



Title	Inhibition of Human BK Polyomavirus Replication by Small Noncoding RNAs
Authors(s)	Tikhanovich, I., Liang, B., Seoighe, C., et al.
Publication date	2011-05-04
Publication information	Tikhanovich, I., B. Liang, C. Seoighe, and et al. "Inhibition of Human BK Polyomavirus Replication by Small Noncoding RNAs." American Society for Microbiology, May 4, 2011. https://doi.org/10.1128/JVI.00547-11 .
Publisher	American Society for Microbiology
Item record/more information	http://hdl.handle.net/10197/4996
Publisher's version (DOI)	10.1128/JVI.00547-11

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Inhibition of human BK polyomavirus replication by small non-coding RNAs

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Running title:

Small non-coding RNAs inhibit BKV DNA replication

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ABSTRACT

Small non-coding RNAs regulate a variety of cellular processes including genomic imprinting, chromatin remodeling, replication, transcription, and translation. Here we report small replication-regulating RNAs (srRNAs) that specifically inhibit DNA replication of the human polyomavirus BK (BKV) *in vitro* and *in vivo*. srRNAs from FM3A murine mammary tumor cells were enriched by DNA replication assay-guided fractionation and hybridization to the BKV Non-Coding Control Region (NCCR), and synthesized as cDNAs. Selective mutagenesis of the cDNA sequences and their putative targets suggests the inhibition of BKV DNA replication is mediated by srRNAs binding to the viral NCCR, hindering early steps in the initiation of DNA replication. Ectopic expression of srRNAs in human cells inhibited BKV DNA replication *in vivo*. Additional srRNAs were designed and synthesized that specifically inhibit SV40 DNA replication *in vitro*. These observations point to novel mechanisms for regulating DNA replication and suggest the design of synthetic agents for inhibiting replication of polyomaviruses and possibly other viruses.

INTRODUCTION

Small non-coding RNAs (ncRNAs) have important roles in eukaryotic gene expression determining developmental processes and growth and differentiation (reviewed in (1, 8, 20, 27, 30, 50)). In addition, certain ncRNAs (e.g. Y-RNAs) are required for chromosomal DNA replication and proliferation of mammalian cells (5, 23), however, the mechanisms by which the latter act are not yet understood. Roles for ncRNAs in regulating viral infections of mammalian cells were uncovered with the discovery of virus- and host-encoded RNAs controlling viral gene expression (reviewed in (10, 41)), and with the recognition that recruitment of the cellular Origin Recognition Complex (ORC) to the viral origin of replication (OriP) by Epstein-Barr virus (EBV) nuclear antigen 1 (EBNA1) and initiation of EBV DNA replication is RNA-dependent (38).

BK virus (BKV) infects nearly the entire human population, but generally is innocuous unless viral replication is activated following kidney transplantation and immune suppression, when it may cause Polyomavirus Associated Nephropathy (PVAN) and allograft loss (14, 19). Polyomavirus DNA replication *in vitro* and *in vivo* requires one viral protein, the large T antigen (TAg), with other factors supplied by the host cell (25, 28). Polyomavirus DNA replication initiates with TAg binding at the viral origin of replication (39, 44), and recruitment of host factors such as replication protein A (RPA), DNA polymerase α -primase (Pol-primase) and topoisomerase I (2, 45), followed by Pol-primase catalyzed *de novo* synthesis of nascent primers for DNA replication. Early initiation products, the nascent DNA primers, are then extended by DNA polymerase δ and proliferating cell nuclear antigen (PCNA) and the loading factor replication factor C on the leading and lagging strands (37, 55). Although the DNA replication machineries are conserved between primates and rodents, BKV does not replicate in mouse cells as a result of species-specific interactions between TAg and cellular Pol-primase, analogous to what has been observed with simian virus 40 (SV40) (26, 28, 31, 32, 42, 49, 53). In contrast to what was observed with SV40, however, dominant negative factor(s) present in mouse cell extracts act at the BKV origin of replication to inhibit DNA replication, even in an otherwise supportive environment (28, 53). The studies reported here identify small ncRNAs as being responsible for the observed inhibition of BKV DNA replication by mouse cell extracts. Notably, these small replication-regulating RNAs

(srRNAs) can dramatically inhibit BKV DNA replication when ectopically expressed in human cells.

MATERIALS AND METHODS

DNAs

pUC18-based plasmid DNAs with complete viral origins included pOriBKV (containing sequences of the archetype BKV Dik strain) (28); pOriSV40 (SV-S strain (42)), and BKV-mPyV (containing chimeric BKV and murine polyomavirus origins) (28). All DNAs for replication assays were purified with Midiprep kits (Qiagen, Germany). pU6 was constructed by insertion of a U6 promoter DNA fragment cut out with Bgl II and EcoR I from a modified pSirenRetreQ vector into the BamH I and EcoR I digested pUC18 vector. pU6-B-5-1 and pU6-mY1 were constructed by insertion of annealed synthetic oligonucleotides of DNA encoding B-5-1 and mY1 RNA sequences into pU6 vectors using BamH I and EcoR I sites. Sequences of oligonucleotides for B-5-1 are 5' GATCACCGGTCTCACGACGACATTCCAGACGTGGCCTCGTGGGTGCTTCCACGTTGCGAACACCCCGATTTCCTCCGGTCCCTTTTTTGGAA 3' and 5' AATTTTCCAAAAAAGGGACCGGGAAATCGGGGTGTTTCGCAACGTGGAAGCACCCACGAGGCCACGTCTGGAATGTCGTCGTGAGACCGGT 3'; for mY1: 5' GATCGGCTGGTCCGAAGGTAGTGAGTTATCTCAATTGATTGTTACAGTCAGTTACAGATTGAACTCCTGTTCTACTTTCCCCCTTCTC ACTACTGCACTTGACTAGTCTTTTTTGGAA 3' and 5' AATTTTCCAAAAAAGACTAGTCAAGTGCAGTAGTGAGAAGGGGGGAAAGTGTAGAACAGGAGTTCAATCTGTAAGTACTGACTGTGAACAATCAATTGAGATAACTACTACCTTCGGACCAGCC 3'.

Purification of proteins and the inhibitory activities (IAs).

Human recombinant topoisomerase I (48), Pol-primase (43), RPA (35, 40), BKV TAg and SV40 TAg (4, 28, 36) were expressed, purified and their concentrations and activities determined as previously described (21).

To purify IAs of BKV DNA replication extracts from FM3A cells (57) were prepared as previously described (28). FM3A cell extracts (5 mg) were initially passed over Affigel blue resin (0.5 ml, Biorad) under gravity flow and then over a phosphocellulose P11 resin (Whatman). The final flow-through was collected and applied to 1 ml of ssDNA-cellulose equilibrated with 20 mM HEPES-KOH, pH 7.5, 100 mM NaCl, and 0.03 mg/ml BSA, and incubated for 1 h at 4°C. Resin was washed with 20 column volumes of same buffer and IAs were eluted with step gradient using increasing salt concentrations of 250 mM NaCl, 500 mM NaCl, 750 mM NaCl and 1

M NaCl. Fractions eluted with 500-750mM salt concentration containing IAs were collected, dialysed to remove excess salt or alternatively diluted with salt-free buffer and used for anion exchange chromatography. The pooled IA fractions were loaded on a Q Sepharose column (bed volume 1ml) equilibrated with 20 mM HEPES-KOH, pH 7.5, 100 mM NaCl and washed sequentially with 5 column volumes of buffer containing 250 mM NaCl. Bound material was eluted in 750 mM NaCl, 20 mM HEPES-KOH, pH 7.5, diluted with salt free buffer and loaded on to Mono Q HR 5/5 column using the ÄKTA Explorer FPLC system (buffer A: 20 mM HEPES-KOH, pH 7.5, 50 mM NaCl and 1 mM EDTA; buffer B: 20 mM HEPES-KOH, pH 7.5, 1 M NaCl and 1 mM EDTA). IAs were eluted with a salt gradient. Peak fractions were dialysed and tested for BKV DNA replication inhibition or alternatively extracted with 1 volume phenol, 1 volume chloroform and precipitated with 2-propanol.

