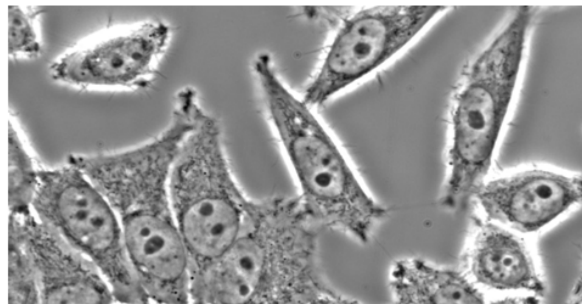
UNIVERSITY OF TROMSØ

MASTER'S THESIS IN MEDICAL BIOLOGY

STUDY OF THE INTERACTION BETWEEN BK VIRUS LARGE T-ANTIGEN AND AGNOPROTEIN



BY

KOMAN MIREILLE SOPHIE CHINAN ADOU

HOST MICROBE INTERACTION

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UNIVERSITY OF TROMSØ

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ABSTRACT

Human polyomavirus BK (BKV) is a non enveloped virus with a double-stranded, circular DNA genome. BKV infects >70% of the human population world-wide. Infection occurs predominantly during childhood and the virus remains in a latent state throughout life in the immune competent individuals. In the context of immunosuppression, however, reactivation occurs and can lead to renal stenosis and interstitial nephritis in kidney transplant patients, and hemorrhagic cystitis in bone narrow transplant patients. Moreover, BKV has been associated with several human cancers, but its causal role remains disputed. One of BKV's protein known as agnoprotein may play a role in these pathogenic processes. To develop antiviral therapy it is required to elucidate the exact biological function of this protein. One way to examine the function of agnoprotein is by identifying possible cellular interaction partners. Another way is to understand agnoprotein's role in the viral life cycle. Thereto, we examined the interaction of agnoprotein with another viral protein, large T-antigen (LT-ag) and the functional implication of this interaction. First, we investigated the effect of agnoprotein on the transcriptional activity of LT-ag on the BKV early promoter by transient transfection studies in HEK293. Our results revealed that LT-ag affects BKV early promoter in a concentration-dependent manner with low concentrations of LT-ag inhibiting, while high concentrations stimulated BKV early promoter activity. Co-expression of agnoprotein repressed LT-ag-induced activation of the BKV early promoter, suggesting that agnoprotein may exert a negative regulatory effect on transactivation by LT-ag. To test whether agnoprotein mediates its effect through direct interaction with LT-ag, we studied a possible association between these proteins. GST pulldown, co-immunoprecipitation (*in vivo* and *in vitro*), and mammalian two hybrid studies confirmed an interaction between LT-ag and agnoprotein.

ABBREVIATIONS

AMP: Ampicillin

BKV: BK Virus

BMT: Bone Marrow Transplant

Bp: base pair

CDK: Cyclin-Dependent Kinase

CDK: Cyclin-dependent kinase

cDNA: complementary DNA

CNS: Central Nervous System

CoIP: Co-immunoprecipitation

C-terminal end: Carboxyl terminal end

DBD: DNA BindinDomain

DTT: Dithiothreitol

GST: Glutathione –S-Transferase

HC: Hemorrhagic Cystitis

HEK 293: Human Embryonic Kidney cells

HeLa: Henrietta Lacks

HpyV6-9: Human Polyomavirus 6-9

IPTG: Isopropyl-ß-D-Thiogalactosidase

JCV: JC Virus

kDa: Kilo Dalton

KIPyV: KI Polyomavirus Virus

LB: Luria-Bertani broth

LDS: Lithium Dodecyl Sulphate

LPV: Lymphotropic Polyomavirus

LT-ag: Large T antigen

MCC: Merkel Cell Carcinoma

MCPyV: Merkel Cell Polyomavirus

mRNA: messenger Ribo Nucleic Acid

NaOH: Sodium Hydroxide

Nbs: Nijmegen breakage syndrome protein

NCCR: Non coding Control Region

NCRR: Non coding Control Regulatory Region

N-terminal end: Amino terminal end

OBD: Origin Binding Domain

ORFs: Open Reading Frame

PBS: Phosphate Buffered Saline

PBST: Phosphate Buffered Saline Tween

PCNA: Proliferating Cell Nuclear Antigen

PML: Progressive Multifocal Leukoencephalopathy

pRb: protein Retinoblastoma

PyVAN: Polyomavirus Associated Nephropathy

PyVHC: Polyomavirus associated Hemorrhagic Cystitis

RPETECs: Renal Proximal Tubular Epithelial Cells

SDS: Sodium Dodecyl Sulfate

SDS-PAGE: Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis

Smt-ag: Small t antigen

SV40: Simian Virus 40

TATA box: Goldberg- Hogness box

TSV: Trichodysplasia Spinulosa–associated Virus

VERO: Verda Reno (green kidney) a cell line

VP1-VP4: Viral capsid protein 1-4

WAF-1: Wild –type p53 Activated Fragment 1

WB: Western BLOT

WUPyV: WU Polyomavirus (Washington University)

YB-1: Y-box binding transcription factor

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1. INTRODUCTION

1.1 History of the Human Polyomavirus

Polyomaviruses were originally discovered in 1953 when Ludwig Gross was studying leukemia induced after injection of the murine leukemia virus into newborn mice. He observed that some animals inoculated with this retrovirus also developed adenocarcinomas of the parotid gland. Extracts of the tumor contained a virus, which induced the formation of a variety of solid tumors in newborn mice; hence the virus was named polyomavirus [1]. Until recently, polyomaviruses and papillomaviruses were designed as the two subfamilies of the family *Papovaviridae* but the term *papovavirus* has now been abandoned, and each subfamily is elevated to the status of family. Viruses of the two families are unrelated immunologically and genetically and also have different biological characteristics [2].

Several other polyomavirus have been isolated from different species after the first discovering. The polyomaviruses have a limited host range and can productively infect only they genuine host. The different polyomaviruses are widely distributed among vertebrates [3].

The polyomavirus family includes several human viruses. The first to be discovered was JC virus (JCV) and BK virus (BKV), both of which were isolated in 1971 from immunocompromised patients [4]. JCV was recovered from the brain of a patient (with the initials J.C.) who died of progressive multifocal leukoencephalopathy (PML), a demyelinating disorder of the central nervous system (CNS) [5]. BKV was isolated from the urine of a Sudanese renal transplant patient (with the initials B. K.) who developed ureteral stenosis and was shedding inclusion-bearing epithelial cells in his urine [6].

In the late 1950s and early 1960s, millions of people around the world were inadvertently exposed to a third polyomavirus, simian virus 40 (SV40) of rhesus macaques, due to administration of contaminated polio vaccines [7]. This virus, Simian virus 40 (SV40), is a natural infectious agent in rhesus macaque (*Macaca mulatto*). Recent studies revealed the presence of SV40 DNA in healthy individuals that were never vaccinated with contaminated vaccines or that had never been in contact with monkeys. Seroepidemiological studies revealed that up to 15% of the human population contains antibodies against Simian virus 40, thus supporting the possibility that SV40 can spread in human by the mean of horizontal infection and vertical transmission [8].

In the last few years, several new human polyomaviruses have been isolated. In 2007 and 2008 the isolation of two new human polyomavirus from nasopharyngeal samples by sequencing cDNA libraries was reported. KI virus (KIPyV) isolated by a group at Karolinsha Institute and WU virus (WUPyV) which was found by a group at Washington University [9, 10]. Another novel HPyV was discovered by Feng and his colleagues obtained after pyrosequencing more than 380,000 cDNA sequences from Merkel cell carcinoma. They succeeded in obtaining the complete genome which was named Merkel cell polyomavirus (MCPyV) [11].In August, 2010, a sixth polyoma virus, trichodysplasia spinulosa-associated polyomavirus (TSV), was discovered by Van der Meijden and colleagues in the proliferative skin lesion termed trichodysplasia spinulosa seen in immunosuppressed patients. [12]. HPyV6 and HPyV7 are most closely related to KI and WU viruses and were discovered by Schowalter and colleagues in 2010. The seroprevalence is 69% for HPyV6 and 35% for HPyV7 in the population [13]. A novel human polyomavirus was identified with generic PCR in a kidney transplant patient under immunosuppressive treatment. Its genome was completely amplified and sequenced. It appeared as the closest relative to the African green monkey-derived lymphotropic polyomavirus (LPV). The virus was tentatively named Human Polyomavirus 9 (HPyV9) [14].

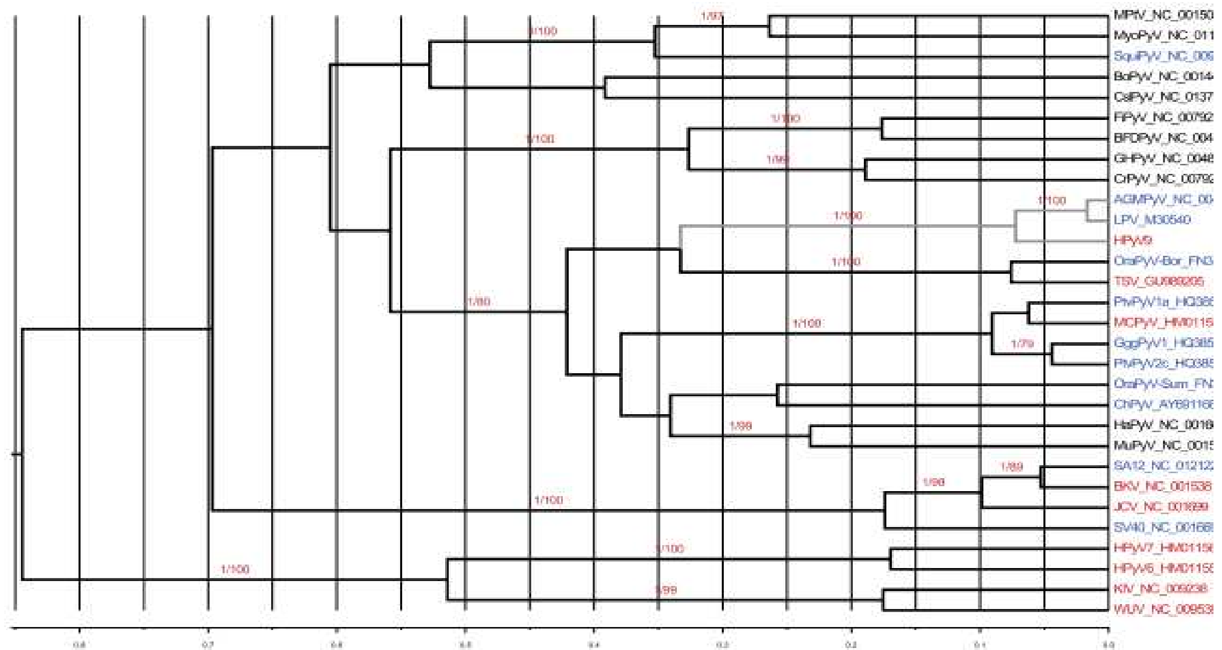


Figure 1: A schematic representation of the evolutionary relationships animal and mammalian polyomavirus. The human polyomavirus are shown in red and those from nonhuman primates are in blue. The figure is retrieved from [14].

1.2 Human polyomaviruses associated diseases

The most studied human polyomaviruses are BK virus and JC virus. The route of infection remains unknown, but respiratory, oral, body fluids, and renal tansplacental transmission has been suggested [15]. BKV is a nephrotropic virus, but nucleic acid sequences and proteins can be detected in other tissues like blood, brain, liver, heart, lung and gonads [17], while JCV nucleic acid can be found in the kidney, blood, urogenital system cells and the gastrointestinal tract [18].

Infections with both JCV and BKV are common in childhood and are largely asymptomatic. For BKV, 90% of the children aged 5-9 years are seropositive, while for JCV 50-60% is seropositive after they turn 10 years old. The amounts of JCV antibodies remained stable while the ones of BKV decrease by ageing. A person that is seronegative for BKV is more likely to be seropositive for JCV than a person that has the antibodies against BKV. It seems that both viruses have different routs of transmision, or that the seropositively of JCV might protects against the infection of BKV [15].

The primary infection with BKV and JCV seems to be asymptomatic and the virus establishes a harmless life-long latent infection in the host, but reactivation of the virus in immunosuppresed individuals can lead to illness. BKV is associated with nephropathy (PyVAN) in renal transplant patients and hemorrhagic cystitis (PyVHC) in bone narrow transplants [19, 21]. PyVAN was first reported in 1995; 1-10% of all renal transplant patients are found to have PyVAN within the first year after the transplantation [22]. The majority of PyVAN is caused by BKV but there are also a few cases caused by JCV. The disease is caused by high level BKV replication in epithelial cells lining the renal tubules. This leads to necrosis and results in renal dysfunction and sometimes also graft loss. Due to improved diagnostic technique, graft loss rate is now reduced to 10-30% [23]. Hemorrhagic cystitis (HC) is an inflammation of bladder mucosa leading to dysuria, hematuria and hemorrhage resulting in clot retention and renal failure. It is a major complication of high dose chemotherapy in bone marrow transplant (BMT) patient [24], causing morbidity and occasional mortality [25]. The incidence of PyVHC after bone marrow transplantation is 5-20% [26]. A high BKV load in urine has been correlated with an increased risk of PyVHC [27].

JCV is causative agent of progressive multifocal leukoencephalothy (PML) a fatal progressive demyelinating disease of the central nervous system due to viral replication in the oligodendrocytes [20].

1.3 Human polyomavirus and cancer

The polyomaviruses JCV, BKV, and SV40 have been implicated in several human diseases and are undergoing increased scrutiny as possible cofactors in human cancer [28]. These viruses can induce tumors in several rodent species, and can be detected with higher frequency in certain tumors compared to the corresponding healthy tissue (Table1) [29]. Whether these viruses are the true cause of these tumors remains controversial. Studies on the presence of nucleic acid or proteins from the noval polyomavirus KIPyV, WUPyV, HPyV6, HPyV7, HPyV9, and TSPyV in human cancer are lacking. Nevertheless MCPyV was found in tumor cells in patient suffering from Merkel cell carcinoma [11]. Later, several groups have evaluated the presence of MCV DNA in MCC and have found MCV in 40-100% of MCC primary tumors, reviewed in [30]. The large T antigen from MCV positive MCC tumors were found to have multiple mutations resulting in truncation of the LT-ag. The truncated LT-ag is unable to stimulate viral genome replication but retain its ability to bind retinoblastomaprotein, and is suggested to be a specific signature of Merkel cell polyomavirus persistence in human cancer cells [31, 32].

