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DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

CONTRIBRUTION OF BK VIRUS miniT PROTEIN TO VIRAL ONCOGENIC ACTIVITY

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ABSTRACT

BK virus (BKV) is one of five pathogenic human viruses in the *Polyomaviridae* family. This polyomavirus infects more than 80% of the human population. Most clinical studies suggest that in immunocompetent hosts, the virus persists in many organs following a primary infection, although the asymptomatic infection mainly involves the kidneys and peripheral blood leukocytes. On the other hand, in a patient with an underlying immunocompromising condition, often involving T cell deficiency, reactivation of the viral infection may occur, leading to serious complications such as hemorrhagic cystitis in bone marrow recipients, and BKV nephropathy, a major cause of graft dysfunction and rejection in renal transplant recipients. Experimental in vitro and in vivo models have also shown BKV to be an oncogenic agent; BKV induces tumors in rodents, and is associated with several human cancers. BKV, a double stranded DNA virus, produces three early proteins: large, small, and mini TAg (TAg, tAg, miniT). These three proteins are produced by alternative splicing of a common precursor mRNA. In this study I have demonstrated that miniT contributes to BKV transformating ability. I completed the sequence of the BKV(WT9) genome, and created a miniT null mutant (BKV(WT9)-Δ4366) by introducing a point mutation at the donor splice site for the miniT transcript. This point mutation disrupted the consensus donor sequence for miniT mRNA without altering the coding sequence of TAg. I used a dense focus assay to compare the ability of the wild type and mutant forms of the virus to induce transformation of the rodent cell line, Rat 2. My experiments suggest that miniT protein does indeed contribute to viral transforming efficiency. Isolated cells transformed by wild type vs. mutant viruses produced the expected early viral proteins.

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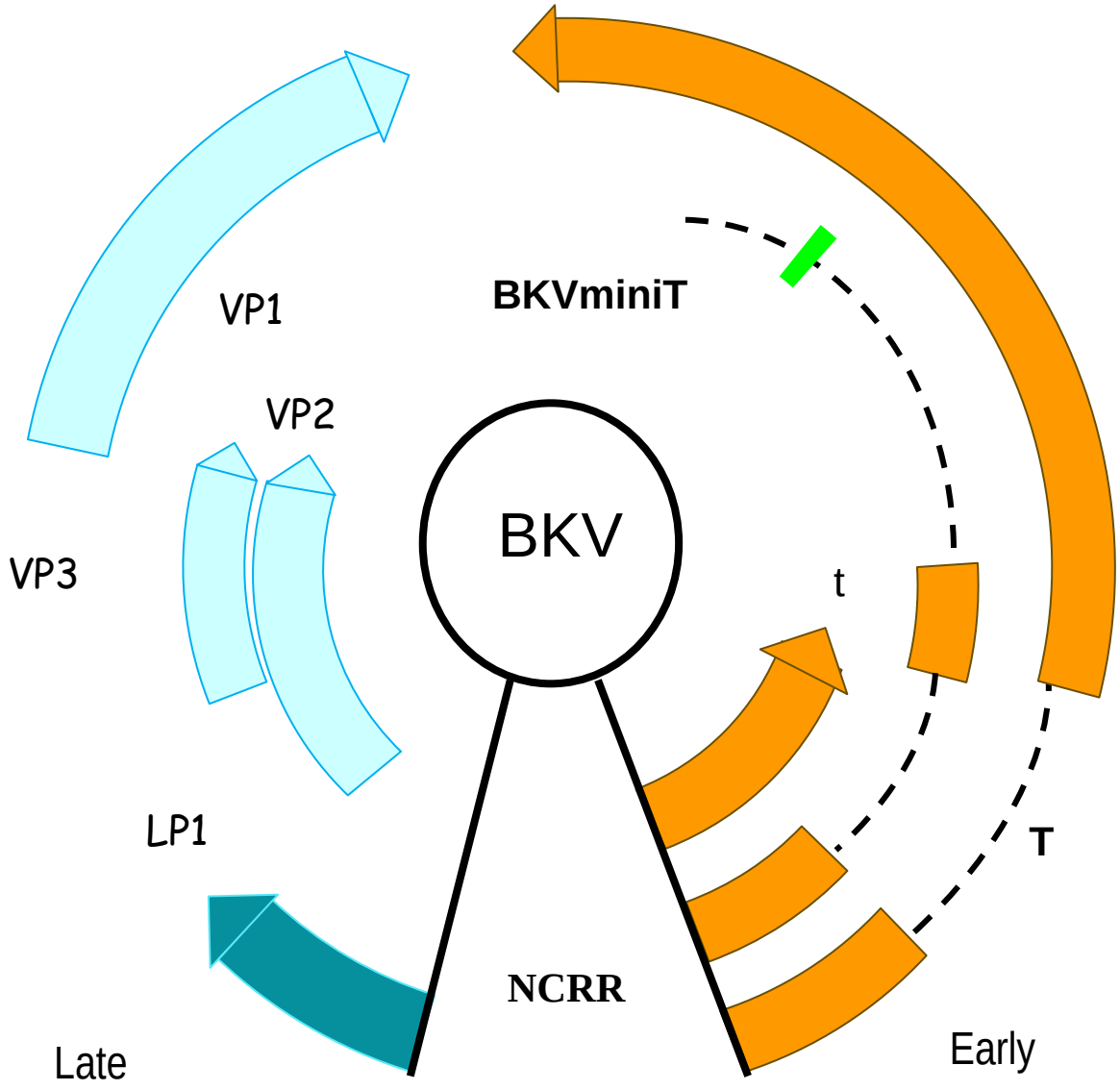
INTRODUCTION

BK virus (BKV), a member of the *Polyomaviridae* family, is one of five pathogenic human viruses in the family [1]. It was first isolated in 1971 from the urine of an immunocompromised renal transplant patient with the initials B.K. [1, 2]. While more than 80% of the adult human populations test positive serologically for BKV, most primary infections occur during childhood [2]. Clinical pathologic studies suggest that in immuncompetent hosts, the virus persists in many organs after the primary infection, but the asymptomatic persistent infection mainly involves the kidneys and peripheral blood leukocytes [1, 3-4]. On the other hand, in patients with an immunocompromising disease, especially involving T cell deficiency, reactivation of the virus can occur [5, 6]. Reactivation of BKV infections can lead to serious complications such as hemorrhagic cystitis in bone marrow recipients, and BKV nephropathy, the major cause of graft dysfunction and rejection in renal transplant recipients [7-9]. Experimental in vitro and in vivo models have implicated BKV as an oncogenic agent; BKV induces tumors in rodents, and is associated with several human tumors [4, 10-14].

