Insights into *Polyomaviridae* MicroRNA Function Derived from Study of the Bandicoot Papillomatosis Carcinomatosis Viruses

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Several different members of the *Polyomaviridae*, including some human pathogens, encode microRNAs (miRNAs) that lie antisense with respect to the early gene products, the tumor (T) antigens. These miRNAs negatively regulate T antigen expression by directing small interfering RNA (siRNA)-like cleavage of the early transcripts. miRNA mutant viruses of some members of the *Polyomaviridae* express increased levels of early proteins during lytic infection. However, the importance of miRNA-mediated negative regulation of the T antigens remains uncertain. Bandicoot papillomatosis carcinomatosis virus type 1 (BPCV1) is associated with papillomas and carcinomas in the endangered marsupial the western barred bandicoot (*Perameles bougainville*). BPCV1 is the founding member of a new group of viruses that remarkably share distinct properties in common with both the polyomavirus and papillomavirus families. Here, we show that BPCV1 encodes, in the same orientation as the papillomavirus-like transcripts, a miRNA located within a long noncoding region (NCR) of the genome. Furthermore, this NCR serves the function of both promoter and template for the primary transcript that gives rise to the miRNA. Unlike the polyomavirus miRNAs, the BPCV1 miRNA is not encoded antisense to the T antigen transcripts but rather lies in a separate, proximal region of the genome. We have mapped the 3′ untranslated region (UTR) of the BPCV1 large T antigen early transcript and identified a functional miRNA target site that is imperfectly complementary to the BPCV1 miRNA. Chimeric reporters containing the entire BPCV1 T antigen 3′ UTR undergo negative regulation when coexpressed with the BPCV1 miRNA. Notably, the degree of negative regulation observed is equivalent to that of an identical reporter that is engineered to bind to the BPCV1 miRNA with perfect complementarity. We also show that this miRNA and this novel mode of early gene regulation are conserved with the related BPCV2. Finally, papillomatous lesions from a western barred bandicoot express readily detectable levels of this miRNA, stressing its likely importance in vivo. Combined, the alternative mechanisms of negative regulation of T antigen expression between the BPCVs and the polyomaviruses support the importance of miRNA-mediated autoregulation in the life cycles of some divergent polyomaviruses and polyomavirus-like viruses.

MicroRNAs (miRNAs) are small, approximately 22-nucleotide (nt) regulatory RNA molecules that have been shown to play an important role in numerous fields, including virology and immunology (reviewed in references 3, 9, 37, and 43). Virus-encoded miRNAs that target viral or host transcripts have been identified (35, 38). In some cases, mutant viruses defective for miRNA production have been shown to have altered activities of obvious relevance to virus infection. Some activities associated with virus-encoded miRNAs include evasion of the innate immune response (36) and control of the switch from latent to lytic infection (24, 26). Thus, miRNAs represent a strategy used by several different virus families for optimizing their infectious life cycle.

miRNA biogenesis has been reviewed in depth (see references 12 and 22). In brief, miRNAs are derived from longer primary transcripts (pri-miRNAs) that contain a hairpin structure called a precursor miRNA (pre-miRNA). The nuclear endonuclease Drosha “liberates” the hairpin pre-miRNA (23, 47), which is then exported to the cytoplasm and further processed into the 22-nt form by the Dicer endonuclease (8, 20). This final miRNA product is stabilized within the cytoplasmic multiprotein RNA induced silencing complex (RISC) (18). RISC-bound miRNAs associate with mRNA target transcripts, typically by binding to the 3′ untranslated (UTR) region with imperfect complementarity (3). This results in inhibition of protein production and the indirect nucleolytic turnover of the targeted transcripts. However, though it is rare in animals, miRNAs encoded by plants and some viruses can also bind to target mRNAs with perfect complementarity and direct a specific, RISC-mediated endonucleolytic cleavage of that targeted transcript. Typically, this cleavage occurs in the coding portion of the transcript. In this mode, miRNAs act via a mechanism that is indistinguishable from small interfering RNA (siRNA)-directed transcript cleavage (12).

Polyomaviruses are small, nonenveloped viruses that contain double-stranded circular DNA genomes of approximately 5 kb and have been identified from various avian and placental mammalian hosts (reviewed in references 14 and 21). We have previously shown that several members of the *Polyomaviridae*, including three human pathogens, encode miRNAs that lie antisense to and with perfect complementarity to the early transcripts (11, 33, 34, 40, 41). The early transcripts give rise to the tumor antigen proteins (T antigens). These immunogenic proteins perform various cell cycle regulatory and other func-
tions to initiate and carry out viral genome replication. We have identified miRNAs from human host viruses (JC virus [JCV] [34], BK virus [BKV] [34], and Merkel cell carcinoma-associated polyomavirus [MCV] [33]), Old World monkey host viruses (SA12 [11] and simian virus 40 [SV40] [40]), and the murine polyomavirus (muPyV) [41]. In all cases, these miRNAs lie antisense to the early transcripts, albeit their position within the genome can vary by thousands of nucleotides. Analysis of early transcripts and proteins, as well as chimeric reporters, confirms that these miRNAs can direct sRNA-like cleavage of the early T antigen transcripts. This can result in a reduction in early protein levels in cell culture models of lytic infection (40, 41) and thus represents a form of viral autoregulation of gene expression.

The fact that such evolutionarily divergent members of the Polyomaviridae all encode autoregulatory miRNAs underscores the likely importance of this mode of gene expression control. However, there are several observations that make the importance of this regulation unclear. First, it is possible that some polyomaviruses might not encode miRNAs. For example, computational prediction using the Vmir software package (11, 17, 39) fails to identify strong pre-miRNA candidates from the WU virus (unpublished observations). Second, the degree of negative regulation imparted on the early gene products is only partial; abundant early protein levels are detectable at late times of lytic infection with wild-type virus, and inhibition of these miRNAs leads to only minor (a few fold or less) increases in early protein levels (34, 40, 41). Third, lytic infection of cultured cells with mutant SV40 or muPyV viruses (that are defective for miRNA production) shows kinetics and virus yields similar to those for infection with wild-type viruses (40, 41). Finally, acute in vivo infection of mice with the miRNA mutant muPyV shows virus yields that are similar to those of wild-type virus (41). In summary, the conserved autoregulatory activity of the polyomavirus miRNAs implies importance; however, numerous observations (as discussed above) make the degree and context of this importance unresolved.