Table 1: Human cancers in which human polyomaviruses have been detect. Retrieved from [1].

Virus	Cancer	Viral Products	Virus	Cancer	Viral products
BKV	Brain tumors	DNA, RNA, Protein	**JCV**	Brain tumors	DNA, RNA, Protein
	Bone tumors	DNA, RNA		Lymphoma (Hodgkin)	DNA, Protein
	Insulinomas	DNA		Leukemias	DNA
	Kaposi's sarcoma	DNA		Prostate cancer	DNA, protein
	Adrenal tumor	DNA		Colorectal carcinoma	DNA, Protein
	Genital tumor	DNA		Gastric Cancer	DNA
	Renal carcinoma	DNA		Lung cancer	DNA, RNA, Protein
	Prostate cancer	DNA, Protein		Tongue carcinoma	DNA, Protein
	Urinary tract tumor/ Bladder tumor	DNA, RNA, Protein		Esophageal carcinoma	DNA, Protein
			KIPyV	not done	not done
SV40	Brain tumors	DNA, RNA, Protein		not done	not done
	Lymphoma (Hodgkin)	DNA, Protein	**WUPyV**	not done	not done
	Leukemias	DNA			
	Lymphomas	DNA,	**MCPyV**	not done	not done
	Bone tumor	DNA,			

	Breast cancer	DNA, Protein	**TSV**	not done	not done
	Urotheliomas	DNA	**HPyV9**	not done	not done
HPyV6	not done	not done	**HPyV7**	not done	not done

1.4 Mechanism by which HPyV can induce cell transformation

The three regulatory proteins (LT-ag, st-ag and agnoprotein) of BKV, JCV, and SV40 can contribute to cellular transformation. The HPyV regulatory proteins can interfere with the cell cycle by upregulating expression and activity of cyclin/cyclin-dependent kinase (CDK) complexes, and by inactivating cell cycle inhibitors like p53, the retinoblastoma family members and CDK-inhibitors [29].

The viral oncoproteins, the MAP kinase, Wnt, Notch, STAT, and IRS /PI3-K/AKT pathways can stimulate several signaling pathways involved in cell cycle regulation. Another mechanism is to modulate the activity of general transcription factors like TBP, TAF1, TAF4, SP1, AP-1, AP-2 and Oct6. LT-ag can functionally replace TAF1 and can also affect DNA methylation by enhancing the expression of DNA methylating enzymes. The regulatory proteins of HPyV may also immortalize cells by avoiding apoptosis and by increasing telomerase activity. Moreover LT-ag, st-ag and agnoprotein can induce mutations in the genome by disrupting the normal function of DNA repair system. Finally LT-ag can perturb the activity of mitotic spindle checkpoint proteins Bub1 and Bub3, and suppress the inhibitory activity of Nijmegen breakage syndrome protein 1(Nbs), allowing re-initiation of DNA synthesis during the S phase [29]. In the MCPyV positive MCC, LT-ag seems to be truncated in the C-terminal part, but the putative retinoblastoma-binding site is retained and these truncated LT-ag mutants can still interact with retinoblastoma but can no longer sustain viral replication. The oncogenic potentials of KIPyV and WUPyV have not yet been shown, but their LT-ags contain putative retinoblastoma and p53 binding sites, which may possess oncogenic properties [29].

1.5. BKV virion

The BK virion is a non-enveloped viral particle with an approximately 42nm icosahedral capsid protecting the double stranded circular DNA genome (Figure 2). The surface consists of 72 capsomers, where each capsomer is made of five molecules of the major capsid protein VP1. Below each capsomer is a single molecule of the minor proteins VP2 and VP3. The virion consists of 88% protein and 12% DNA [33].

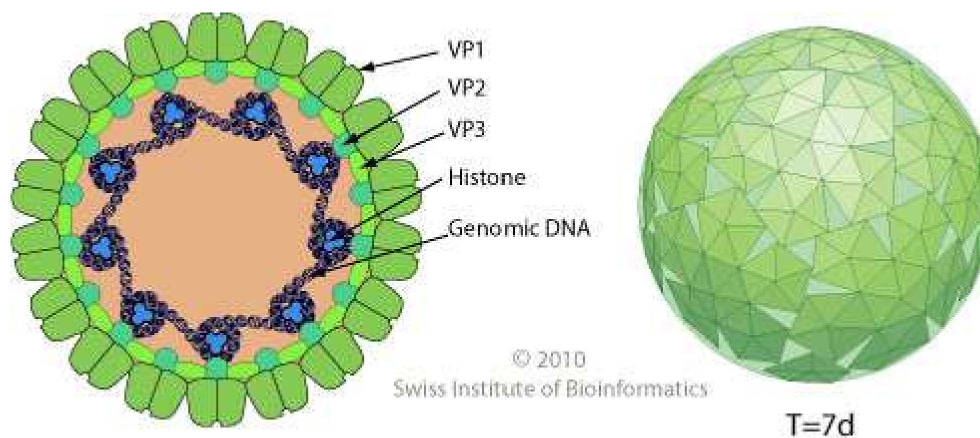


Figure 2: Schematic diagram of BK virion surrounded by capsid proteins**.** Figure retrieved from (www.expasy.org/viralzone)

1.5.1 BKV genome

The genome of BKV is a single copy of a circular double stranded DNA with average size of 5300 base pairs (bp). The BKV genome is 70% and 75% homologous to the SV40 and JCV genome, respectively [33]. The viral chromatin is similar to the host chromatin, with a length of 200 bp DNA wrapped around the host cell histones H2A, H2B, H3 and H4 to form 20 nucleosomes. The genome of polyomavirus is called the mini-chromosomes. Although the viral genome is small, its coding capacity is extended by the use of overlapping open reading frames (ORFs) located on both DNA stands, and by the use of alternative translation codon. The genome (Figure 3) can functionally be divided in three functional regions: non-coding control region (NCRR), early region and late region [34].

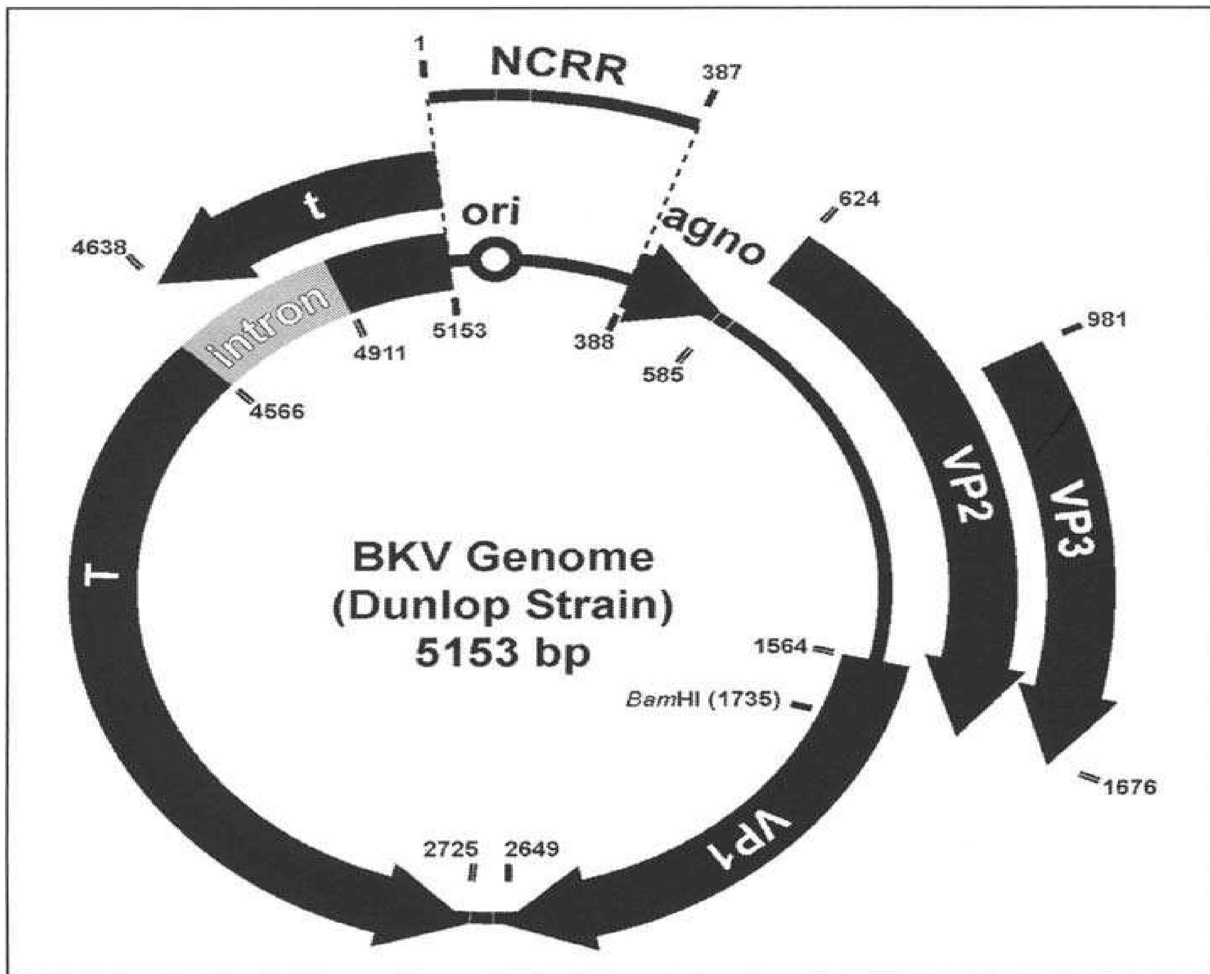


Figure 3: Genomic map of BK virus Dunlop strain.

The BKV genome is a closed circular, double-stranded DNA molecule ~5 kb in size. The coding regions for the early genes, large and small T antigens (T and t), are transcribed in a counterclockwise direction, and the late genes, agnoprotein (agno) and VP1 through VP3, are transcribed in a clockwise direction. The non-coding regulatory region (NCRR) is ~387 bp and includes the origin of replication (ori) and binding sites for various transcription factors. The figure is retrieved from [35].

1.5.2 Genomic organization

1.5.2.1 The non-coding control region (NCCR)

The bidirectional NCCR controls the transcription of both the early and late promoters and also contains the origin of replication, which regulates the initiation of viral DNA synthesis.
It is defined as the region between the ATG start codon for T antigen and the start of the agnogene region, which encodes agnoprotein. A comparison of the NCCRs of JCV, SV40, and BKV, is shown in (Figure 4).

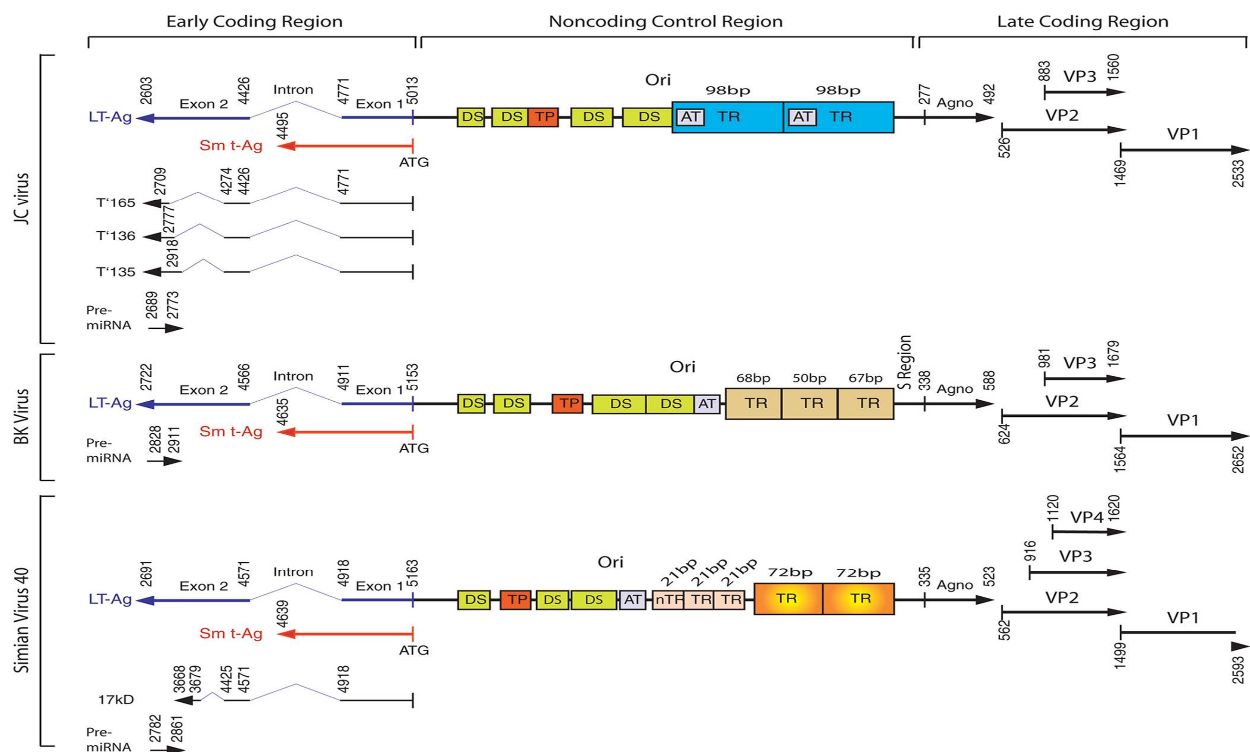


Figure 4: Comparison of the genomes of JCV, BKV, and SV40. The circular genomes of the three polyomaviruses are shown as a linear schematic diagram (not to scale) with the NCCR at the center flanked by the coding regions. The early region on the left, which is transcribed from right to left, and the late region on the right, which is transcribed left to right. The early region of each virus encodes two primary regulatory proteins, large T antigen (LT-Ag) and small t antigen (Smt-Ag). JCV and SV40 early regions also encode additional regulatory proteins. JCV encodes T'135, T'136, and T'165 (119), and SV40 encodes an additional 17-kDa protein (130). The late region of each virus, on the other hand, encodes three structural capsid proteins (VP1, VP2, and VP3). SV40 has also recently been shown to encode an additional very late protein, VP4, which functions in virus-mediated cell lysis. The late coding region of each virus also encodes a regulatory protein known as agnoprotein (agno). Also shown in the NCCR are regions with dyad symmetry (DS), true palindromes (TP). The figure and figure legend are retrieved from [36].

