Expression and Function of MicroRNAs in Viruses Great and Small

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Since they employ host gene expression machinery to execute their genetic programs, it is no surprise that DNA viruses also encode miRNAs. The small size of viral genomes, and the high degree of understanding of the functions of their gene products, make them particularly favorable systems for the examination of miRNA biogenesis and function. Here we review our computational and array-based approaches for viral miRNA discovery, and we discuss the structure and function of miRNAs identified by these approaches in polyomaviruses and herpesviruses.

MicroRNAs (miRNAs) mediate posttranscriptional gene regulation in most eukaryotes and have been shown to play important regulatory roles in many cellular processes, including development, differentiation, metabolic control, apoptosis, and tumorigenesis (for review, see Du and Zamore 2005; Hammond et al. 2005; Kim 2005). In the human genome alone, more than 460 miRNAs have been identified (miRBase, <http://microrna.sanger.ac.uk/sequences/index.shtml>) (Griffiths-Jones 2004). MicroRNAs are derived from primary nuclear pol II transcripts (pri-miRNAs), which can be thousands of nucleotides in length. Processing of these RNAs by the nuclear microprocessor complex (which includes the enzyme Drosha) yields 60–80-nucleotide imperfect hairpins known as pre-miRNAs (Lee et al. 2002, 2003; Denli et al. 2004; Gregory et al. 2004; Han et al. 2004; Landthaler et al. 2004; Zeng et al. 2005). These pre-miRNAs are transported to the cytosol (Yi et al. 2003; Bohnsack et al. 2004; Lund et al. 2004; Zeng and Cullen 2004), where another cellular enzyme, Dicer, processes them to result in the mature approximately 22-nucleotide miRNA (Bernstein et al. 2001; Grishok et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001; Chendrimada et al. 2005; Forstemann et al. 2005; Gregory et al. 2005; Jiang et al. 2005; Saito et al. 2005). The resulting miRNA enters the multiprotein RNA-induced silencing complex (RISC), where it is hypothesized to scan translating RNAs and direct their cleavage if found to have a perfect match (similar to siRNAs), or translational repression if bound to the RNA with imperfect homology (Hamilton and Baulcombe 1999; Tuschl et al. 1999; Zamore et al. 2000; Grishok et al. 2001; Hutvagner et al. 2001; Doench et al. 2003; Zeng et al. 2003). Because DNA viruses generally employ host pol II machinery to express their genes, it is expected that many such viruses will encode miRNAs—a prediction that was validated by Pfeffer et al. (2004), who first cloned miRNAs from cells infected with several herpesviruses (Pfeffer et al. 2004, 2005).

Despite rapid progress in understanding miRNA biogenesis, the functions of the vast majority of miRNAs remain unknown. The large size of the human genome and the incompletely understood nature of the events governing target recognition are the principal reasons that the targets of most cellular miRNAs are not yet known. The fact that many cellular cDNAs are of unknown function further complicates the task of deciphering the biology of many host miRNAs. In contrast, viral genomes are small, and a substantial percentage of their gene products have well-understood activities or function in known pathways. This makes viral genomes a favorable place to study the biogenesis and function of miRNAs (Sullivan and Ganem 2005). With this in mind, we have begun to search for miRNAs in DNA viruses and to capitalize on the well-understood biology of their viral progenitors to explore their biological function.

To this end, we developed a computer program, called v-miR, to screen viral genomes for inverted repeats with the properties of pre-miRNAs (as defined from a library of known human miRNAs that had been identified by cDNA cloning). On the basis of these properties, each predicted hairpin is assigned a numerical score; the higher the score, the greater the similarity of the hairpin to known pre-miRNAs (for details of the algorithm, see Grundhoff et al. 2006; the program is available to all labs on request). The program typically identifies many more hairpins than are actually involved in generating miRNAs in vivo, but it represents a useful screening tool. To evaluate the utility of the program, we decided to examine its performance on a small (5 kb), well-understood DNA virus, SV40.