BKV is a non-enveloped virus with a double stranded, circular DNA genome. The BKV genome is divided into the early coding region, the late coding region and the non-coding control region (NCCR) [Fig.1]. The NCCR includes the origin of DNA replication, along with binding sites for regulatory factors involved in transcription and replication [15]. The early region encodes two regulatory proteins, large T antigen and small t antigen (TAg and tAg, respectively). These proteins regulate viral replication and promote transformation of non-permissive cells [16, 17]. The late region encodes agnoprotein and the capsid proteins VP1, VP2, VP3 which are expressed after replication of the genome has been initiated. A third BKV T antigen protein was discovered in 2001, this protein is called miniT [22]; it is also known as TrunctAg [18].



[Fig. 1] BKV Genome. BKV has a circular, double stranded DNA genome. The genomic map shows two protein coding regions separated by a non-coding regulatory region [NCRR]. The early region encodes proteins TAg, tAg, and miniT, while the late region encodes proteins VP1, VP2, VP3, and LP1.

Early proteins [TAg, tAg] regulate viral replication and are involved in oncogenic activity.

Late proteins [VP1, VP2, and VP3] are expressed after genomic replication has initiated and encode viral capsid proteins.

Late protein [LP1] is a regulatory protein.

BKV miniT protein consists of 136 amino acids, and shares 133 amino-terminal amino acids with TAg. These shared amino acids contain an Hsc70-binding J domain and an LxCxE binding region for RB tumor suppressor family proteins; miniT protein also contains an E3 ubiquitin ligase binding domain called CUL 7, and a nuclear localization signal (NLS) [18]. TAg induces transformation through the actions of the J, LxCxE, and p53 domains; miniT lacks the p53 binding sequence. Studies have demonstrated that polyomavirus TAgs bind RB proteins (pRb, p130, p107) through their LxCxE domain. Once bound, TAgs recruit the molecular chaperone Hsc 70 through the J domain, which then promotes the release of members of the E2F family of transcription factors [20-23]. The RB proteins are active during the G_0/G_1 cell phase and prevent E2F-dependent transcription of genes needed for cell progression towards S phase. However, through their J domain and LxCxE motif, the polyomavirus TAgs deregulate the cell cycle, inducing premature progression of cells through the S phase and thus promoting transformation of non-permissive cells [19-22]. Inappropriate cell cycle activation can lead to p53-induced apoptosis, an activity which is controlled by the third TAg domain that binds and inactivates p53. Other domains, such as the CUL 7 binding motif, have also been suggested to influence TAg- induced transformation by inhibiting apoptosis [23].

BKV miniT protein is homologous to JCV T'_{135}, T'_{136}, T'_{165} and to SV40 17KT. Much like miniT, these latter proteins share amino acids with their respective TAg, and possess a J domain, an LxCxE motif, and a NLS. JCV T' proteins have transformation potential, with T'_{136} being the predominant T' protein expressed in transformed cells [22]. SV40 17KT has been shown to rescue TAg dnaJ mutants, reduce p130 levels, stimulate cell entry into S phase by releasing E2F, and induce transformation of human fibroblasts [24]. Therefore, although TAg is primarily responsible for the immortalization and transformation of non-permissive cells, transformation assays have

demonstrated that JCV T' proteins (T'_{135}, T'_{136}, and T'_{165}) and SV40 17KT protein do contribute to a transformed phenotype [21-22]. Based on sequence homology and on previous transformation experiments with T'_{135}, T'_{136}, T'_{165} and 17KT, we hypothesize BKV miniT influences oncogenic transformation.

My project was designed to test whether the miniT protein plays a role in the transformation process. I have utilized a strain of BKV called pBKV(WT9). Both wild type and mutant forms of this BKV strain have been examined for the ability to induce transformation of the rodent cell line, Rat 2, using a dense focus assay. Initial studies were performed with pBKV(AS), but I have repeated 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

In order to initiate this study, restriction enzyme digests were performed on pBKV(AS), pBKV(WT9), and clones pBKV(WT9)- BMSKS # 5 and # 7. 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 (BMSKS), 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 amounts of DNA, double distilled H_20 (ddH_20), enzyme and buffer added to the reaction, and the amount of 10X loading buffer added after the reaction.

	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
10X 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	1	0	0	0	1	0	0	0	1	0	0	1	0
Pst I	0	0	0	1	0	0	0	1	0	0	0	1	0	0	1
10X Loading Buffer	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

*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 at 120V for 30-60 min.

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 Nucleic Acid Facility at The Pennsylvania State University. The pBKV(WT9) sequence was compiled and compared to the pBKV(AS) sequence [26] utilizing 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

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 [3.5nMoles, 10mM], 1µl of reverse primer [5.6nMoles, 10mM], 2µl of magnesium [Mg^{2+} 1.5mM], and 5µl of 10X reaction buffer in a 50µl reaction mix with 1µl of 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 (1µl) was transfected into Z competent [100µl] 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]. The bacterial cells were incubated on ice for 20 minutes. Super optimal broth with catabolite (SOC) media [400µl] was added to each sample at room temperature; 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

Large scale DNA preparation of pBKV(WT9) and mutant pBKV(WT9)-ΔminiT were prepared using the Qiagen maxi-prep kit. Mutant pBKV(WT9)-ΔminiT DNA was sent for sequencing to verify the G to A point mutation at base pair 4366. Due to changes that could have also occurred in the pBKV(WT9)-ΔminiT genome during site-direct mutagenesis, only a fragment of the genome (2,202bp) was sequenced which included the introduced point mutation, and the restriction enzyme sites for BglII. Both pBKV(WT9) and mutant pBKV(WT9)-ΔminiT were then digested with BglII to cleave each genome into two fragments.

For this enzyme reaction, 10µg of pBKV(WT9) DNA and 20 µg of pBKV(WT9)-ΔminiT DNA were digested individually in a 100µl mixture containing 4µl of Bg1 II, 10µl of 10X buffer and appropriate amounts of ddH_2O. Samples were incubated for 90 minutes, and then electrophoresed on 0.8% agarose gel. A BglII digestion fragment (2,202bp) from pBKV(WT9)-ΔminiT was isolated and purified using The Wizard Plus Gel Purification System Kit (Promega). The backbone (5,974bp) containing the pBKV(WT9) sequence in BluescriptKS (BSmKS) vector, without the BglII fragment, was also isolated and purified.

Creation of the Final pBKV(WT9)-ΔminiT Clone

To assemble the pBKV(WT9)-Δ4366 construct, the mutated DNA fragment isolated from pBKV(WT9)-ΔminiT was inserted into the pBKV(WT9)- BSmKS backbone lacking the smaller BglII fragment. The two purified Bgl II fragments were ligated with T4 DNA ligase at room temperature for 24 hours. Ligated product was subjected to restriction enzyme digests with BglII to verify ligation of the 2,202bp fragment to the 5,974bp fragment; and, with XbaI to identify the orientation of the ligated fragment in the final construct.

