, these miRNAs may represent a response from the host to modulate genes involved in the immune response.

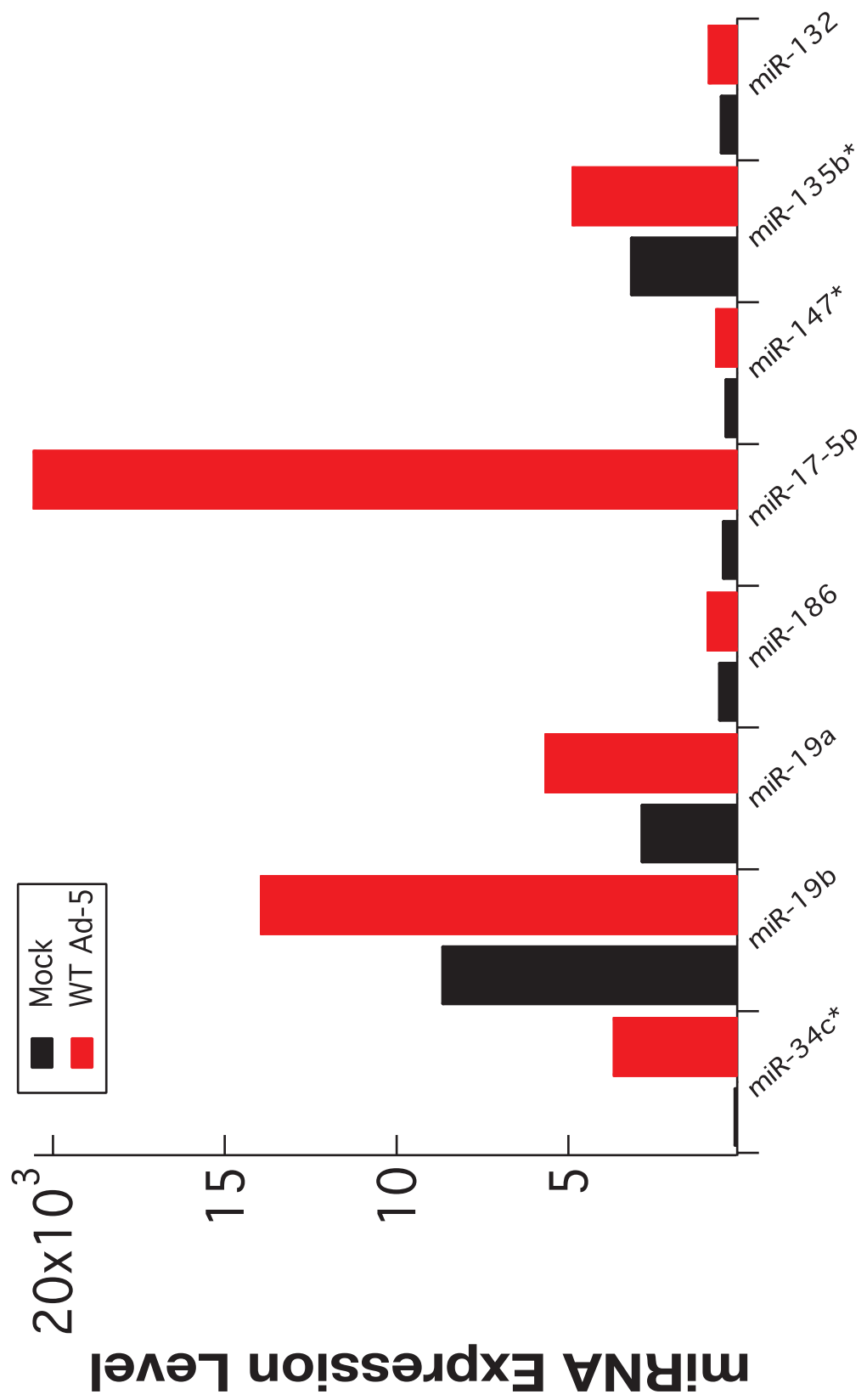
Interestingly, three of the five miRNAs upregulated by adenovirus infection, miR-17-5p, miR-19a, and miR-19b have previously been experimentally validated as key regulators of genes involved in cell cycle progression (138). These miRNAs exist in a miRNA polycistron (miR-17-92) of 7 total miRNAs, whose expression is misregulated in certain tumors (23). These so called “oncomirs” have been shown to regulate expression of E2F1, and expression of the miRNA locus itself is regulated by the c-myc transcription factor (138).

There are also many other interesting potential cellular mRNA targets for the subset of misregulated miRNAs. Unlike our previous attempts to define a cellular target for miR-VA, these predictions will prove much more reliable, as conservation of the cellular miRNA and its perspective targets across a panel of species can be utilized in the algorithm. Additionally, recent refinements to the target prediction algorithm have been made to more accurately predict target genes (72, 100). A listing of the misregulated



**Fig. 4.15: Cellular miRNAs downregulated during Adenovirus infection.**

A miRNA gene array (LC Sciences, Houston, TX.) was performed on the small RNA fraction isolated from mock-infected or adenovirus-infected HeLa cells. miRNA that were significantly downregulated in infected cells are shown above.



**Fig. 4.16: Cellular miRNAs upregulated during Adenovirus infection.** A miRNA gene array (LC Sciences, Houston, TX.) was performed on the small RNA fraction isolated from mock-infected or adenovirus-infected HeLa cells. miRNA that were significantly upregulated in infected cells are shown above.

miRNAs with some of their predicted cellular targets is provided in Table 4.2 and Table 4.3. These lists include only high scoring predictions, and they contain some intriguing potential regulators of immune defense, cell cycle control, and even RNA silencing components. Additionally, we observed that one of the miRNAs downregulated during infection, miR-28, bears perfect complementarity in its seed sequence to the 3' end of miR-VA "A" (Table 4.2). What significance this complementarity has is unknown, but we do find this to be a very interesting observation to further pursue.

This microarray analysis provided us with a wealth of information to use for follow up studies. Obviously, all of these potentially misregulated miRNAs will need to be validated with further northern blot and microarray analysis. However, these preliminary findings have the exciting potential to uncover new aspects of adenovirus infection, as well as the host response to virus.

