Supporting Information

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SI Materials and Methods

DNA Constructs, Transfection, Infection, and Luciferase Assays. Site-directed mutagenesis was performed using the QuikChange II Site-Directed Mutagenesis kit (Stratagene) as described previously (1) on both pGEM-Dik and -Dunlop (2) to create the miRNA mutant in the archetype (A) and rearranged (R) background, respectively. Three point mutations were created in the precursor-microRNA (pre-miRNA) sequence G13A, U43G, and U46G (where 1 is the 5′ start of the pre-miRNA) using primers A, B, and C and their reverse complements (Table S1). Mutations and the integrity of the early and late coding regions were confirmed by DNA sequencing.

Constructs pcDNA3.1puro, pcDNA3.1dsRluc, pcDNA3.1dsFFluc, and pcDNA3.1BKV miRNA were kindly provided by C. Sullivan (University of Texas at Austin, Austin, TX) (3). The region of the BK polyomavirus (BKPyV) genome corresponding to the miRNA target sequence in the TAg mRNA was PCR amplified and subcloned into the 3′ UTR of pcDNA3.1dsRluc as previously described, as a single target as opposed to a concatemerized target, using primers D, E, F, and G (Table S1). Site-directed mutagenesis was used as above to create the mutated BKPyV miRNA expression vector pcDNA3.1BKVmiRNA mutant.

293 cells were plated into 12-well dishes and transfected with Turbofect (Fermentas). Cells were transfected with 100 ng pcDNA3.1dsRlucBKV TAg, 50 ng pcDNA3.1dsFfluc (transfection control) and 1,850 ng pcDNA3.1BKVmiRNA or pcDNA3.1BKVmiRNAmutant expression vector for a total of 2 μg DNA. Cell lysates were harvested 48 h posttransfection (hpt) and analyzed using the dual-luciferase reporter assay system according to the manufacturer's instructions (Promega) and read on a Promega GloMax 96 microplate luminometer.

The archetype virus and rearranged variant NCCR were cloned into the phRG promoter construct, kindly provided by H. Hirsch (University of Basel, Basel, Switzerland), as previously described (4). Constructs were confirmed by DNA sequencing.

NCCR flip constructs were created using the Dik-3 site (A) and Dunlop-3 site (R) swap vectors (5). These vectors contain SpeI and SacII sites in the O and S blocks of the NCCR, respectively. The NCCR structure was flipped by adding a SacII to the O block using primer H and a SpeI site to the S block using primer I (Table S1). This resulted in a flipped NCCR structure where the top strand becomes the bottom strand and vice versa. PCR products were gel purified and ligated into digested Dik- and Dun-3–site backbones using T4 DNA ligase. The flip constructs were confirmed by DNA sequencing.

Southern Blotting. Southern blotting was performed as described previously described (5). Plasmid DNA, pGEM-Dik WT and miRNA mutant, were digested with DraI. The blots were probed under Northern blotting conditions as previously described (6). The Southern blot was quantified using a Typhoon phosphorimager and Imagequant software (GE).

Quantitative PCR. All RNA was harvested at the indicated time points as described in *Materials and Methods*. RNA integrity was confirmed by electrophoresis on an agarose gel. To quantify the BKPyV 5 prime (5P) miRNA, a stem-loop reaction (7) was performed with 1 μg of total RNA, 0.5 μL 10 mM dNTP mix, 1 μL of 1 μM BKPyV 5P stem-loop reverse transcription (RT) primer L, the negative control RT primer recognizing the middle of the mature miRNA primer M, or the human miRNA control *hsa-let-7a* RT primer N (Table S1). The stem-loop primer annealing program is 5 min at 65 °C and 2 min on ice. The stem-loop product is then added to an RT reaction using SuperScript III reverse transcriptase (Invitrogen) containing 4 μL first-strand buffer, 2 μL 0.1 M DTT, 0.1 μL SUPERase (Ambion), and 0.25 μL (50 units) SuperScript III reverse transcriptase. The pulsed RT program was performed with the following parameters: 30 min at 16 °C, followed by 60 cycles of 30 s at 30 °C, 30 s at 42 °C, and 1 s at 50 °C, and then 5 min at 85 °C. Next, real-time PCR was performed with the RT product in a total volume of 20 μL using 10 μL Power SYBR green PCR master mix (Applied Biosystems), 2 μL RT product, 1 μL 10 μM primers O and P (Table S1), and 6 μL H_2O. Amplification was performed in 96-well PCR plates using the iCycler iQ5 real-time detection system (Bio-Rad) with the following program: 5 min at 95 °C, 35 cycles of 95 °C for 5 s, 60 °C for 10 s, and 72 °C for 1 s. Analysis of serial dilutions of RNA from infected renal proximal tubule epithelial (RPTE) cells show that from 1 ng to 1 μg, a range which includes the conditions used in our experiments, the BKPyV 5P stem-loop assay is linear ($R^2 = 0.991$) and that from 0.5 ng to 500 ng, the *hsa-let-7a* stem-loop assay is linear ($R^2 = 0.998$). Results are represented as relative transcript levels, where transcript is normalized to *hsa-let-7a* and the A WT sample is arbitrarily set to 1.