A bacterial transformation was performed using ligated DNA product to obtain recombinant DNA clones; clones were screened to find the expected mutant. A culture of bacteria containing the construct pBKV(WT9)-Δ4366 was grown overnight and used to produce large-scale preparation of DNA. pBKV(WT9)-Δ4366 DNA was sent to the Nucleic Acid Facility of The Pennsylvania State University to be sequenced with primers mT4f 5'- GCC CTT GGT TTG GAT AGA TTG C – 3' and mT4r 5'- CAC ATC CTC ACA CTT TGT CTC- 3' to verify the expected sequence.

Cell Culture

Rat2 cells were propagated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), penicillin (100U/ml), streptomycin (100U/ml) and L-glutamine (2mM), and incubated at 37˚ C in an atmosphere containing 10% CO_2.

DNA Transfection

Rat 2 cells ($4x10^5$) were seeded on 60mm plates. After incubating cells overnight, medium (DMEM + 10% FBS with antibiotics) was changed four hours prior to transfection. Cells were transfected using the calcium phosphate procedure [27]; 1μg of plasmid DNA per 60mm plate. Four hours after transfection, cells were rinsed with DMEM, and medium (DMEM + 10% FBS with antibiotics) was added. After 24 hours medium was changed to DMEM + 5% FBS with antibiotics. This medium (4ml) was changed every 5 days thereafter. Rat 2 cells were transfected with SV40, pBKV(WT9), pBKV(WT9)-Δ4366, and calf thymus (CT) DNAs.

Transformation was measured by the appearance of dense foci on the monolayer of transfected cells [28]. Transformed cells growing in 60mm plates were fixed with 3.5% formaldehyde in PBS for 60 minutes, and stained with hematoxylin for 45 minutes. Transformed cells were rinsed twice with ddH_2O. Hematoxylin staining was fixed by adding ammonium hydroxide, diluted 1:40 with ddH_2O. 60mm plates were inverted, allowed to dry, and dense foci were counted.

Transformed Rat 2 cell lines were isolated from dense foci growing on a monolayer of untransformed Rat 2 cells, and single cell clones were obtained through dilution. Each cell line expressed viral TAg, as detected by immunofluorescent staining.

Immunopercipitation/Western Blotting of Viral Proteins

To obtain cell lysates, cells growing on 100mm plates were washed twice with 4ml of cold STE (0.15M NaCL; 0.02 M Tris, pH 7.2; 1mM EDTA, pH 7.2), then treated with 1ml EBC buffer (50mM Tris; 500mM NaCl; 1% NP-40) containing protease inhibitors (leupeptin, 2µg/ml; Aprotinin, 1µg/ml; E-64, 2µg/ml; Pefabloc, 1mM), and phosphatase inhibitors (EDTA, 5mM; β-glycerophosphate, 25mM; sodium vanadate, 1mM; sodium fluoride, 5mM) . Plates were rocked for 15 minutes and lysed for 20 minutes at 150 rpm at 4°C. Collected extracts were centrifuged in a microfuge for 20 minutes at 4°C, clarified lysates stored at -80°C.

Viral proteins were immunopercipitated by incubating cell lysates for 60 minutes at 4°C with 10µl each of monoclonal α-T antibodies, PAb 416, PAb 2003, and PAb 2024 [25]. Staph A cells were activated by replacing the storage buffer with EBC buffer containing BSA (1mg/ml) and incubating the cells at room temperature for 15 minutes. To the activated staph A, 9µl of α-mouse IgG (2µg/µl) were added, and cells were incubated for 30 minutes on ice. Activated staph A cells (15µl) were added to cell extracts and incubated at 4°C for 30 minutes. Immune complexes were pelleted by centrifugation for 1 minute, and washed three times with EBC buffer containing leupeptin (2µg/ml). Sample buffer was added (12µl) to collect immune complexes. Samples were vortexed, heated for 5 minutes at 95°C and electrophoresed on 18% SDS-polyacrylamide gel at 190V for 100 minutes.

The SDS-polyacrylamide gel was soaked in Hoffer's transfer buffer (25mM Tris, 193mM Glycine, 20% methanol, 0.01% SDS) for 20 minutes, and proteins were transferred to a nitrocellulose membrane overnight at 30V. The membrane was rinsed with ddH_20 and soaked in blocking buffer (6.5% Non-fat dry milk, 0.1% Tween-20, 10X TBS) twice for 30 minutes. The membrane was then incubated with 10ml of blocking buffer containing a monoclonal antibody

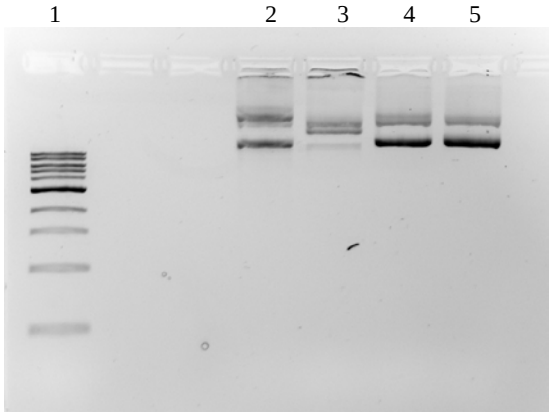
mix (40µl each of PAb 416, PAb 962, PAb 901, PAb 2001, PAb 2024, PAb 2030) and rocked for 120 minutes. Blots were washed three times in 60ml of wash buffer (0.1% Tween-20, 10X TBS) for 5 minutes. Blots were then incubated with 10ml blocking buffer containing the secondary antibody conjugated to alkaline phosphatase (Sigma, dilution 1:4000) for 75 minutes. Blots were washed as previously described and developed in 9ml of premixed BCIP/NBT solution (Sigma) for 35 minutes.

Immunofluorescent Staining

A sparse layer of cells growing on a glass cover slip was fixed for 10 minutes with a 1:1 mixture of acetone and methanol. TAg was detected after the cells were incubated for 30 minutes with a mixture of α-T antibodies (PAb 416, PAb 962, PAb 901, PAb 2001, PAb 2003, PAb 2024, PAb 2030). After washing thoroughly, cells were incubated for 30 minutes with fluorescein-conjugated α-mouse immunoglobulin G antibody.