The early proximal side of the NCCR is highly conserved between different strains of the same virus, contains the origin of viral DNA replication, and almost never undergoes rearrangement. The late proximal side of the NCCR contains the repetitive enhancer elements and undergoes rearrangements, including mutations, deletions, and duplications, that account for most of the differences between different strains of the same virus [36].

The JCV NCCR is variable in nature due to rearrangements and yet largely confers the tissue-specific expression of the viral early and late genes [37, 38]. A comparison of NCCR sequences among a number of JCV isolates revealed that most of the variability is confined to the 98-bp tandem repeat region. Based on the occurrences of deletions and duplications, JCV isolates are assigned to two classes [37, 38]. The class I viruses are characterized by the presence of the 98-bp tandem repeat within the NCCR, which is the prototypical strain of JCV. The class II viruses contain strains that exhibit variations from the NCCR of class I with deletions and insertions.

In the case of the SV40 NCCR, there is a TATA box just upstream from the start site for the early transcription region, which is involved in fixing the site of transcription initiation precisely [39]. Further upstream is the "promoter" region, which contains two 21-bp tandem repeats and a 22-bp element that has a very similar sequence. The promoter region contains six GC-rich motifs that are binding sites for Sp1 and are indispensable for gene expression [39, 40, and 41]. There is an enhancer region that contains two 72-bp perfect repeats.

The BKV NCCR is characterized by the highest degree of variation between strains due to the occurrence of multiple rearrangements in the late proximal enhancer element observed for different isolates. The archetypal or unrearranged BKV NCCR (WW strain), which is predominant in the urine and is the transmissible form of the virus [42, 43], is arbitrarily divided into five regions, named the O, P, Q, R, and S elements [44 45,46]. The O element is the early proximal element between the T-antigen start codon and the 5 end of the enhancer element and includes the origin of DNA replication, the start site for early transcription, and the early 5 untranslated region, followed by the enhancer elements P, Q, R, and S. S is the leader region of the late transcript leading up to the agnoprotein translation start codon. BKV, early promoter activity is dependent upon elements that lie both upstream and downstream of the transcription start site [46].

1.5.2.2 The early region

The early region of the BKV genome with 2.4 kb long located on the upstream side of the origin is involved in transcription and translation in the BKV life cycle. That region encodes two regulatory proteins: Large tumor antigen (LT-ag) and small tumor antigen (st-ag) have a common pre messenger RNA (mRNA) precursor and are produced by alternative splicing. Transcription of the early regions proceeds on one DNA strand in a counterclockwise direction [47, 48].

1.5.2.3 The late region

The late region of BKV is 2.3 kb long, resides downstream of the origin of replication and encodes three structural proteins VP1, VP2, VP3, and one non-structural agnoprotein. These proteins are also translated from different mRNA produced from a common pre-mRNA transcript by alternative splicing. Transcriptions of the late region proceed on the complementary strand of the early transcription in a clockwise direction. VP1 is translated in from a different spliced mRNA while VP2, VP3 are translate from the same mRNA and in the same reading frame, but with alternative start codons [49] (Figure 3). The viral proteins encoded by BK virus are summarized in Table 2.

Table 2: Overview of the six BKV encoded viral proteins. Table modified after [50, 51].

Proteins	Transcription	Cellular localization	Molecular mass	No. amino-acids	Function
Large T	Early	Nucleus	80.5	695	Initiates viral replication; stimulate host synthesis; regulates viral transcription
Small T	Early	Nucleus/cytoplasm	20.5	172	Facilitates viral replication
VP1	Late	Nucleus	40.1	362	Major capsid protein, enable entry, mediates hemaglutination

VP2	Late	Nucleus	38.3	351	Minor capsid protein
VP3	Late	Nucleus	26.7	232	Minor capsid protein; subset to VP2
Agnoprotein	Late	Cytoplasm/Perinuclear	7.4	66	Facilitates capsid assembly

1.5.2.4 Large T antigen (LT-ag)

LT-ag is a multifunctional protein mediating host cell activation and preventing apoptosis, participates in early and late BKV transcription and replication of the viral genome. The different regions of LT-ag have different function in DNA replication [52]. LT-ag contains three main functional domains: the N-terminal J-domain, the central origin binding domain (OBD), and the helicase domain. The C-terminal region determines the host range. LT-ag is the only viral protein involved in viral DNA replication serving as an initiator protein and as replicative helicase [reviewed 53]. LT-ag localizes in the nucleus during the early phase of infection, interacts with a number of the host cellular proteins that regulate cell cycle, transcription and metabolism. LT-ag first binds to and inactivates two host cell tumor suppressor proteins retinoblastoma protein (pRb) and p53. pRb controls cell cycle and inhibits S-phase gene expression, whereas p53 blocks the cell cycle and can induce apoptosis. Inactivation of these two proteins leads cell to enter into cell cycle S phase from quiescent cell G phase. Also LT-ag must bind to the origin of replication and orchestrates the bidirectional DNA replication in the presence of the host proteins like DNA polymerase α-primase, topoisomerase I, nucleolin, proliferating cell nuclear antigen (PCNA), and replication protein A [54]. LT-ag also binds to the NCCR, thereby controlling viral replication and transcription of viral genes [53].

1.5.2.5 VP1

VP1 is the largest capsid protein and it forms the outer shell of the capsid. VP1 accounts for 75% of the virion protein mass, it contains a variable antigen binding region, which acts as the

major ligand for viral attachment and mediates capsid binding to membrane receptors of susceptible cells [55]. VP1 contains epitopes for hemaglutination inhibition and neutralization [50]. The C-terminal end of VP1 is thought to bind the VP1 pentamers to each other [56].

1.5.2.6 VP2, VP3 and VP4

VP2 and VP3 are minor structural proteins that make a link between VP1 and the genome in the virion they make [57]. The C-terminal end of the VP2 is the entire sequence of VP3; VP2 has also 119 amino acids additionally to this on N-terminal end [58]. Recently, a study showed that SV40 late region encodes another protein referred to as VP4 which is translated from another internal start codon in VP2/VP3 mRNA. Although BKV and JCV contain a putative open reading frame for this peptide, expression of VP4 has not been confirmed. For SV40, VP4 has been found to not be capsid protein but a regulatory protein that enhances lysis of the infected host cell and release of the mature virus particles [57].

1.5.2.7 Agnoprotein

Agnoprotein is one of the six major BKV proteins with 8kDa and expressed as a late protein together with the three capsid proteinsVP1, VP2 and VP3, after the early genes large T-antigen and small T-antigen at around 36 h post infection. The agnoprotein of JCV, BKV and SV40 have a high degree of sequence conservation, especially in the N-terminal half of the protein where 31 or 25 amino acids out of 37 are identical between BKV and JCV or BKV and SV40, respectively. The C-terminal part on the other hand has greater variability in the amino acid sequence, and may be involved in virus specific processes (Figure 5).

```
              10        20        30        40        50        60        70
JCV   MVLRQLSRKASVKVSKTWSGTKKRAQRILIFLLEFLLDFCTGEDSVDGKKRQRHSGLTEQTYSALPEPKAT
      ||||||||:|||||:|||:|||||||||:||:||:||:|| ||||||||:::
BKV   MVLRQLSRQASVKVGKTWTGTKKRAQRIFIFILELLLEFCRGEDSVDGKNKSTTALPAVKDSVKDS
      ||||:|||:|||||  ::|: :|| |||:::|:||:||:|| |||:|||| ::
SV40  MVLRRLSRQASVKVRRSWTESKKTAQRLFVFVLELLLQFCEGEDTVDGKRKKPERLTEKPES
              10        20        30        40        50        60
```

Figure 5: Comparison of the amino acid sequence of JCV, BKV and SV40 agnoprotein. Sequence identity is indicated with a tilde (l). Conservative sequence changes are indicated with a colon (:). Amino acids that are identical in all three polyomaviruses are shown in bold face. Figure and figure legend are retrieved from [59].

The agnoprotein has been shown to reside predominantly in the cytosol and in the perinuclear region in association with the outer nuclear membrane, but a minor fraction can also be

detected in the nucleus of SV40 and JCV infected cells [59]. Studies in JCV and SV40 have suggested that agnoprotein is involved in nuclear egress [60], gene expression, viral release, virion assembly and maturation [59]. Safak and co-workers have shown that JCV agnoprotein negatively regulates DNA replication and transcription in human glioblastoma cells. JCV agnoprotein was found to interact with LT-ag, and the authors discussed whether agnoprotein may thereby prevent the disproportional production of DNA and capsid proteins in order to optimize the virion formation [61]. The agnoprotein of JCV, SV40 and BKV polyomaviruses has been found to be phosphorylated *in vivo* [59]. A recent study revealed that Ser-7, Ser-11 and Thr-21 are phospho-acceptor sites in JCV and BKV agnoprotein. Mutation of these sites into non-phosphorylable alanine resulted in disturbed viral propagation compared to the wild type [62]. The role of phosphorylation of agnoprotein is poorly understood, but agnoprotein seems to affect viral propagation and the transcriptional transactivation property of agnoprotein [63]. Protein-protein interaction studies have demonstrated that agnoprotein of JCV could bind directly to p53, increase the activity of the CDK inhibitor p21/ wild type p53 activated fragment1 (WAF1), and cause cell cycle arrest in G2/M phase [64]. A study revealed that JCV agnoprotein increased chromosome fragmentation and micronucleus formation and sensitized cells to the cytotoxic effects of the DNA damaging agent cisplatin. Whereas cisplatin-treated control cells accumulated in S phase, cells expressing agnoprotein did not. Agnoprotein expression correlated with impaired double-strand-break repair activity in cellular extracts and reduced expression of the Ku70 and Ku80 DNA repair proteins. The authors concluded that agnoprotein inhibits DNA repair after DNA damage and interferes with DNA damage-induced cell cycle regulation [65].

The function of BKV agnoprotein in the viral life cycle is less studied. BKV deficient virus propagates in cell culture although to a lesser extent than wild type [64]. BKV agnoprotein has also been found to interact with three unidentified proteins of 50, 70 and 100 kD, and to co-localize with lipid droplets [66, 67]. The functional implications of these interactions have not been investigated so far. Data from JCV and SV40 may give indication on the functions of the BKV agnoprotein since the similarities between them is high.

1.5.3 BKV life circle

Almost everyone is exposed to the polyomavirus as a child [68]. The primary infection is either completely asymptomatic or takes the form of mild respiratory illness. The virus then

enters a latent state and resides primary in the kidney, where it is shown to infect renal tubular cells, the parietal epithelial layer of Bowman's capsule and the transitional epithelium (Figure 6) [69]. There is some evidence that it may hibernate in peripheral blood lymphocytes [70].

BK (Dunlop) requires approximately 48-72 hours completing its life cycle in primary human renal proximal tubular epithelial cells (RPETECs) [71]. The BKV life cycle is completely dependent upon host cellular machinery. Viral transcription, translation and DNA replication require cellular enzymes and co-factors.

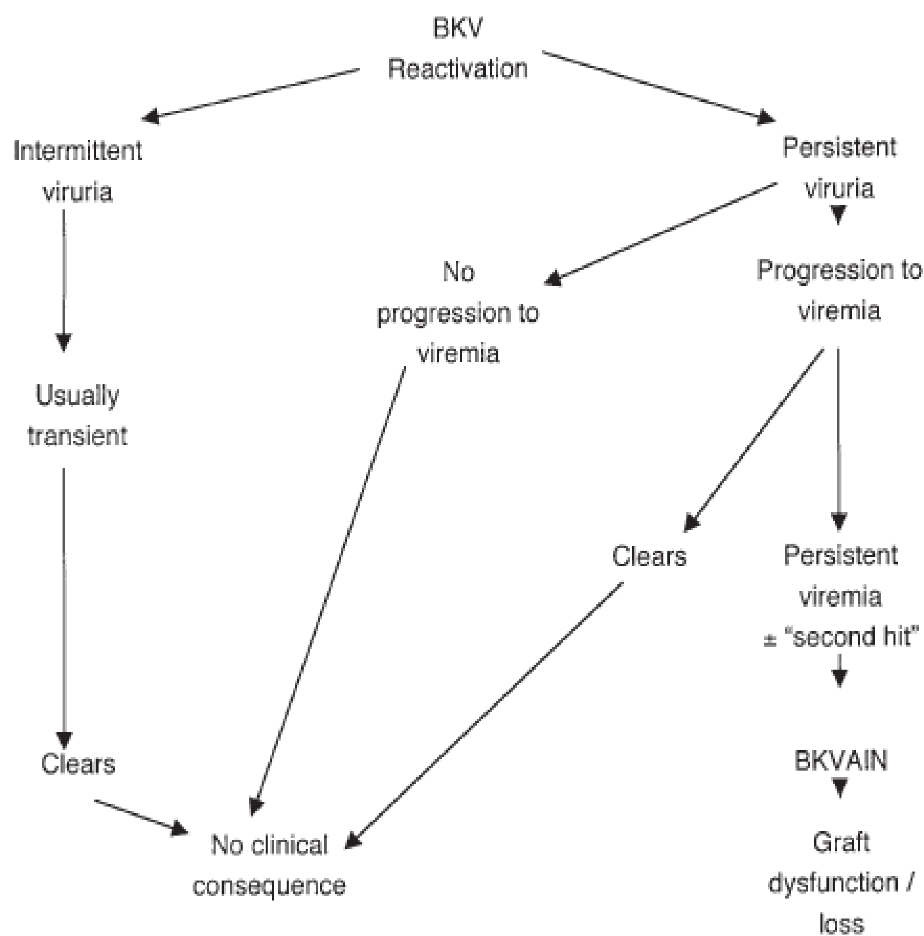


Figure 6: Life cycle of BKV. Figure retrieved from [73].