To quantify viral early and late transcripts, total RNA was harvested and treated with DNase (Promega) to eliminate contaminating DNA. To generate cDNA, a reverse transcription reaction was performed with 50 ng of RNA template using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed in a total volume of 25 μL using 12.5 μL Power SYBR green PCR master mix (Applied Biosystems), 2.5 μL cDNA template, and 300 nM each primer: for early transcript, primers Q and R; for late transcript, primers S and T; and for GAPDH, primers U and V (Table S1). Amplification was performed in 96-well PCR plates using the iCycler iQ5 real-time detection system (Bio-Rad) with the following program: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 58 °C (for early mRNA) or 57C (for late mRNA) for 30 s. Results are represented as relative transcript levels, where transcript is normalized to GAPDH and the A WT sample is arbitrarily set to 1.

1. Abend JR, Imperiale MJ (2008) Transforming growth factor-beta-mediated regulation of BK virus gene expression. *Virology* 378(1):6–12.
2. Broekema NM, Imperiale MJ (2012) Efficient propagation of archetype BK and JC polyomaviruses. *Virology* 422(2):235–241.
3. Seo GJ, Fink LH, O'Hara B, Atwood WJ, Sullivan CS (2008) Evolutionarily conserved function of a viral microRNA. *J Virol* 82(20):9823–9828.
4. Gosert R, et al. (2008) Polyomavirus BK with rearranged noncoding control region emerge in vivo in renal transplant patients and increase viral replication and cytopathology. *J Exp Med* 205(4):841–852.
5. Broekema NM, et al. (2010) A system for the analysis of BKV non-coding control regions: Application to clinical isolates from an HIV/AIDS patient. *Virology* 407(2): 368–373.
6. McClure LV, Lin YT, Sullivan CS (2011) Detection of viral microRNAs by Northern blot analysis. *Methods Mol Biol* 721:153–171.
7. Varkonyi-Gasic E, Wu R, Wood M, Walton EF, Hellens RP (2007) Protocol: A highly sensitive RT-PCR method for detection and quantification of microRNAs. *Plant Methods* 3:12.

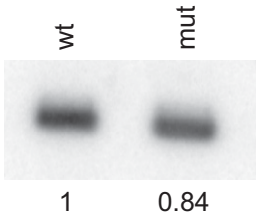

Fig. S1. WT Northern blot probe recognizes the mutant miRNA sequence. Equal amounts of WT and miRNA mutant plasmid DNA were digested with DraI and run on an agarose gel, blotted, and probed under Northern blotting conditions using the 5P probe. Numbers indicate the quantification of band intensity normalized to the WT. WT, pGEM-Dik; mut, pGEM-Dik miRNA mutant.

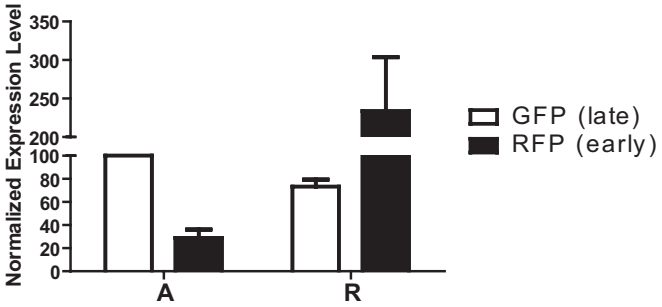

Fig. S2. Promoter activity of archetype virus vs. rearranged variant. The 293 cells were transfected with the phRG reporter plasmid containing either the archetype (A) or rearranged (R) NCCR. Expression level is the percentage of GFP (late promoter) or RFP (early promoter) positive cells normalized to GFP positive cells transfected with phRG containing the A noncoding control region (NCCR). The archetype expression level was arbitrarily set to 100. A minimum of 100 foci were counted for each fluorescence channel in each independent experiment. Each bar is the average from three independent experiments and the error bars are SD.