RESULTS

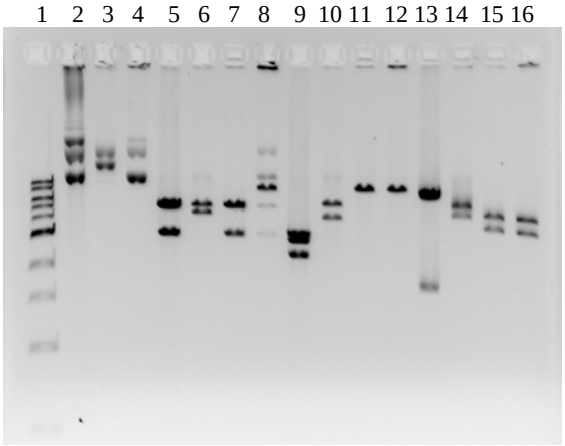
Before I performed my initial restriction enzyme digestion, I electrophoresed all four viral DNAs in a 1% agarose gel to verify that the viral DNAs where in the correct vector: Bluescript as opposed to pBR322. As can be seen from the gel photograph below, BKV (AS) and clones BKV (WT9) - #5 and #7 have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



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

[Fig. 2] Bluescript Vector Placement of BKV. The following gel picture demonstrates that both mutant pBKV(WT9)- #5 and #7 are in Bluescript, the vector desired for this experiment.

Restriction enzyme digestion of BKV (AS), BKV (WT9), and clones pBKV (WT9) #5 and #7 allowed me to determine the orientation of the viral DNA in the vector. The following picture is of a 1% agarose gel, through which the DNA samples were electrophoresed for one hour.



1. Marker

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

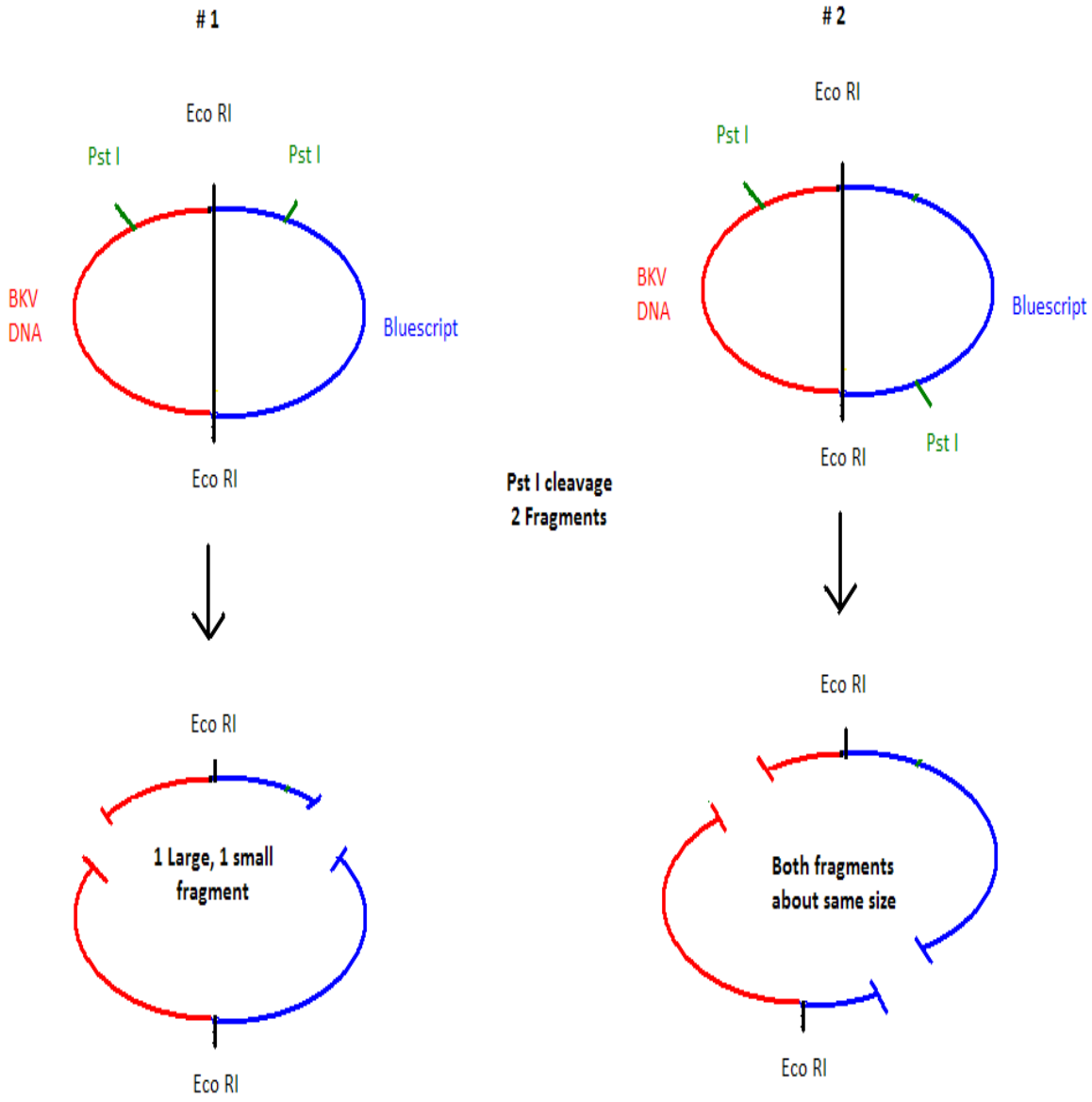
Digested with EcoRI
5. BKV (AS)
6. BKV (WT9)
7. BKV (WT9)-#5
8. BKV (WT9)-#7

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

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

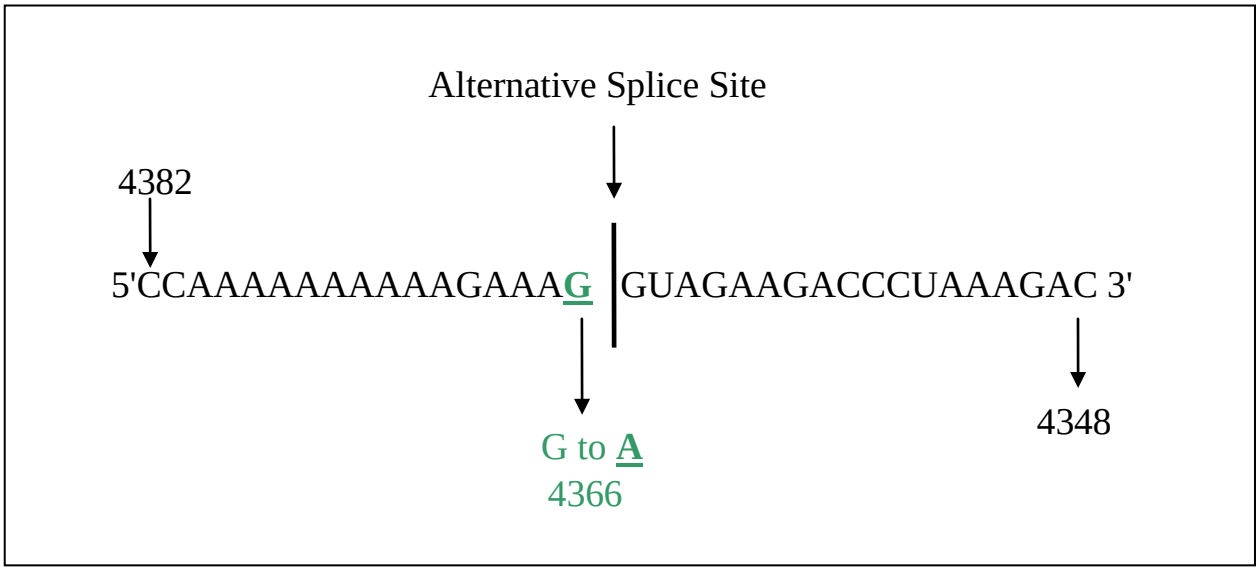
[Fig. 3] Restriction enzyme digestion of pBKV(AS), pBKV(WT9), and clones pBKV(WT9) #5 and #7. The restriction enzyme digest demonstrates the orientation viral DNA in the vector.