***In vitro* DNA replication assays**

Replication of DNAs *in vitro* was assayed as previously described (28, 53) with slight modifications. Briefly, the reaction (40 μ l) contained 20 mM HEPES-KOH, pH 7.8, 7 mM MgAc 1 mM DTT, 4 mM ATP, 200 μ M each CTP, UTP, and GTP, 50 μ M dCTP, 100 μ M each dATP, dTTP, and dGTP; 40 mM creatine phosphate (pH 7.8), 40 μ g/ml creatine kinase and 5 μ Ci of [α^{32} P] dCTP (3000 Ci/mmol, Perkin-Elmer); 0.25 μ g origin-containing plasmid DNA; and human HeLa cell extract (100 μ g of total protein). The small RNAs were added to the assay as indicated in the figure legends. The reaction was started by the addition of 0.2 μ g of purified BKV TAg. After incubation for 60 min at 37°C, reaction products were precipitated with cold 10% (w/v) trichloroacetic acid containing 2.5% (w/v) sodium pyrophosphate and spotted on glass fiber filters (GF/C; Whatman), washed with 1 M HCl and analyzed by scintillation counting.

***In vivo* DNA replication assays**

Human kidney proximal tubular epithelial cells (HK-2 cells) were grown in RPMI-1640 with 10% FBS, seeded in 12-well plates (1×10^5 cells/well), and incubated overnight at 37°C. Cells were transfected with expression vector for TAg pCMV-BKTA_g (10 ng), template plasmid pOriBKV (10 ng), srRNA expression vectors pU6-mY1/pU6-B-5-1 or empty control vector pUC (200 ng), and pBC plasmid vector (500

ng) as carrier DNA using Fugene HD (Roche) transfection reagent. Replicated template DNA was extracted and analyzed by southern blotting as previously described (28). Independent assays were performed in triplicate and yielded equivalent results.

Monopolymerase DNA replication assay

The BKV and SV40 monopolymerase replication assay contained 0.25 μg of pOriBKV DNA (28) or 0.5 μg of pOriSV40 (42), respectively. The assay was supplemented with 50 ng topoisomerase I, 100 ng Pol-primase and 1 μg RPA, in 30 mM HEPES-KOH, pH 7.8, 7 mM MgAc, 0.1 mM EGTA, 0.5 mM DTT, 200 μM each UTP, GTP, and CTP, 4 mM ATP, 100 μM each dATP, dGTP, and dTTP, and 10 μM dCTP, 40 mM creatine phosphate, 1 μg creatine kinase, 0.1 mg/ml heat-treated BSA, and 5 μCi [$\alpha^{32}\text{P}$]-dCTP (3000 Ci/mmol, Perkin-Elmer) in 40 μl . The small RNAs were added to the assay as indicated in the figure legends. Purified BKV or SV40 TAg (0.2 μg) was added to start the reaction as indicated, and after incubation for 60 min at 37°C, the reaction products were precipitated with cold 10% (w/v) TCA containing 2.5% (w/v) sodium pyrophosphate and spotted on glass fiber filters (GF/C; Whatman), washed with 1 M HCl and analyzed by scintillation counting.

Initiation of replication of BKV DNA

Initiation reactions were performed according to (47, 53). In detail, the initiation reaction mixtures contained 0.25 μg of origin containing DNA, 1.6 μg of BKV TAg, 1 μg of RPA, 30 mM HEPES-KOH, pH 7.8, 7 mM magnesium acetate, 1 mM EGTA, 1 mM dithiothreitol, 0.2 mM UTP, 0.2 mM GTP, 0.01 mM CTP, 4 mM ATP, 40 mM creatine phosphate, 1 μg of creatine kinase, 0.3 μg of topoisomerase I, 0.2 mg/ml of bovine serum albumin (BSA), and 10 μCi of [$\alpha^{32}\text{P}$] CTP (3,000 Ci/mmol; Perkin-Elmer). The reaction was started by the addition of 100 ng recombinant human Pol-primase. The small RNAs were added to the assay as indicated in the figure legends. The reaction mixture was spotted on DE81 paper (Whatman), and then unincorporated NTPs were removed by washing six times with 0.5 M Na_2HPO_4 , twice each with water and ethanol. The incorporation of NMPs was analyzed by scintillation counting.

Unwinding assay

The unwinding of origin containing plasmid DNA was carried as previously described (56) with slight modifications. The SV40 and BKV unwinding assay contained 0.5 µg of pOriBKV DNA or 0.5 µg of pUC-HS DNA (or pOriSV40) (42), 50 ng topoisomerase I, 1 µg RPA, in 30 mM HEPES-KOH, pH 7.8, 7 mM MgAc, 0.1 mM EGTA, 0.5 mM DTT, 4 mM ATP, 40 mM creatine phosphate, 1 µg creatine kinase, 0.1 mg/ml BSA in 40 µl. Purified BKV or SV40 TAg (0.2 µg) was added to start the reaction, and after incubation for 60 min at 37°C, the reaction mixture was supplemented with 4 µl of proteinase K/EDTA solution (10 mg/ml proteinase K, 0.1 M EDTA) and 3 µl of 1% SDS and incubated for 30 minutes at 37°C. After that, 2 µl of 3M NaAc and 70 µl of 2-propanol were added to precipitate DNA. The mixture was incubated for 1 h at -70°C. DNA precipitate was collected by centrifugation (22,000xg for 15 minutes at 4°C) washed with 70% ethanol, briefly air dried, resuspended in 5 µl of 1x agarose loading buffer and separated in 1.5% agarose (low melting point quality) gels at 9 V per cm. Gels were stained with 0.05 mg/ml of ethidium bromide and photographed under UV irradiation. For quantification the stained gels were scanned using a FLA5100 imager and the ImageGauge software (both Fuji). The unwinding activity is defined as the amount of unwound DNA relative to the total DNA (supercoiled plus unwound) determined in the reaction. Percentage of inhibition is $100 - 100 \cdot a_i/a_0$ with a_i being the activity in the presence of inhibitory RNA and a_0 the activity without RNA.

Cloning of small RNA

Synthesis of cDNA clones from purified RNA preparations was performed according to Lau et al. (24) with modifications. Total RNA (10^8 cells) was separated on Mono Q. RNA fractions containing RNA that inhibit BKV DNA replication were combined. In the initial step, RNA was ligated to an activated miRNA cloning linker containing Ban I restriction site AppCTGTAGGCACCATCAddA (IDT Inc.) in buffer without ATP. RNA greater than 40 nt was purified from the gel. Alternatively RNA was separated from unligated 3' adaptor by using G-50 spin column. Purified RNA was used for 5' adaptor ligation (ATCGTtaggcaccugaaa, RNA/DNA version (lowercase RNA)). RNA mixture greater than 40 nt was purified as described above. Purified RNA was used for RT-PCR (RT primer ATTGATGGTGCCTAC, PCR primers ATTGATGGTGCCTACAG, ATCGTAGGCACCTGAAA). Following 25 cycles of

PCR products were subsequently cloned into pGemT-Easy vector (Promega).

***In vitro* RNA synthesis**

For *in vitro* RNA synthesis PCR was performed using primers containing T7 or Sp6 promoter sequences. Alternatively predigested plasmid DNA or synthetic oligonucleotide (template single stranded oligonucleotide with annealed promoter sequence containing primer) was used as a template. Template DNA was transcribed using T7 or Sp6 RNA polymerase (Roche). RNA was purified by isopropanol precipitation (1 volume) following acid phenol, phenol/chloroform, and chloroform extraction.

Enrichment of RNAs binding to origin DNA

An RNA pool was produced by *in vitro* transcription of PCR products of cloned small RNA and then was used for selection of origin-binding species. Denatured RNA (0.5 μ g) and 50 ng of denatured BKVori (Dik strain 5031-282) were hybridized at 48⁰C in SELEX buffer. Resulting mixtures were separated in 2% agarose gel and RNA/DNA hybrids were purified from the gel using a gel extraction kit (Sigma) and then subjected to RT-PCR amplification followed by *in vitro* transcription. The enriched RNA pool was used for the next round of selection. Procedure was repeated up to 12 times. PCR products after enrichment steps 5, 8 and 12 were cloned into pGemT-Easy vector, selected and inserts were sequenced.