To better understand polyomavirus miRNA function, we sought to determine if an evolutionarily distant group of viruses that retain some features of the Polyomaviridae might also encode miRNAs and employ similar modes of gene regulation. Bandicoot papillomatosis carcinomatosis virus types 1 and 2 (BPCV1 and BPCV2) comprise a fascinating group of marsupial viruses that share distinct characteristics of both the Polyomaviridae and Papillomaviridae (7, 46). The BPCV viruses encode T antigen early proteins and have a genomic organization similar to that of polyomaviruses. Like all polyomaviruses, the BPCV genomes consist of an origin flanked with promoters of opposing orientation that encode either the early regulatory or late structural gene products. However, the size of the BPCV genome and the genes encoding the late proteins (L1 and L2) are most similar to those of the papillomaviruses. It has been theorized that the BPCVs are derived from an ancient coinfection recombination event between a polyomavirus and a papillomavirus (5).

Bandicoots comprise a genus of small to medium-sized omnivorous marsupials. BPCV1 infects western barred bandicoots (Perameles bougainville), an endangered species whose only remaining natural range is on the Bernier and Dorré Islands in Western Australia. Infection with BPCV1 is associated with an often fatal papillomatosis and carcinomatosis disease that is endemic among the western barred bandicoots and has therefore hindered repopulation efforts (45). BPCV2 is a related virus that was isolated from a papillomatous lesion from a southern brown bandicoot (Isoodon obesulus) (7). BPCV1 and -2 display the hallmarks of a common evolutionary ancestor, possessing similar genomic organizations and gene products, and are ~85% identical in nucleotide sequence. Here, we show that both BPCV1 and -2 encode evolutionarily conserved miRNAs that bind to and negatively regulate transcripts containing the 3′ UTR of the large T antigen transcripts. This novel mechanism of T antigen regulation lends strong support to the importance of miRNA-mediated autoregulation in the polyomaviruses and polyomavirus-like viruses.

MATERIALS AND METHODS

Ethics statement. The use of animals was noted as tissue samples, blood samples, and cadavers collected at the Kanyana Wildlife rehabilitation center, and therefore, a formal approval application was not necessary as determined by the Murdoch University Department of Environment and Conservation Animal Ethics Committee.

Cell culture and RNA isolation. Human embryonic kidney (HEK) 293 and 293T cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Cellgro, Virginia). Total RNA was harvested using an in-house PIG-B solution (2 M guanidinium thiocyanate [EMD, New Jersey], 20 mM citrate buffer, pH 4.5, 5 mM EDTA [Fisher Scientific, New Hampshire], 0.25% Sarkosyl [Sigma Aldrich, Missouri], 48% saturated phenol, pH 4.5 [Amresco, Ohio], 2.1% isomyl alcohol [Fisher Scientific], 0.5% β-mercaptoethanol [Sigma Aldrich], 0.1% 8-hydroxyquinoline [EMD], and 0.0025% Coomassie blue [EMD]) as described previously (25a, 34, 44a).

Vector construction, transfection, and Northern blot analysis. All DNA vector constructs were confirmed by sequence analysis through the Institute of Cellular and Molecular Biology Sequencing Facility at the University of Texas at Austin. A plasmid containing the entire BPCV1 genome (kindly provided by Lucy Woolford, Murdoch University, Australia) was digested with the restriction enzyme BglII/XhoI sites of the pcDNA3.1neo expression vector, removing the cytomegalo virus (CMV) promoter in the process. The primers used were as follows: BPCV1 NCR2 forward primer, ATCGATCGAAGATCTGGAGAAAGTTCTTGTACGAGACGAC and BPCV1 NCR2 reverse primer described above to generate the second fragment. The plasmid pcDNA3.1-BPCV1-NCR2, which contains the entire BPCV1 noncoding region 2 (NCR2) and partial UTR of the large T antigen, was cloned into the XhoI site of the pcDNA3.1puro expression vector. The resulting BPCV1 expression vectors were named pBPCV1-Early and pBPCV1-Late. The plasmid pcDNA3.1-BPCV1-NCR2, which contains the entire BPCV1 noncoding region 2 (NCR2) and partial large T antigen and L1 open reading frame, was generated by cloning an approximation of 1,500-bp PCR-amplified fragment of the BPCV1 genome into the BglII/Xhol sites of the pcDNA3.1neo expression vector, removing the cytomegalovirus (CMV) promoter in the process. The primers used were as follows: BPCV1 NCR2 forward primer, ATCGATCGAAGATCTGGAGAAAGTTCTTGTACGAGACGAC, paired with the BPCV1 NCR2 reverse primer, TAGCTAGCTCGAGGCCAACGCCTAAAGCAGAACTTG.

The plasmid pcDNA3.1-BPCV1-Dpro, in which the putative promoter in NCR2 is replaced with a unique KpnI restriction site, was generated through a three-way ligation between two PCR-amplified NCR2 fragments into the BglII/Xhol sites of the pcDNA3.1neo expression vector. The primers used were as follows: BPCV1 NCR2 KpnI reverse primer, ATCGATCGAAGATCTGGAACATCCCCTAAC, paired with the BPCV1 NCR2 forward primer described above to generate the first fragment, and the BPCV1 NCR2 KpnI forward primer, ATCGATCGAAGATCTGGAGAAAGTTCTTGTACGAGACGAC, paired with the BPCV1 NCR2 reverse primer described above to generate the second fragment. The plasmid pcDNA3.1-BPCV1-DproNCR2, in which the entire NCR2 is deleted except for the BPCV1 promoter, was generated by digesting pcDNA3.1-BPCV1-NCR2-Dpro using KpnI and BglII. The digested vectors were blunt ended using Klenow fragment (New England Biolabs) and 10 mM deoxynucleoside triphosphates (dNTPs) (Invitrogen, California) at 37°C for 30 min. Following Klenow fragment fill-in, the vectors were gel purified (Fermentas, Maryland) and self-ligated using T4 DNA ligase (New England Biolabs). All constructs were linearized by Sall restriction digestion prior to transfection into 293 cells. The chimeric truncation construct containing an S40 early promoter is the nonlinearized form of pcDNA3.1-BPCV1-DproNCR2.

