Contribution of BK Virus miniT Protein to Viral Oncogenic Activity

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Introduction:

BK Virus (BKV), a human polyomavirus, was first isolated in 1971 from a renal transplant patient who was shedding inclusion-bearing epithelial cells in his urine (Gardner et al., 1971). More than 80% of the adult human populations test positive serologically for BKV and most primary infections occur in childhood (Gardner et al., 1971). Clinical pathologic studies suggest that in immuncompetent hosts, the virus persists in many organs after the primary infection, but the asymptomatic infection mainly involves the kidneys and peripheral blood leukocytes (Ashan and Shah, 2005; Bam, 2005; Tognon, 2003). On the other hand, in patients with an immunocompromising disease, especially involving T cell deficiency, activation of the virus occurs (Sundsfjord et al.,1994; Markowitz et al., 1993). BKV has been associated with hemorrhagic cystitis in bone marrow recipients, and BKV nephropathy, the major cause of graft dysfunction and rejection in renal transplant recipients (Hashida et al., 1976; Binet et al., 1999; Nickeleit et al., 2003). BKV is also an oncogenic agent that induces tumors in rodents, and has been associated with human cancer (Tognon, 2003).

BKV is a non-enveloped virus with a double stranded, circular DNA genome that encodes two regulatory proteins, large T and small T (TAg and tAg, respectively). These proteins regulate viral replication and promote transformation of non-permissive cells (Moens et al, 1995). Recently, a third BKV protein was discovered, which is hypothesized to influence oncogenic transformation; this protein is called miniT (Prins, Bam and Frisque, unpub. data).

My project is designed to test whether the miniT protein plays a role in the transformation process. I am utilizing two strains of BKV, pBKV(AS) and pBKV(WT9). Both wild type and mutant forms of these strains will be examined for the ability to induce transformation of the rodent cell line, Rat2, using a dense focus assay. Initial studies were performed with pBKV(AS), but I will repeat this work with pBKV(WT9) because it transforms the rat cells more efficiently, thus allowing us to observe greater differences in transformation potential of wild type versus miniT mutant virus.

Methods:

Viruses / DNAs

To initiate this study, restriction enzyme digests were performed on pBKV(AS), pBKV(WT9), and clones pBKV(WT9)- BMSKS # 5 and # 7 to identify the direction in which the pBKV(WT9) DNA has been inserted in the Bluescript vector. Twelve 20 µl DNA samples were prepared for digestion, three samples (pBKV(AS), pBKV(WT9),

pBKV(WT9)-BMSKS #5) were left uncut to serve as size markers for supercoiled DNA. The enzymes utilized for the digestion were EcoRI, BamHI, and Pst I; the last two, allowed us to determine the orientation of the two viral DNAs in the Bluescript Vector, a high copy number plasmid. One microliter(µl) of the appropriate digestion buffer was added per sample; for those samples being digested by Bam HI, an additional 1 µl of BSA was added to enhance enzyme activity. The following table indicates the amount of DNA, double distilled water (ddH_20), enzyme, buffer, and 10X loading buffer utilized per sample to make a 20 µl solution:

	pBKV(AS)				pBKV(WT9)				pBKV(WT9)-BMSKS # 5				pBKV(WT9)-BMSKS # 7		
Samples	Uncut	1	2	3	Uncut	1	2	3	Uncut	1	2	3	1	2	3
DNA	1	1	1	1	4.8	4.8	4.8	4.8	0.5	0.5	0.5	0.5	0.5	0.5	0.5
ddH2O	17	16	16	16	13.2	12.2	12.2	12.2	17.5	17	16.5	16.5	16.5	16.5	16.5
Enzyme Buffer	0	1	1	1	0	1	1	1	0	1	1	1	0	1	1
EcoRI	0	1	0	0	0	1	0	0	0	1	0	0	1	0	0
Bam HI	0	0	2	0	0	0	2	0	0	0	2	0	0	2	0
Pst I	0	0	0	1	0	0	0	1	0	0	0	1	0	0	1

*All volumes are in microliters.

Restriction enzyme digests were performed under the conditions recommended by the manufacturer (New England Biolabs). The samples were electrophoresed in a 1% agarose gel containing the intercalating dye ethidium bromide at 120V for 30-60 min. DNA fragments were visualized by UV light and photographed.

The second step in this project was to complete the nucleotide sequence analysis of the pBKV(WT9) DNA that had been initiated earlier by other members of the laboratory. To do so, a sequencing primer was designed [5'—CTG GTG TAG ATC AGA GGG—3'] and sequence analysis of the pBKV(WT9) DNA was performed by the Penn State DNA sequence facility. The pBKV(WT9) sequence obtained was compared to the pBKV(AS) sequence utilizing a sequencing software (NCBI Nucleotide Blast), since the DNA sequence for these two viruses was expected to differ by about 50-100 nucleotide base pairs.