RESULTS

Inhibitory activities for BKV replication in mouse cell extracts are small ncRNAs specific for the BKV NCCR

In vitro BKV DNA replication assays using the monopolymerase assay (28) were used to guide biochemical fractionation to purify IAs (28, 53) from mouse FM3A cell extracts. Fractions of all purification were analyzed by SDS PAGE, and after Mono Q chromatography, no specific protein bands (stained with silver) correlated with IAs (data not shown). The Mono Q purified fractions of IA occurred as one single peak whose A254/A280 was approximately two, suggesting that IAs are predominantly comprised of nucleic acids. These fractions were analyzed by agarose gel electrophoresis followed by ethidium bromide staining and were found to contain nucleic acids whose migration on a gel suggests a size of less than 100 nt (Fig. 1A). RNase treatment but not DNase treatment of the fractions with maximal inhibitory activity abolished inhibition of BKV DNA replication *in vitro* (Fig. 1B) indicating the IA is composed of small RNAs, hereafter called srRNAs. While these findings do not rule out the presence of accessory proteins (such as RNPs), no evidence has been obtained suggesting the requirement for such.

To assess the specificity of the fractionated srRNAs, the highly purified Mono Q fractions were added to monopolymerase replication assays with either BKV or SV40 TAg and DNA containing their cognate NCCRs. These analyses revealed that the inhibition of BKV replication by srRNAs is specific for the BKV NCCR and is independent of the source of TAg (the SV40 TAg can act at the BKV origin (28); Fig. 1C and data not shown). Notably, the full replication activity of the monopolymerase assay with the SV40 template indicates these srRNAs neither inhibit enzymatic activities of cellular replication factors nor those of TAg. RNAs of other mouse cells and human cells were analyzed for inhibitory activities: cellular RNAs of NIH-3T3 cells, prior to and after purification by Mono Q chromatography, significantly inhibited BKV DNA replication in the monopolymerase assay, whereas no inhibition (and even some stimulation) was detected with unfractionated human HeLa-S3 and HEK293S cell RNAs and RNAs purified by Mono Q chromatography (Figs. 2B to 2E; and data not shown). Fractions A2 and A3 from NIH-3T3 cells (Figure 2C) were the most inhibitory and the profile of IAs differed among the cell lines, suggesting the expression and composition of the srRNAs is cell line-specific.

Enrichment, cloning and sequence analysis of murine srRNAs

The MonoQ-purified small RNAs described in Figure 1A were cloned according to Lau et al. (24) and the recovered plasmids were sequenced. In total 71 cDNA sequences were obtained having an average length of 47 ± 13 nt and an average GC content of $57\pm 10\%$. These cDNAs were ligated to a T7 promoter and transcribed, and the transcripts tested in various replication assays. One srRNA (Seq3) significantly inhibited BKV replication in the monopolymerase assay and expressing Seq3 in human cells inhibited BKV DNA replication *in vivo* by about 50%, whereas Seq3 did not inhibit SV40 replication in the monopolymerase assay (summarized in Table 1 and data not shown). Structure predictions using the RNA fold program (<http://www.tbi.univie.ac.at/RNA/man/RNAfold.html>) suggested that Seq3 contained a hairpin with a ΔG of -10.9 kcal/mol and bioinformatic analyses of Seq3 showed small similarities to origin sequence but did not identify a sequence annotation in published databases (data not shown).

To obtain additional replication inhibitory RNA sequences at a higher frequency than by direct cloning, we developed an alternative strategy involving an adaptation of SELEX (Systematic Evolution of Ligands by EXponential enrichment; (12, 17) Previous observations that templates containing BKV core origins with heterologous murine polyomavirus (mPyV) origin flanking sequences can be replicated by FM3A mouse cell extracts supplemented with human Pol-primase complex (53) suggest the inhibitory activity of FM3A extracts (and thus the srRNAs) act through the viral NCCR. Accordingly, cDNAs derived from purified srRNA fractions were ligated to a T7 promoter and transcribed *in vitro*, and RNAs capable of binding to the denatured BKV NCCR were amplified by RT-PCR, repetitively). The inhibitory activity of the amplified RNA pool significantly increased with the number of SELEX cycles (data not shown): after 5 (B-5), 8 (B-8) and 12 selection cycles (B-12), cDNA sequences from the SELEX generated srRNAs were cloned and sequenced (Table 1 and data not shown).

BLAST searches of mouse and human genomes and transcriptomes were performed with the SELEX-selected cDNA sequences as queries, but no significant similarity was found. However, transcripts from repetitive sequences and modified non-coding RNAs are difficult to determine by BLAST searches (11), so the RepeatMasker

program (46) was employed to search mouse and human genome databases (genome plus transcripts), and similarities were identified with RNAs from diverse genomic repeats, snRNAs and tRNAs (data not shown). It is noteworthy that some of these RNAs, of approximately the same size as the srRNAs, are derived from heterochromatic satellite sequences at pericentromeric regions of chromosomes, and these RNAs are enriched in human and murine cancer cells, especially by DNA stressors and inducers of apoptosis (3, 22, 54).

B-5-1 and B-5-8 srRNAs are BKV-specific and expressed in mouse cells *in vivo*

The SELEX-selected B-5-1 and B-5-8 srRNAs inhibited *in vitro* BKV DNA replication to a greater extent than other RNAs (Fig. 3A, summarized in Table 1). Bioinformatic analyses using calculations of hybridization energy with data sets for RNA-DNA hybrids of Sugimoto et al (51, 52) revealed that srRNAs contain at least two sequence elements complementary to independent regions located on opposite strands of the BKV NCCR. The calculated hybridization energies in dependence of the nucleotide location of the BKV NCCR for the two RNAs B-5-1 and B-5-8 are shown as examples (Fig. 3B): both RNAs contain two such sequences described above, highlighted by different color-coding. Expression of these srRNAs in mouse FM3A cells was confirmed by RT-PCR (Fig. 3C). The amplified PCR products from FM3A cells were cloned and sequenced, and found to have identical sequences to B-5-1 or B-5-8 RNAs. However, similar amplification products were not detected with cellular RNAs from NIH 3T3 cells or human HeLa cells. A weak cDNA product band was detected with cellular RNAs from human 293S cells, using the B-5-8 specific primers (Fig. 3C), and verified by sequencing the cDNAs. These B-5-8 RNAs of 293S cells were reproducibly detected, but in much lower concentrations than those of FM3A cells, and their presence may explain why the total RNA of 293S cells did not stimulate BKV DNA replication or even slightly inhibited replication, whereas HeLa cells, which lack this RNA, stimulated BKV DNA replication (compare Figure 2E with 2D).

Inhibitory srRNAs block BKV DNA replication at an initiation step

To determine whether the unwinding or the initiation reaction is the target for inhibition, these initiation steps were biochemically analyzed. Addition of inhibitory

srRNA partially reduced the level of unwound origin DNA (Fig. 4A, summarized in Table 1). A slight decrease in unwinding of origin-containing DNA was determined with total FM3A RNA, which was in the range of that observed with B-5-8, but no significant reduction of unwound DNA was determined with B-5-1 and B-8-1 RNA (although both significantly inhibited BKV DNA replication (Fig. 3A and Table 1). Despite that multiple RNAs including total FM3A RNA reduced unwinding of BKV DNA, the extent of this inhibition (always lower than 15%) cannot solely account for the observed inhibition of viral replication. In contrast to the slight effect on unwinding of BKV origin-containing DNA, the initiation of BKV DNA replication was reproducibly and efficiently inhibited by srRNAs B-5-1 and B-8-1 (Fig. 4B, Table 1). In summary, these data reveal that srRNAs can inhibit multiple stages of DNA replication but that the initiation reaction is the main target of the inhibition.

Interactions of srRNAs with BKV NCCR DNA

Previous investigations revealing that sequences flanking the BKV core origin are required for function of the inhibitory activity in FM3A extracts (53) and the observation of interactions of srRNAs with origin DNA (Fig. 3B), led to the design of experiments exploring the regions in srRNAs B-5-1 and B-5-8 that are responsible for inhibition. Deletion or modification of sequences at the 3' or 5' end of B-5-8 RNA, predicted to hybridize to the BKV NCCR, abolished the ability of RNAs to inhibit the BKV monopolymerase assay (Fig. 5). Furthermore, RNAs that should bind to the BKV NCCR (e.g. B-5-8(1-17) and B-5-8(37-60)) but that lack a connecting sequence did not inhibit DNA replication (data not shown). Deletions of B-5-1 RNA at the 3'- or 5'-termini did not affect its inhibitory activity, supporting the suggestion that binding sites are situated within the 23-50 nt central region of B-5-1 RNA (Table 1). This sequence was synthesized *in vitro*, and B-5-1(23-50) transcripts were found to exhibit an inhibitory activity equal to that of the parent molecule B-5-1 (Fig. 5B). Closer analysis of the B-5-1(23-50) sequence revealed that two sites (underlined nucleotides of B-5-1 and B-5-1(23-50) in Table 1) should hybridize to the BKV core origin on opposite strands. Introduction of a point mutation into the most 3' site of the two hybridization sequences abolished the inhibition (Fig. 5C, Table 1, B-5-1(23-50)mut. In contrast, RNA B-5-1(23-50)rc with reverse complementary sequence to B-5-1(23-50), which should hybridize to the reverse complementary sites of the origin DNA (i.e. sites opposite to the original hybridization sites), exhibited inhibitory

activity similar to B-5-1 (Fig. 5C). This suggests that inhibition of replication initiation is mediated by simultaneous binding of one srRNA molecule to opposite strands of the NCCR.