Based on the base pair length of the fragments digested with the Pst I enzyme cut, the orientation of vector for pBKV(WT9) and pBKV(AS) is represented in Figure 4.

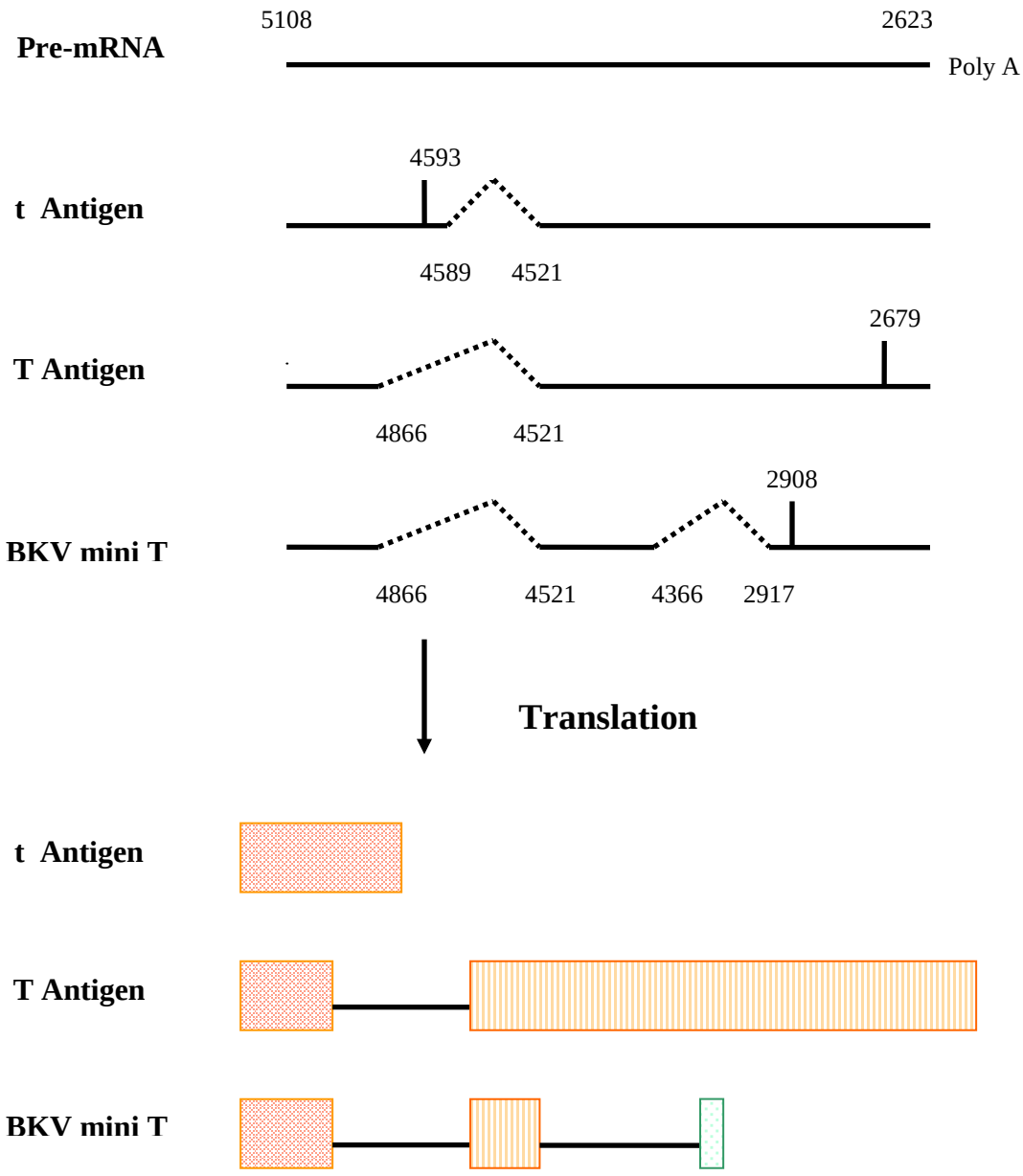


[Fig. 4] Vector orientation for pBKV(WT9) and pBKV(AS). The circle represents double- stranded DNA. The black line in the middle of each circle serves to separate the viral DNA (left, red line), and the Bluescript Vector (right, blue line). Depending on the orientation of the viral DNA in the vector, 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.

A partial DNA sequence for pBKV(WT9) had been obtained earlier and I completed the remaining sequence. To permit comparisons with previously sequenced pBKV(AS), pBKV(WT9) was sequenced with primers T3 and T7, and results were compared with the parental pBKV(WT) sequence (12 nucleotide differences out of 5216 nucleotides). Based on the DNA sequencing results of clone pBKV(WT9) and the blast comparison (using NCBI Nucleotide Blast) to pBKV(AS), the donor-splice site for the miniT mutation was identified at nucleotide 4366 [Fig. 5]. The introduction of a G to A point mutation at the miniT donor splice site prevented the generation of a miniT transcript, and thus, the miniT protein could not be produced. This mutation did not alter the amino acid coding sequence for TAg protein. The pBKV(WT9) sequence data, also enabled the mapping of the alternative splicing of the early coding mRNA and their corresponding proteins [Fig. 6].



[Fig. 5] BKV miniT mRNA donor-splice site. The BKV miniT sequence around the protein splice site is depicted above in the 5 prime to 3 prime direction; the point mutation is demarcated in green at nucleotide 4366.