SV40, a member of the polyomavirus family, causes a largely asymptomatic renal infection in its natural simian host. (However, when injected into rodents, in which it cannot efficiently complete its full replicative cycle, it causes fibrosarcomas—and it is this unnatural property, rather than its authentic biology, that has attracted the most experimental interest.) The SV40 replicative cycle is one of the best understood genetic programs in animal virology

(for review, see Cole 1996). The circular 5-kb dsDNA genome directs expression of two major transcription units, early and late. Early mRNAs represent a family of spliced transcripts that encode large and small T antigens, regulatory proteins whose role in productive infection is primarily to promote viral DNA replication (for review, see Sullivan and Pipas 2002). Following T-antigen accumulation, multimers of large T bind to the viral origin of DNA replication and trigger genomic replication. Following the onset of DNA synthesis, the late transcription unit is activated, generating a series of spliced mRNAs encoding the viral structural proteins. Following accumulation of the late proteins, mature virus particles are assembled, and cell lysis releases the infectious progeny viruses. As shown in Figure 1, the early and late mRNAs are transcribed from opposite strands on the circular genome and overlap one another in the region bounded by their respective polyadenylation signals. These poly(A) signals are not 100% efficient, and longer primary transcripts resulting from readthrough of these signals have been detected in infected cells (Acheson 1978).

Figure 2A shows the readout of the v-miR program on the late strand of the SV40 genome, with hairpin scores displayed as a function of map position. The highest-scoring hairpin in Figure 2A maps just downstream from the late poly(A) site (Fig. 1). Although another equally high-scoring hairpin was identified on the early strand (not shown), only the late-strand candidate pre-miRNA was detected by northern blot analysis. When infected cells are examined with probes from the region of the hairpin, species with the mobilities of pre-miRNA and miRNAs are readily identi-

fied (Fig. 2B). As expected from their polarity and map position, they accumulate at late times after infection (Fig. 2C). Close inspection of the northern reveals that multiple species of miRNA are visible in the 20–24-nucleotide region of the gel, and nuclease mapping confirms that both strands of the hairpin give rise to miRNAs that can be incorporated into RISC (Sullivan et al. 2005).

The location of these miRNAs indicates that they possess perfect complementarity to early (T antigen) mRNAs (Fig. 1). As such, they would be expected to trigger cleavage of those mRNAs, much as would a siRNA. Northern blotting for early mRNAs revealed the presence of a collection of small (~300 nucleotides) polyadenylated RNA fragments that accumulated preferentially at late times (Fig. 3); mapping of these fragments identified it as the probable 3' product of miRNA-mediated cleavage of early mRNA, since their 5' ends mapped to the regions of miRNA complementarity (not shown). To verify this, we constructed a mutant of SV40 that was incapable of generating the miRNAs. This was done by engineering multiple point mutations into the predicted pre-miRNA hairpin so as to disrupt its structure and prevent Dicer-mediated processing to the mature miRNAs. As shown in Figure 3, this mutant SV40 virus was unable to generate the predicted cleavage product following infection; as expected, mutant-infected cells accumulated enhanced levels of T-antigen mRNA and proteins. However, the mutant had no growth defect: A careful one-step growth curve reveals wild-type and mutant viruses to grow to identical titers with identical kinetics (Fig. 4). This indicates that the excess T antigen generated by the mutant serves no replicative purpose.

Why, then, has evolution selected for the production of the miRNA? One explanation relates to the fact that T antigen appears to be the major target of cytotoxic T lymphocytes (CTLs) directed against the virus. If so, then down-regulation of T-antigen synthesis might be expected to reduce susceptibility to CTL-mediated lysis. To test this, we infected simian cells bearing murine MHC-I chains with wild-type or mutant SV40, and examined susceptibility to lysis by murine CTLs directed against several epitopes of T antigen, using ^{51}Cr release assays. Figure 5 shows that cells expressing the miRNA are indeed less susceptible to lysis by CTLs; this effect could be overcome by high multiplicity of infection (not shown), suggesting that it results from reduced antigen levels, and not from some special immunomodulatory effect of the miRNA.

The SV40 miRNAs described here are conserved in all SV40 isolates, and orthologs are found in most primate polyomaviruses but are not conserved in murine polyomavirus (Py). However, examination of the murine Py sequence with v-miR (Fig. 6) reveals that the top-scoring hairpin is in a different genomic location but is also found on the late strand, 3' to the late poly(A) site, although much farther downstream from it than the SV40 miRNA (Fig. 1). Interestingly, 25 years ago, R. Treisman, while mapping 5' and 3' ends of Py late RNAs, identified ends consistent with a structure identical to this hairpin, and speculated that they might have been generated by an RNase-III-like enzyme (a prescient suggestion that foreshadowed the fact that Drosha is an RNase III family

Figure 1. Transcript map of SV40. Shown are the early (left) and late (right) transcripts (depicted as closed arrows); coding regions are shown as open arrows. The SV40 and Py pre-miRNA hairpins are shown; both are of late polarity, but map to the central (Py) or distal (SV40) regions of the T antigen. Note that the pre-miRNAs are antisense to the early transcripts encoding T-antigen proteins. (Modified, with permission, from Sullivan et al. 2005 [Nature Publishing Group]).