Development of an srRNA that inhibits SV40 DNA replication *in vitro*

B-5-1(23-50) RNA is predicted to have equivalent hybridization sites to the SV40 NCCR, due to the high homology between BKV and SV40. However, the putative SV40 hybridization sites overlap in the RNA sequence, so one RNA molecule would not hybridize to both strands of the SV40 origin at the same time. To examine whether srRNAs inhibitory to SV40 can be developed from this molecule, mutant RNA B-5-1(23-50)SV40 was designed by introducing four nucleotides in the 3' part of the RNA, to create an additional hybridization site for the SV40 core origin (and concomitantly, destroying the hybridization site to the BKV core origin; Fig. 5C, summarized in Table 1). In full agreement with the prediction and in contrast to B-5-1(23-50), RNA B-5-1(23-50)SV40 effectively inhibited SV40 but not BKV DNA replication (Fig. 5C, Table 1).

De novo design of srRNAs that inhibit BKV DNA replication *in vitro*

These results indicate that RNAs hybridizing to the upper and lower strand of the BKV or SV40 NCCR, within or close to the core origin, will inhibit DNA replication. To test this further, we designed RNAs *de novo* having both hybridization sites within the BKV core origin, or having one site within the core origin and a second site complementary to a sequence 100 nts distant, within the late enhancer sequence. RNAs D1 and D2, respectively (Fig. 6A, Table 1) were synthesized and tested for inhibitory activity. Alternatively the complementary sequences were synthesized in a reverse complementary fashion (Fig. 6A, RNA D3, Table 1). All three RNAs also contain a quadruple A stretch to connect the two binding sites. As predicted, these three RNAs significantly inhibited BKV DNA replication in the monopolymerase assay (Fig. 6B, summarized in Table 1), indicating that RNAs with two complementary sites to the viral NCCR are sufficient to inhibit the viral DNA replication *in vitro*.

srRNA inhibits BKV DNA replication *in vivo*

To ascertain if srRNAs function *in vivo*, the B-5-1 srRNA sequence was placed downstream of the U6 promoter (pU6-B-5-1) and transiently expressed in human kidney tubular epithelial cells (HK-2 cells), together with template DNAs and a DNA expressing BKV TAg. In multiple independent assays, BKV DNA replication was consistently inhibited by up to 70% compared to transfections with the vector DNA (pUC) or the vector expressing an unspecific mouse Y-RNA (pU6-mY1) (Fig. 7), indicating srRNAs that inhibit the BKV replication *in vitro* also inhibit BKV DNA replication *in vivo*.

DISCUSSION

That ncRNAs can control both cellular and viral DNA replication is a significant recent development in our understanding (5, 23, 38). In prior studies of BKV replication, we discerned the presence of diffusible inhibitors in mouse FM3A cell extracts that had not been detected in similar studies of the replication of other polyomaviruses (28, 53). Further characterization reveals these inhibitors to be small RNAs, termed srRNAs that are origin-specific and can function both *in vitro* and *in vivo*. During our purification we could not detect any specific proteins associated with these srRNAs. Furthermore, inhibition of BKV DNA replication by srRNA was observed in the monopolymerase assay, indicating other accessory proteins are not absolutely required for the inhibition (28, 53).

It is important to contrast previous studies of RNA aptamers that inhibit the viral helicase (17), with our observations that srRNAs do not inhibit helicase activity of viral TAg. Moreover, the primase activity of Pol-primase itself is not blocked directly by srRNAs, since the same enzyme complex initiates SV40 DNA replication but not BKV DNA replication in the presence of these srRNAs. This is consistent with our previous findings that the inhibitory activities are specific for BKV DNA replication but do not interfere with SV40 DNA replication (28, 53). However, the *de novo* synthesis of RNA primers during the initiation of BKV DNA replication by Pol-primase may be inhibited by sequence-specific hybridization of the srRNAs.

Bioinformatic analyses of the inhibitory srRNAs suggest that inhibition of BKV DNA replication requires a single molecule of RNA to anneal to both strands of the viral non-coding control region. This leads to the hypothesis that inhibition of replication is mediated by hybridization of srRNAs to sequences on opposite strands close to or within the BKV core-origin so as to form a replication-inactive initiation complex (Fig. 8). Interestingly many srRNAs have the feature that just one region of the RNA hybridizes to both strands of the SV40 origin at neighboring sites. Since the SV40 core origin pentamer elements form a more perfect palindrome than the BKV core origin elements, the srRNAs inhibitory for BKV replication do not inhibit SV40 DNA replication. The isolation of identical sequences for both RNAs B-5-1 and B-5-8 suggest that they do not belong to a family of highly repeated genomic sequences, but the identification of genomic sequences encoding these RNAs will require sequencing the FM3A tumor cell genome or deep sequencing of its ncRNAs. The binding of

srRNAs to the viral origin DNA is reminiscent of the interaction of antigene RNAs to promoters, but is likely to involve different biochemical mechanisms and pathways, since the argonaute protein is most likely involved in the promoter-dependent inhibition of transcription but not in the action of srRNAs (6, 7, 18, 58).

The mechanism of inhibition of BKV DNA replication by srRNAs was examined in various ways: deletion mutants of the srRNAs encompassing or exchanging nucleotides within predicted binding sites abolished the inhibition of DNA replication by the srRNAs. In addition, an artificial RNA was designed that specifically inhibits SV40 but not BKV DNA replication, and the *de novo* design of RNA sequences inhibited BKV DNA replication. That srRNAs are expressed in tumor cells, and also that the small DNA polyomaviridae have very successfully served as model systems to study viral and cellular functions including the molecular mechanisms and regulation of host DNA replication and transcription (9, 15) lead us to speculate about a role of srRNAs in cellular control pathways: The negative regulatory srRNAs reported here, together with the recently described positive regulatory RNAs (5, 23, 38) provide novel and additional levels of control to eukaryotic DNA replication and may lead to the development of new strategies to inhibit replication of viruses or other infections agents. Thus, as for ncRNAs in gene expression (3, 13, 16, 20), srRNAs may contribute to regulation of DNA replication in addition to the well-known factors such as posttranslational modifications, protein-protein interactions and protein stability (8, 27, 29, 33, 34).

ACKNOWLEDGMENTS

We thank Drs. T. Krude, A. Samali, S. Gupta and C. Morrison for reagents, advice on small RNA cloning, and critical reading of the manuscript. This work was supported by grants of Science Foundation Ireland (HPN), a NUIG student fellowship, a Thomas Crawford Hayes fellowship (both IT), and funds from the University of Missouri-Columbia (WF). C.S. is a Science Foundation Ireland Stokes Professor of Bioinformatics.

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FIGURE LEGENDS

Figure 1 Determination of nature of inhibitory activity. **A.** Fractions F7-F12 corresponding to the Mono Q elution peak analyzed by electrophoresis in 2% agarose gel followed by staining with EtBr. The inhibitory activity (IA) toward BKV DNA replication, and the 100 bp marker are indicated on the top and the right of the panel, respectively. **B.** Mono Q chromatography-purified IA from mouse FM3A cells was treated with RNase or DNase, and then tested in BKV monopolymerase replication assays. **C.** srRNA fraction purified from FM3A extracts were added to the monopolymerase replication assay using DNA containing the BKV origin of replication with SV40 TAg, or DNA containing the SV40 origin of replication with BKV TAg (75 ng of RNA with an estimated length of 50 nt (length estimation of RNA is derived from the cloning results without SELEX) yield a 50% inhibition with an 36-fold molar excess of RNA over origin DNA). Reactions with BKV TAg and DNA containing the BKV origin of replication served as controls. The incorporation of dNMPs into the BKV origin DNA was detected by acid precipitation and scintillation counting. DNA synthesis was determined in duplicates and repeated three times. The average of incorporation of dNMPs and the standard deviations are presented.