1.5.3.1 Attachment

Viral attachment to susceptible host cell is acquired for the virus to enter the cell. All the susceptible host cells have specific receptors utilized by different virus. BKV interactions with host cellular receptors have been the subject of only limited investigations. The primary receptor binding determinant on BKV is the VP1 protein. The host cell receptor for BKV appears to be an N-linked glycoprotein, in which GT1b and GD1b have been identified as component gangliosides [72]. Both these gangliosides have an α-(2-8) linked di-sialic acid-motif as a common feature. An α-(2-3) sialic acid linkage has also been shown to be important [72, 73].

Despite considerable homology at the genetic level, BKV differs from other polyomaviruses with regard to the chemical nature of its receptor. Thus, the JCV receptor is an N-linked glycoprotein containing terminal α-(2-3) and α-(2-6)-linked sialic acids. The mouse polyomavirus binds to receptors containing α-(2-3) linked sialic acid N-glycoproteins as well as a 4b1 integrins. SV40 VP1 interacts with major histocompatibility class I proteins and O-linked glycan molecules [74].

1.5.3.2 Cell entry and intracellular trafficking

The mode of BKV entry into the cell and routes of intracellular trafficking are currently being clarified. Electron microscopy observations on human biopsy material show that BKV entry into host cells is similar to SV40, and mediated by non-clathrin coated vesicles resembling caveolae. In contrast, JCV enters the cell by clathrin-dependent endocytosis. The mechanisms of endocytosis and intra-cellular trafficking utilized by BKV have not been investigated in detail. However, it has been established that the route from cell membrane to the nucleus includes the endoplasmic reticulum and microtubules [75, 76]. There may also be participation of the Golgi apparatus, and other cytoskeletal elements such as actin, and microfilaments. The mechanism by which polyomavirus traverses the nuclear envelope to enter the nucleus is only partially understood. VP2 and VP3 contain a nuclear transport signal that may facilitate nuclear targeting of the viral mini-chromosome. Nucleoporin, a protein associated with the nuclear pore complex, has also been implicated. The uncoating process of polyomaviruses has been stated to occur after the virions have entered the cell nuclei, but it has been shown for SV40 virus that some disassembly can occur in the endoplasmic reticulum [77]. After the genome reaches the nucleus, the viral transcription and replication can occur.

1.5.3.3 Viral gene expression, DNA replication, viral assembly and release

The nucleus of the cell is the site of viral replication and virion assembly for all DNA viruses except poxvirus. BKV replication begins with the transcription of the early genes that encode the LT-ag and st-ag that are expressed soon after infection of the host cell. As stated previously, LT-ag and st-ag are differentially translated by alternative splicing of the early mRNA transcript. Removal of the LT-ag intron splices the first exon with the next exon allowing translation of LT-ag. Alternatively, retention of the intron allows translation to reach a termination codon within the intron resulting in st-ag. It is the production of the LT-ag that causes quiescent cells to reenter the cell cycle and thus begin replication of cellular DNA [78]. LT-ag autoregulates its own transcription by negative feedback [79] and is largely responsible for the cell transforming potential of BKV [80]. This transforming potential of LT-ag is attributed to its ability to bind and inhibit the function of host tumor suppressor proteins including p53 and pl05RBl [81, 82]. In permissive host cells, the LT-ag acts as a regulatory protein, directing the remaining events of viral replication that result in a productive infection. Replication of the BKV genome occurs within the nucleus well after the transcription and translation of the early genes has begun. Replication begins at ori (Dunlop strain sequence) within the NCRR and proceeds in both directions and is completed when the replication forks meet on the opposite side of the genome (Figure 3). The late genes, consisting of the structural proteins VPl, VP2 and VP3, and the agnoprotein genes, are predominately expressed after genomic replication has been initiated. The completion of viral replication process concludes when the VP1, VP2 and VP3 proteins that will constitute the capsids are transported to the nucleus and the viral capsomeres assemble around the newly replicated genomes, forming stable virus particles [3]. The replication rate of BKV in vivo is not well known, however, in vitro replication of BKV strongly depends on the host cell and is efficient only in primary cultures of human fetal kidney or neuralgia cells [83, 84] and requires 3 to 4 weeks to reach a maximum virus titer [84, 85]. The mechanisms for viral release are less studied. Some previous studies for SV40 indicate involvement of the secretory machinery for release [86], while more recent studies suggest contribution of VP4 [57].

1.5.3.4 Regulation of viral gene expression of Large T-antigen

As LT-ag is no part of the virion, expression of LT-ag starts after infection due to presence of cellular transcription factors. Its initial levels are low early in infection, but LT-ag stimulates its own production and levels will increase. Initially, there is no expression of the late region because a cellular transcription factor belonging to the steroid receptor family occupies late promoter and therefore blocks transcription of late genes. The concentration of this cellular factor is, however, limited and as viral DNA starts to replicate it will be diluted out and viral genomes in which the late promoter is not blocked by this cellular factor are accessible for late gene expression. At lower concentrations, LT-ag will bind binding site II because it has a higher affinity for this site. This will stimulate transcription of the early genes (auto stimulation of LT-ag expression). The amount LT-ag raises and LT-ag will also binds to LT-binding site I, and inhibits its own synthesis (auto-repression; Figure 7). DNA replication starts when LT ag binds to LT binding site II. DNA replication results in increased amount of viral genomes and release of repression of late genes and the transcription of these starts [87].

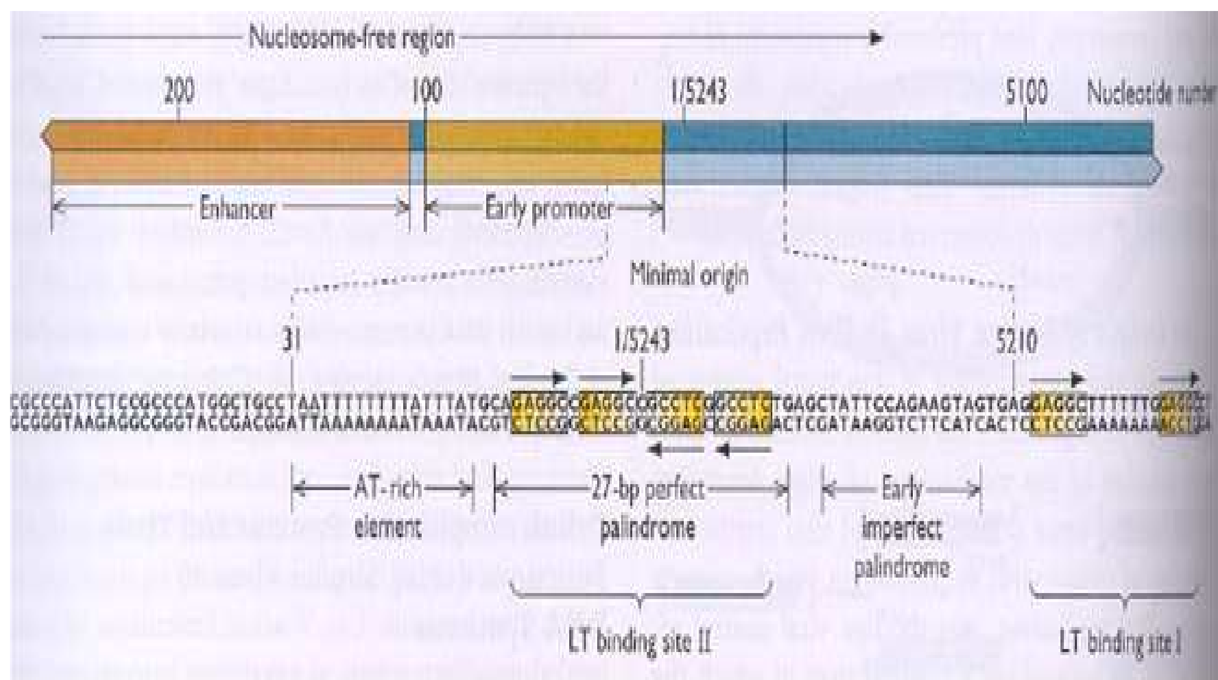


Figure 7: NCCR of SV40 contain a minimal origin; with LT-binding sites in yellow, AT-rich element and an early imperfect palidome. Figure retrieved from [87].

2 Aim of the study

Human polyomavirus BK (BKV) is a non enveloped virus containing a double stranded, circular DNA genome. BKV infects population all over the world. The infection occurs in 70% of the human population during childhood and remains in the latent state throughout life in the immune competent individuals, but in the context of immunosuppression, is largely responsible for the diseases renal stenosis, interstitial nephritis in kidney transplant patients and hemorrhagic cystitis in bone narrow transplant patients. Moreover, BKV has been associated with several human cancers. One of the viral proteins is agnoprotein. The exact function of BKV agnoprtoein remains elusive, but it may play a role in these malignant processes. BKV's role in the viral life cycle is not completely understood. This study was initiated to elucidate the biological role of agnoprotein during viral replication. JCV agnoprotein was shown to interact with and to suppress LT-ag mediated transcription of the viral promoter. The aims of this study were:

- Can BKV agnoprotein interfere with LT-ag's effect on early promoter activity?
- Can BKV agnoprotein physically interact with LT-ag?
- Evaluate whether the interaction between those two proteins is important for viral propagation.

3 MATERIALS AND METHODS

3.1 MATERIALS

Table 3: Growth media for bacteria used in this study

Growth medium	Purpose	Contents
LB+(Luria Bertani)	Overnight culture of bacteria for plasmid purification	4000ml H_20, 100g Trypton, 50g yeast extract, 11g glucose, 100g NaCl, H_20 up to 5000ml, pH=7.4 and 100 µl l/ml Ampicillin
LB plates (Luria Bertani)	Short term storage of bacterial strains prior to inoculating LB+ Plasmids purification	10g agar, 500ml H_20, 500ml LB, 5ml $CaCl_2$ and 100 µl / Ampicillin or Kanamycin
NZCYM	Overnight culture of bacteria for GST-protein purification	900ml d H_20, 10g Trypton, 5g NaCl, 1g Casaminoacid, 5g yeast extract, 2g $MgS0_4$-H_20 adjusted to 1000ml, pH=7.5 and 100 µl /ml Ampicillin

Table 4: Kits used in this study

Kit	Manufacturer	Purpose
NucleoSpin R Plasmid kit	Machery Nagel	Plasmid purification, small quantities
Nucleobond R Xtra Midi Kit	Machery Nagel	Plasmid purification, medium quantities

Table 5: Plamids constructs used in this study

Plasmids	References or source	Purpose
RcCMV	Invitrogen	Transfection
RcCMV-Agno	[64]	Transfection

LT-agVP16	Moens, unpublished	Transfection
pcDNA3	Invitrogen	Transfection
pRcCMV-BLT	[104]	Transfection
pcDNA3-1-VP1	Moens, unpublished	Transfection
EGFP-C2	clontech	Transfection
pVP16-AD	Clontech	Transfection
pM-BKV-agno	Moens, unpublished	Transfection
pM	Clontech	Transfection
Gal_5E1b.-Luc	[105]	Transfection
pNCCR_EL-Luc	Moens, unpublished	Transfection
pM-P53	clontech	Transfection
pG-TCR-LE-Luc	Moens, unpublished	Transfection

Table 6: buffers and solutions used in the study

Method	Buffer	Contents
Washing of cells **Washing of membranes** **Washing of GST-proteins General buffer**	Phosphate buffered saline (PBS)	137mM Nacl, 2.7mM KCl, 10mM Na_2HPO4, 2mMKH_2HPO_4
DNA purification	LYS buffer	
	NEU buffer	
	EQU buffer	
	ELU buffer	
	WASH buffer	
	RES+RNase buffer	

	TE buffer	10mM Tris-HCl pH8, 1mMEDTA
Splitting of cells	Trypsin	0.25% Trypsin in PBS, 0.05%Na_2-EDTA
Transfection of cells	Optimen	GlutaMax^{TM} I, 2400mg/L sodium Bicarbonate, HEPES, Sodium Purivate, Hypoxanthine, Thymidine, Trace Elements, Growth Factor,1.1mg/L Phenol Red (Gibo)
Growth of cells	Growth medium: DMEM	Various salts ,amino acids, vitamins , glucose and phenol red, Penicillin, Streptomycin and 0.3 or 10% Foetal Bovine Serum
SDS-PAGE gel electrophoreses	LDS Nupage Loading Buffer	Invitrogen
	SDS running buffer 20x	C.B.S scientific company inc 40ml 20xrunning buffer, 760ml H_20.
Staining of SDS-PAGE gels	Coomassie Blue Staining	50% methanol, 10% Acetic Acid, 0.05% coomassie Brillant Blue R-250, H_20
	Fixation solution	50% methanol, 10% Acetic Acid, dH_20
	Destaining solution	5% methanol, 7% Acetic Acid, dH_20
Western Blot		
	Washing buffer	100mMTris-HCl pH9.5 1mM EDTA, 100mM NaCl and 10mM $MgCl_2$
	CDP star assay buffer	5ml DEA, 420ml with dH_20,
	CDP star	5ml DEA and 420ml dH_20 with
	Blocking buffer	150ml PBS,7.5g milk powder and 0.150ml tween