*Infection by another DNA virus, human cytomegalovirus (HCMV), produces a pattern of miRNA regulation distinct from that of adenovirus*

We also performed miRNA microarray analysis on RNA from cells infected with another unrelated DNA virus, human cytomegalovirus (HCMV). HCMV is a member of the Herpesvirus family, which contains some of the largest and most complex viruses. Of this family of viruses, CMV is the largest member, with a genome size of 230 kb. Accordingly, CMV encodes a wealth of viral gene products that subvert the host cell into a machine for virus production. Numerous immunomodulatory activities have been

**Table 4.2: Putative Cellular Targets of miRNAs Downregulated During adenovirus Infection**

miRNA	Putative cellular target
<b>miR-98</b>	<ul style="list-style-type: none"> <li>- Myc proto-oncogene (c-myc)</li> <li>- Ras-related protein Rab-14</li> </ul>
<b>miR-423</b>	<ul style="list-style-type: none"> <li>- Bcl-2-related proline-rich protein</li> </ul>
<b>miR-25</b>	<ul style="list-style-type: none"> <li>- Interferon-induced, double-stranded RNA-activated protein kinase (PKR)</li> <li>- Bcl-2/adenovirus E1B 19-kDa protein-interacting protein</li> <li>- E2F transcription factor 3</li> </ul>
<b>miR-224</b>	<ul style="list-style-type: none"> <li>- Bcl-2/adenovirus E1B 19-kDa protein-interacting protein</li> </ul>
<b>miR-181b</b>	<ul style="list-style-type: none"> <li>- Ribonuclease III (EC 3.1.26.3) (Drosha)</li> <li>- Homo sapiens TAR DNA binding protein</li> </ul>
<b>let-7i</b>	<ul style="list-style-type: none"> <li>- TAR RNA-binding protein 2</li> <li>- E2F transcription factor 5</li> </ul>
<b>miR-28</b>	<ul style="list-style-type: none"> <li>- 2-5A-dependent ribonuclease (RNase L)</li> </ul> <p>5' <b>AAGGAGCUCACAGUCUAUUGAG</b> 3' human miR-28  3' <b>UUCCUCGUGAGGGGCACAGA</b> 5' miR-VA "A"</p>

**Table 4.3: Putative Cellular Targets of miRNAs Upregulated During adenovirus Infection**

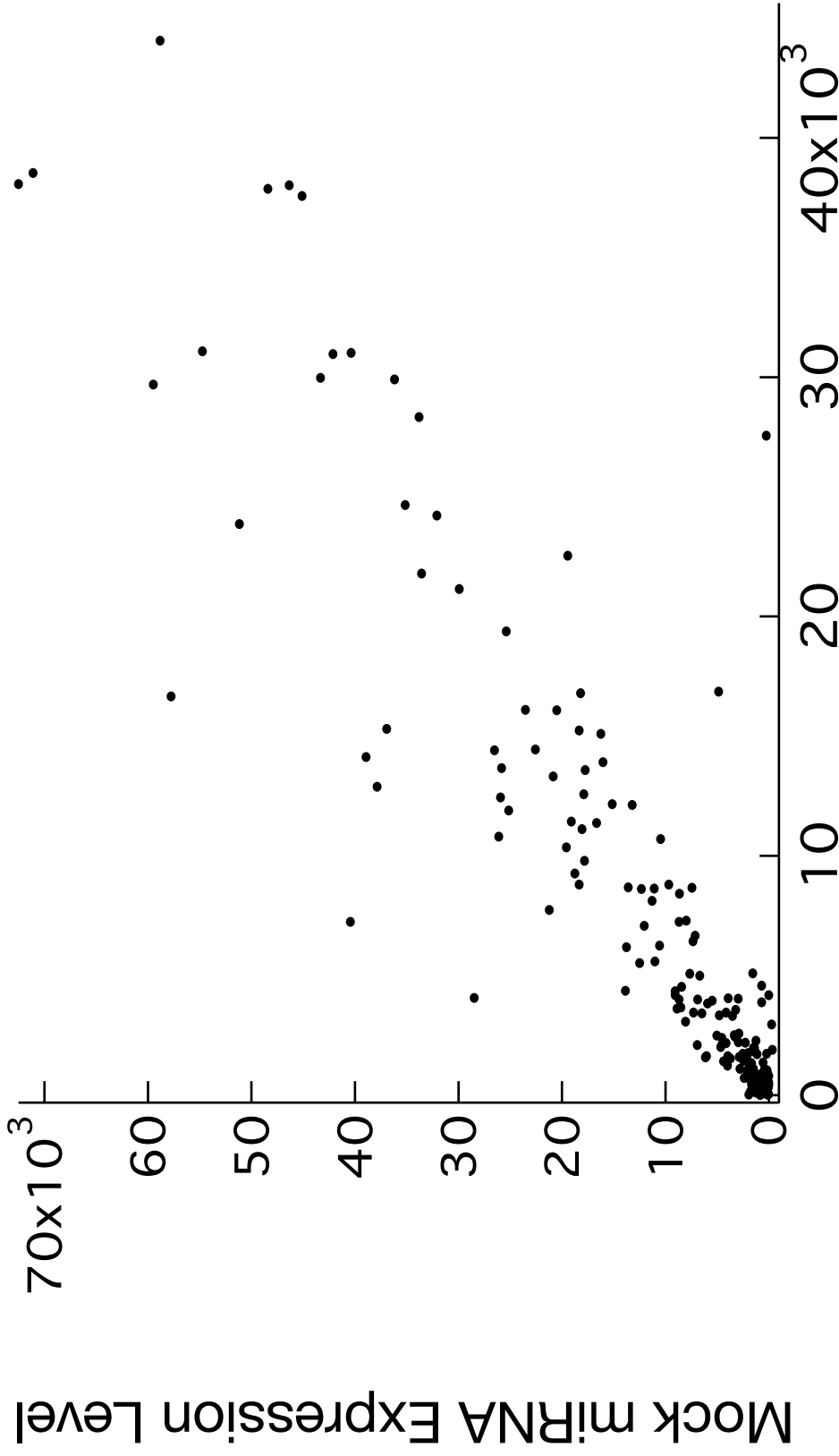
miRNA	Putative cellular target
miR-132	<ul style="list-style-type: none"> <li>- Ribonuclease III (EC 3.1.26.3) (Drosha)</li> <li>- H. sapiens mitogen-activated protein kinase kinase kinase (MAP3K10)</li> </ul>
miR-186	<ul style="list-style-type: none"> <li>- Interferon-induced with helicase C domain protein 1 (EC 3.6.1)</li> <li>- Lupus La protein</li> <li>- Homo sapiens TAR DNA binding protein</li> </ul>
miR-17-5p	<ul style="list-style-type: none"> <li>- E2F1 transcription factor (experimentally validated)</li> </ul>
miR-19a & b	<ul style="list-style-type: none"> <li>- Components of miRNA cluster associated with oncogenesis</li> </ul>



described for this virus. We performed miRNA microarray analysis for HCMV infection of HEL cells, harvesting RNA at 48 hours post infection. Once again, we used specific probes to detect all known miRNA and miRNA\* sequences at the time of the array.