Figure 2 Purification of inhibitory srRNAs. **A.** Mono Q fractions of total RNAs from FM3A cells were analyzed by electrophoresis in an 1.5% agarose gel. In parallel, fractions A1, A2, A3, A4 and A8 of FM3A (**B**), NIH 3T3 (**C**), HeLa (**D**) and HEK293 (**E**) cells corresponding to 0.6 M, 0.62 M, 0.64 M, 0.66 M and 0.73 M NaCl. The incorporation of dNMPs into the BKV origin DNA was detected by acid precipitation and scintillation counting. DNA synthesis was determined in duplicates and repeated three times. The average of incorporation of dNMPs and the standard deviations are presented.

Figure 3 Replication of polyomavirus DNA in the presence of srRNAs. **A.** srRNAs were transcribed *in vitro* using oligonucleotides with an SP6 promoter; the sequence coding for the indicated RNAs as templates were assayed for inhibition of BKV DNA replication either in HeLa extract or in the monopolymerase replication assay. DNA synthesis was determined in duplicates and repeated three times. The average of incorporation of dNMPs and the standard deviations are presented. **B.**

Calculation of hybridization energies of B-5-1 and B-5-8 and their interaction with the BKV NCCR. The energy was calculated using the nearest-neighbor method with data sets for RNA-DNA hybrids of Sugimoto et al. (51, 52). Each determination of hybridization energy lower than -6 kcal/mol and -4 kcal/mol (upper and lower panel, respectively) was plotted versus position on the origin of replication. Blue and red colors mark the binding of RNA to the upper and the lower DNA strands. **C.** 25 ng of total RNA from FM3A, NIH 3T3, HEK293S and HeLa cells was reverse transcribed using random decamers as primer at 42°C. Reverse transcription products were PCR amplified using specific primers for B-5-1 and B-5-8 sequences. For B-5-1 specific product 47 nt of nested PCR is presented. FM3A reverse transcription product without reverse transcriptase served as a negative control (-RT).

Figure 4 Unwinding of BKV DNA and initiation of BKV DNA replication in the presence of srRNAs. **A.** Unwinding of BKV and SV40 origin-containing DNAs (lanes 1 to 11 and lanes 12 to 16, respectively, as indicated) with BKV large TAg in the absence of RNA (lanes 1 and 12 as indicated by BKV and SV40) and in the presence of 10 or 20 ng B-5-1, B-5-8, B-8-1, or total FM3A RNA as indicated. The B-5-8 srRNA does not inhibit SV40 origin-dependent unwinding suggesting that the helicase function of BKV large TAg is not inhibited by this RNA. Percentage of inhibition in the presence of RNAs relative to samples without RNA is given in the diagram. **B.** Increasing amounts of B-5-1 RNA, B-5-8 RNA, B-8-1 RNA, total RNA from NIH 3T3 cells, or origin-based SELEX step 12 RNA, B-12, were added to the initiation assay with DNA containing the BKV origin of replication. Incorporation of labeled nucleotide in the absence of large TAg served as a background control and was subtracted.

Figure 5 Modulation of BKV and SV40 DNA replication in the presence of mutant srRNAs. **A.** and **B.** Wild type and deletion mutants of B-5-1 and B-5-8 RNA were added to the monopolymerase BKV DNA replication assay in the indicated amounts (the numbers in brackets were used for the description of the mutants and refer to the nucleotide regions of B-5-1 and B-5-8 which are transcribed *in vitro* using T7 RNA polymerase). **C.** Small RNAs added to the monopolymerase DNA replication assay with SV40 or BKV origin of replication and the respective TAg. The mutants derived from B-5-1 RNA B-5-1(23-50), B-5-1(23-50)mut, B-5-1(23-50)rc

(reverse complement of B-5-1(23-50)) and B-5-1(23-50)SV40 (mutant of B-5-1(23-50) having two binding sites SV40 NCCR) putatively involved in hybridization to the SV40 or BKV origin of replication in the monopolymerase DNA replication assay containing SV40 or BKV origin of replication in the presence of the purified recombinant SV40 or BKV TAg, respectively. The incorporation of dNMPs into the BKV origin DNA was detected by acid precipitation and scintillation counting. DNA synthesis was determined in duplicates and repeated three times. The average of incorporation of dNMPs and the standard deviations are presented. The data are summarized in Table 1.

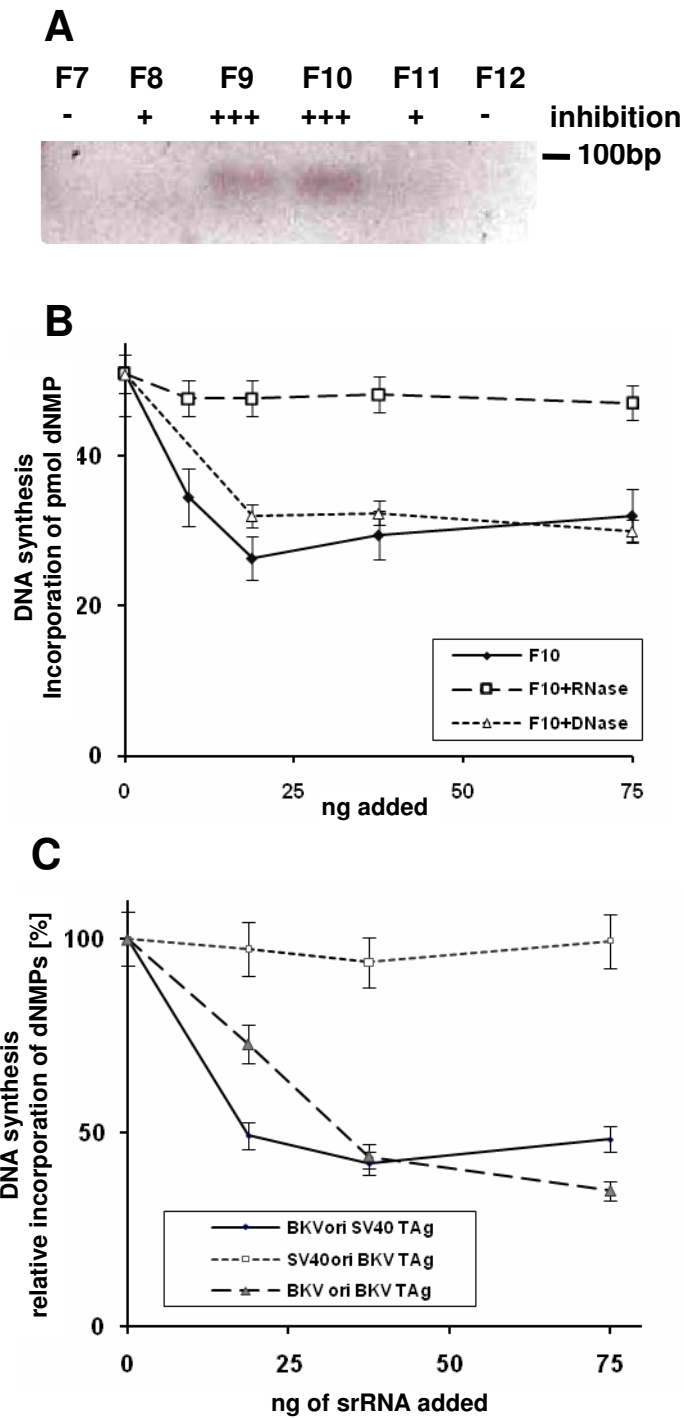
Figure 6 Inhibition of BKV DNA replication in the presence of newly designed inhibitor RNAs. **A.** Following the hypothesis that RNA must contain two sites complementary to the opposite strands of the BKV NCCR to inhibit BKV DNA replication, the RNA sequences D1, D2 and D3 were designed with D3 containing the binding sites of D1 in opposite orientations. The energy was calculated using the nearest-neighbor method with data sets for RNA-DNA hybrids of Sugimoto et al. (51, 52). Each determination of hybridization energy lower than -7 kcal/mol was plotted versus the position at the BKV origin of replication. Blue and red colors mark the binding to the upper and the lower DNA strands. **B.** RNA sequences D1, D2 and D3 were added to the monopolymerase BKV DNA replication assay as indicated. The incorporation of dNMPs into the BKV origin DNA was detected by acid precipitation and scintillation counting. DNA synthesis was determined in duplicates and repeated three times. The average of incorporation of dNMPs and the standard deviations are presented.