	Blotting buffer	5.8g Tris,29g glycin, 200ml Methanol, 800ml dH_20
	PBST	PBS and 0.1% tween
Stripping	Stripping 0.2 M NaOH	pH9.5, 0,2M Na0H, 75ml dH_20
Co-immunoprecipitation	Loading buffer	10µl LDS Nupage sample buffer (invitrogen), 2µl 1M DTT, 8µl dH_20
	coIP buffer	50mM Tris-HCl pH8, 150mM NaCl, 0.5% Triton-X-100, 1mM EDTA,1mM DTT, 1xprotease inhibitor solution
	Sepharose beads	50% sepharose beads in coIP buffer
GST purification		
	Isopropyl β-D-1-thiogalactopyranoside (IPTG)	1 M IPTG
	Glutathione beads	50% gluthatione beads in PBT
	Protease Inhibitor solution	1tablet protease inhibitor+ 2ml dH_20
	PBST	PBST, 1% Triton-x-100, protease inhibitor
	DTT	1M DTT
	LDS sample buffer	Invitrogen
	Elution Buffer	5mM reduced glutathione 50mM Tris-HCL pH 8.0
GST Pulldown		
	PBST	9.9ml PBS,100 µl Triton-X-100, 400 µl protease inhibitor
	Glutathione beads	50% gluthatione beads in PBST

Table 7: Mammalian cells line used in this study

Cell-Line	Organism	Organ	Reference	Purpose
HEK293	Human	Kidney	ATCC **CRL-1573™**	Transfection
VERO	African green Monkey	Kidney	ATCC CCL-81	Transfection
HeLA	Human	Cervical	ATCC CCL-2	Transfection

Table 8: Transfection reagents used in this study

Transfection reagent	Manufacturer	Purpose
Lipofectamine™ 2000	Invitrogen, cat # 11668-019	Transfection for subsequent Luciferase assays and Co-ip
Metafectene® Pro	Biontex, cat # T040-2.0	Transfection for subsequent Luciferase assays and Co-ip

Table 9: Antibodies used in this study

Antibody	Manufacturer or source
Anti-LT-antigen	[66]
Anti-Agno	[66]
Goat anti-rabbit IgG-AP conjugated human	Southern Biotech
Anti-p53	Cell Signaling Technology

3.2 METHODS

3.2.1 Plasmids DNA purification and isolation with the Nucleobond protocol

Plasmid DNA was prepared using the Nucleobond Xtra midi kit based on alkaline lysis of the bacterial cells followed by absorption of DNA onto a silica membrane in the presence of high salt. The procedure is on four basic steps: preparation of a bacteria lysate, absorption of DNA onto the Nucleobond membrane, washing and elution of plasmid DNA [88]. All the steps were performed at room temperature.

A single colony was picked from an LB plate with appropriate antibiotics and inoculated in 100ml LB+ selective medium in a flask. The flask was incubated on a shaker at 37°C overnight. The overnight culture was transferred in a tube and centrifuged at 6000xg at 4°C for 20minutes. The supernatant was discarded and the pellet was re-suspended in 8 ml cold RES+RNase buffer (table 6). The solution was afterwards transferred into a new 50ml centrifuge tube and 8ml LYS buffer was added. The tube was inverted 6 times and incubate on the bench for 5min to allow the denaturation chromosomal DNA and of the proteins in the solution (Macherey-Nagel). Meanwhile the coloum was equilibrated by addition of 12ml the EQU buffer (table 6) was added to the top of edge of the filter in the column, and the column was emptied by gravity flow. 8ml of the NEU buffer was then added to the lysate and the tubes were inverted 15 times. The addition of NEU stopped the denaturizing process and precipitated SDS, proteins, chromosomal DNA and other cell debris [88]. The lysate was added to the filter in the column. The columns were allowed to go empty by gravity flow and added 5ml EQU buffer to the edge of the filter. The column was emptied by gravity flow and the filter containing cellular debris was removed. The column, which now contained plasmid DNA was then washed with 8ml of WASH buffer (table 6) and drained by gravity flow. The column was placed in high speed glass centrifuge tubes. The DNA plasmid was eluted with 5ml ELU buffer (table 6) and the column emptied by gravity flow. Afterwards the DNA was precipitated with 3.5ml Isopropanol and left for 2minutes at room temperature before centrifuging for 20minutes at 20000xg at 4°C. The supernatant was discarded carefully and the DNA was washed by adding of 2ml of 70% ethanol followed by centrifugation for 5minutes at 20000xg at 4°C. The supernatant was carefully removed and the DNA pellet was dried for 10 minutes at the room temperature. The final step was to dissolve the DNA pellet in 100µl of TE buffer and the purified DNA was transferred to a 1.5ml eppendorftube. The DNA concentration was measured by use of spectrophotometer ND-1000.

3.2.2 Mammalian cells cultures

Mammalian cell culture in this study could be divided into two main component parts. These include maintaining cells in culture and manipulation of cultured cells. The cells used in the study were Vero, HEK 293 and HeLa.

Vero cell is derived from African green monkey kidney cells. The *Vero* lineage was isolated from Kidney epithelial cells extracted from an African green monkey (Cercopithecus aethiops). The lineage was developed on 27 March 1962, by Yasumura and Kawakita at theChiba University in Chiba Japan. The original cell line was named "Vero" after an abbreviation of "Verda Reno", which mean "green kidney" in esperanto, while "vero" itself means "truth" [89]. Figure 8 show how Vero cells look like in culture.

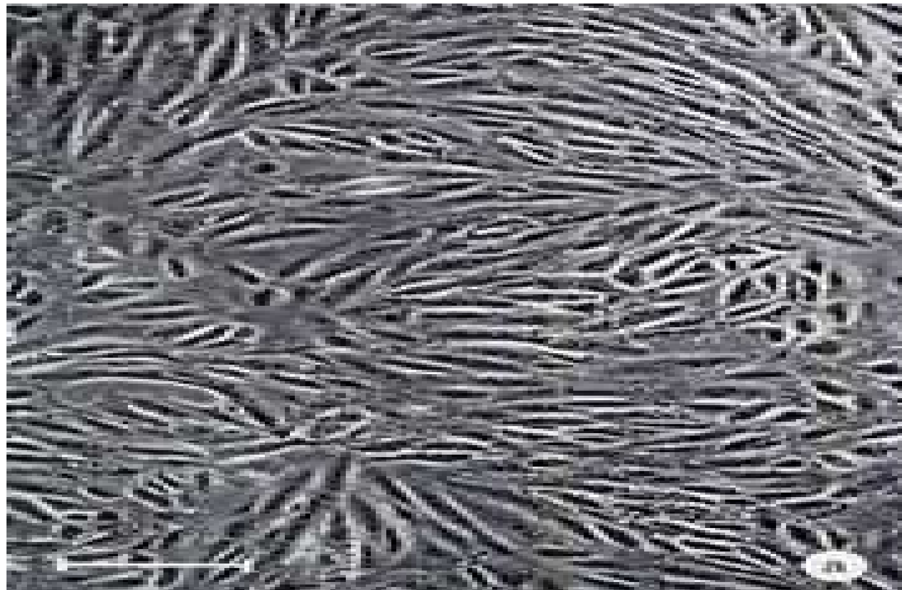


Figure 8: Confluent Vero cells Image from Zeltus.com © 2011 William Parker

Human Embryonic Kidney 293 cells, also often referred to as HEK 293 are a specific cell line originally derived from human embryonic kidney cells grown in tissue culture. HEK 293 cells were generated in early 70s by transformation of cultures of normal human embryonic kidney cells with sheared adenovirus 5 DNA in Alex Van der Eb's laboratory in Leiden, Holland. The human embryonic kidney cells were obtained from a healthy aborted fetus and originally cultured by Van der Eb himself, and the transformation by adenovirus was performed by Frank Graham who published his findings in the late 1970s after he left Leiden for McMaster University in Canada. They are called HEK for human embryonic kidney,

while the number 293 comes from Graham's habit of numbering his experiments; the original HEK 293 cell clone was simply the product of his 293rd experiment [90].

HEK 293 cells are very easy to grow and transfect very readily and have been widely-used in cell biology research for many years. Figure 9 shows the HEK293 cells

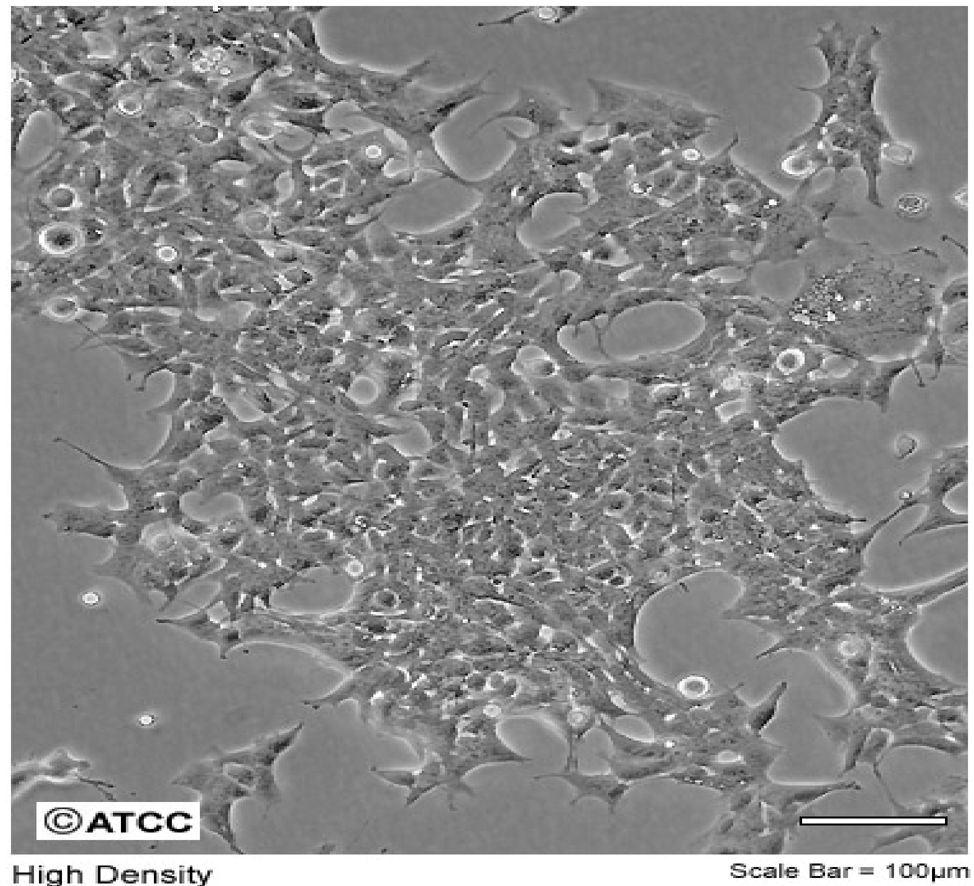


Figure 9: HEK cells grown in a flask containing growth media. Image from ATCC

HeLa cell is a cell type in an immortal cell line used in scientific research. It is one of the oldest and most commonly used human cell lines. The line was derived from cervical cancer cells taken from Henrietta Lacks, a patient who eventually died of her cancer on October 4, 1951. The cell line was found to be remarkably durable and prolific as illustrated by its contamination of many other cell lines used in research. The cells were propagated by George Otto Gey shortly before Lacks died in 1951. This was the first human cell line to prove successful in vitro, which was a scientific achievement with profound future benefit to medical research [91].

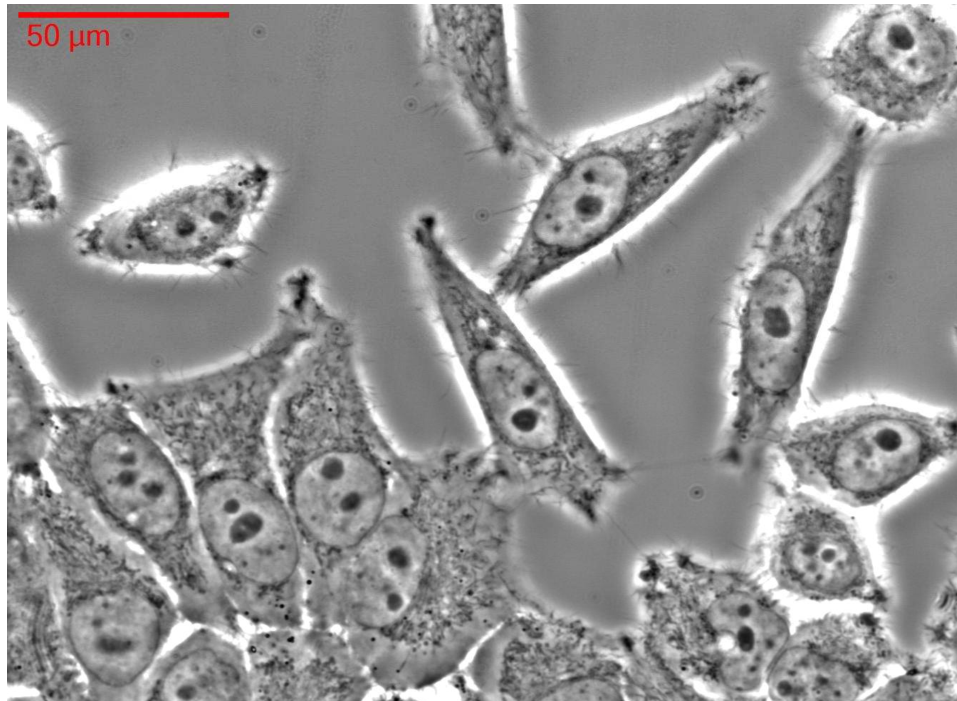


Figure 10: Hela cells grown in flask containing growth media. Image from infobarrel.com

All procedure was performed in SCANLAF bench. All solutions to be used including the growth medium, PBS and trypsin were pre-warmed to 37°c before using.