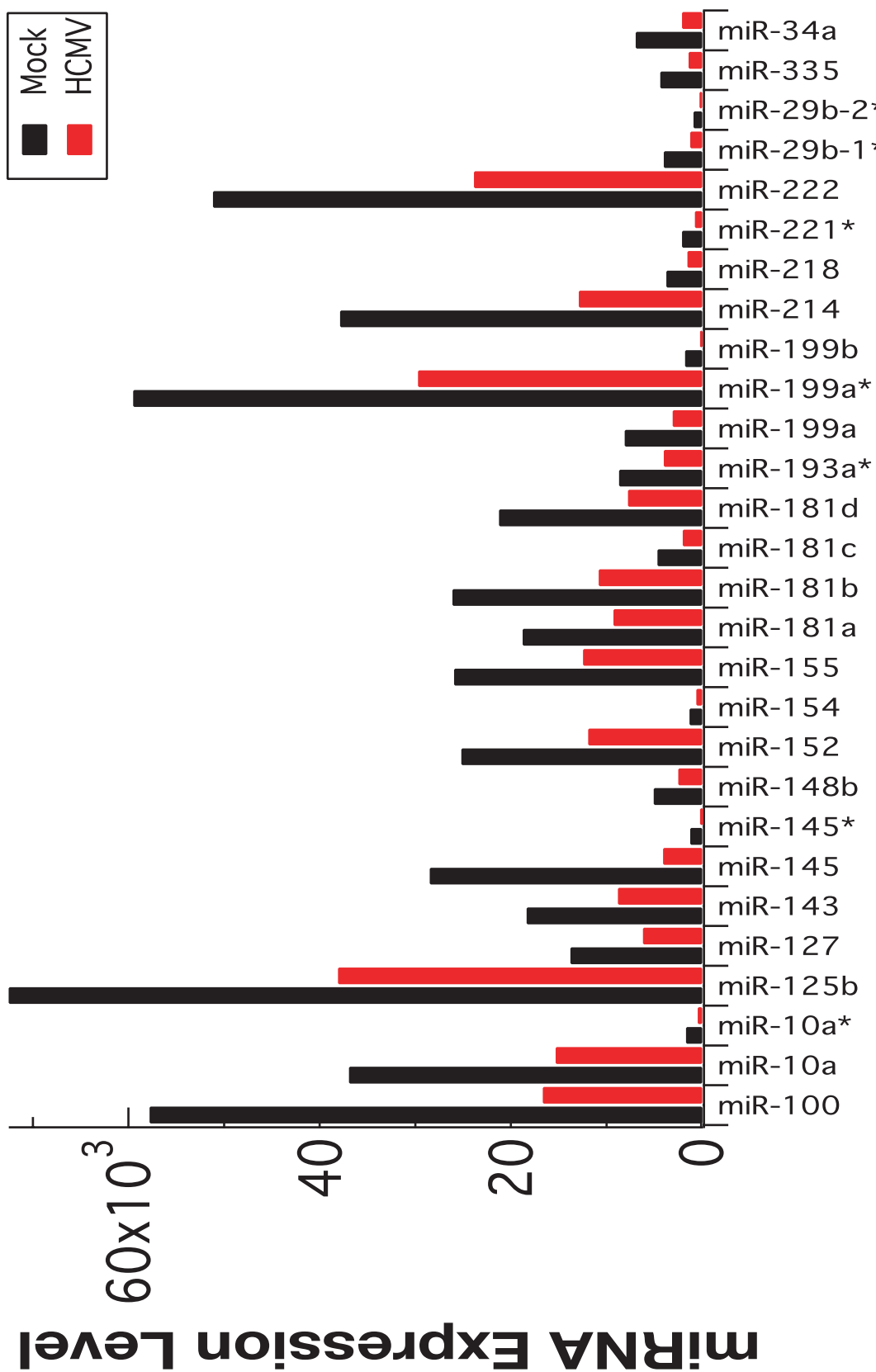
Similar to the adenovirus array, we saw that the majority of cellular miRNAs are unaffected by virus infection at this timepoint, as most miRNAs in a space plot of mock versus infected samples fell along a linear trace (Fig. 4.17). Not surprisingly, a higher percentage of miRNAs had significant changes in their expression than was seen with the adenovirus array (Fig. 4.18a/b & Fig. 4.19). Most of these miRNAs with altered expression levels were downregulated in response to infection (Fig. 4.18a/b). Interestingly, only one miRNA was similarly misregulated in both HCMV and adenovirus infection. This miRNA, miR-181b, was downregulated during both infections, raising the possibility that this miRNA regulates factors that are important for a host response to infection. Also, unlike the upregulation of oncomirs in adenovirus infection, we observed that no miRNAs previously associated with cancer signatures were significantly upregulated in response to CMV (187). However, we did observe that several members of miRNA families displayed coordinated downregulation in response to virus (Table 4.4). Unlike the miR-17-92 polycistron, many of the miRNAs in miRNA gene families are not in close proximity. In fact, many reside on different chromosomes. Therefore, coordinated regulation of these miRNAs would underscore the importance of these particular gene families in the context of infection.

An attractive hypothesis from these microarray analyses is that different viruses induce different miRNA signatures in their host cells, and that these patterns of miRNA



### HCMV Infected miRNA Expression Level

**Fig 4.17: Global Analysis of miRNAs expression during HCMV infection.** RNA taken from mock-infected or HCMV (Ad-169)-infected HEL cells at 48 h.p.i. was analyzed by miRNA microarray (LC Sciences). A space plot displaying mock and virus infected expression levels for each miRNA detected is shown.



**Fig. 4.18a: Cellular miRNAs downregulated during HCMV infection.**

A miRNA gene array (LC Sciences, Houston, TX.) was performed on the small RNA fraction isolated from mock-infected or HCMV-infected HEL cells. miRNA that were significantly downregulated in infected cells are shown above.