Figure 2. SV40 encodes a miRNA. (A) v-Mir prediction of pre-miRNAs for the SV40 genome in the late orientation. Each dot represents a candidate pre-miRNA. The vertical axis shows the v-Mir score; the higher the score, the more likely a candidate is a bona fide miRNA. Circled is the confirmed SV40-encoded pre-miRNA. (B) Northern blot analysis confirms SV40 encodes a miRNA. The left panel diagrams the three probes used in this figure. Arrows identify miRNAs generated from each arm of the pre-miRNA hairpin structure (5' and 3' probe). The control probe (TL probe) that is directed against the terminal loop only recognizes the pre-miRNA 57-nucleotide band and not the ~22-nucleotide miRNAs, demonstrating the specific processing of the stem into miRNAs. (C) Northern blot analysis of RNA harvested from cells at various times postinfection. Arrows indicate bands that correspond to miRNAs.

Figure 3. SV40 miRNA directs cleavage of early transcripts. Shown is northern blot of poly(A) purified RNA from cells infected with wild type (WT) or mutant (SM) virus at various times postinfection. The band that corresponds to the cleavage fragment is marked with an asterisk. (Modified, with permission, from Sullivan et al. 2005 [Nature Publishing Group].)

Figure 4. The SV40 miRNA mutant (SM) virus grows as well as wild type (WT) in cultured cells. Shown is a one-step growth curve of virus harvested at various times postinfection from Bsc40 monkey kidney epithelial cells that were infected at a multiplicity of infection of 5 plaque-forming units per cell. (Modified, with permission, from Sullivan et al. 2005 [Nature Publishing Group].)

Figure 5. Cells infected with SV40 miRNA mutant (Sm) are less susceptible to cytotoxic T lymphocyte (CTL)-mediated lysis. Simian cells which express a murine class I allele were infected at a multiplicity of infection of 1 plaque-forming unit per cell with either the miRNA mutant virus (Sm, *black bars*) or wild-type virus (WT, *white bars*) at various ratios of murine CTLs (that recognize an epitope in large-T antigen) to target infected cells. (Modified, with permission, from Sullivan et al. 2005 [Nature Publishing Group].)

member) (Treisman 1981; Treisman and Kamen 1981). In 1982, Fenton and Basilico identified a fragment of early mRNA from this region that is exactly the size predicted for cleavage generated from the predicted miRNA; as expected, this fragment was detected only at late times postinfection (Fenton and Basilico 1982). We have verified that this miRNA is indeed made (C.S. Sullivan, unpubl.); together with the cleavage fragments identified by Fenton and Basilico (1982), this strongly indicates that the overall strategy of down-regulating early mRNA at late times with miRNA-directed cleavage is a conserved feature of polyomavirus biology.

The demonstrated utility of v-miR on small DNA virus genomes emboldened us to examine its ability to identify miRNAs in herpesviruses, a family of large, enveloped DNA viruses whose genomes encode 100–150 genes. We chose two herpesviruses for study—Kaposi's sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV). Both are lymphotropic DNA tumor viruses that reside in B lymphocytes and are linked to B-cell lym-

phomas; KSHV also produces the endothelial neoplasm KS. Both viruses are known to produce miRNAs. Exhaustive cloning in KSHV-infected B cells had previously identified 11 miRNAs (Cai et al. 2005; Pfeffer et al. 2005; Samols et al. 2005), and cloning from EBV-infected lymphoblastoid cells resulted in identification of 5 miRNAs (Pfeffer et al. 2004). Thus, an empiric database of identified miRNAs existed, against which we could calibrate our approach.