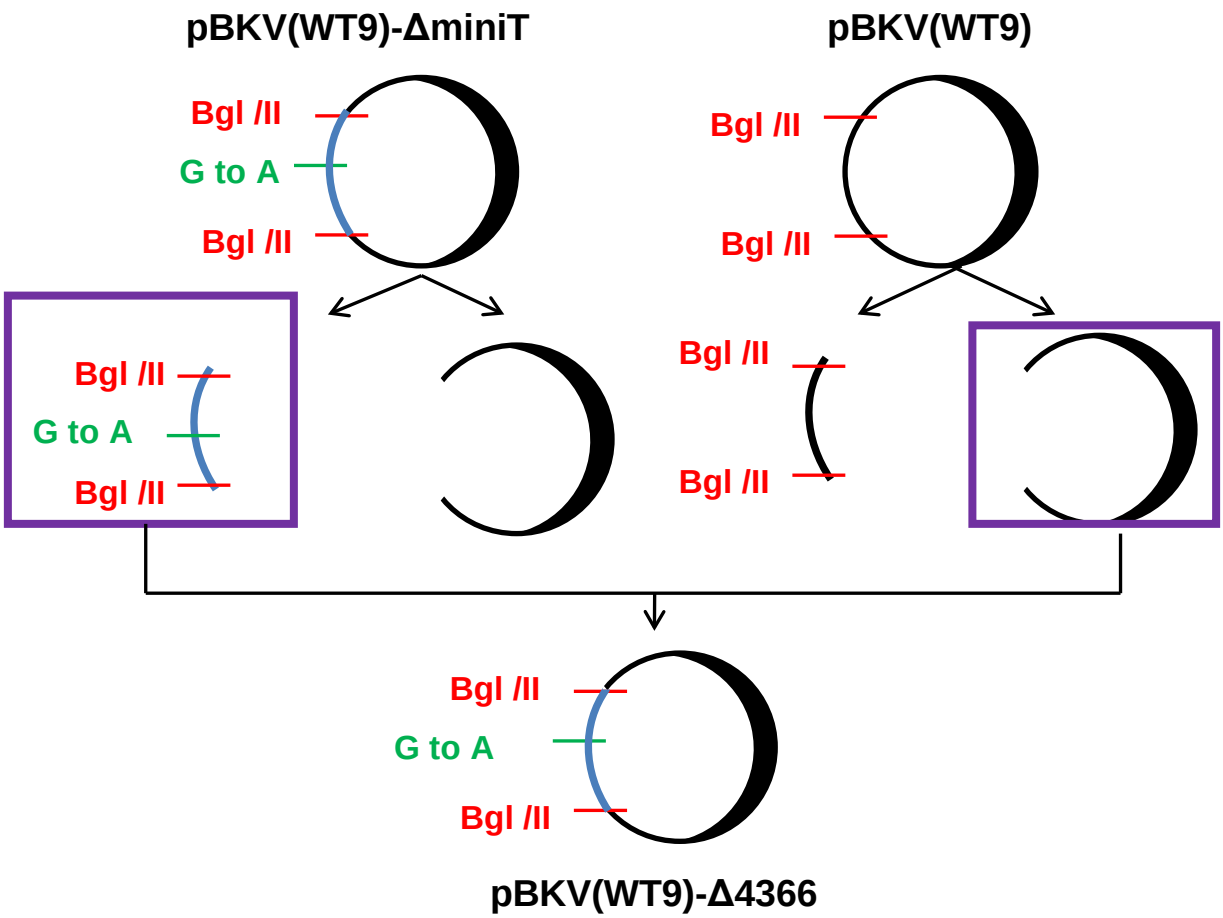


[Fig. 6] Alternative splicing of pBKV(WT9) early mRNA. In the top portion of the figure, the pre-mRNA is shown as a solid black line; while the mRNA for each respective protein is depicted by the combination of solid and dashed lines. The solid lines represent the exons, while the dashed lines represent the introns; underneath the dashed lines, the nucleotide numbers for the donor and acceptor splice sites are shown. Vertical black lines indicate the locations of the stop codons. The bottom portion of the figure depicts the translated proteins from their respective mRNAs.

The primary objective of my project was to abolish 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 this BKV strain 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 mRNA in pBKV(WT9), we predicted that we would be able to detect differences, if they existed, 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 already created mutation into the pBKV(WT9) DNA. However, sequence differences were present in the two viral DNAs near the donor site, so this approach was rejected. Instead I repeated the mutagenesis procedure with pBKV(WT9)-BMSKS as the template and the long PCR-Site Directed Mutagenesis protocol.

Verification of the mutation in pBKV(WT9)-ΔminiT was accomplished by sequencing using primers mT1, mT2, and mT3. Once confirmed, maxi-preps of the mutant and the wild type DNAs were prepared. Since the PCR Mutagenesis technique might cause unexpected changes elsewhere in the genome, DNAs were digested with Bg1II to obtain the mutated fragment from pBKV(WT9)-ΔminiT and this fragment was joined to the backbone fragment from pBKV(WT9) [Fig. 7].

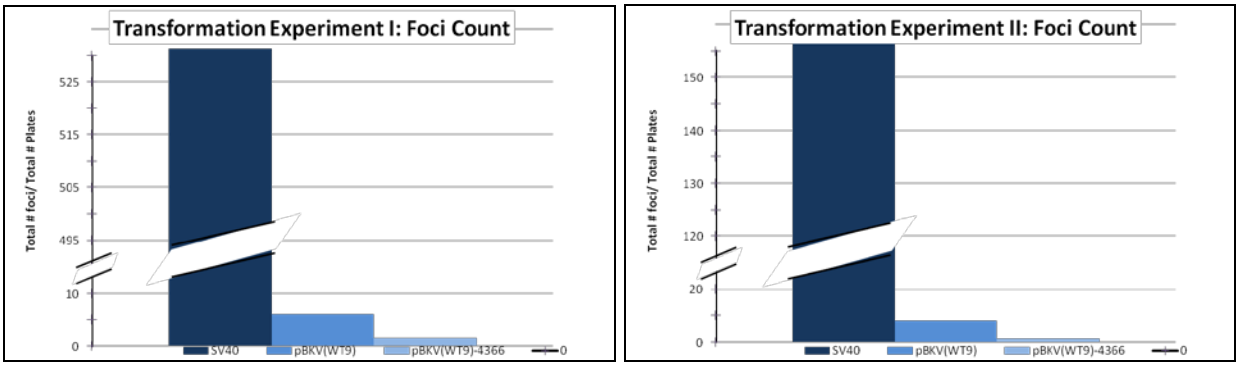
To ensure the identity of the mutant, DNA was sent for sequencing, and confirmed to be the correct construct. Upon obtaining the final recombinant DNA, the mutant and the parental DNAs were prepared for transfection into the Rat2 cell line to test transforming ability.