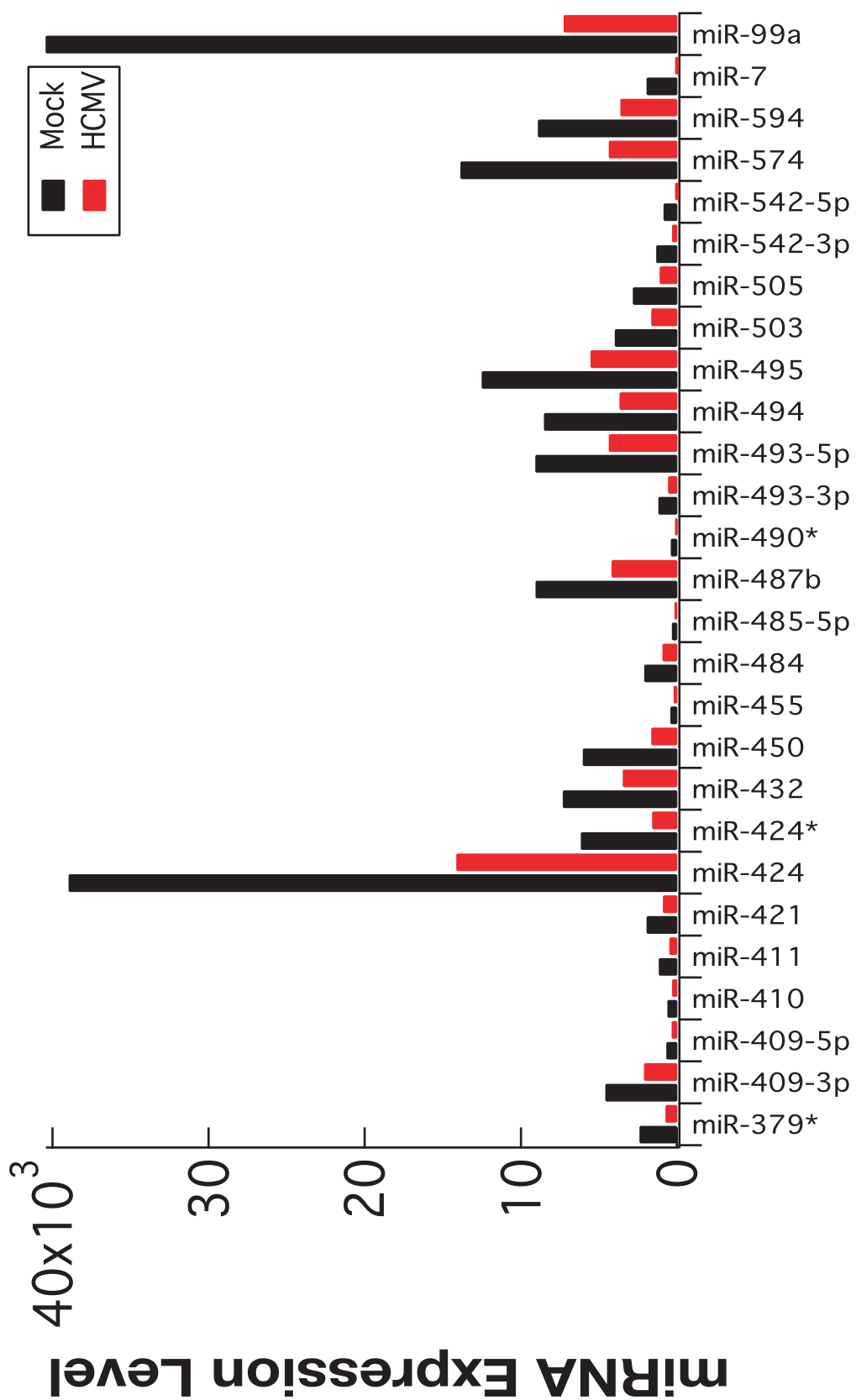
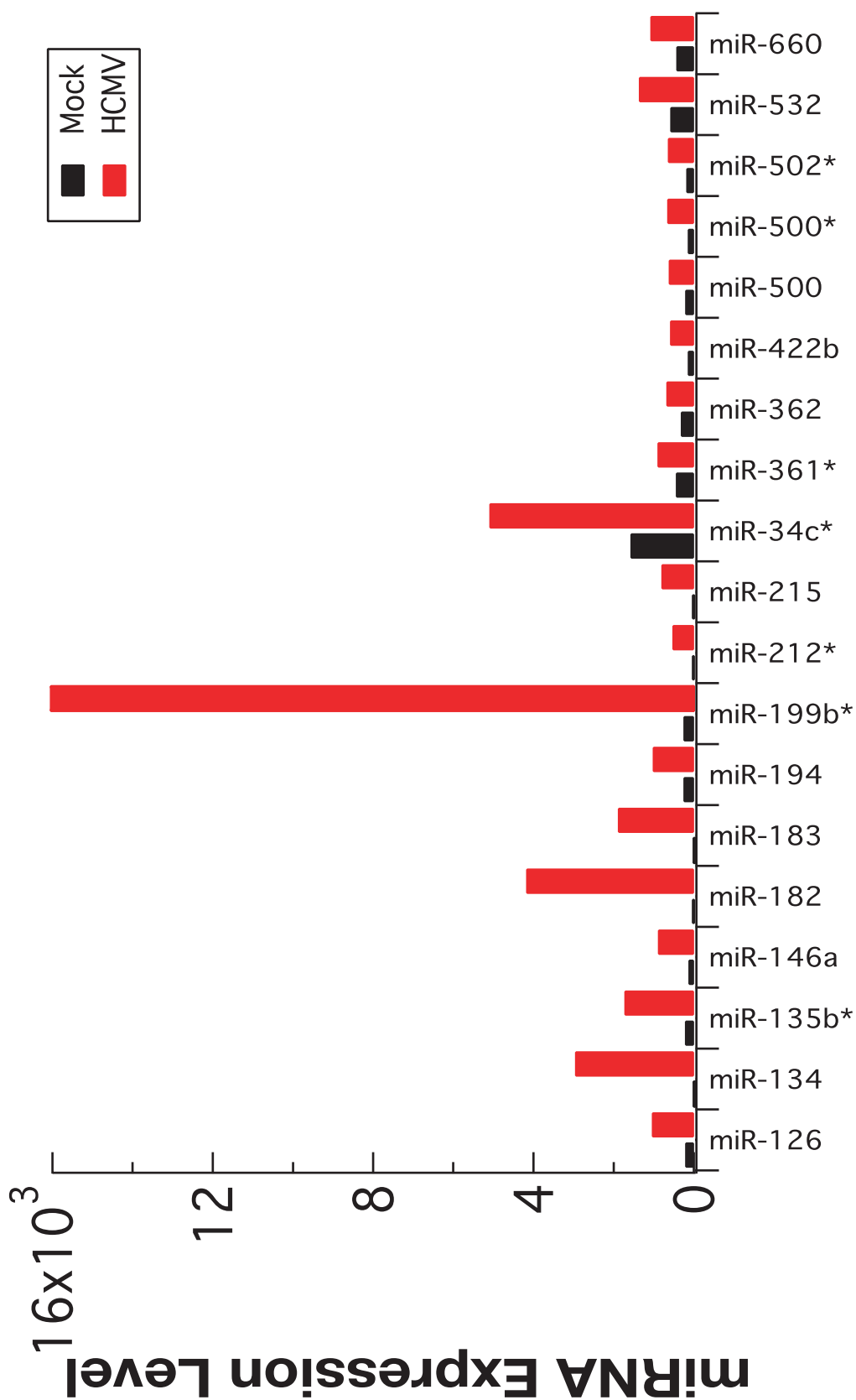


Fig. 4.18b: Cellular miRNAs downregulated during HCMV infection (cont.).