The large size of these viral genomes (~165 kb) indicated that v-miR would identify too many hairpins to consider using northern blotting as the secondary screen, as we had earlier done for polyomaviruses. In fact, with screening parameters (filters) similar to those used for SV40 (Fig. 1), more than 3000 hairpins were identified in KSHV alone. More advanced computational strategies and stringent filtering (based in part on the expanded number of cellular miRNAs that have been cloned) reduce this number considerably, but still leave many hairpins to screen. We therefore turned to a microarray-based approach, details of which can be found in Grundhoff et al. (2006). Briefly, for KSHV we constructed two custom arrays: (1) a "hairpin array," made up of the 3000 hairpins predicted by v-miR; and (2) a "tile array," produced by tiling across the viral genome with 50-nucleotide oligonucleotides, in 500-nucleotide steps. RNA was prepared from a KSHV-positive lymphoma cell line (BCBL-1), and from KSHV-negative BJAB cells, then differentially labeled and hybridized to the arrays. In addition, we prepared BCBL-1 RNA corresponding to the 20–25-nucleotide fraction (enriched for bona fide miRNAs, as well as containing nonspecific RNA degradation products) and to the 30–40-nucleotide fraction (a control fraction representing nonspecific degradation products alone). Again, these two preps were differentially labeled and hybridized to the arrays. Viral sequences that hybridized preferentially to infected cell RNA over uninfected cell RNA, and to the probes from the 20–25-nucleotide fraction over the 30–40-nucleotide fraction, were selected for further analysis. This involved northern blotting of BCBL-1 RNA (as compared to BJAB

Figure 6. Polyomavirus is predicted to encode a miRNA. v-Mir prediction of pre-miRNAs for the PyV genome in the late orientation. See legend for Fig. 2A. Circled is the top-scoring predicted pre-miRNA, identical to the hairpin structure originally hypothesized by Treisman (see Treisman and Kamen 1981) to be processed by an RNase-III-like enzyme. The position of this hairpin on the genome is shown in Fig. 1.

RNA) looking for virus-specific bands of 20–25 nucleotides. Figure 7 shows the results of these analyses, for both KSHV and EBV. The KSHV experiments identified 9/11 previously known miRNAs, plus one additional species that had escaped earlier detection for trivial reasons (it harbors a cleavage site for a restriction enzyme that had been used in the cloning procedure). In EBV, 18 miRNAs were identified—a large increase over earlier cloning experiments, although most of this difference is attributable to the fact that the EBV strain used previously harbors a large deletion in a region that encodes a large cluster of miRNAs. Although the functions of all of these herpesviral miRNAs remain unknown, our results establish that v-miR can be useful in the screening of large viral genomes, when used in conjunction with additional molecular screening methods.

What does the future hold for the study of viral miRNAs? Now that good methods exist for identification of such RNAs, we can expect an avalanche of new miRNA sightings. The challenge now is to discern their function(s), a mission that will begin with identification of their molecular targets. For those miRNAs with host RNA targets, this exercise will likely be as difficult as it is for cellular miRNAs. However, we can anticipate that many viral miRNAs will have viral targets—as in the polyomaviruses. For these, not only will target identification be simpler, but divining the biological significance of the interaction should also be more straightforward, since the pathways in which many viral genes function are already

known. But this, of course, is nothing new: It is precisely these features of viral genomes—small size, limited complexity, and exploitation of host functions—that brought them (as phages) to the attention of geneticists 50 years ago, at the dawn of the age of molecular biology.

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Figure 7. Identification of novel miRNAs in gamma-herpesviruses using a combined computational / microarray approach. Candidate pre-miRNAs were predicted using v-Mir, and those candidates that scored positive on microarray analysis were further validated via northern blot analysis. Northern blots using different probes identify miRNAs expressed by (A) Kaposi's sarcoma-associated herpesvirus (KSHV), and (B) Epstein-Barr virus. Candidates were considered bona fide miRNAs if they showed a distinct band around 22 nucleotides that was not detectable in RNA from uninfected cells. In each panel, RNA from uninfected (left lane) and infected (right lane) cells was probed. EtBr staining of low-MW 5S rRNA and tRNA is shown as a load control (bottom panels). (Modified, with permission, from Grundhoff et al. 2006 [©RNA Society].)

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Cold Spring Harbor Symposia on Quantitative Biology

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Cold Spring Harb Symp Quant Biol 2006 71: 351-356

Access the most recent version at doi:[10.1101/sqb.2006.71.065](https://doi.org/10.1101/sqb.2006.71.065)

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