Figure 7 *In vivo* BKV DNA replication in the presence of srRNA. BKV DNA replication *in vivo* was measured by transfecting HK-2 cells with BKV-origin containing plasmids and BKV TAg expression vector together with an empty vector (pUC, lane 1), or a control vector expressing mouse Y1-RNA (pU6-mY1, lane 2; mouse Y1-RNA also does not inhibit BKV DNA replication *in vitro* (Tikhanovich and Nasheuer, unpublished data)) or B-5-1 expression vector (pU6-B-5-1, lane 3). Cells were harvested 48 hours post transfection and replicated BKV DNA was analyzed by EcoR I and Dpn I digestion, agarose gel electrophoresis and southern blotting as previously

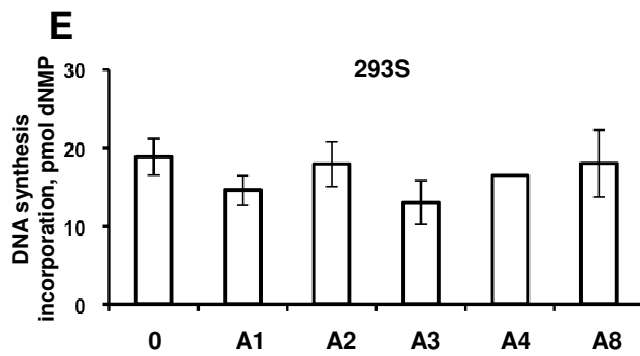
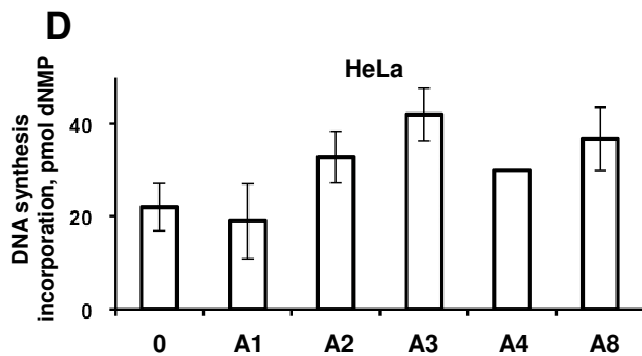
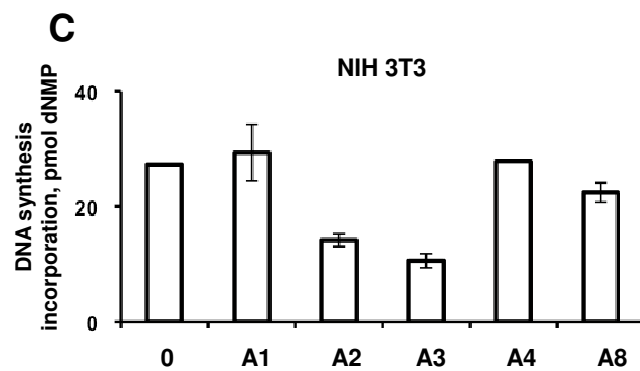
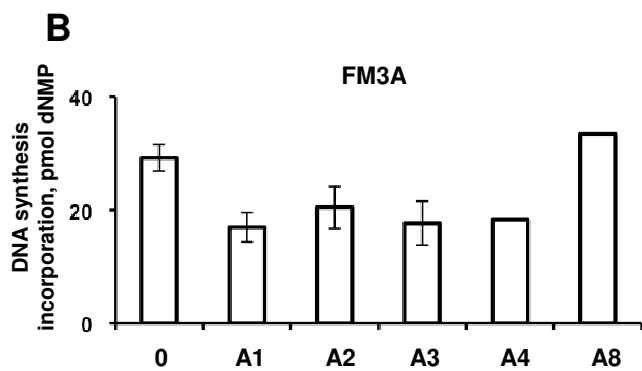
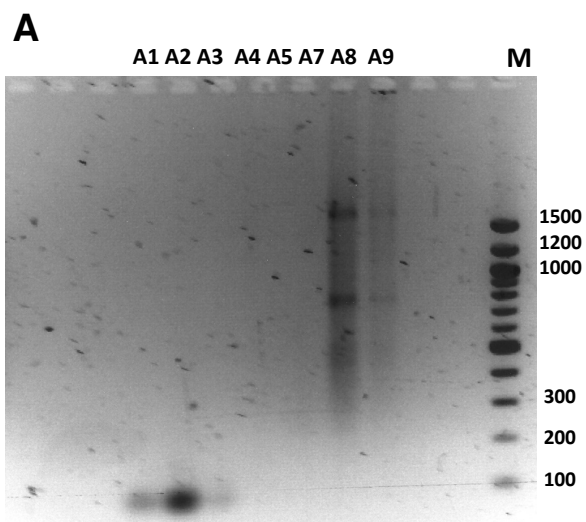
reported (28). The average quantification of band density of three *in vivo* DNA replication experiments and the standard deviation are presented in panel B. The amount of Dpn I-resistant replication products in the transfection of pUC empty vector were arbitrarily set to 100%.

Figure 8 Model of inhibition of BKV DNA replication by srRNAs. An srRNA molecule anneals to opposite strands of the BKV origin of replication and interferes with primer synthesis by Pol-primase by changing the structure of the initiation complex. Leading and lagging strands of DNA are brought together by interaction with srRNA, and primer synthesis is blocked while unwinding is undisturbed.