**Fig. 4.19: Cellular miRNAs upregulated during HCMV infection.**

A miRNA gene array (LC Sciences, Houston, TX.) was performed on the small RNA fraction isolated from mock-infected or HCMV infected-HEL cells. miRNA that were significantly upregulated in infected cells are shown above.

miRNA Family	miRNA Family Members Coordinately Regulated On Array
miR-181	miR-181a, miR-181b, miR-181c, miR-181d
miR-148	miR-148b, miR-152
miR-221	miR-221, miR-222
miR-154	miR-154, miR-409-5p, miR-409-3p, miR-410, miR-487b, miR-494

**Table 4.4: miRNA gene families misregulated in response to HCMV infection.**

Global miRNA microarray analysis was performed on RNA from mock-infected or HCMV-infected HEL cells. Family members that were similarly downregulated in response to HCMV infection are shown.

expression are important regulators of genes involved in the process of infection. We are currently verifying and extending our microarray analysis by comparing miRNA profiles from adenovirus and CMV infections at similar timepoints in the same cell line (HEL). Our hope is that these results will shed light on key miRNAs involved in host response to infections, as well as distinct miRNAs that define specific viral infections.

## Discussion

### *Defining a role for miR-VA*

Our work has demonstrated that adenovirus produces a miRNA species, miR-VA, from the VA RNA I locus during the course of infection. We took several different approaches in an attempt to ascertain the importance and function of this miRNA. First, we utilized a specific 2' oMe antisense inhibitor of miR-VA to block the function of miR-VA in infected cells. When the 2' oMe oligonucleotide was introduced into cells 12 hours prior to infection, it had virtually no effect on virus replication, as growth curves for the viruses in cells treated with the specific inhibitor were identical to those transfected with a control 2' oMe oligonucleotide (Fig. 4.1). These results are in contrast to another study, which showed that 2' oMe inhibition of the miR-VA region produced a modest reduction in virus accumulation (6). Several important differences exist in the protocols used in this study versus ours, including the cell line used, size of the 2' oMe inhibitor, concentration of inhibitor, and method of virus collection. Our study used HeLa cells, a 20 nM final concentration of a 2' oMe inhibitor antisense to the 22 nucleotide miR-VA "A", and collection of supernatant and cellular fractions for titering. The other group utilized 293 cells, a 100 nM concentration of 2' oMe inhibitor covering the miR-VA "A" sequence plus an additional 8 nucleotides upstream of this sequence, and a collection of supernatant for titering. Any or all of these differences in the protocol could contribute to the discrepancies between the experiments. Therefore, it would be beneficial to repeat



the experiment using the same conditions used in their study. However, from our initial analysis, it does not appear that miR-VA affects virus replication in this cell culture setting.

We also attempted to ascertain the function the miR-VA by identifying cellular mRNA targets that would be subject to miR-VA regulation. The TargetScan algorithm produced a high scoring match for miR-VA "A" to regulate cellular Dicer (Fig. 4.2). However, a series of tests for the ability of miR-VA to regulate these target sites in vitro and in cell culture failed to support the bioinformatics prediction (Figs. 4.5- 4.7). In all likelihood, the incorrect target prediction suffered from the fact that one of its most important parameters for significance, conservation of miRNA and target sequence across multiple species, had to be removed for this analysis. This was due to the fact that the human adenovirus exhibits species-specific tropism.

These experiments failed to uncover functional significance for miR-VA in our cell culture infection model. However, it is possible that the miRNA is not necessary for the virus in this artificial infection setting. To date, the examples of viral miRNAs from animal viruses appear to play important roles for the virus in the context of a host organism. The EBV-encoded miRNAs are alternately expressed in various stages of the virus life cycle, and look to be important factors in the establishment and maintenance of latency (147). Similarly, a miRNA produced from the herpes simplex virus latency-associated transcript (LAT) regulates apoptosis in infected cells, contributing to the persistence of the virus (57). The SV40-encoded miRNAs regulate viral transcript expression, thus limiting the level of T antigen produced by the virus in a mechanism

which renders the infected cells less immunogenic (171). All of these examples of viral miRNAs involve the regulation of factors involved in persistence and/or latency for the virus in a host. Therefore, it is possible that the role of miR-VA may not be properly studied in a cell culture setting. There is precedence for adenovirus to establish a persistent state of infection, as studies have described the isolation of adenovirus from cultured adenoid and lymphoid tissue of human subjects (181). Studies also demonstrated that the virus grew very slowly in infections of human lymphocytes, which may mimic the persistent state of infection (87). Perhaps it is in settings such as this that miR-VA will display its importance and function.

*Adenovirus affects Dicer at multiple levels*

Our studies to determine if miR-VA "A" regulated cellular Dicer levels initially led to the confusing result that both Dicer mRNA and protein are reduced during the course of adenovirus infection, though not through the direct action of miR-VA "A" (Figs. 4.3 & 4.4). This does not appear to be due to a global effect on host transcription, as data from several microarrays indicates that wholesale changes in gene expression do not occur during virus infection. Rather, at 20 hours post infection only ~100 cellular genes are significantly modulated in their expression (34, 50). We noticed in both qRT-PCR and western blot analysis that prior to the decrease in the levels of Dicer, there was an initial increase at ~12 hours post infection. This trend correlates well with the expression pattern seen for a variety of immunomodulatory genes initially upregulated by

adenovirus infection (50). This is suggestive that perhaps Dicer itself is a component of the host immune response, as it is being targeted for inhibition by the virus, and its expression patterns are similar to those seen for other immune defense genes.

Our analysis of adenovirus infections in different cell lines indicated that the observed reduction in Dicer levels inversely correlated with virus replication (Figs. 4.8 & 4.9). In 293 cells, where the dl331 virus displays a growth defect, we saw that Dicer levels were not compromised. In contrast, the dl331 virus induced a reduction in Dicer protein levels in HeLa cells, where the virus replicates at similar levels to WT Ad-5. It is likely that a viral gene product expressed at high levels in the setting of active virus replication is responsible for the observed effect on Dicer. Experiments using mutant virus deleted for various adenovirus genes would be useful in helping to determine which gene product causes this effect. Likely candidates would be the viral E3 gene, which performs a variety of immunomodulatory functions for the virus, or the E1A protein, which is responsible for the activation and repression of a wide assortment of host genes (165).

Interestingly, a published study reports that VA RNA suppresses the RNA silencing response, because extracts from 293 cells infected with WT virus are far less efficient at dicing a dsRNA substrate in *in vitro* reactions than extracts from dl331-infected 293 cells (4). However, our data showing Dicer protein levels decreasing in cells undergoing high levels of replication indicates that the effect described in this study may actually be due to low levels of Dicer in the WT extract in relation to the dl331 extract. Our testing of this hypothesis has been compromised by the fact that we are currently

unable to detect *in vitro* dicing activity in extracts from any cell line other than 293 cells, which appear to have higher levels of endogenous Dicer activity than the other human cell lines examined (Fig. 4.10).