Mutagenesis

In order to produce the G to A mutation that would disrupt splicing of the miniT mRNA, the following PAGE- purified forward and reverse primers were utilized, respectively: BKSDMf 5'-CCA AAA AAA AAA GAA A**A**G TAG AAG ACC CTA AAG AC-3' and BKSDMr 5'-GTC TTT AGG GTC TTC TAC **T**TT TCT TTT TTT TTT GG-3' (altered nucleotide underline and in bold). A PCR reaction was carried out using 1µl of *Pfu* turbo polymerase [2.5U/µl], 1µl of dNTP mix [10mM], 1µl of forward primer [3.50nMoles, 10mM], 1µl of reverse primer[5.60nMoles, 10mM], 2 µl of magnesium [Mg^{2+} 1.5mM], and 5µl of 10X reaction buffer in a 50µl reaction mix with the pBKV(WT9) [139ng/µl] template.

The PCR sample was electrophoresed on a 1% agarose gel to confirm that PCR product was generated. PCR product was then treated with Dpn I for 1 hour at 37° C to digest the wild type BKV DNA produced in bacterial cells. The original template

[pBKV(WT9)-BMSKS # 5],the undigested PCR product [uncut 3], and the digested PCR product [DpnI 3] were electrophoresed on an 0. 8% agarose gel to determine if most of the digested PCR product resisted Dpn I cleavage.

PCR product was transfected into competent bacterial cells. Three samples were used: pBKV(WT9)-BMSKS #5 [10ng/µl], the undigested PCR product [uncut 3], and the digested PCR product[DpnI 3]. Z competent cells [100µl] were used for each transformation. One µl of each sample was added to 100µl competent cells, and the mixture was incubated on ice for 20 minutes. Room temperature super optimal broth with catabolite (SOC) media [400 µl] was added per sample; samples were then placed for 45-60 minutes in the 37° C shaker. Each sample was plated [200µl] on two Luria-Bertani agarose with ampicillin (LB +Amp) plates and incubated overnight at 37° C. Individual colonies from the digested PCR product [DpnI 3] plate were selected and DNA was extracted using the Wizard Plus SV Minipreps DNA Purification System Kit.

To verify that the correct mutation was introduced into pBKV(WT9) DNA, the mT1 sequencing primer was designed 5'- CAG TGG TTT GGC TTA GAC C-3' and DNAs prepared from colonies 4, 5, and 6 on the digested PCR product [DpnI 3] plate were taken to the sequencing facility. Several sequencing reactions were performed to identify a DNA containing the desired sequence mutation.

Large scale preparation (maxi-prep) of DNA

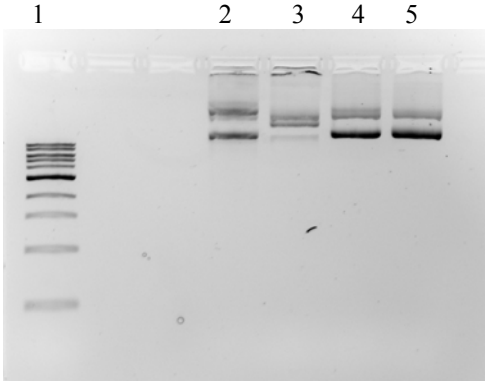
Maxi-preps of pBKV(WT9) and mutant pBKV(WT9)-ΔminiT were prepared using the Quiagen maxi-prep kit. Samples were then digested with Bgl II to cleave the desired fragment of DNA from pBKV(WT9)-ΔminiT, containing the mutation, and pBKV(WT9), without the mutation. For this enzyme reaction, 10µg of pBKV(WT9) DNA and 20 µg of pBKV(WT9)-ΔminiT DNA were digested individually in a 100 µl mixture containing 4µl of Bg1 II, 10 µl of 10X buffer and appropriate amounts of ddH_2O. Samples were incubated for 1.5 hours, and electrophoresed on 0.8% agarose gels. The desired fragments were removed from the agarose gel and purified using The Wizard Plus Gel Purification System Kit.

Following the construction of the mutant, Rat2 cells will be transfected with wild type and mutant miniT DNAs representing both pBKV(AS) and pBKV(WT9) genomes using a calcium phosphate technique. The time of appearance of dense foci (evidence of transformed cells) will be noted and the number of foci will be counted following staining of the cells with a dye. Cells from a dense focus will be isolated with pipette tips for further analysis.