3.2.2.1 Thawing of cells

The mammalian cells are stored at -196°C in the nitrogen tank. The cells are stored in serum containing DMSO as cryoprotector. Prior to use, cells should be thawed rapidly. Thereto, the vial containing the frozen cells was removed from liquid nitrogen storage and quickly thawed the cells by gently swirling the vial at water bath with a temperature of 37°C until there is just a small bit of ice left in the vial. The vial was wiped with 70% ethanol, and transferred the vial it into a 15 ml tube containing pre-warmed complete growth medium in a laminar flow hood. Before opening, wipe the outside of the vial with 70% ethanol. Transfer the desired amount of pre-warmed complete growth medium appropriate for your cell line drop wise into the centrifuge tube containing the thawed cells. The cell suspension was centrifuged at approximately 200 × g for 5 minutes. After the centrifugation, the clarity of supernatant and visibility of a complete pellet were check. The supernatant was removed by use of a sterile Pasteur pipette. The cell, which now have been washed to remove the DMSO, were suspended in complete growth medium, and transferred into the appropriate culture vessel and

placed into the recommended culture environment. The appropriate flask size depends on the number of cells frozen in the cryovial, and the culture environment varies based on the cell and media type, and medium is changed the next day. The cells are grown for3 -4 day passages before using in experiments.

3.2.2.2 Splitting of cells

When the cells were confluent, they needed to be spitted. The media was then removed from the cells in the flask and the cells were wash with pre-warmed 10ml 1xPBS (37^oC). To detach the cell from the flask, 2ml of pre-warmed trypsin was added and left for 3-5minutes until all the cells rounded up. 10ml of pre-warmed DMEM containing 10% FBS was added and the cells were re-suspended by pipetting several times carefully. The trypsin activity was neutralized by adding fresh medium containing the FBS. In a new flask, the appropriate volume of fresh medium was added in desired ratio of the suspension solution. The flask was kept in the CO_2 incubator at 37^oC until the next splitting day which usually is 3 days from the first splitting day.

3.2.2.3 Seeding out cells

The pre-warmed PBS was used to wash the cells and the trypsin was added to detach the cells from the bottom of the flask. The growth media was added in the appropriate ratio. The number of cell was evaluated by counting with the Bucker chamber. One drop of the suspension cell was put in the Bucker chamber, 3 A-quadrant were counted using the microscope and the mean value was calculated. This value, X, corresponds to Xx 10^4 cell/ml. the appropriate number of cells was seeded in wells and incubated at 37^oC. In this work 200000cells/ml were used per well in a 6 well dish plate while $2x10^6$ cells were used in 9 cm dishes

3.2.2.4 Transfection of the cells with Plasmids

The process of introducing nucleic acids into eukaryotic cells by nonviral methods is defined as transfection. Using various chemical, lipid or physical methods, this gene transfer technology is a powerful tool used to study gene function and protein expression in the context of a cell. Development of reporter gene systems and selection methods for stable maintenance and expression of transferred DNA have greatly expanded the applications for transfection.

For those applications a high transfection rate is needed. This can be done by a variety of methods Electroporation, Heat shock nucleofection, magnetofection, lipofection and metafection among others [92].

During lipid mediated transfection, cationic lipids form vesicles with a bilayer lipid sheet, known as liposomes in aqueous solutions. When liposomes encounter nucleic acids they re-form into nucleic acid lipid complexes called lipoplexes which can be actively taken up by eukaryotic cells by means of endocytosis. In this case, the lipoplex enters into the cell cytosol via the endosomes.

Mechanism:

Transfection active lipoplexes spontaneously form from cationic lipids and negatively charged DNA. Lipoplexes are taken up by endocytosis. The DNA is released by destruction (osmotic effects and fusion) of the endosome membrane. The DNA enters the cell nucleus during mitosis (not in RNA Transfection) [93].

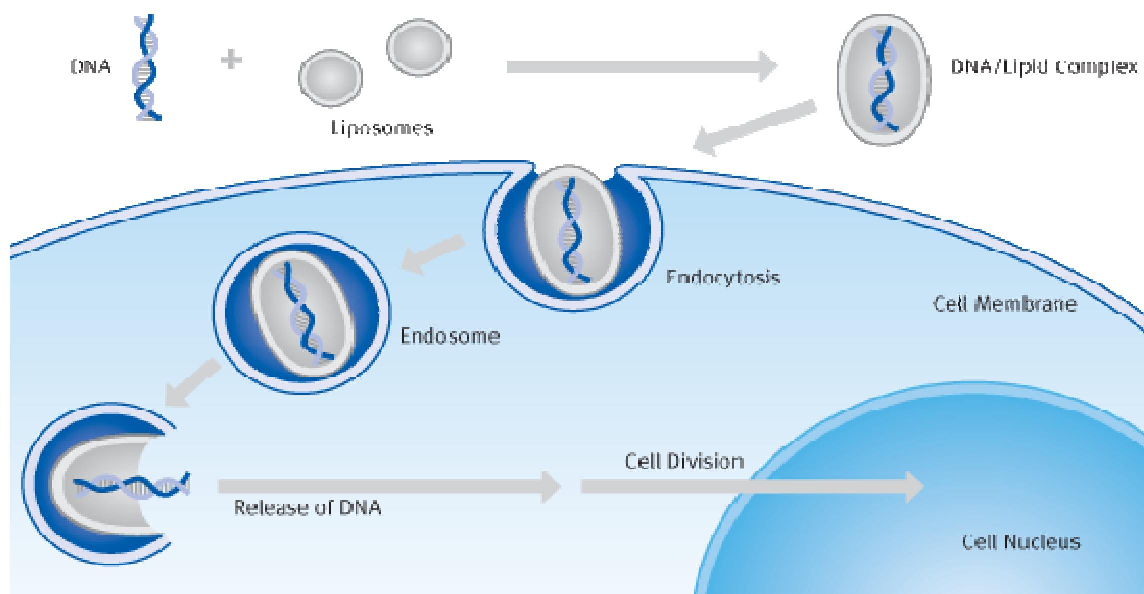


Figure 11: Mechanism of transfection. DNA is mixed with liposomes, resulting in DNA/lipid complex which then are endocytosed by the cell. Copyright © 2005 – 2011 Biontex Laboratories GmbH. All rights reserved.

3.2.2.5 Transfection by using Metacfectin

The day before the transfection 2 ml 100000cells/ml were seeded out in each well in a 6-well plate. At the time of the transfection, the cells were about 50-80% confluence. For each transfection, the DNA and the metafectin were prepared as follows:

15ml tubes were marked and added 750µl of optimen and metafectin (2.5 times amount of metafectin per µg DNA) Then the DNA was added, and. the tube were incubated for 20 minutes at room-temperature. Then the media was removed from the cell and added 500µl optimen followed by 250µl of the transfection mixture. The 6-well dishes were then put back to incubation. After 4hours the optimem was removed, and replaced by 2ml of DMEM containing either 10% or 0.3%FBS prior to mammalian two-hybrid studies or transactivation reporter studies, respectively.

3.2.2.6 Harvesting cells

Before further analysis, the cells need to be harvested. Cells were washed once with PBS and added to either 100µl LDS sample buffer or lysis buffer for western blot or luciferase measurement, respectively. The cells were detached from the 6 well dishes by use of a scraper, and the lysate was put in pre-cold 1.5 eppendorftube. Sample for western blots were incubated for 10 minutes at 4°C, and sonicated for 3-4 seconds, while the samples for luciferase measurement were centrifuged 10minutes at 4 ^{o}C. All the samples were stored at -20°C until used.

3.3 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

This is the most widely used method for qualitative analysis of proteins and monitoring of their purity. It is based on separating proteins according to size and estimates the molecular weights of proteins. SDS (Sodium Dodecyl Sulphate-polyacrylamide) is an anionic detergent that binds to and denatures proteins, which confer negative charge to the polypeptide chain. LDS (Lithium Dodecyl Sulphate) and the bromophenol blue and the DTT (Dithiothreitol) are added to the sample [94].

DTT reduces disulfide bridges that may be necessary for the tertiary structure of proteins. The dye bromophenol blue allows for monitoring of the migration of the proteins. Upon application of the samples, an electric field is applied. The negative charged molecules migrate towards the anode according to their sizes. Smaller molecules migrate faster than

bigger molecules. The gel is composed of a stacking and a separating gel made by polymerization of acrylamide monomers and the choice and concentration hence pore size of the gels depend on the protein to be analyzed [95].

Samples are treated with LDS and the reducing agent DTT and boiled for 10 minutes. The gel between the glass plates was then placed in a chamber with the electrophoresis running buffer. The samples are loaded along with the Magic Maker. The gel was run for 50minutes with 20 mA. The proteins can then be visualized in the gel by staining with Coomassie blue, western blotted as the case in this study.

3.3.1 Staining Protein Gels with Coomassie Blue

The Coomassie dyes (R-250) bind to proteins through ionic interactions between dye sulfonic acid groups and positive protein amine groups as well as through Van der Waalsn attractions. Coomassie R-250, is a sensitive stain for protein detection in PAGE gels. Coomassie staining gives blue bands on a clear background, with a sensitivity of 50 - 100ng/band [96].

The SDS-PAGE gel to be stained was covered with a fixation solution and was left for 1 hour at room temperature with careful shaking. The fixation solution was removed and the coomassie blue R-250 was added and kept for 30minutes at room temperature with careful shaking. The gel is then rinse with a fixation solution, afterward the destaining solution was added and left at room temperature on the shaker. The destaining solution was change regularly; in order to improve the destaining efficiency a small piece of paper was added. When the bands were visible a picture was taken and the result was analyzed.

3.3.2 Western Blot

Western blot analysis can detect specific protein in a mixture of any number of proteins. This method is dependent on the use of a high-quality antibody directed against a desired protein.

The proteins in cell lysate are separated using SDS-polyacrylamide gel electrophoresis (also known as SDS-PAGE). This separates the proteins by size. The gel is placed onto a nitrocellulose membrane and using electrophoresis, drives the protein bands onto the nitrocellulose membrane. Afterwards, the nylon membrane is incubated in a blocking buffer for 1 hour. This step is included to prevent unspecific binding between the membrane and the antibody. Thereafter, the membrane is added to a blocking buffer containing primary

antibody**.** The primary antibody, which is the specific antibody, sticks to the protein and forms an antibody-protein complex with the protein of interest. The membrane is rinsed and incubated with asecondary antibody. The secondary antibody is an antibody-enzyme conjugate against the primary antibody. The secondary antibody will stick to the primary antibody. The conjugated enzyme which is link to the secondary antibody will allow visualizing on a machine (LAS-3000) [97].

3.3.3 Clear page system

Proteins samples were prepared by taking 15ul of the lysate, 2ul 1MDTT and 5.5ul LDS Nu Page Loading buffer into an eppendorftube. The samples were incubated at 70°C for 10 minutes before the loading. In this study the ClearPage gel 10-20% with 12 and 17 wells were used. The gel was removed from the plastic and rinsed with water; the wells were rinse with the Running buffer. Afterward the gel was put in the XCell SureLockTM chamber. The inner and outer chamber was filled with SDS running buffer. 15ul of the sample was loaded per well and the gel was run for 50 minutes at 200V. The gel was then stained by commassie blue dye (section 3.3.1) or used for western blot (section 3.3.2)

3.3.4 Stripping

The membrane can be re-used in presence of other antibodies. The membrane need to be stripped in order to remove the previous attached antibodies. The membrane should then be put in 0.2M NaOH for 5 minutes at room temperature on the shaker. The membranes were washed 3x5 minutes with 25ml PBST. Finally, was blocked in 25ml blocking buffer for 1 hour on a shaker at room temperature and procedure completed as mentioned above (section 3.3.2).

3.4 Co- immunoprecipitation

Co-immunoprecipitation (Co-IP) is a technique for the analysis of protein interaction. The procedure includes: An antibody specific to the protein of interest is added to a cell lysate. The antibody-protein complex is then precipitated usually using protein-G or protein-A sepharose which binds most antibodies. If there are any protein/molecules that bind to the first protein, they will also be precipitated. Co-precipitated protein can then be identified by Western blot analysis or by sequencing a purified protein band [98].

In this study the *in vitro* and *in vivo* Co-immunoprecipitation were performed

3.4.1 *In vivo* Co-immunoprecipitation

One day before the co-immunoprecipitation, 200.000 cells were seeded out in two 10cm dishes. The next day the transfection was done with empty vector and/or expression plasmids encoding agnoprotein or LT-ag and the cells were incubated over night. The cells were then washed twice with warmed pre-PBS and harvested in 300µl lysisbuffer. The lysates were centrifuged at 13000rpm for 10 minutes at 4^oC in order to remove cellular debris. The supernatant contains the intracellular proteins. An input control was taken, that is 30µl of the supernatant was put into another eppendorftube and added 2µl 1M DTT and 8µl LDS loading buffer. The tube was incubated for 10 minutes at 70^oC and stored at -20^oC. To the rest of the supernatant an antibody not important for the experiment was added, and the tube was put on the rotator for 1 hour at 4^oC. Then 60µl of 50% sepharose G beads were added, the tubes were put back on the rotator for 1 hour at 4^oC. The beads were spinned down and the supernatant was transferred into a new eppendorftube. This preclearing step removes eventual proteins that unspecifically would bind to the beads or immunoglobulin. Then, the antibody against agnoprotein was added in the tubes and the tubes were put back on the rotator for 1 hour at 4^oC. Then the tubes were added 80µl of 50% sepharose G beads, before incubation at the rotator for 1 hour at 4^oC. The beads were washed 3x1ml lysis buffer and washed once more with 2x1ml 50mM Tris HCl pH=8. Afterward all the buffer was removed from the beads and 10µl LDS loading buffer+ 2µl 1MDTT+8µl H_20 were added. The proteins were released from the beads by incubation at 70^oC for 10 minutes. The samples were stored at -20^oC until further analysis on western blot.