Finally, we tested the effect of the level of Dicer protein in infected cells on virus propagation. This was initiated by the observation that dl331 virus has a growth defect in 293 cells, which in addition to expressing higher levels of PKR than HeLa cells, also contain higher levels of Dicer activity. We tested the role of Dicer during infection in these cells by either knocking down expression of Dicer with siRNAs (Fig. 4.11), or overexpressing Dicer cDNA in cells prior to infection (Fig. 4.12). We observed that neither pre-treatment of cells to distort Dicer levels made an appreciable change in the level of virus produced in the cells. Again, during this rapid cell culture model of infection there may not be enough time for the secondary effects of Dicer inhibition or overexpression to be realized.

#### *Assessing the impact of adenovirus on the host miRNA pathway*

The accumulating data suggesting that adenovirus targets human Dicer for regulation by several methods indicates that this molecule is an important host cell regulator in the context of infection (4, 111). A prediction from the studies would be that miRNA biogenesis would be affected in infected cells since Dicer function is impaired. We undertook several approaches to address this prediction. Our initial studies focuses on several individual miRNAs. If Dicer function is impaired in cells, then we would

expect to see an increase in the levels of pre-miRNAs and a concurrent decrease in the levels of mature miRNAs. We were initially unable to reliably detect human pre-miRNAs using DNA or RNA-based probes. However, we were successful in detecting miR-17-5p using an LNA probe (Fig. 4.13). Analysis of the pre-miR-17-5p did not reveal an increase in the precursor during infection. Instead, pre-miRNA levels appeared to decrease during adenovirus infection. We also did not observe a dramatic change in mature miRNA levels during infection. However, since this is only an analysis of a single miRNA species, we cannot make any broad conclusions about impact on miRNA biogenesis. A RISC-mediated target cleavage assay was next used to measure the level of endogenous let-7 activity in infected cells as well (Table 4.1). The measured concentration of let-7 in HeLa cell extract was in agreement with a previous study (65). We did not detect a significant change in the level of let-7-mediated target cleavage in infected cells. These results from a limited sampling of cellular miRNAs suggest that the cell culture model of infection for adenovirus may not produce large-scale perturbations of cellular miRNA biogenesis, despite the observed effects on Dicer. Although it has not been properly tested, it is believed that miRNA-RISC complexes are very stable, with a half-life that could be on the order of days to weeks (52). Therefore, the impact on RISC-associated miRNAs may not be fully realized at the observed timepoints in these infection studies.

We performed a more comprehensive analysis of the cellular miRNA pool in the face of infection by utilizing microarray technology to detect all known miRNAs and miRNA\*s. In confirmation of the northern blot analysis and let-7-RISC activity assay,

we saw that most cellular miRNAs did not change their expression significantly at 24 hours post infection (Fig. 4.17). However, we observed a small subset of miRNAs whose expression was misregulated during infection (Figs. 4.15 & 4.16). These miRNAs may prove to be important components in the establishment of an environment conducive to adenovirus infection. Or, alternatively, they may represent an attempt by the host cell to regulate specific genes as a defense mechanism during infection. Intriguingly, three of the five miRNAs specifically upregulated during adenovirus infection belong to the miR-17-92 oncogenic cluster. Adenovirus is characterized as a small DNA tumor virus, as selected strains of adenovirus can induce tumors in rodents (175). Additionally, adenoviruses transform cultured cells by the action of the viral oncogenes, E1A and E1B (33, 49, 164). Therefore, upregulation of these specific miRNAs fits into the transforming, oncogenic profile of adenovirus. Additional experiments need to be performed to determine the significance of these miRNAs during adenovirus infection.

Analysis of miRNA expression during HCMV infection revealed that, like adenovirus, the majority of host miRNAs were not significantly altered during infection (Figs. 4.17- 4.19). This larger, more complex DNA virus altered the expression of more miRNAs than adenovirus, and there was surprisingly little overlap in those miRNAs and miRNA\*s that were misregulated. Only miR-181b, miR-34c\*, and miR-135b\* were coordinately regulated in adenovirus and HCMV infection. If considerable overlap occurred in the characterization of miRNA upregulated or downregulated in response to both viruses, it might be concluded that those miRNAs regulated host defense factors. However, based on our results, it appears that viruses may induce specific miRNA

signatures. It will be interesting to perform similar experiments in a variety of cell lines to determine if these miRNA signatures persist. If so, then these patterns of regulation will provide important new clues about genes and pathways involved in virus infection.

# **CHAPTER V:**

# **DISCUSSION**



*A. dsRNA and the host response to virus*

Much like the non-specific mammalian immune response to dsRNA, the basic principle underlying the RNAi silencing mechanism observed in invertebrate and plant species is the general recognition of dsRNA as “foreign” material that must be dealt with rapidly. This cellular reaction likely stems from a universal association of dsRNA species with viral pathogens, since viral dsRNA species are spuriously produced during genome replication and bi-directional transcription activities. Therefore, viruses must find ways to circumvent this molecular bias against their dsRNA intermediates if they are to successfully establish infection of the host cell. To this end, numerous examples of inhibitors from mammalian viruses have been identified which perturb the actions of the innate dsRNA sensing molecules, such as PKR, 2-5A, RIG-I, and the TLRs (45, 76, 119, 120, 132, 152, 185). A similar story has emerged in plants and invertebrates, where it appears that RNA silencing pathways constitute the major anti-viral response. In response to this host defense system, inhibitors of various RNA silencing mechanisms have been detected in a wide range of insect and plant viruses (186).

The relationship between a virus and its host is like that of chess match, with each combatant strategically attempting to attack its opponent, and at the same time maintain an impenetrable defense. For nearly every host defense mechanism, there is a counter defense system implemented by the virus. Classic examples of this phenomenon include the multiple counter mechanisms that HCMV has developed to defend against multiple stages of MHC class presentation of viral antigen, and the multiple mechanisms utilized

by poxviruses to prevent interferon activation (133, 162). Adenoviruses similarly utilize both E1A-mediated repression of interferon response genes and VA RNA inhibition of PKR to abrogate the cellular interferon response (58, 149, 165). The underlying implication is that the more essential and powerful an immune response against a virus, the more the virus will need to adapt ways to circumvent the response(s). Using this line of thinking and considering the established precedence of virally-encoded suppressors of RNA silencing in plant and insect viruses, the hypothesis can be made that if RNA silencing is involved in the immune response to mammalian viruses, then mammalian viruses will correspondingly encode inhibitors of RNA silencing. However, in contradiction to this idea is the observation that some mammalian viruses encode miRNAs, which require RNA silencing activity for their function. This would imply that mammalian viruses would not desire to subvert the host silencing pathways.