The plasmids pcDNA3.1-BPCV1-mir-B1 and pcDNA3.1-BPCV2-mir-B1, which express the BPCV1 miRNA and BPCV2 miRNA, respectively, were gen-
erated by cloning an ~400-bp PCR-amplified fragment of either the BPCV1 genome or the BPCV2 genome into the KpnI/XhoI sites of the pcDNA3.1neo expression vector. The primers used were as follows: BPCV1-miR-B1 forward primer, ATCGATCGAGGTACCCCGGATGTCGGCAGTATTTTGCTTATGTG, and BPCV1-miR-B1 reverse primer, ATCGATCGACTCGAGGAAGCTTAACTTATGACTCC; BPCV2-miR-B1 forward primer, ATCGATCGAGGTACCCCGGATGTCGGCAGTATTTTGCTTATGTG. 293T cells were plated in 6-well plates and transfected using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. 293T cells were transfected with empty pcDNA3.1neo vector as a negative control. Total RNA was harvested at 48 h posttransfection. Ten micrograms of total RNA was separated on a Tris-borate–EDTA–Urea–15% polyacrylamide gel. The gel was transferred onto an Amersham Hyper N+ membrane (GE Healthcare, Illinois) and probed for miRNA as previously described (17). The probe sequences used were as follows: BPCV1 3p probe, GGCACAATCTCAGCAATGAT, BPCV1 5p probe, AAGCTACTAAGGCTGAGTGTCGGCAGTATTTTGCTTATGTG, and BPCV1 loop probe, TATTATACCTCGGGTATTAAAAAG TACTA; BPCV2 downstream flank probe, GAGATTTTCTTATGATGTCATTACGTAAGG.

**Computational prediction of viral pre-miRNAs, promoters, and polycadenylation sites.**

The viral pre-miRNA prediction algorithm, Vmir (24, 25), was used to obtain candidate pre-miRNAs from the genomes of BPCV1 and BPCV2 (genomic accession number NC_010107.1 and NC_010817, respectively). The secondary structure of pre-miRNAs were predicted via the mfold RNA folding prediction web server (48). The intrinsic promoters for both BPCV1 and BPCV2 were predicted via the Berkeley Drosophila Genome Project web server (7, 46). The polycadenylation sites for both BPCV1 and BPCV2 were determined with a default minimum promoter cutoff score of 0.80 (between 0 and 1.00). To predict the polycadenylation recognition and/or cleavage site of the BPCV T antigen transcripts, two different algorithms were used (13, 42).

**Small RNA library generation and computational analysis of sequencing reads for SOLID.**

293T cells were plated in 75-flasks and transfected with either pBPCV1-Early or pBPCV1-Late, using the Lipofectamine 2000 reagent as described above. Total RNA was harvested at 48 h posttransfection as described above. One hundred sixty micrograms of total RNA was gel fractionated to isolate small RNAs. The gel fraction containing the ~10- to 70-nt size ranges was excised. The excised gel fragment was cut into smaller pieces and soaked in 30 ml of 1 M NaCl (Ambion, Texas) for ~40 h at 4°C while under constant rotation using a LabQuake shaker rotisserie (Thermo Fisher Scientific). The supernatants were concentrated through centrifugation with the Vivaspin 15R concentrator (Sartorius, Germany) at 3,000 × g for 40 h at 4°C while under constant rotation. The RNA was precipitated with 10- to 70-nt size ranges was excised. The excised gel fragment was cut into smaller pieces and soaked in 30 ml of 1 M NaCl (Ambion, Texas) for ~40 h at 4°C while under constant rotation using a LabQuake shaker rotisserie (Thermo Fisher Scientific). The supernatants were concentrated through centrifugation with the Vivaspin 15R concentrator (Sartorius, Germany) at 3,000 × g for 40 h at 4°C while under constant rotation. The RNA was precipitated with 1 M NaCl (Ambion, Texas) for 40 h at 4°C while under constant rotation. The RNA was precipitated using a Luminoscan Ascent microplate luminometer (Thermo Fisher Scientific). Results from the miRNA Renilla luciferase were normalized to the firefly luciferase readings, and the ratios were plotted as a bar graph relative to the empty vector control.

The luciferase reporters contain internal deletions of the 3p miRNA binding site. The positive control reporters contain a destabilized version of the 3p miRNA binding site. The positive control reporters contain a destabilized version of the 3p miRNA binding site. The positive control reporters contain a destabilized version of the 3p miRNA binding site. The positive control reporters contain a destabilized version of the 3p miRNA binding site. The positive control reporters contain a destabilized version of the 3p miRNA binding site. The positive control reporters contain a destabilized version of the 3p miRNA binding site. The positive control reporters contain a destabilized version of the 3p miRNA binding site. The positive control reporters contain a destabilized version of the 3p miRNA binding site. The positive control reporters contain a destabilized version of the 3p miRNA binding site. The positive control reporters contain a destabilized version of the 3p miRNA binding site. The positive control reporters contain a destabilized version of the 3p miRNA binding site.

**northern blot analysis.**

An adult western barred bandicoot (Perameles bougainville) severely affected by multicompartimental and squamous cell carcinomas associated with BPCV1 infection was euthanized for humane reasons. Cutaneous papillomas and non-lesional skin biopsy specimens were collected in sterile tubes and rapidly frozen in liquid nitrogen. Skin samples were finely minced using sterile scalpel blades, and then 20 mg of tissue was mixed with 350 µl of RTL-β-ME solution (1 ml buffer RTL [Qiagen] with 10 µl of β-mercaptoethanol) and homogenized using a Heidolph DIAx 600 homogenizer (John Morris Scientific P/L, Bentley, Australia) for 5 min on the highest setting (24,000 min–1). The lysate was centrifuged at maximum speed in a microcentrifuge for 3 min and transferred to a fresh microcentrifuge tube. Ethanol (100%) was added to the cleared lysate to bring the final concentration up to 60% ethanol. The next, the samples were applied to an RNaseasy (Qiagen) mini-spin column to purify the total RNA according to the manufacturer’s instructions, except that after the final wash step, the samples were stored at approximately 4°C for several days while still on the column. The final elution steps were conducted with one volume of nuclease-free water and then repeated with one volume of nuclease-free Tris-EDTA (TE), pH 7.