Results:

Before the enzyme digestion was performed, I electrophoresed all four viral DNAs in a 1% agarose gel to verify that the viral DNAs where in the correct vector: Bluescript as opposed to pBR322. As can be seen in Figure 1, pBKV(AS) and clones pBKV(WT9) - #5 and #7 have the same gel migration patterns, indicating that the clones were in the Bluescript vector.

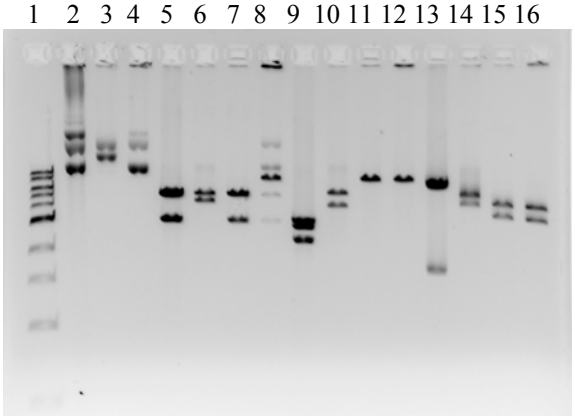
[Fig.1] The following gel picture demonstrates that both mutant pBKV(WT9)- # 5 and # 7 are in Bluescript, the vector desired for this experiment.



1. Marker
2. BKV(AS) ~ Bluescript
3. BKV(WT9) ~ pBR322
4. BKV(WT9) - #5
5. BKV(WT9)- # 7

Restriction enzyme digestion of pBKV(AS), pBKV(WT9), and clones pBKV (WT9) #5 and #7 with BamHI or PstI demonstrated the orientation of the clones in the vector. Figure 2 is a picture of a 1% agarose gel, through which the DNA samples were electrophoresed for one hour.

[Fig. 2]



1. Marker

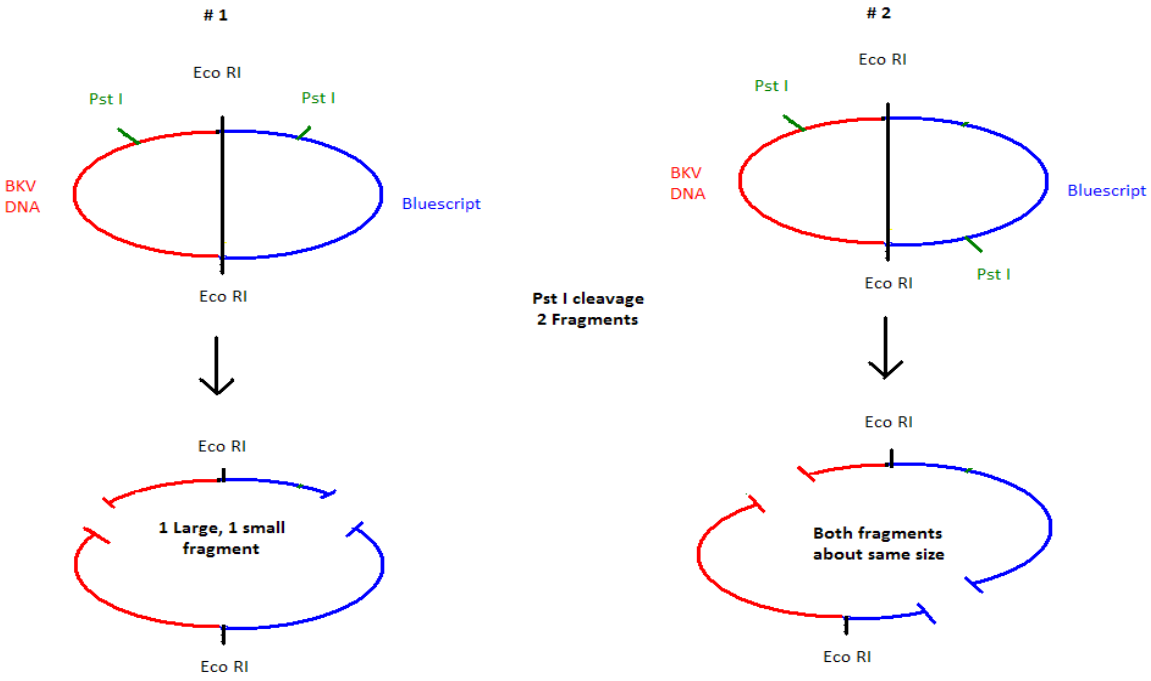
Uncut Samples
2. BKV (AS)
3. BKV (WT9)
4. BKV (WT9)-# 5

Cut with EcoRI
5. BKV (AS)
6. BKV (WT9)
7. BKV (WT9)-#5
8. BKV (WT9)-#7

Cut with BamHI
9. BKV (AS)
10. BKV (WT9)
11. BKV (WT9)-#5
12. BKV (WT9)-#7

Cut with Pst I
13. BKV (AS)
14. BKV (WT9)
15. BKV (WT9)-#5
16. BKV (WT9)-#7

Based on the base pair length of the Pst I enzyme cut, the orientation of vector for pBKV(WT 9) and pBKV(AS) is represented in Figure 3.