The work presented in this dissertation explores the relationship between the host RNA silencing response and mammalian viruses, using adenovirus as a model virus. Our data as well as others suggests that the relationship between mammalian viruses and RNA silencing is more complex than what is seen in plants and insects. From our own work, we observe that adenovirus encodes miRNAs, yet infection by adenovirus also leads to a dramatic decrease in cellular Dicer levels. Reports in the literature have detailed examples of RNA silencing suppressors from mammalian viruses, as well as virally-encoded miRNAs. We will explore each of these aspects separately.

### **Mammalian viruses encode suppressors of RNA silencing**

Viruses are extremely efficient obligate parasites of host cells, as they maintain their coding information in compact genomes. Because of this, viruses do not encode factors that are non-essential to their replication and fitness in some cellular context. Viral genes that directly inhibit the function of a cellular gene product or pathway provide a strong indication that the inhibited host factor or pathway would normally serve to limit the infection of the virus. Therefore, the presence of virally encoded inhibitors of RNA silencing would imply that silencing pathways play a role in defense against the virus. In support of this idea, suppressors of RNA silencing have been identified in mammalian viruses. Studies using the E3L and NS1 dsRNA-binding proteins from vaccinia virus and influenza virus, respectively, demonstrated that these proteins could suppress anti-viral silencing mechanisms in plants and insect cells (21, 30, 103). These results do not point directly to a similar role in mammalian cells, since the experiments were performed in other systems. Indeed, this observed inhibition could be non-specific or not relevant to an infection in a mammalian cell where the primary role of these proteins is to block PKR activation. However, the presence of PKR and the IFN response in mammalian cells preclude the obvious experiment of performing analogous studies in mammalian cells. A more appropriate study provided two pieces of data indicating that RNA silencing responses were relevant to a host response to virus. It was demonstrated that a cellular miRNA was able to restrict the growth of primate foamy virus (PFV) in human cells. The virus was found to have a complementary site to the endogenous miR-

32 that is present in all viral transcripts. The virus was also found to encode an RNA silencing suppressor, allowing the virus to replicate in cells expressing miR-32. The suppressor protein, Tas, was also shown to inhibit silencing in mammalian cells as well as plant cells (91). These findings strongly suggested that RNA silencing is involved in a host response to virus.

Our work, along with others, has demonstrated that adenovirus uses multiple mechanisms to perturb Dicer expression and function. We find that following infection, an early increase in Dicer mRNA and protein levels is followed by a dramatic decrease (Fig. 4.3 & 4.4). This type of regulation is similar to what is seen for immune mediators in response to cytopathic adenovirus infection (50). Others have demonstrated that VA RNA can act as a suppressor of pre-miRNA and dsRNA processing (4, 111). The fact that the virus employs multiple mechanisms to counteract the expression and function of Dicer implies that this protein plays a role in host defense against the virus. In our hands, artificially misregulating Dicer expression prior to adenovirus infection did not affect the rates of virus replication (Fig. 4.11 & Fig. 4.12). This result implies that an RNAi-like response against viral dsRNA generated during infection is not a major determinant of the ability of the virus to grow in the 293 or HeLa cell settings. If viral siRNAs produced from viral dsRNA actively worked to limit virus transcription, then we would have expected to see an effect on virus replication when Dicer was overexpressed or repressed prior to infection. It is also formally possible that the MOI of virus used in these studies (MOI=3) and the subsequent rate of viral replication was too high for us to see an effect of Dicer misregulation. A lower MOI or a cell culture setting allowing for slower

replication of the virus may provide evidence of a Dicer-related effect on virus replication.

Taken as a whole, the results of our work and others, indicates that RNA silencing can impact the course of mammalian virus infection. However, at this point there is not enough evidence to suggest that RNA silencing is responsible for a general anti-viral role against mammalian viruses. Further experiments will be needed to test this theory. For our experiments, it would be valuable to establish an adenovirus infection setting that more closely approximates what occurs *in vivo*. Additional studies of mammalian viruses should also be performed to try to identify an anti-viral role for RNA silencing. Most plant viruses are RNA viruses, and therefore, most examples of plant virus-encoded silencing suppressors derive from RNA viruses. As such, it would be advantageous to look for evidence of silencing inhibitors in rapidly replicating RNA viruses. Further studies of DNA viruses should include vaccinia virus, since the E3L protein has been demonstrated to act as silencing suppressors in other systems and because the large genome of poxviruses encodes numerous immunomodulatory genes. The biggest obstacle to studying the effects of RNAi in mammalian cells is the presence of PKR. However, murine knockout PKR cells exist (167). It would be informative to perform experiments similar to our Dicer knockdown and overexpression studies in these cells and examine the effect on the growth of various viruses. We are unable to perform these studies with adenovirus type-5, since the virus is tropic for humans, not rodents. Similarly, other RNA silencing proteins, such as Ago-2 can be misregulated prior to infection in this system. However, a potential drawback to these studies is that it may be

difficult to achieve high levels of transfection efficiency in these cells. However, these experiments have the potential to shed light on an anti-viral role for RNA silencing in mammals.

### **Mammalian viruses encode miRNAs**

Just as the demonstration that viral suppressors of RNA silencing imply a role for those pathways in host defense, the presence of viral miRNAs in mammalian viruses implies that viruses may be dependent of RNA silencing for viral fitness. Several mammalian viruses have been shown to encode miRNAs through both experimental and computational methods (22, 29, 54, 57, 146, 147, 171). All of these miRNAs derive from DNA viruses, which typically have larger genomes and encode more genes than RNA viruses. Additionally, a cellular miRNA that appears to assist replication of hepatitis C virus in liver cells has been described (73). Our own studies of adenovirus VA RNA led to the identification of previously unidentified miRNAs, miR-VAs, encoded within VA RNA genes. Other groups have described these miRNAs as well (6, 158). We demonstrated that these miRNAs are loaded into Ago-2 RISC complexes and could perform cleavage of a target RNA, providing evidence that these are authentic miRNAs. The identification of viral miRNAs and demonstration that they contribute to viral fitness in some settings provides compelling evidence that mammalian viruses utilize and in some instances, require host RNA silencing pathways.

At this point, the validated targets of viral miRNAs have been involved in the development of a persistent or latent state of infection. The EBV miR-BART2 has perfect complementarity to the DNA polymerase gene, BALF5. The slicing of this viral transcript by miR-BART is thought to be an important autoregulatory function of the virus as it establishes latency (147). The SV40 virus miRNAs are thought to help the virus induce a persistent state of infection by slicing early mRNAs bearing perfect complementarity, thus reducing the level of immunogenic T antigen on the cell surface and making the cells less susceptible to attack by cytotoxic T cells (171). HSV encodes miRNAs from their latency-associated transcripts (LATs), and these miRNAs downregulate expression of cellular genes that induce apoptosis, thus allowing the latent virus to remain in its host cell (57).