**Northern blot analysis.**

The final blot was reacted with the 3p probe, stripped with boiling hot
A diagram showing the expression of T antigens and L1/L2 proteins from the BPCV1 Early and Late promoters. The diagram includes sequences for T antigens, NCR, L1/L2, pBPCV1-Early, and pBPCV1-Late.

B. A graph showing the relative coverage of L2, L1, lTag, and sTag with early and late promoters.

C. A sequence alignment of the 5p and 3p probes for loop probe experiments.

D, E, F, G. Gel images showing the results of experiments with 5p, Loop, 3p, and Flank probes.
stripping buffer (0.1% sodium dodecyl sulfate [J.T. Baker, New Jersey] in double-distilled water), and then probed with the control terminal loop probe. Due to the small amount of nonlesional RNA negative-control sample that was available, a second Northern blot analysis was conducted using only 180 ng of nonlesional RNA sample and lesional sample 1. The blot was first probed with the 3p probe, stripped as described above, and then probed with the loading control probe (hsa-let-7a). The probe sequence for the hsa-let-7a probe was complementary to TGAGGTAGTAGGTTATAGT.

RESULTS

Discovery of a BPCV1-encoded miRNA. To identify miRNAs encoded by BPCV1, we cloned the entire viral genome downstream of a heterologous CMV promoter. Two different vectors were created, each designed to drive expression of transcripts from either the early or late orientation (pBPCV1-Early and pBPCV1-Late) (Fig. 1A). Next, we transfected human embryonic kidney cells (293T) with either vector and then isolated total RNA. The RNA was size fractionated to isolate small RNAs (between ~10 to 70 nt in length), which were then subjected to next-generation sequencing via the massively parallel SOLiD platform (2). Analysis of the pBPCV1-Early and pBPCV1-Late sequencing results showed 39,390 and 82,208 reads, respectively, that mapped perfectly to the BPCV1 genome. Surprisingly, the vast majority of the reads from both the pBPCV1-Early and pBPCV1-Late-transfected samples mapped to a single 22-nt RNA encoded in the late (papillomavirus-like) orientation at nucleotides 4963 to 4984. This region of the genome lies within the second noncoding region (NCR2) that is located between the 3’ ends of the T antigens and L1/L2 (Fig. 1B). Although the distribution of reads from both samples was surprisingly similar, we confirmed that the sequencing reads were obtained from correct transfection events, since we were able to isolate a few reads that spanned the junctions of the plasmid vector backbone and the viral genome (data not shown). The relative abundance and size of this small RNA suggested it as a good candidate miRNA.

We then analyzed the genomic region surrounding this abundant 22-nt RNA and predicted the secondary structure (Fig. 1C). This analysis revealed a predicted hairpin structure that possesses features common to many pre-miRNAs, including a low ΔG of −41.20 kcal/mol, and a long stem portion (>30 nt) with few large asymmetrical bulges. The abundant 22-nt RNA maps to the top portion of the 3’ arm of the stem portion of the predicted hairpin (Fig. 1C, indicated in bold). These results suggest that the abundant 22-nt RNA could be a miRNA derived from this hairpin pre-miRNA. Upon closer inspection of the sequencing reads, we observed additional evidence consistent with miRNA production. First, we observed an enrichment in 35mer reads (the maximum read length of our library) initiating at positions 4934 to 4936, with the highest number of reads in both samples initiating at position 4934. These full-length reads are consistent with being derived from the 5’ end of the predicted pre-miRNA hairpin structure. Second, we observed a small number of 21- to 23-nt reads with 5’ start sites coterminal with the full-length reads that mapped to the 5’ arm of the predicted hairpin stem, consistent with it being a passenger strand derivative of a putative pre-miRNA (typically, processing of a pre-miRNA yields one abundant derivative, the “guide strand miRNA,” and a less-abundant derivative from the other arm of the stem called the “star strand” (+) or “passenger strand” (−) miRNA). Combined, these results strongly suggested that we had identified a pre-miRNA (and its derivatives) encoded by BPCV1.

To confirm the authenticity of this candidate miRNA, we conducted Northern blot analysis using several different probes (diagrammed in Fig. 1C). When probing with a radiolabeled oligonucleotide that is perfectly complementary to the abundant 22-nt RNA, we observed a prominent band migrating at 22 nt (Fig. 1F). In addition, we saw a fainter band migrating at approximately 60 nt that is consistent with a pre-miRNA (Fig. 1F). Next, we probed for the other, less abundant strand and observed a banding pattern completely consistent with it being a passenger-strand RNA. Note that there are bands present at ~22 nt and ~60 nt but that the ratio of the smaller band to the larger band is much lower than when probing for the guide-strand derivative (Fig. 1D). We utilized two negative-control probes, one designed to recognize the loop portion of the pre-miRNA (which should not be processed into a stable, smaller ~22-nt RNA) and one designed to a flanking region just outside the predicted hairpin region. As expected, the “loop” probe detected a band consistent with the pre-miRNA but did not detect any bands migrating at ~22 nt (Fig. 1E). The negative-control “flank” probe did not detect any specific bands (Fig. 1G). These results strongly suggest that the ~22- and ~60-nt bands we detected are not due to nonspecific degradation or siRNA generation, since in either of these scenarios flanking genomic regions should also produce detectable small RNAs. Thus, we conclude we have identified a bona
fide BPCV1-encoded miRNA, and according to the conventions of miRBase (15a), we name it “BPCV1-miR-B1.”