[Fig. 7] Construction of pBKV(WT9)-Δ4366. The right-half of the circle represents the Bluescript plasmid vector, and the left- half represents the double-stranded viral DNA. The BglII digest fragment containing the mutation is shown in blue, and it is demarcated with the introduced G to A mutation in green. Restriction sites for BglII are shown in red. The arrows display the separation and union of viral fragments to produce the final viral construct. As demonstrated in the diagram, the digested BglII fragment from pBKV(WT9)-ΔminiT containing the mutation was inserted into the unaltered pBKV(WT9) backbone to produce the construct pBKV(WT9)-Δ4366; this recombinant construct does not express miniT protein.

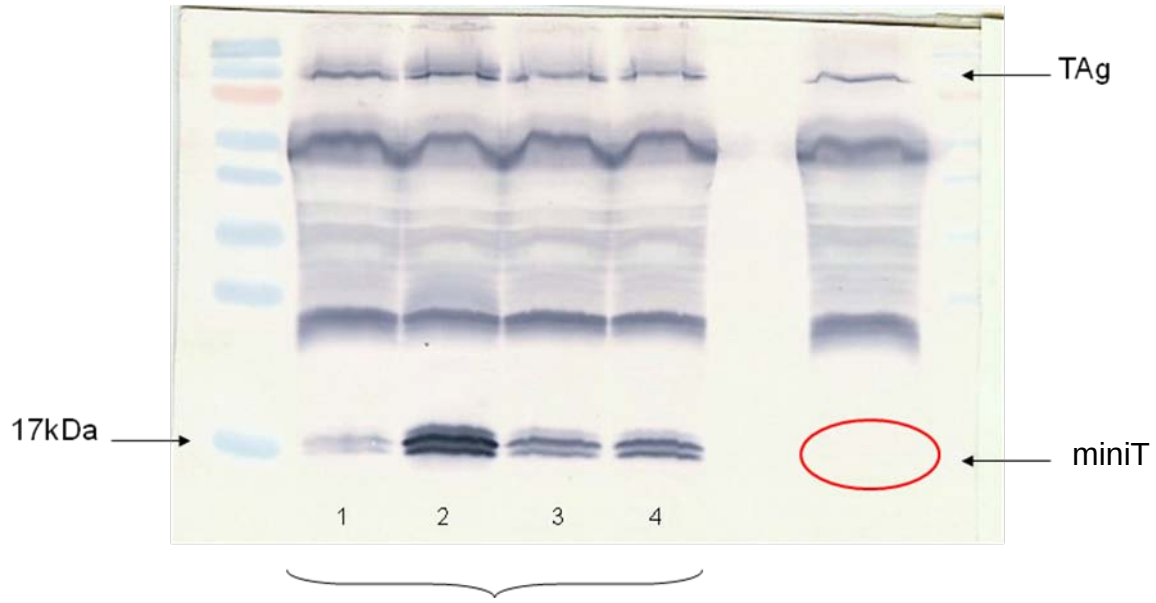
Transfected Rat2 fibroblasts were analyzed for the formation of dense foci. The number of foci generated is a measure of viral transforming efficiency. Focus formation was observed in the BKV(WT9) transformed cells 35 days p.t., however, in the BKV(WT9)-Δ4366 transformed cells, initial focus formation was seen 40-days p.t. To evaluate transformation efficiencies between WT and mutant viral genomes, foci were counted on each plate. Following hematoxylin staining, I counted an average number of 8 foci per plate for BKV(WT9), and 2 foci per plate for

BKV(WT9)-Δ4366. I performed a second transformation assay and obtained comparable numbers for BKV(WT9) and BKV(WT9)-Δ4366 transfected cells, 10 and 2 foci respectively [Fig.8]. No foci appeared on the control plates in either experiment.



[Fig. 8] Transformation Assays Foci Count. The graph demonstrates the total foci count for each experiment. In transformation assay I the foci count was 529 for SV40, 8 for BKV(WT9), 2 for BKV(WT9)-Δ4366, and zero for CT. For transformation assay II the foci count was 160 for SV40, 10 for BKV(WT9), 2 for BKV(WT9)-Δ4366, and zero for CT.

To ensure that the observed foci represented virally transformed cells and not spontaneous transformation events, foci where isolated from plates transfected with either the mutant or the parental BKV genome. Protein expression in the cells was analyzed via immunoprecipitation and Western blotting using monoclonal antibodies that recognize BKV T proteins [Fig.9]. I observed that cells from all foci isolated expressed the BKV tumor proteins. Cells transformed by the BKV mutant do not produce of miniT protein, and the absence of this protein did not appear to alter the expression of BKV TAg.



[Fig. 9] Expression of BKV early proteins in BKV-transformed Rat 2 cells. Protein (4µg) from cell lysates of BKV-transformed cells was immunoprecipitated and immunoblotted with a mixture of monoclonal antibodies that recognize the amino terminus of BKV TAg. The miniT protein is absent in cells transformed by the BKV mutant. This protein is present in cells transformed by the wild type virus and is seen as three differentially phosphorylated forms [3]. The 17kDa molecular marker is denoted by the label.

CONCLUSION

In this study I confirmed that the two pBKV(WT9) DNA clones, 5 and 7, were assembled correctly and are in the desired Bluescript vector. Restriction enzyme digestions 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 4. I chose pBKV(WT9) for additional studies, including the completion of the genomic sequence.

Sequence comparisons revealed numerous variations between pBKV(AS) and pBKV(WT9) within the region containing the mutation, leading me to use a site-directed mutagenesis method to create the pBKV(WT9)-ΔminiT mutant. I performed PCR mutagenesis utilizing primers BKsdmf and BKsdmr, and obtained a mutant which, after analysis, was confirmed to have the correct sequence. Large scale preparations of the BKV DNAs were made and digested with Bgl II to generate fragments that were used to create the final mutant construct, pBKV(WT9)-Δ4366. The correct construction of the mutant was confirmed, and both mutant and parental DNAs were transfected into the Rat 2 cell line. Dense foci appeared on the plate 35-40 days later and more foci were observed in cultures transfected with wild type BKV. All foci picked from the plates expressed BKV tumor proteins, indicating cellular transformation was virally induced and not spontaneous. Notably, miniT protein was shown to contribute to the oncogenic process.