In our experiments, we were unable to describe a target of miR-VA and did not observe a noticeable effect when we abrogated miR-VA function prior to infection. However, in our experimental setting, we were attempting to test the function of miR-VA under conditions where the lytic virus rapidly destroys its host cells. Based on the precedence of the other aforementioned viral miRNAs, it may be more appropriate to study the function of miR-VA in a context where adenovirus replicates more slowly. Reports of persistent adenovirus infection of adenoid and lymphoid tissues suggest that lymphocytes in these tissues may harbor the virus (181). Evidence from the literature further suggests that cultured human lymphocytes potentially provide a more appropriate setting to examine a persistent state of adenovirus infection (87). Studies in such a quasi-persistent setting may help us elucidate a function and/or cellular targets for miR-VA that

is masked in our highly lytic infection setting. It will also be beneficial to examine other mammalian viruses for the presence of miRNAs. To date, all the examples of viruses producing miRNAs are DNA-based and are capable of entering a persistent or latent stage of infection. Seemingly good candidates for analysis would be other viruses that have been demonstrated to show persistent or latent infections, such as parvoviruses, or the hepatitis B and hepatitis D viruses. For potential RNA viruses that produce miRNAs, retroviruses would seemingly be the most obvious candidates, since they integrate into the host genome, and their candidate miRNA genes could be expressed and processed just like endogenous cellular miRNAs.

### **The role of RNA silencing in the response to mammalian viruses**

The seemingly contradictory findings that some mammalian viruses encode RNA silencing suppressors and other viruses encode miRNAs makes it difficult to interpret the role of RNA silencing in mammalian virus infections. Certainly, the answer is not as straight forward as in plants and insects, which appear to use RNA silencing as a robust anti-viral response. In examining the data thus far, it would seem that the viral miRNA demonstrations provide compelling evidence that viruses may utilize host silencing responses to their own advantage. The evidence for the some of the potential silencing suppressors is clearly weakened by the fact that silencing suppression assays were performed in plants and insects due to the limitations of studies in human cells. It could be that RNA silencing mechanisms, although present, do not contribute strongly to an



anti-viral response. Mammals have evolved a complex and specific immune defense against viruses, with the early innate response to dsRNA followed by a specific, cell mediated response. Perhaps the effects of an RNAi response is negligible in this context. Additionally, the mammalian RNA silencing pathways are far less complex than those of plants, and even insects, which use the silencing pathways for viral defense. This again, raises skepticism about the robustness of an RNA silencing anti-viral response in mammals. Despite all these caveats, it is still difficult to overlook some of the data indicating that RNA silencing plays an active role in immune defense. The miRNA-based regulation of PFV virus and its corresponding silencing suppressor are strong indicators of an active role of RNA silencing in host defense. Additionally, in the case of HCV, a cellular miRNA appears to help define cellular tropism for the virus. It is easy to imagine that many cellular miRNAs are capable of targeting viral genes as well.

In actuality, it may be naïve to assume that RNA silencing either works “for” or “against” the mammalian viruses. Based on the current research, it appears that RNA silencing pathways play different roles for different viruses. The fact that all described viral miRNAs are encoded by DNA viruses which are able to attain a persistent or latent state of infection indicates that it is perhaps only these viruses which co-opt the use of the RNA silencing pathway for their own viral fitness. In contrast, RNA silencing machinery may play a role in defense against non-latent viruses like vaccinia and influenza. Selective action of an particular immune response against a virus are not unprecedented, as some mammalian viruses are clearly more susceptible to cell-mediated immunity and generate negligible antibody response. In other instances of viral defense, neutralizing

antibody responses play more prominent roles. It is not trivial to think that viruses may have similarly varying susceptibility to RNA silencing.

Even in our own experiments with adenovirus, we observe the seemingly contradictory findings that adenovirus produces miRNAs, but also limits Dicer expression during replication. One interpretation of this data would be to that the virus is interfering with RNA silencing pathways, and that the processing of miR-VA by Dicer and subsequent incorporation into RISC is simply a consequence of the virus attempting to saturate host silencing machinery. This model implies an anti-viral activity of the RNA silencing pathway, which adenovirus is non-specifically inhibiting by its expression of large amounts of VA RNA.

Given the data from other persistent/latency-associated DNA viruses which encode viral miRNAs, it seems that the production of miR-VA by adenovirus is not just a coincidental artifact to VA RNA saturating the RNAi pathway. Rather, it may be part of the mechanism that helps adenovirus establish a persistent infection in appropriate settings. Our kinetic analysis of miR-VA accumulation versus full length VA RNA accumulation correlates well with the observed decrease in Dicer protein levels over the course of infection (Figs. 3.5 & 4.3/4). In another model, we would propose that VA RNA is processed to miR-VA at very early times post infection. We observe that the accumulation of miRNA accumulation levels off quickly, possibly indicating saturation of RNA processing components. Since miRNA-RISC complexes appear to be highly stable, the miR-VA incorporated into RISC at these early times post-infection would provide adenovirus with a pool of functional miRNA to target specific cellular genes that

help the virus maintain a persistent infection (52). Our kinetic analysis of miR-VA versus full length VA RNA accumulation also indicates that when miR-VA accumulation is leveling off (~ 12 h.p.i.), the production of VA RNA is greatly increased. At these times, very high levels of viral transcription are occurring, thus increasing the likelihood that dsRNA intermediates are being produced. The production of high levels of VA RNA at this time allows the VA molecules to saturate PKR, thus preventing recognition of viral dsRNA and the shutoff of host cell translation. This model provides the rationale for how the virus would quickly produce a miRNA promoting persistent infection before Dicer levels are reduced transcriptionally, and also allows for the virus to maintain regulation of PKR at appropriate times during infection. This model additionally supports the idea that the RNA silencing pathways can be both exploited by a virus to perform a function that aids in viral persistence/fitness, and perform anti-viral regulatory functions.

## **Summary**

In summary, the data from this thesis presents evidence that human adenoviruses encode miRNAs, produced from the VA RNA locus of the virus. Additionally, we show that virus infection leads to a decrease in expression of Dicer, a key component of mammalian gene silencing pathways. While the role of these interactions with RNA silencing pathways remains to be fully elucidated, evidence from other viruses indicates that these interactions may have important ramifications for viral fitness and immune

evasion. Finally, global studies of miRNA expression profiles during viral infections indicate that viruses may produce specific miRNA signatures. These studies implicate a potential role of RNA silencing mechanisms as participants in the host response to animal viruses.

## **CHAPTER VI:**

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