**Evolutionary conservation of the BPCV1 miRNA.** BPCV2 is the only other known virus closely related to BPCV1, possessing ~85% nucleotide identity and a similar genomic organization. An alignment of both genomes shows that the region spanning the pre-miRNA shares 89.5% (153/171 nt) identity (data not shown). These data imply a high likelihood that BPCV2 would encode a miRNA homologous to BPCV1-miR-B1. To test this notion, we first utilized a bioinformatic approach using the Vmir miRNA prediction software (17, 39, 40). This analysis showed that BPCV1-pre-miR-B1 scored as the fifth-highest candidate in the late orientation and the 11th-highest-scoring overall candidate (Fig. 2A, top panel; also data not shown). Interestingly, this analysis showed that an analogous region of the genome in BPCV2 predicted a candidate pre-miRNA that scored even higher as the best candidate in the late orientation and the second-best overall candidate (Fig.

![FIG. 2. BPCV miRNAs are evolutionarily conserved.](image-url)

(A and B) Vmir predictions for BPCV1 and BPCV2 pre-miRNAs. The top panels show the Vmir-predicted pre-miRNA candidates from BPCV1 and BPCV2. The y axis indicates the Vmir scores of the candidate pre-miRNAs. The x axis indicates the genomic positions for BPCV1 and BPCV2, respectively. Candidate pre-miRNAs of the late orientation for both BPCV1 and BPCV2 are indicated by triangles. Verified BPCV1-miR-B1 and BPCV2-miR-B1 are marked with a circle. The secondary structure predictions for BPCV1 and BPCV2 pre-miRNAs are shown below each Vmir plot. The sequences of 3p miRNAs are indicated in bold. (C) Northern blot analysis confirms that BPCV2 encodes a miRNA. The analysis was performed as described in the legend for Fig. 1.
we name this miRNA “BPCV2-miR-B1.”

miRNA homologous to that of BPCV1 (Fig. 2C). Accordingly, Northern blot analysis of this RNA confirms that BPCV2 encodes a promoter, transfected cells, and harvested total RNA. Northern blot analysis of this RNA confirms that BPCV2 encodes a miRNA homologous to that of BPCV1 (Fig. 2C). Accordingly, we name this miRNA “BPCV2-miR-B1.”

Biogenesis of BPCV1-miR-B1. As described above, we obtained numerous sequencing reads for BPCV1-miR-B1 irrespective of whether the RNA was harvested from cells transfected with the early- or late-orientation expression vector. Furthermore, Northern blot analysis readily identified the miRNA from both constructs (Fig. 1C). Our ability to detect the miRNA from the early-orientation construct implies that an intrinsic robust promoter activity is present in the late (papillomavirus) orientation of the BPCV genome that drives expression of BPCV1-miR-B1. Since both the BPCV miRNAs are found within NCR2, and this region is not predicted to encode any proteins (7, 46), we speculated that it may serve as a cassette that contains a promoter to drive expression of the primary transcripts that give rise to the BPCV miRNAs. To test this hypothesis, we first subjected the NCR2 sequences of both BPCV1 and BPCV2 to bioinformatic analysis (31) to identify candidate promoter regions (Table 1). The highest-ranked candidate promoter regions for both BPCV1 (nt 4769 to 4818) and BPCV2 (nt 4745 to 4794) were found to be in similar genomic locations, approximately 60 nt upstream of each respective BPCV pre-miRNA. Both of these candidate promoters contain a putative TATA box (Table 1). A sequence alignment between NCR2 of BPCV1 and that of BPCV2 revealed that despite having only 73.9% (7) sequence identity in this region, the pre-miRNA and putative promoter regions are highly conserved (~90%; 187/211 nt) (Fig. 3A), implying a conserved function. To test for functional activity of these putative promoters, a series of constructs was created (Fig. 3B). We first cloned the entire NCR2 of BPCV1, as well as various truncations/internal deletions of this region. We individually transfected each of these constructs and then conducted Northern blot analysis for BPCV1-miR-B1 3p miRNA. The construct containing the full-length NCR2 (named “1” in Fig. 3B and C) expressed robust levels of the miRNA (Fig. 3C). However, the truncation construct pcDNA3.1-BPCV1-ΔproΔNCR2, which has eliminated the predicted promoter and all upstream sequences within NCR2 (nucleotides 3758 to 4808), showed a dramatic reduction in the expression of the miRNA (Fig. 3C). To rule out the possibility that this truncation mutation somehow compromised important features of the pre-miRNA, we generated a chimeric construct containing an SV40 early promoter upstream of this truncation (named “3” in Fig. 3B and C). This chimeric construct was able to rescue readily detectable levels of the miRNA. These data argue that all essential cis processing elements for the pre-miRNA lie within ~60 nt of the base of the stem portion of the predicted hairpin pre-miRNA. To determine if removal of the predicted promoter region (nucleotides 4769 to 4808) was sufficient to dramatically reduce transcription, an internal deletion mutant that deleted just the predicted promoter (preserving the rest of the NCR) was created (pcDNA3.1-BPCV1-ΔproΔNCR2, named “4” in Fig. 3B and C). This mutant construct showed greatly reduced transcription, on par with that of the large deletion mutation (pcDNA3.1-BPCV1-ΔproΔNCR2, named “5” in Fig. 3B and C). The results confirm the proximity of the promoter to the hairpin stem.