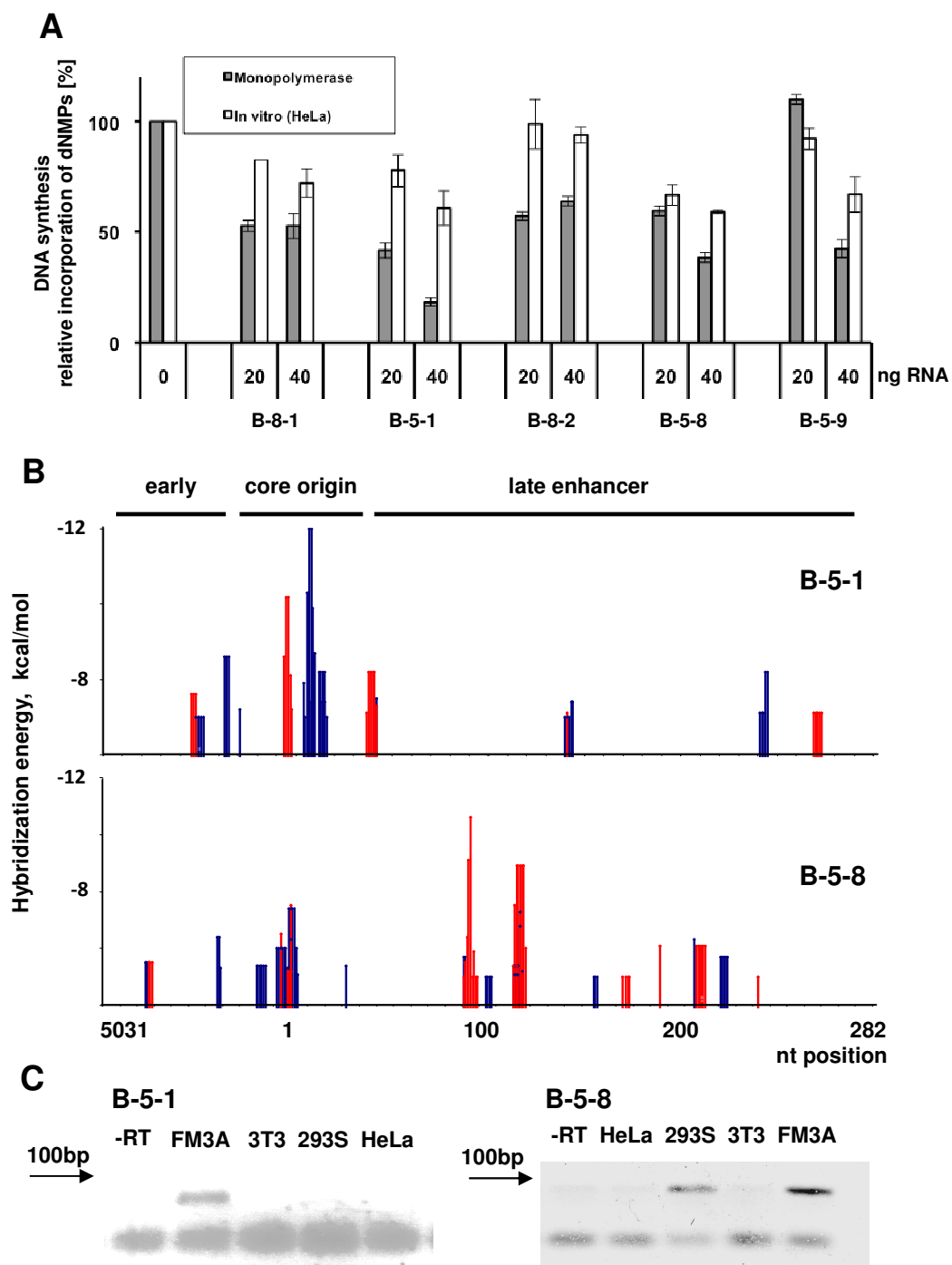
Tikhanovich et al., Figure 1



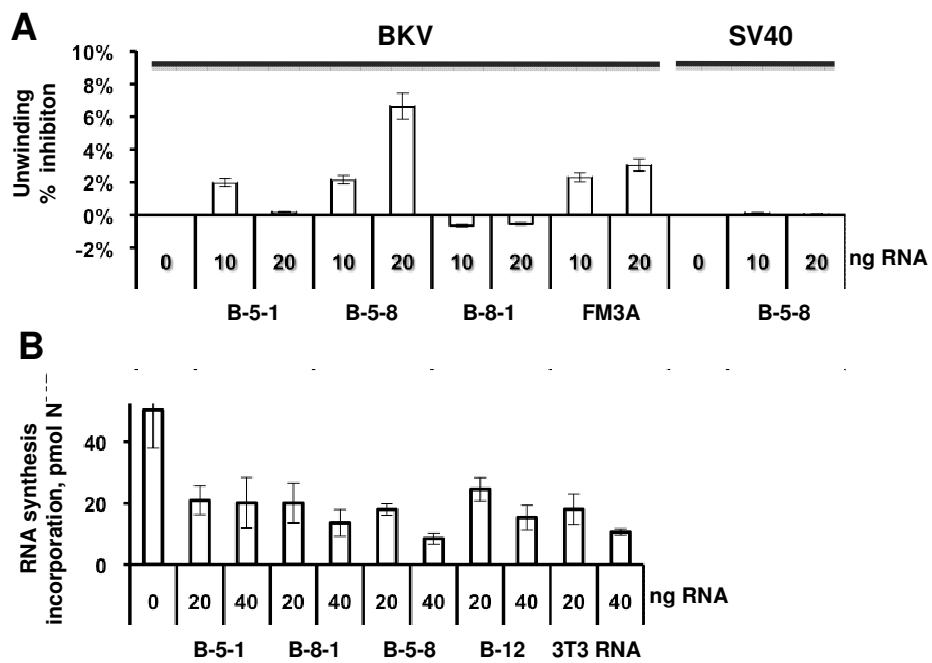
Tikhanovich et al., Figure 2



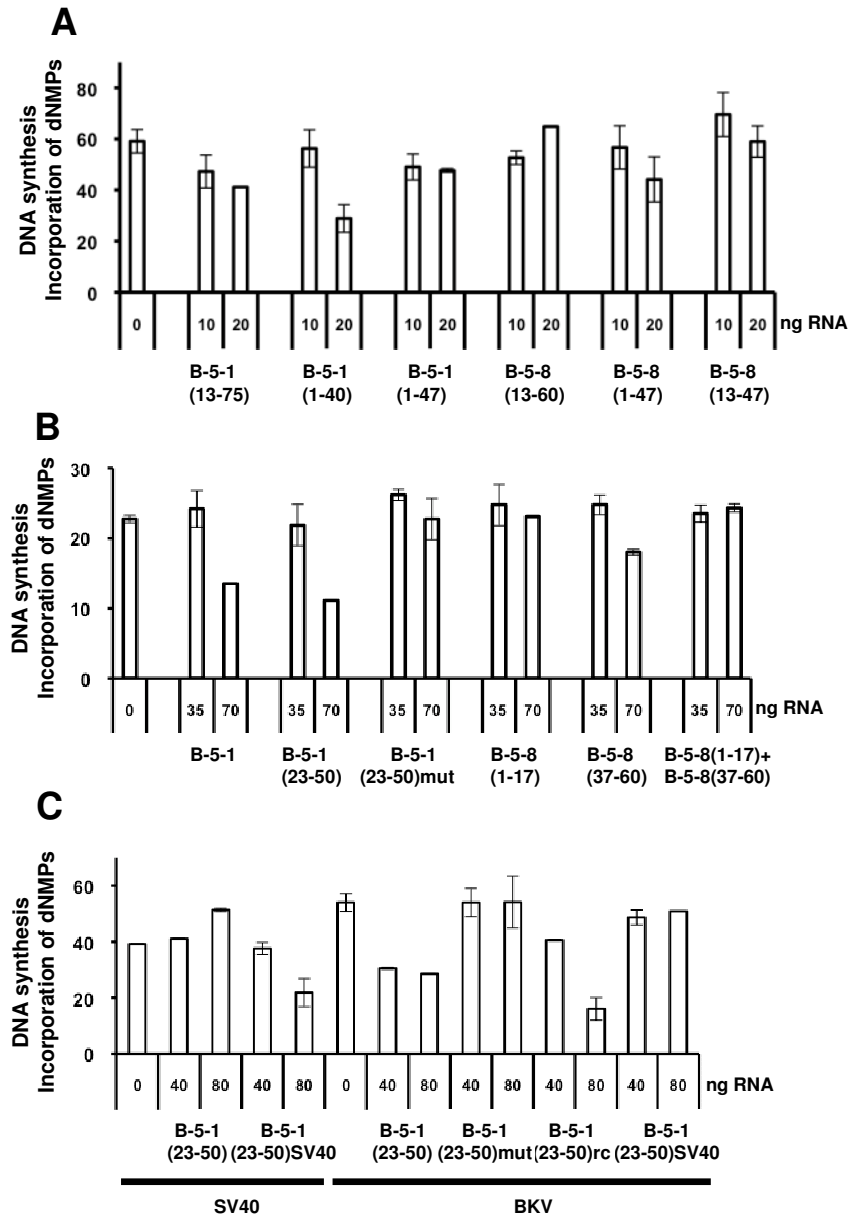
Tikhanovich et al., Figure 3



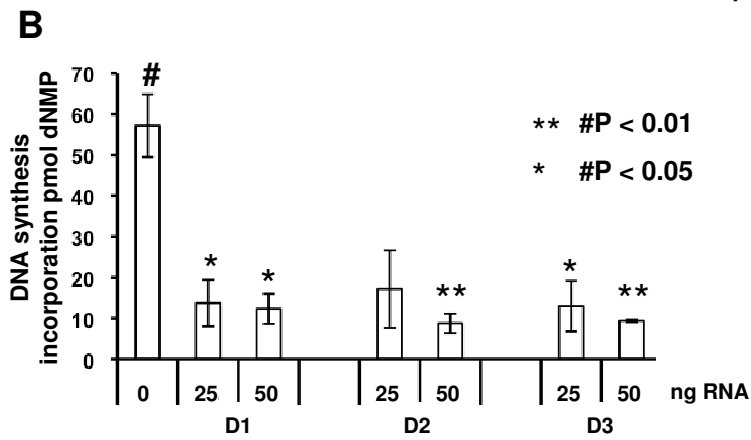
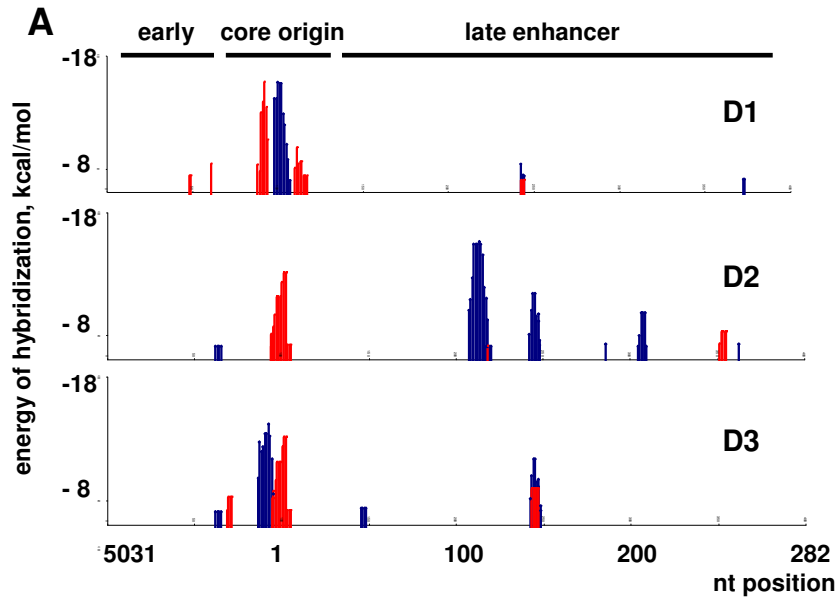
Tikhanovich et al., Figure 4