Table S1. List of primers and oligos used in this study

Oligonucleotides	Name	Sequence (5′–3′)
Primers used for cloning and site-directed mutagenesis*		
A	G13Amut	GCTGAAGTATCTGAGACTTG**A**GAAGAGCATTGTGATTGGG
B	U43Gmut	GGGATTCAGTGCT**G**GATCCATGTCCAGAG
C	U46Gmut	GGGATTCAGTGCT**G**GA**G**CCATGTCCAGAG
D	TAg miRNA FOR	GAAGGCACCATGGGGAAATGTATTCTTGAT
E	TAg miRNA REV	GAAGGTGCCTGACCTTTGGGAATCTTCAGC
F	TAg concat FOR	ATGCTCGAGCGGCCGCCAGTGTGATGGATA
G	TAg concat REV	GCATCTAGAGTAACGGCCGCCAGTGTGCTG
H	OflipLate	ATTATACCGCGGGATTTCCCCAAATAGTTTTGC
I	SflipEarly	ATGGTAACTAGTCTCGCAAAACATGTCTGTC
J	phRG NCCR MluI	AAAAACGCGTCATTTTTGCAAAAATTGCAAAAGAATAGG
K	phRG NCCR BssHII	TTTTGCGCGCCTGGCGCAGAACCATGGCCTT
Primers used for real-time PCR†		
L	5P miRNA stem loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGA TACGACATGCTC
M	5P neg contol stem loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGA TACGACCCAAGT
N	5P hsa-let-7a-1 stem loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACTAT
O	miRNA qPCR FOR	GCCTCGATCTGAGACTTGGGAA
P	Stem loop qPCR REV	GTGCAGGGTCCGAGGT
Q	Early FOR	AAGGAAAGGCTGGATTCTGA
R	Early REV	TGTGATTGGGATTCAGTGCT
S	Late FOR	ACAGGCCACAAATATCAGCA
T	Late REV	TGTGACCAACACAGCTACCA
U	GAPDH FOR	GCCTCAAGATCATCAGCAAT
V	GAPDH REV	CTGTGGTCATGAGTCCTTCC
RNA oligos used for miRNA stem-loop qPCR standard curves		
	bkv-miR-B1-5P	AUCUGAGACUUGGGAAGAGCAU
	hsa-let-7a 5P	UGAGGUAGUAGGUUGUAUAGUU

*Restriction enzyme sites are underlined and point mutations are shown in bold in the primer sequence.
†Underlined portion corresponds to the part of the primer that binds to the 3′ end or the middle (negative control) of the 5P miRNA.

Table S2. miRNA stem-loop qPCR average Ct and copy no./ng RNA

Figures	BKPyV Ave Ct	BKPyV copy no./ng RNA	hsa-let-7a Ave Ct	hsa-let-7a copy no./ng RNA
Fig. 2*C*				
Mock	ND	ND	24.43	2.04×10^5
A WT	22.62	5.17×10^3	24.31	2.23×10^5
A mut	ND	ND	24.30	2.20×10^5
R WT	19.63	3.32×10^4	24.45	1.96×10^5
R mut	ND	ND	24.70	1.7×10^5
Fig. 3*A*				
Mock	ND	ND	23.32	1.63×10^5
A WT	30.19	3.59×10^1	23.36	1.62×10^5
A mut	ND	ND	24.05	9.52×10^4
R WT	19.92	3.39×10^4	24.15	8.84×10^4
R mut	ND	ND	23.66	1.26×10^5
Fig. 4*B*				
Mock	ND	ND	25.61	1.15×10^5
A WT	25.62	7.26×10^2	25.86	9.37×10^4
A flip	29.31	6.29×10^1	25.72	1.03×10^5
R WT	28.29	1.31×10^2	26.36	7.14×10^4
R flip	25.46	8.77×10^2	25.81	1.02×10^5

ND, not detected.