TABLE 1. Predicted promoter sequences in BPCV1 NCR2 and BPCV2 NCR2a

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<th>Region</th>
<th>Position (nt)</th>
<th>Score</th>
<th>Rank</th>
<th>Predicted promoter sequence</th>
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<td></td>
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<td>0.84</td>
<td>5</td>
<td>AGCAGCACAGCGACAGCACAGCAA</td>
</tr>
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a Sequences from both BPCV1 NCR2 and BPCV2 NCR2 were subjected to promoter prediction via the Neural Network Promoter Prediction program (31). The predictions were ranked according to their scores, from 0 to 1.00, with an arbitrary cutoff of 0.8. The start and end columns indicate the genomic location of the predictions in BPCV1 and BPCV2. The nucleotides in bold are the predicted transcription start sites. The row shaded in gray represents the region containing the putative TATA box (Table 1). The nucleotides in bold are the predicted transcription start sites. The row shaded in gray represents the region containing the putative TATA box (Table 1).
FIG. 3. An evolutionarily conserved promoter in NCR2 drives the expression of the BPCV miRNAs. (A) Sequence alignment plot between BPCV1 NCR2 and BPCV2 NCR2 revealed a highly conserved region upstream of BPCV1-miR-B1. The percentage of identity between BPCV1 and BPCV2 is calculated over a window of 20 nt and is plotted on the y axis. The x axis represents the genomic position of NCR2, with 1 being the first nucleotide immediately downstream of the large T antigen stop codon. Black horizontal bars represent BPCV1-miR-B1-3p and -5p miRNAs. The gray bar represents the position of the conserved promoter. (B) Deletion constructs used to map NCR2 promoter activity. The construct, pcDNA3.1-BPCV1-NCR2, included flanking regions from both the L1 and large T antigen genes. Various internal deletion or truncation constructs (as diagrammed) were assayed. The dotted lines represent the deleted region from NCR2, and the dashed lines represent vector sequences. The column on the right indicates the expression of the miRNA relative to the FL construct, “+++” represents abundant miRNA expression, “++” represents intermediate miRNA expression, and “-” represents no detectable miRNA expression. (C) Northern blot analysis from various NCR2 constructs identifies promoter activity at nucleotides 4769 to 4818. The 3p miRNA probe was used for the analysis. The loading control, shown in the bottom panels, is ethidium bromide-stained low-molecular-weight RNA. The miRNA band is indicated by the black arrowhead. (D) Northern blot analysis shows NCR2 promoter activity is conserved between BPCV1 and BPCV2. Analysis and labeling are as described for panel C, above. The miRNA band is indicated by the black arrowhead.
named “2” in Fig. 3B and C). Combined, these data support the existence of a strong promoter in the BPCV1 genome that lies upstream of the pre-miRNA, with essential elements at nucleotide positions 4769 to 4818.

Since there is a high degree of conservation of the BPCV1 miRNA promoter region with BPCV2 (Fig. 3A), we examined whether expression of the BPCV2 miRNA is driven by a similar promoter. We subcloned the entire BPCV2 NCR2, containing both the putative promoter region and pre-miRNA regions, into a vector that does not contain a mammalian promoter. This construct drove high expression of the BPCV2 miRNA to levels comparable to those for BPCV1 (Fig. 3D). We therefore conclude that NCR2 of BPCV1 and BPCV2 each contain a robust transcription-inducing activity, making the BPCV1 and -2 miRNAs some of the only viral miRNAs with well-defined miRNA-specific promoters.

miRNAs encoded by BPCV1 and BPCV2 negatively regulate transcripts containing the large T antigen 3′ UTR via a manner distinct from that of all known polyomaviruses. Because all known Polyomaviridae miRNAs are encoded antisense to the early transcripts, we wondered if this might also be the case for the BPCV1 miRNA. Inspection of the genomic location of BPCV1-miR-B1 shows that the miRNA lies 85 nt downstream from the large T antigen stop codon (nucleotides 5070 to 5072). Two different algorithms predict a strong polyadenylation recognition site at nucleotides 5023 to 5028 in the early orientation (13, 42; data not shown). If these predictions are correct, it would mean that the early transcripts would likely terminate before they reached the BPCV1 miRNA genomic location encoded on the opposite strand. This would render antisense miRNA-mediated cleavage of the early transcripts impossible. We therefore performed 3′ rapid amplification of cDNA ends (3′ RACE) analysis on RNA harvested from cells transfected with pBPCV1-Early to map the 3′ end of the BPCV early transcripts. We sequenced PCR products as well as individual clones from the RACE reaction. The results were identical in both types of analyses—all sequencing reactions, including the direct PCR products, as well as 9 out of 9 individual clones mapped to the same site. These analyses mapped a polyadenylation cleavage site between nucleotides 5008 and 5010 (since this region of the genome contains three thymidines, it is unclear if the first three thymidines mapped are derived from the genomic template or are added by the cellular polyadenylation machinery) (Fig. 4A). This site is consistent with the computationally predicted polyadenylation site (13, 42) and lies greater than 20 nt away from BPCV1-miR-B1. As described in a later section, we were able to obtain RNA from a natural BPCV1 infection of a western barred bandicoot, and mapping of the 3′ end of the early transcripts from this source further confirms the polyadenylation cleavage site we mapped in RNA harvested from transfected cells (sequencing of the direct PCR products and 7 out of 7 individual clones mapped a single location identical to the one mapped for the transfected samples [Fig. 4A]). Combined, these data demonstrate that unlike all known Polyomaviridae miRNAs, BPCV1-miR-B1 is not encoded antisense to the early miRNAs but rather lies in a separate downstream portion of the genome. Therefore, BPCV1-miR-B1 does not possess perfect complementarity to the early miRNAs and is less likely to direct siRNA-like, miRNA-mediated cleavage of these transcripts (Fig. 4B).

During the course of analyzing the sequencing data, we noted that performing a local BLAST search (1) of BPCV1-miR-B1 to the BPCV1 genome produced two significant “hits” at different locations within the genome. As expected, one hit displayed a perfect 22-nt match and mapped to BPCV1-miR-B1 itself. Unexpectedly, the other hit displayed a 20-of-22-nt match that mapped to the 3′ UTR of large T antigen (Fig. 4A). Several reasons suggest this would be a likely target site for regulation by BPCV1-miR-B1. First, the portion of complementarity to the seed region (nucleotides 2 to 7, known to play an especially important role in miRNA target recognition) (25) is a perfect match (Fig. 4C). Second, as discussed in a later section, the BPCV1 T antigen 3′ UTR complementary region is 100% conserved (22 of 22 nt) with the related BPCV2 early 3′ UTR, while the remaining portions of the 3′ UTRs share only 76.7% identity (33 of 43 nt). This implies a conserved function in the putative miRNA target portion of early transcript 3′ UTRs. Third, miRNA target sites are thought to be more active when located close to the ends of the 3′ UTR but greater than 15 nt from the stop codon (16), and the putative BPCV1-miR-B1 target site matches these criteria (close to both ends of the 3′ UTR: only ~20 nt from the 3′ terminus of the early transcripts and 19 nt downstream from the stop codon). Therefore, we set out to explore whether BPCV1-miR-B1 regulates transcripts containing the early gene 3′ UTR.