3.4.2 *In vitro* Co-immunoprecipitation

Purified proteins are added alone or in combination with another protein in a buffer during an *in vitro* co-immunoprecipitation experiment. Two tubes were marked, and added 400 ul lysis buffer and purified LT-ag and/or agnoprotein. The tubes were incubated for 10minutes on the rotator at the room temperature. Then an imput control was taken as described in (section 3.4.1). 1100µl of 50% sepharose G beads were added to the rest of the supernatant, and the tubes were incubated on the rotator for 20 minutes at 4^oC. The beads were spined down and the supernatant was placed in a new tubes. Antibodies against either LT-ag or agnoprotein were then added to the tubes, before a new incubation for 1 hour on the rotator at 4^oC. Then

70µl of 50% sepharose G beads were added in the tubes before a final incubation on the rotator at 4°C for 1 hour. The beads were washed 3x1ml with the buffer and washed once more with 2x1ml 50mM Tris HCl pH=8. All the buffer was removed from the beads and 15µl LDS loading buffer+2µl MDTT+3µl H_20 were added prior to incubation at 70°C for 10 minutes. The samples were stored at -20°C until further analysis on western blot

3.5 Mammalian Two-Hybrid System

The mammalian two-hybrid system allows characterization of mammalian protein-protein interactions within a cellular environment that mimics native conditions. The mammalian two-hybrid system is similar to the yeast two-hybrid system in that both are based on the fact that eukaryotic transcription factors are comprised of two distinct physical and functional domains: a DNA binding domain (DBD) and an activation domain (AD). The DBD recognizes a specific DNA sequence, which could be a GAL element. The AD coordinates the assembly of the general transcription factors which are required when RNA polymerase II transcribe the specific reporter gene, like luciferase which are localized downstream of theDNA binding domain, GAL [102].The mammalian two-hybrid system relies on three plasmids that are co-transfected into mammalian cells. Each plasmid has unique features. One plasmid contains a strong transcriptional activation domain (TAD) upstream of coding sequences for the prey protein (for example VP16- LT-ag; where VP16 is the TAD and LT-ag is the prey). The second vector contains a DBD upstream of coding sequences of the bait protein (for example gal-agnoprotein, where gal is the DBD and agnoprotein is the bait. The third vector contains five DNA binding sites for GAL upstream of a minimal TATA box, which is upstream of a specific reporter gene like luciferase. Interaction between proteins like LT-ag and agnoprotein result in presence of both a DNA binding domain (gal) and transactivation domain (VP16) at the reporter- resulting in increased transcription of the firefly luciferase reporter gene [99]. The principle of mammalian two-hybrid assay is illustrated in figure 12.

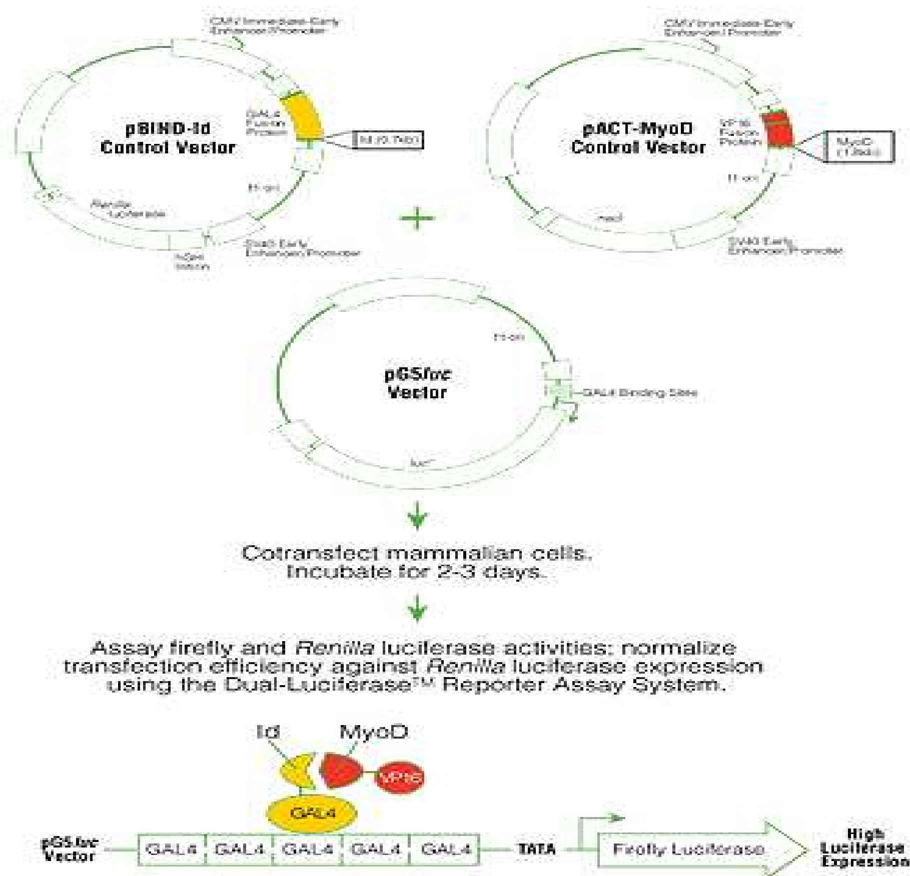


Figure 12: Schematic representation of a positive control reaction in the CheckMateTM Mammalian Two-Hybrid System for protein:protein interactions. For a positive control reaction, the pBIND-Id and the pACT-MyoD Control Vectors are cotransfected into mammalian cells along with the pG5luc Vector. The pG5luc Vector contains five GAL4 binding sites upstream of a minimal TATA box that is upstream of the firefly luciferase gene. The cells are incubated for 2-3 days, lysed and then assayed for firefly and Renilla luciferase using the Dual-LuciferaseTM Reporter Assay System (a), In positive control experiments, interaction between the two proteins, Id and MyoD, in the GAL4-Id and VP16-MyoD fusion proteins .results in high levels of luciferase expression from the pG5luc Vector [100].

Genetic reporter systems are widely used to study eukaryotic gene expression and cellular physiology. Applications include the study of receptor activity, transcription factors, intracellular signaling, mRNA processing and protein folding. Dual reporters are commonly used to improve experimental accuracy. Luciferase is a generic term for the class of oxidative enzymes used in bioluminescence and is distinct from a photoprotein. One famous example is the firefly luciferase in luminescent reactions, light is produced by the oxidation of a luciferin (a pigment) [101].

The reaction catalyzed by firefly luciferase takes place in two steps:

- luciferin + ATP → luciferyl adenylate + PP_i
- luciferyl adenylate + O_2 → oxyluciferin + AMP + light

3.6 Gluthatione-S-Transferase purification

Glutathione-S-Transferase (GST) fusion protein is a 26kDa protein and the GST fusion coding sequence is cloned into an Isopropyl-ß-D-Thiogalactosidase (IPTG) inducible expression vector. This fusion protein is exprexed in bacteria with a protease deficient strain and purified by affinity chromathography on glutathione agarose beads. The target proteins are lysates of cells. The cells lysates and the GST fusion protein are incubated together with glutathione-agarose beads. Complexes recovered from the beads are resolved by SDS-PAGE and analyzed by western blotting [102].

An *E.coli* colony containing the transformed plasmid was inoculated into 10 ml LB media with 100µg/ml ampicillin and the culture was grown at 37°C overnight at with shaking of 250 rpm. The next day, 10 ml of the overnight culture was used to inoculate 100 ml NZCYM with Amp. The culture were allowed to grow at 37°C until OD_{600}= 0.6. The expression of the GST-agno-protein was then induced by addition of IPTG to a final concentration of 1.0mM followed by continues incubation for 2-3 hours at 37°C. The cells were poured into 50 ml centrifuge tubes and pelleted at 4000 rpm for 20 minutes. The supernatant was discarded. The cell pellet could be stored at -20°C for later use or used immediately. The cell pellet was re-suspended in 2ml PBST containing 200µl protease inhibitor cocktail. In order to lyse the cells, the suspension was sonicated 3x10 seconds on ice. The cellular debris was spined down at 13000g during 10 at 4°C, while the supernatant was transferred to a new eppendorf tube and 100µl 50% gluthatione beads were added. The tube was placed on the rotator for 30-60minutes at 4°C. During this period, the GST-agnoprotein would bind to the GST-beads. The beads were washed twice with PBST and once with PBS. The GST-agnoprotein was eluted from the beads by addition of 2x250µl 5mM gluthatione in 50mMTris pH=8.0. The gluthation would compete for binding to the GST-beads, thereby releasing GST-agnoprotein. All protein fractions and the remaining beads could be stored at -20°C until use. For evaluations of the results, the samples were prepared as follow: A 15µl aliquote of eluate was added 2µl 1MDTT and 5.5µl LDS-buffer, while the beads were added 3µlH_20+ 2µl 1MDTT+5.5µl LDS. The samples were then incubated for 10minutes at -70°C. All samples including beads

were run on SDS-PAGE gel to check for presence of GST or the fusion protein GST-agnoprotein. The principle behind GST purification is illustrated in figure 13

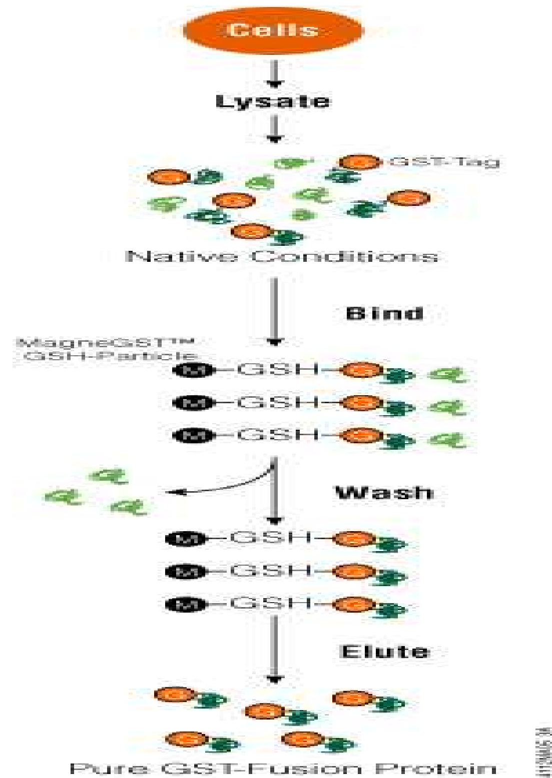


Figure 13: Schematic diagram of the MagneGST™ Protein Purification System. Bacterials cells containing a GST-fusion protein are lysed using the provided MagneGST™ cell Lysis Reagent or with an alternative lysis method and the MagneGST™ particles are added directly to the crude lysate. GST-fusion proteins bind to the MagneGST™ particles. Unbound proteins are washed away and the GST-fusion target protein is recovered by elution with 50mM gluthione (Adapted from Promega guide protocol).

3.7 Gst pulldown and detection

The pull-down assay is an in vitro method used to determine a physical interaction between two or more proteins. Pull-down assays are useful for both confirming the existence of a protein-protein interaction predicted by other techniques like co-immunoprecipitation and as an initial screening assay for identifying previously new protein-protein interactions. The assay requires the availability of an affinity support, immobilized tagged protein (fusion protein) which is used to capture or pull-down a protein binding partner that may be a recombinant or *in vitro* translated protein. The entire complex can be eluted from the affinity

support by the use of SDS-PAGE loading buffer. Protein complexes in the eluted can be visualized by SDS-PAGE gel and western blot detection with antibodies [103].

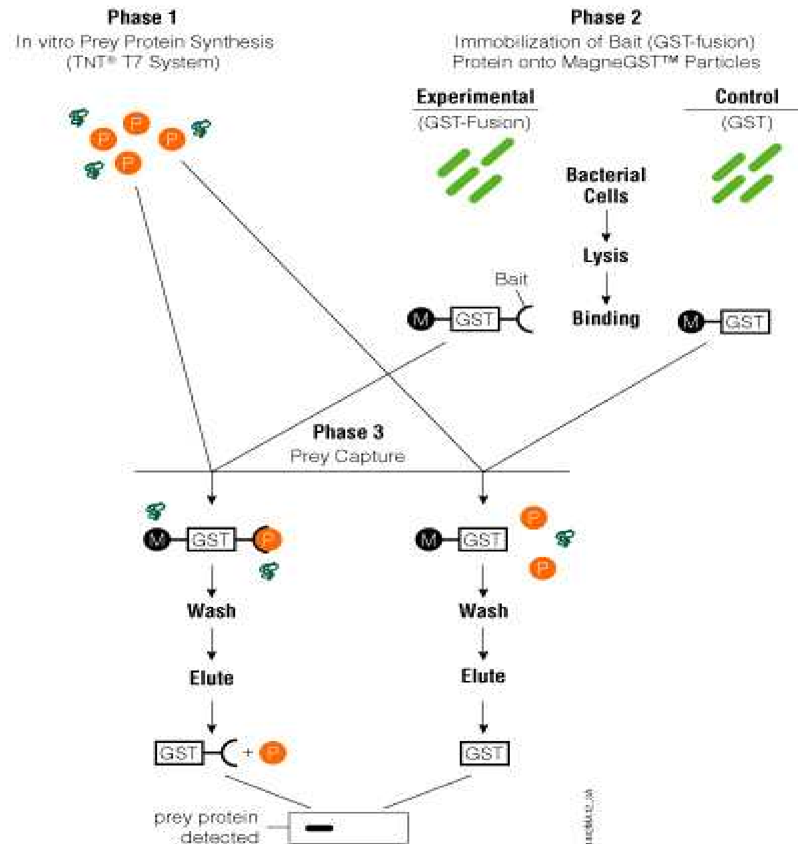


Figure 14: Schematic diagram of the MagneGST™ Pull-Down System protocol. The prey protein is expressed in transcription /translation reaction , the E:coli expressed GST-fusion (bait) protein is immobilized onto the MagneGST™ particles and the prey protein is added to MagneGST™ particles carrying bait and captured through bait prey. Nonspesifically bound proteins are then washed away and the prey protein is analyzed. Phases 1 and 2 are performed simultaneously (Adapted from Promega guide protocol).