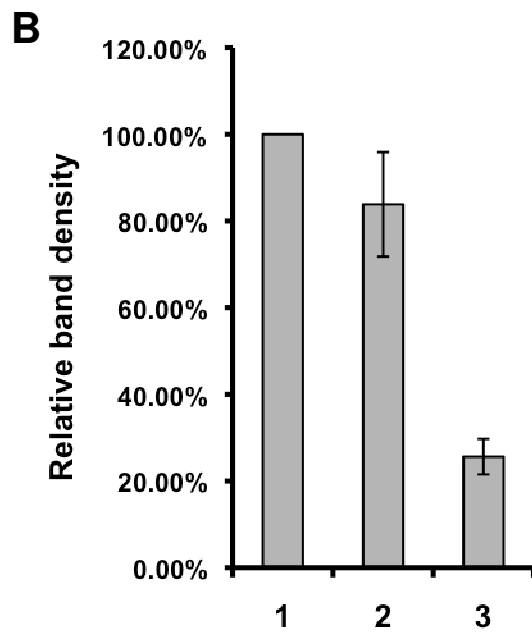
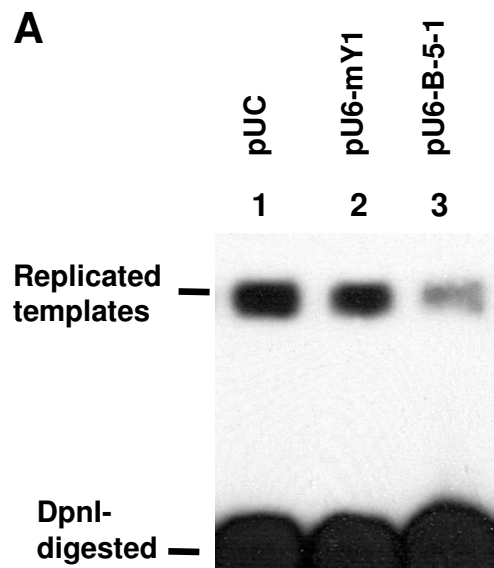
Tikhanovich et al., Figure 5



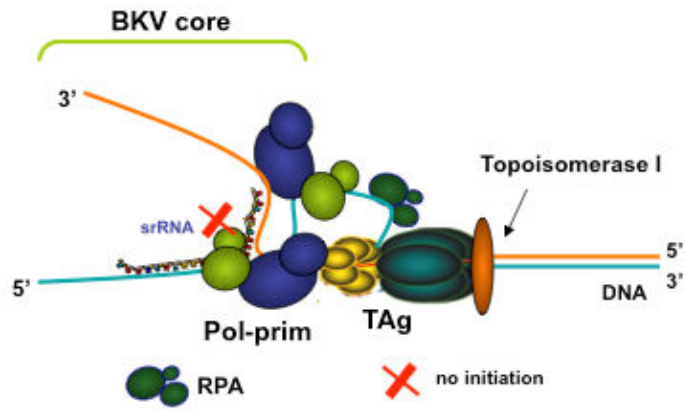
Tikhanovich et al., Figure 6



Tikhanovich et al., Figure 7



Tikhanovich et al., Figure 8



RNA sequences (all but 23-50mut and 23-50SV40 contain two complementary sites to the NCCR of BKV DIK strain between nt 5031-5141 plus 1-281)	srRNA name	Inhibition of assay			
		Monopol. BKV	Unwind. SV40	Init. BKV	Init. BKV
<u>AGGCGCCCGGGUUCGACUCCCGUGUGGGAACCA</u>	Seq3	++	—	n. d.	n. d.
<u>CCGGUCUCACGACGACAUUCCAGACGUGGCCUCGUGGGUGCUUCCACGUUGCGAACACCCCGAUUUCGGUCCC</u>	B-5-1	+++	—	+/-	+++
<u>GACAUUCCAGACGUGGCCUCGUGGGUGCUUCCACGUUGCGAACACCCCGAUUUCGGUCCC</u>	13-75 [#]	++	—	n. d.	n. d.
<u>CCGGUCUCACGACGACAUUCCAGACGUGGCCUCGUGGGUGCUUCCACGUUGCGAACACCCCG</u>	1-60 [#]	++	—	n. d.	n. d.
<u>CCGGUCUCACGACGACAUUCCAGACGUGGCCUCGUGGGUGCUUCCAC</u>	1-47 [#]	—	—	n. d.	n. d.
<u>GACGUGGCCUCGUGGGUGCUUCCACGUU</u>	23-50 [#]	+++	—	n. d.	n. d.
<u>GACGUGGCCUCGUGGGUGCUU</u> A CACGUU	(23-50)mut ^{#*}	—	—	n. d.	n. d.
<u>GACGUGGCCUCGUGGG</u> GGCUUCUGA GUU	(23-50)SV40 [#]	—	+++	n. d.	n. d.
<u>AACGUGGAAGCACCCACGAGGCCACGUC</u>	(23-50)rc [#]	+++	—	n. d.	n. d.
<u>GGCUGGCACUCUGGACUCUGAAUCCAGCGAUCGAGUUCAUAUCUCGGUGGGACCUCCA</u>	B-5-8	+++	—	+	+++
<u>CCCAGGACCAUCUCCAUUUUUAAGUUGAGGGACCACUGUGGGUCAGUGGGGUCACUUGGAGCCAAGACUCACAUAU</u> <u>AAGCUUCCUGGA</u>	B-5-9	++	—	n. d.	n. d.
<u>CCUCCACCUUGGUGGCUGGAUGGCCGAUGGCCUUCUCUCUCCAUCUGGGCAAGAACCAGCUUGGUUGACUGUGUG</u> <u>AUUUUGAGGUCCCUCUCCC</u>	B-8-1	++	—	—	+++
<u>CGAUCUGUCGCACGACAGCGCCGUGCAGUGAUCGCGCAGUAGCGCUUCCAGUGCUUCUGAGUAUUCGUUGUACA</u> <u>UGGUGCGUCCCUAAUCCCUUAAGCGUC</u> <u>CCUCGUCCCUGCAUCCC</u>	B-8-2	++	—	n. d.	n. d.
(5129–5139) <u>GGAGGCAGAGGCGG</u> <u>aaaaCUCCACCCUUUCUC</u> (5132–5119)	D1**	++++	—	n. d.	n. d.
(1–5129) <u>CCGCCUCUGCCUCC</u> <u>aaaaCUGGGCAGCCAGCC</u> (101–114)	D2**	++++	—	n. d.	n. d.
(5139–5129) <u>CCGCCUCUGCCUCC</u> <u>aaaaGAGAAAGGGUGGAG</u> (5119–5132)	D3**	++++	—	n. d.	n. d.

Nucleotides predicted to hybridize to the NCCR are underlined; Seq3 was obtained in the original screen for srRNAs without SELEX; [#]mutant forms of B-5-1; * changing residue in red in one of the two hybridization sites of the B-5-1 mutant 23-50 abolishes its inhibitory activity; 23-50SV40 has four nts highlighted in red introduced that destroy complementarity to BKV origin/NCCR but create complementarity to SV40 origin/NCCR. ** D1, D2 and D3 were designed as described in the text, and are not derived from natural RNA sequences; aaaa: spacer containing four As between hybridization sites; n.d.= not determined; Monopol. = monopolymerase; Unwind. = unwinding; Init = initiation