Here, I have demonstrated for the first time that a trunctated T protein enhances transformation by a polyomavirus as measured by a dense focus assay. Studies with SV40 17KT and JCV T' proteins have shown that these proteins are expressed during transformation [22, 29-31]. Nevertheless, the contributions of these proteins to viral transformation ability have not been

directly demonstrated. Although, SV40 17KT has been shown to reduce p130 levels, and rescue TAg dnaJ mutants, indicating the involvement of the protein in the transformation process [24]; experiments demonstrating this 17KT function did not involve the use of the whole polyomavirus genome cloned, but instead relied on individual plasmids encoding each viral T protein. It is important to note that attempts at mutating the donor splice site of 17KT have been unsuccessful as the viruses containing the splice site mutation do not abolish 17KT or its function [24]. In this study, the donor splice site mutation of miniT was successful and demonstrated that miniT directly contributes to the transformation ability of BKV.

Future studies will include transformation assays with pBKV(AS) and pBKV(WT9) parental and mutant genomes to directly compare transformation efficiencies in each strain. Replication assays have been performed with the pBKV(AS) miniT mutant construct, demonstrating that miniT affects viral replication. To assess the effects on replication for the pBKV(WT9) strain, replication assays inhuman glial cultures using both the parental and mutant genomes will be conducted. Lastly, further characterization of the isolated transformed cell lines will allow us to investigate those cellular functions regulated by the BKV tumor proteins during transformation, and should illuminate the role played by the BKV miniT protein.

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EDUCATION

The Pennsylvania State University – University Park, PA
***Bachelor of Science**, Toxicology, anticipated May 2010*
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Schreyer Honors College—University Park, PA
***Honors** in Biochemistry and Molecular Biology*

RESEARCH EXPERIENCE

Undergraduate Research Assistant June 2007 – Present
Supervisor: Dr. Richard Frisque – The Pennsylvania State University

- Investigated miniT protein expression of BK Virus variant BKV(WT9) in a rodent cell line, Rat 2, using both wild type and mutant forms of each virus

PRESENTATIONS

Oral Presentations

Contribution of BK Virus miniT Protein to Viral Oncogenic Activity
18th Annual National McNair Research Conference, University of Wisconsin-Milwaukee, November 2010

BK Virus Transformation in Rodent Cell Line, Rat2
2009 Summer Research Opportunities Program Symposium, The Pennsylvania State University, August 2009

Site-Direct Mutagenesis of the BKV Variant, BKV(WT9)
16th Annual Penn State McNair Scholars Research Conference, Pennsylvania State University, July 2008

A BK Virus Variant BKV(WT9)
The Summer Research Opportunities Program Symposium, The Pennsylvania State University. August 2007

Poster Presentations

Contribution of BK Virus miniT Protein to Viral Oncogenic Activity
2009 CIC/SROP National Conference, The University of Michigan, July 2009
17th Annual Penn State McNair Scholars Research Conference, Penn State University, July 2009

A BK Virus Variant BKV(WT9)
2007 CIC/ SROP National Conference, Purdue University, July 2007

Round Table Presentations

BK Virus Transformation Potential and the Involvement of miniT Ptotein
2009 CIC/SROP National Conference, The University of Michigan, July 2009

A complete sequence of BK Virus Variant, BKV(WT9)
2007 CIC/ SROP National Conference, Purdue University, July 2007

PUBLICATIONS

Barrantes Gomez DP, Bam RA , Frisque RJ. (2010). BK virus miniT protein contributes to viral transforming and replication activities. (Manuscript in preparation).

Barrantes Gomez DP, Frisque RJ. (2009). Contribution of BK Virus miniT Protein to Viral Oncogenic Activity: Part III. The Pennsylvania State University Summer Research Opportunities Program Journal. Retrievable from http://www.gradsch.psu.edu/diversity/srop.html

Barrantes Gomez DP, Firsque RJ. (2008). Contribution of BK Virus miniT Protein to Viral Oncogenic Activity: Part II. The McNair Journal:15, 1-7.

Barrantes Gomez DP, Frisque RJ. (2007). Contribution of BK Virus miniT Protein to Viral Oncogenic Activity: Part I. The Pennsylvania State University Summer Research Opportunities Program Journal. Retrievable from http://www.gradsch.psu.edu/diversity/srop.html

SCHOLARSHIPS, HONORS, & AWARDS

Intramural NIAID Research Opportunities (INRO), National Institutes of Health, 2010

The Mildred S. Bunton and Calvin Waller Undergraduate Fellowship, Penn State University, 2006 - 2010
- Received complete tuition, room and board for 4 years

Smart Grant, The Eberly College of Science, 2009

Summer Research Opportunities Program (SROP) Grant, Penn State University, Summer 2009

Ronald E. McNair Post-Baccalaureate Research Program Grant, Penn State University, Summer 2008

Summer Research Opportunities Program (SROP) Grant, Penn State University, Summer 2007

Dean's List, The Pennsylvania State University, Academic Year 2006, 2008, and Fall 2007

Galen Dreibelbis Endowment for Excellence in Agriculture, The College of Agricultural Sciences**,** 2008 - 2009

Horace T. Woodward Scholarship, The College of Agricultural Sciences**,** 2007

John N. Adam Jr. Scholarship for Excellence in Agriculture, The College of Agricultural Sciences**,** 2007

Latino Academic Excellence, PA Summit on Educational Excellence for Latino Students, 2007

SERVICE ACTIVITIES

President, Bunton-Waller Fellow Student Council August 2008 – May 2010
Eberly College of Science – The Pennsylvania State University
- Coordinated bi-weekly meetings for 10+ undergraduate student representatives
- Assisted with professional development programs focused on resume writing, interviewing, and contract negotiation

- Co-coordinated educational programming for Bunton-Waller Fellows and Lenfest Scholars
- Developed templates for event planning, managing budgets, and other administrative tasks

Program Assistant, The Pennypacker Experience August 2007 – May 2008
Eberly College of Science – The Pennsylvania State University
- Created a supportive environment for residents in the First Year Science and Engineering (FISE), Mildred S. Bunton and Calvin Waller Undergraduate Fellows, and Lenfest Scholars Programs, as well as participants in the Schreyer Honors College
- Planned, developed, and implemented programs that addressed a diverse range of academic, professional, and personal topics
- Reviewed transcripts and aided with four-year academic planning for 45+ undergraduate students

UNIVERSITY SERVICE

Invited Guest Speaker July 2009
Upward Bound- The Pennsylvania State University
- Gave a lecture to low-income and minority high school Upward Bound students about the research process, professional attire, and presentation skills.

Mistress of Ceremony December 2007
College of Education- The Pennsylvania State University
- Hosted the Blue and White Banquet Honoring Pennsylvania's Latino Academic Excellence: Nuestros Estudiantes at The Second PA Conference and Summit on Educational Excellence for Latino Students

TECHNIQUES

- Dense focus transformation assays
- DNA Sequencing
- Immunoflouresence assays
- Immunoprecipitation assays
- Polymerase chain reaction
- Site-directed mutagenesis
- Trasfections using the calcium phosphate technique
- Western blots