[Fig 3] The circle represents double- stranded DNA. The black line in the middle of each circle serves to separate the viral DNA (left, red line), and the Bluescript Vector (right, blue line). Depending on the orientation of the viral DNA in the vector, Pst1 cleavage yields either 2 fragments of dissimilar size (#1) or nearly equal size (#2). pBKV(AS) is oriented in the manner represented by circle 1, and pBKV(WT9) is oriented in its vector the opposite manner, represented by circle 2.

The primary objective of my project is to abolished the expression of pBKV miniT protein by introducing a mutation at the donor splice site. This mutation was previously made in pBKV(AS), but we found wild type pBKV(AS) transforms cells inefficiently. Therefore, because pBKV(WT9) transforms better, we remade the miniT mutant in this BKV strain. By making the mutation at the donor splice site of miniT protein in pBKV(WT9), we predict that we will be able to detect differences in the oncogenic activity of the wild type and mutant viruses. To make the mutation in pBKV(WT9) we considered swapping a restriction enzyme fragment of pBKV(AS) containing the mutation into the pBKV(WT9) DNA. However, sequence differences were present in the two viral DNAs near the donor site, so the mutagenesis procedure was repeated with pBKV (WT9)-BMSKS as the template for long PCR-Site Directed Mutagenesis.

The DNA sequence for pBKV(WT9) was completed. To permit comparisons with previously sequenced pBKV(AS) (using NCBI Nucleotide Blast). pBKV(WT9) -#5 was sequenced with primers T3 and T7, and results were compared with the parental pBKV(WT9) sequence. (12 nucleotide differences out of 5216 nucleotides). Based on the DNA sequencing results of clone pBKV(WT9) -#5 and the blast comparison to pBKV(AS), the donor-splice site for the miniT mutation was identified at nucleotide 4366.

Verification of the mutation in pBKV(WT9) -ΔminiT was accomplished by sequencing using primers mT1, mT2, and mT3, and then, maxi-preps of the mutant and

the wild type DNAs were prepared. Since the PCR Mutagenesis technique might cause unexpected changes else where in the genome, DNAs were digested with Bg1 II to obtain the mutated fragment from pBKV(WT9)-ΔminiT and the backbone fragment from pBKV(WT9). The next step will be to ligate these two DNA fragments together to generate the final mutant containing one nucleotide change. Once ligation is completed, and the final clone is obtained the mutant and the parental DNAs will be transfected into the Rat2 cell line to test transforming ability.

Discussion:

The results generated so far indicate that the two pBKV(WT9) DNA clones, 5 and 7, were assembled correctly and are in the desired Bluescript vector for this experiment. Restriction enzyme digestions have verified the orientation of the viral DNAs within the vector sequence. Although, the initial digest did present some difficulty due either to the impurities in the original DNA sample, or to poor activity of the enzymes, follow-up digestions with newly-prepared DNA and a more optimal enzyme digest indicated that the orientations are indeed as shown in the Figure 3. The results so far have met our expectations, and we can proceed on to the next planned experiments.

Sequences comparisons revealed too many variations between pBKV(AS) and pBKV(WT9) within the region containing the mutation, which led us to use the site-directed mutagenesis method to create the pBKV(WT9)-ΔminiT mutant. We performed PCR utilizing primers BKsdm and BKsdm-r, and obtained a mutant which, after analysis, was confirmed to have the correct sequence. Maxi-preps were successfully prepared and Bgl II digestions led to the generation of the desired fragments to be used to create the final mutant construct, pBKV(WT9)- ΔminiT. Once construction of the mutant is completed, I will pursue the primary goal of determining the role of miniT protein in oncogenic transformation.

Conclusion:

To date, we have completed the sequence analysis of pBKV (WT9) and have verified that DNA clones have been properly assembled in the correct vector. Comparisons of the pBKV(AS) and pBKV(WT9) sequences have been completed and a mutagenesis strategy was devised. A pBKV miniT mutant has been obtained and verified through sequencing. Maxi-preps has been prepared and Bg1 II digestion has been performed to obtain mutant and wild type backbone fragments.

Currently, we do not know if BKV miniT protein influences viral oncogenic activity; however, once cleaved Bgl II fragments are ligated to complete the construction of the final pBKV (WT9) – ΔminiT construct, transfection of the Rat2 cell line will allow us to begin to answer this question.

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