To test the hypothesis that BPCV1-miR-B1 regulates early gene expression, we utilized Renilla luciferase reporter constructs containing the entire BPCV1 3′ UTR. In addition, we generated a negative-control 3′ UTR containing a 2-nt point mutation in the miRNA seed region, a second negative-control 3′ UTR containing a deletion of the entire predicted 22-nt binding region, or a positive-control 3′ UTR containing an engineered binding site that is perfectly complementary to the BPCV1 miRNA (Fig. 5A). Cotransfection of a vector expressing BPCV1-miR-B1 with a reporter plasmid containing the entire BPCV1 early 3′ UTR results in a marked decrease in luciferase expression (Fig. 5B). Notably, cotransfection of either a control vector that does not express any miRNA or a control vector expressing an irrelevant miRNA has little effect on the luciferase activity of the plasmid containing the wild-type BPCV early 3′ UTR, thus implying specificity. Importantly, when we delete the entire putative miRNA target region or, more surgically, alter only two nucleotides within the seed complementary region, we observe no negative regulation. As expected, when we engineered a perfectly complementary reporter, we also observed BPCV1-miR-B1-specific negative regulation. Notably, the degree of regulation observed for this positive-control reporter was similar to that of the natural large T antigen 3′ UTR reporter. These results strongly suggest that BPCV1-miR-B1 can negatively regulate the BPCV1 early transcripts by binding to the 3′ UTR. Thus, although 3′ UTRs represent the most common locale of miRNA-mediated repression in animals, this is the first demonstration of such regulation for polyomavirus-like transcripts.

The BPCV1 large T antigen 3′ UTR shares 84.6% identity (55 of 65 nt) with BPCV2; however, the miRNA binding site is 100% conserved (22 of 22 nt) (Fig. 5C). Therefore, we hypothe-
esized that BPCV2 would undergo miRNA-mediated regulation of its early transcripts. To test this, we conducted experiments identical to those described for Fig. 5A, except that we utilized a reporter vector that contains the entire BPCV2 large T antigen 3′/H11032UTR and a BPCV2 miRNA-expressing vector (Fig. 5D). Strikingly, these results mirrored precisely the trends we observed for BPCV1 (Fig. 5E) and suggest that this novel form of miRNA-mediated regulation of the BPCV T antigens is evolutionarily conserved.

**BPCV1-miR-B1 is expressed in vivo.** Infection with BPCV1 is associated with papillomas and carcinomas in western barred bandicoots (46). PCR detection and *in situ* hybridization analyses demonstrated that the virus is present in the majority of papillomatous and carcinomatous lesions tested (6, 45, 46). Because the western barred bandicoot is an endangered species, obtaining tissue explants from infected animals is a challenge. We were able to obtain both lesional and a small amount of nonlesional tissue samples from a single infected animal that had to be euthanized for humane reasons due to complications arising from BPCV1-associated metastatic squamous cell carcinoma. We harvested total RNA from two different lesional regions on the same animal, as well as nonlesional skin from a different portion of the animal. We were pleased to observe a robust signal showing a specific band from both lesional samples (Fig. 6A). This band comigrates with the BPCV1-miR-B1 band that is detected in the positive-control RNA derived from BPCV plasmid-transfected cells (Fig. 6A).

Probing any of four different negative-control samples, RNA from nonlesional bandicoot skin (Fig. 6C), HEK 293 cells (Fig. 6A), HEK 293T cells (Fig. 6A), or BSC40 African green monkey cells (Fig. 6A), did not lead to detection of any specific bands. As a control to rule out that the band we detected from the lesional samples could be from random degradation of total RNA, we stripped the blot in Fig. 6A and then reprobed with the terminal loop probe (as described in the legend to Fig. 1C). If the band we detect is random degradation of a larger viral transcript, then other probes for RNA sequences proximal to BPCV1-miR-B1 should also provide a signal at ~22 nt. Probing with the terminal loop probe (which recognizes an RNA sequence just proximal to the miRNA) did not detect any bands from the lesional samples (Fig. 6B). This result strongly argues that neither random degradation fragments nor siRNAs...
can account for the miRNA signal we detect migrating at \( \sim 22 \) nt, since both of these processes would be expected to generate Northern blot-detectable bands throughout this region of the genome (and not just from one arm of the pre-miRNA precursor hairpin). It is worth noting that the terminal loop probe recognizes the pre-miRNA band in the positive-control RNA sample but not in the lesional samples. Either of two explanations likely accounts for this: (i) more-efficient processing of...
the pre-miRNA in vivo or (ii) a pre-miRNA nuclease activity that is active in the preparation of the in vivo samples. Regardless, the fact that the loop probe recognizes the pre-miRNA from the positive-control transfected sample rules out any technical artifact that could account for its inability to detect bands from the lesional tissue. Together, these data demonstrate that the BPCV1 miRNA is expressed at high levels in some contexts of in vivo infection.

DISCUSSION

The discovery of new virus-encoded miRNAs and understanding their functions represent an exciting developing subfield of virology. Here we describe miRNAs from a fascinating group of recently described viruses, representing only the sixth virus family/group shown to encode miRNAs. BPCV1 and BPCV2 are remarkable because they represent natural hybrids of two different virus families, the Polyomaviridae and the Papillomaviridae. Like the polyomaviruses, the BPCVs express T antigen proteins and have a genomic organization that consists of an origin of replication flanked by promoters that drive the regulatory T antigen transcripts in one direction around the circular genome and drive the capsid transcripts in the other direction (Fig. 7). However, unlike those of the polyomaviruses, the capsid proteins are clearly related to the papillomavirus capsid proteins L1 and L2. Furthermore, the size of the genome is ~7,300 bp, closer to that of the papillomaviruses (~7 to 8.5 kb) than the polyomaviruses (~5.2 kb). In contrast to the known polyomaviruses and most papillomaviruses, the BPCVs also contain a long untranslated region (NCR2) of
~1.3 kb, which we have shown serves as both a promoter and template to drive the primary miRNA-encoding transcript (Fig. 1 and 3).

We have demonstrated that the BPCV miRNAs can direct negative regulation of transcripts that contain the large T antigen 3’ UTR (Fig. 5A and B). This suggests that similar to what has been observed in the polyomaviruses (33, 34, 40, 41), herpesviruses (4, 15, 28, 44), and ascoviruses (19), BPCV miRNAs play a prominent role in the regulation of viral gene expression. This notion is supported by the observation that the BPCV large T antigens have a relatively small 3’ UTR at ~60 nt (Fig. 4A); however, 20 of 22 are perfectly complementary to the miRNA. Since one of the mismatched target nucleotides could bind as a GU wobble, this potentially leaves only a single unpaired nucleotide between miRNA and mRNA target site (Fig. 4C)—significantly fewer than almost all known animal miRNA-target pairs.

Adding further weight to the model that BPCV1-miR-mediated viral gene regulation is relevant during infection is the fact that 3’ UTR regulation we observe is evolutionarily conserved with BPCV2 (Fig. 5D and E). Using the Vmir miRNA prediction software, we easily identified the pre-miRNA that gives rise to BPCV2-miR-1 (Fig. 2B). Interestingly, the BPCV2 pre-miRNA scores as a higher-ranked candidate than BPCV1-miR-1 (overall rank, 2 versus 11). We previously observed a similar phenomenon for two different strains of MCV (33). These observations have relevance to future attempts of using bioinformatics approaches for identifying new viral miRNAs. Rather than analyzing a single viral genome, future bioinformatics efforts might be better served if a series of closely related viruses, or even different isolates of the same virus, were compared to identify common regions of predicted pre-miRNAs. Finally, the fact that we show the BPCV1 miRNA is abundantly expressed during infection in vivo (Fig. 6) argues that the regulation of the T antigen 3’ UTR we observe in cultured cells likely occurs in at least some in vivo settings.

We have determined that the function of the second non-coding region of the BPCVs includes encoding a promoter and template for the primary transcript that contains the pre-miRNA. We note that we cannot rule out that additional promoters and transcripts may be used to generate this miRNA during some stages of infection. It is interesting to point out that both NCR2 regions of BPCV1 and BPCV2 contain a predicted large T antigen binding site consensus sequence of GAGGC (7, 29, 46). In addition, our observation that this promoter is robustly active in cells derived from placental mammals suggests it is responsive to transcription factors that were present in the last common ancestor of placental and marsupial mammals. Of note, possible host transcription factor binding sites that are predicted in these NCR2 regions include p53, E2F1, p300, and NF-kB, (27; data not shown). These observations suggest that regulation of the expression of the BPCV miRNAs may be complex and affected by numerous trans factors of both host and viral origin. Future experiments are required to test this hypothesis.

We have previously shown that six different polyomaviruses encode miRNAs that negatively regulate, or are predicted to negatively regulate, T antigen transcripts. In all cases, despite some sequence differences, these miRNAs are encoded on the opposite strands and antisense to the T antigen transcripts (11, 33, 34, 40, 41). This conserved mode of regulation implies importance; however, one could argue that since the degree of regulation imparted by these is only partial (40, 41), the polyomaviral miRNA-mediated viral transcript regulation is a secondary consequence of the genomic location of these miRNAs. However, the results we present here strongly imply that some diverse viruses that have polyomaviral or polyomavirus-like genomic organizations are under evolutionary pressure to maintain miRNAs that regulate T antigen expression. Because, unlike the polyomaviral miRNAs, the BPCV miRNAs are encoded in a different region of the genome (Fig. 4) and possess an alternative mechanism of action (3’ UTR regulation versus antisense miRNA-mediated cleavage of the coding sequence), we conclude that the miRNA-mediated regulation of T antigen expression is selected for in diverse viruses. We stress that in no way does this rule out the possibility that important host targets exist for these miRNAs. Indeed, as has been shown for several members of the Herpesviridae, viral miRNAs will target virus-encoded or host-encoded transcripts and sometimes a single miRNA can target both (15, 36).

What is the purpose of polyomaviral and BPCV miRNA-mediated regulation of T antigen transcripts? One possibility is that, similar to the many Herpesviridae miRNAs that play a role in latency, the polyomaviral and BPCV miRNAs may play a role in maintaining persistent infection. In this scenario, these miRNAs would prevent excessive or untimely expression of the T antigens, thereby avoiding the triggering of inappropriate lytic induction or clearance by the adaptive immune response. In this regard, it is interesting to note that a putative T antigen binding site exists in the NCR2 region of both BPCV1 and BPCV2 (7, 29, 46). Thus, BPCV T antigen could play a role in either a positive or negative feedback loop regulating expression of the miRNA. Currently, persistence is a poorly understood facet of the polyomavirus life cycle. To further elucidate the role of virus-encoded miRNAs in regulating persistent and/or lytic infections, future studies will require the development of appropriate in vivo models.

Finally, our results identify the first 2 viruses that encode papillomavirus-like gene products that also encode a miRNA. A previous study of human papillomavirus type 31 strongly suggested that at least some papillomaviruses are not likely to encode miRNAs (10). However, it is still formally possible that other papillomaviruses may encode miRNAs. Our results suggest that these papillomaviruses with multiple noncoding regions, such as the Felis domesticus papillomavirus type 1, belonging to the genus Lambdapatillomavirus (30), would be attractive candidates in a hunt for true papillomavirus-encoded miRNAs.

In summary, we have shown that an interesting new group of viruses encode miRNAs, which has important implications for distantly related viruses, including some human pathogens. In the future, exciting questions will be addressed, such as the role of polyomavirus and polyomavirus-like miRNAs in possibly targeting host transcripts and establishing or maintaining persistent infection. What is clear at this point is that viral miRNA-mediated regulation of viral transcripts is important for divergent viruses, including herpesviruses, polyomaviruses, and the BPCVs.
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