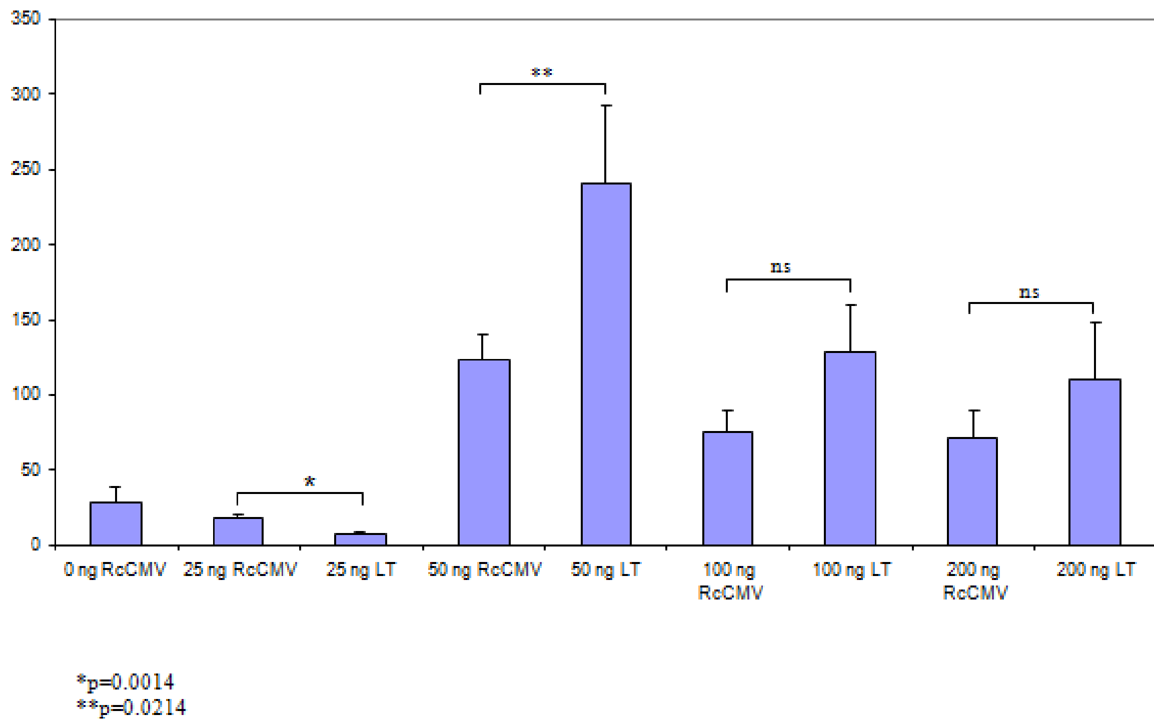
The mammalian cells were seeded in 12wells of 6wells dishes and transfected with RcCMV-LT-ag. The cells were washed once with a warm PBS. One well was a control of transfection efficiency of LT-ag, and was added 80ul lysis buffer, scraped and transferred into an eppendorf tube. The rest of the wells were added 180ul PBST, scraped and transferred in the cold eppendorf tube. The tube was kept for 30minutes on the rotator in a cold room. And there after the cell debris was pelleted by centrifugation for 10 minutes at 13000rpm at 4°C. 20ul of the lysate was taken as an input control and 5.5ul LDS buffer+2ul 1M DTT added, followed by incubation at 70°C for 10 minutes prior to storage at -20°C until use. The rest of the lysate were transferred into 15ml tube and 100ul of 50%GST beads+50ug GST added followed by incubation for 2 hours on the rotator in the cold room. In order to pellet down the beads the

lysate was spined 2minutes at 1000g.This step is a pre-clearing step to remove eventual proteins that unspecifically binds to either GST or the beads. The preclearing step was repeated once. The supernatant was then divided into two eppendorf tubes and added 50ul of 50% GST beads. Then 10ug GST was added in one tube and 10ug GST-agno in the other tube. Both tubes were incubated for 2 hours on the rotator in the cold room. The GST bead was spined down and the supernatant was removed. The beads were carefully washed 3 times with washing buffer. 20ul sample buffer were added in the tubes, all the samples were incubated 10 minutes at 70°C. The samples could be stored at -20C and the samples were then analyzed on western blot.

4 RESULTS

4.1 Effect of Large T-antigen on transcriptional activity of BKV early promoter

Previous studies with JC virus have shown that agnoprotein influences LT-ag mediated transcription of the viral genes and replication of viral DNA [62]. The same group has also found that JCV agnoprotein and LT-ag physically interact with each other [62]. Our research group has also preliminary results which suggest an interaction between BKV agnoprotein and BKV LT-ag (result not shown). First we wanted to evaluate the effect of LT-ag on the BKV early promoter in HEK 293 cells. Therefore we performed co-transfection experiments in HEK 293 cells by using constant amount of luciferase reporter construct (250 ng pGL-TCR-LE-LUC) together with various amounts of expression plasmids or empty vector. As seen in the Figure 15, LT-ag transrepresses and transactivates the early promoter, depending on the amount of expression plasmids used in the transfection. 25ng of LT-ag expression plasmid resulted in transrepression of the early promoter (compare lane 2 and 3), while 50ng of LT-ag expression plasmid resulted in a 2 fold increased in the transcriptional activity (compare lane 4 and 5). When increasing the amounts of a plasmid expressing LT-ag (100-200ng), we did not observe any significant effect on the early transcription (lane 6-9). These results suggest that LT-ag mediated effect on luciferase reporter containing BKV early promoter depends on the concentration of the expressing plasmids.



*p=0.0014
**p=0.0214

Figure 15: Effect of Large T-antigen on transcriptional activity of BKV early promoter. A reporter plasmid (pGL-TCR-LE-LUC) (0.25µg) was introduced in HEK 293 in combination with indicated amounts of plasmids expressing LT-ag or the empty vector pRcCMV. At 24h posttransfection, the cells were harvested and luciferase enzyme activity was determined. Luciferase activity is expressed as relative luciferase units. The results represent the average of three independent parallels. The presented figure is a representative of 10 experiments.

Ns= is not significant.

* = significant different

4.2 Effect of BK Virus agnoprotein on Large T-antigen mediated transcription

Having confirmed that LT-ag influences BKV early promoter activity, we now wanted to address whether agnoprotein could interfere with this. Therefore we performed co-transfection experiment in HEK293 cells by using a reporter construct (pGL-TCR-LE-LUC) in combination with various amounts of expression plasmids for LT-ag and/or agnoprotein and/or empty vector. The amount of DNA in each transfection was kept constant by addition of CT-DNA. Cotransfection with a constant amount of reporter (250ng) and 100ng of LT-ag expression plasmid stimulates BKV early promoter activity sevenfold (Figure 16, compare

lane 2 and 3). The presence of agnoprotein (100ng) reduced the LT-ag-mediated transactivation about two and half fold (compare lane 3 and 4). Similarly, cotransfection with 50ng of expression plasmid encoding LT-ag stimulates BKV early promoter almost two fold compare (lane 6 and 7), and we observe a suppressive effect of agnoprotein on the LT-ag mediated transactivation of the early promoter (compare lane 7 and 8).

The observed effect of agnoprotein on LT-ag mediated transactivation of the BKV early promoter suggests that agnoprotein may exert a negative regulatory effect on transactivation by LT-ag.

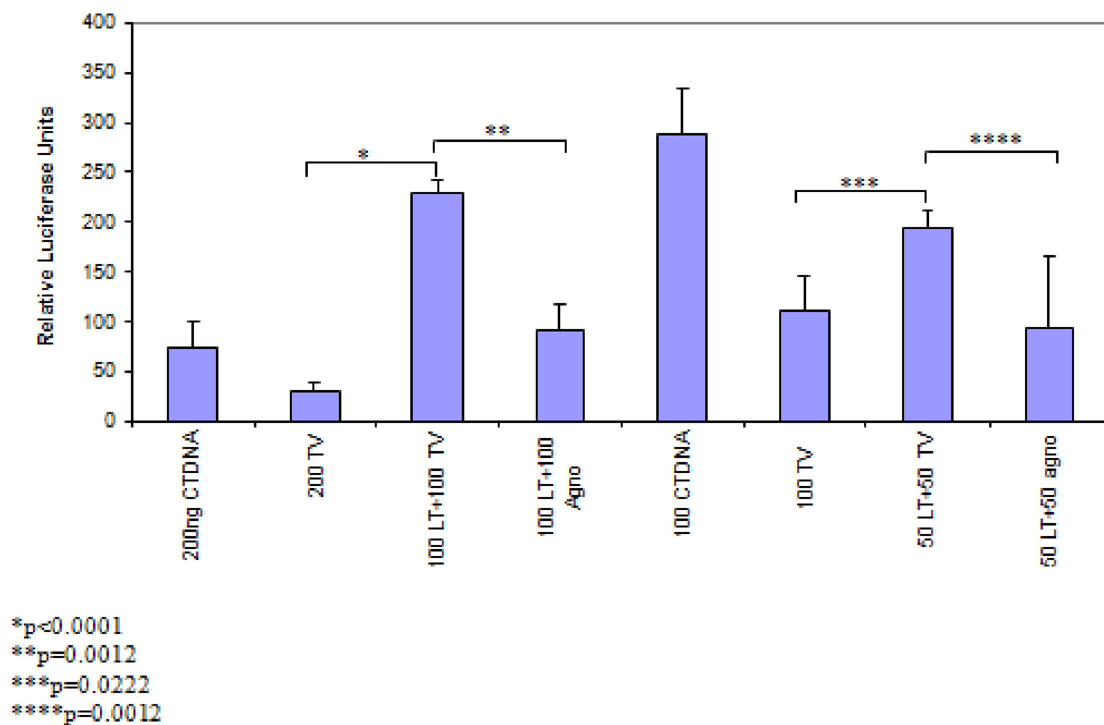


Figure 16: Effect of agno protein on Large T-antigen mediated transcription of BKV early promoter. A reporter plasmid (pGL-TCR-LE-LUC) (0.25µg) was introduced in HEK 293 in combination with plasmids expressing agnoprotein, and/or LT-ag and/or the empty vector pRcCMV. At 24h posttransfection, the cells were harvested and the luciferase enzyme activity was determined. Luciferase activity is expressed as relative luciferase units. The results represent the average of three independent parallels. The presented figure is a representative of 6 experiments.

* = significant different

4.3 Interaction between agnoprotein and LT-ag

The result from agnoprotein on LT-ag mediated transcription studies suggested that BKV LT-ag may physically interact with BKV agnoprotein. An eventual interaction between the viral proteins was analyzed using several methods, including GST pull-downs, co-immunoprecipitation in vivo and in vitro and mammalian two hybrid system as described in section (3.4.1, 3.4.2, 3.5 and 3.7).

4.3.1 Purification of GST and GST-agno

Before performing the GST pull-down, we needed to purify the GST protein and GST-agno-protein. First the plasmid encoding GST-fusion protein was transformed into a protease deficient strain BL21, grown in appropriate media and the expression of fusion protein was induced by IPTG. The GST proteins were then purified as described in method section (3.6). The purification resulted in two elutions; elution 1 and 2, and an extra tube containing the beads with leftovers of protein after the purification. Aliquots of the purified proteins and the leftover from beads were run on SDS-PAGE and stained by coomassie blue. As seen in Figure 17, the GST was successfully purified (elution 1 and 2, lane 5-6), however, even more proteins were left on the beads (lane 3). GST-agnoprotein was found on the beads (lane 8), however the elution fractions 1 and 2 reveal that GST-agno was not successful purified (lane10-11). Purified GST-agnoprotein for further studies was obtained from colleagues in the group. The protein concentration of purified proteins was measured (appendix).

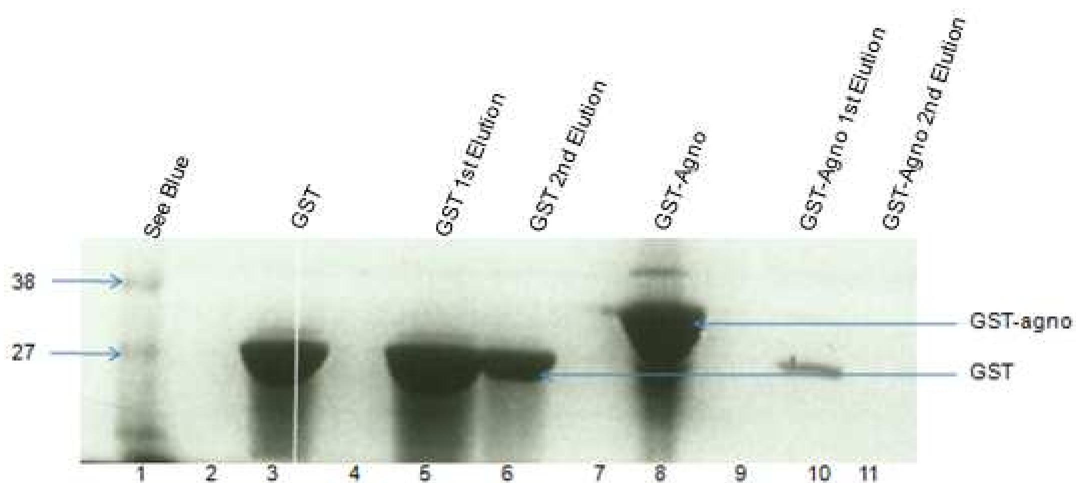


Figure 17: GST-agno and GST purification. Purified GST proteins were separated on SDS PAGE, followed by coomassie blue staining. Lane 1: see blue marker, lane 2: empty well, lane3: GST beads, lane 4: empty well, lane 5: GST 1st elution, lane 6: GST 2nd elution, lane 7: empty well, lane 8: GST-agno, lane 9: empty well, lane 10: GST-agno 1st elution, lane 11: GST agno 2 nd elution.

4.3.2 Interaction between agnoprotein and LT-ag by GST pull down

HEK 293 cells were transfected with expression plasmid encoding LT-ag, and one day post transfection cell lysates were harvested. GST and GST-agnoprotein were added to cell lysates, and GST-pulldown was performed. GST, GST-agnoprotein, as well as bound protein complexes from cell lysates were resolved on SDS-PAGE and analyzed by Western blotting for the presence of specific antibody against LT-ag. As shown in (Figure 18) LT-ag was retained on the gluthatione beads containing GST- agno (lane 10). However, LT-ag could also be found on the GST-beads, which may also suggest non-specific interaction to GST (lane 8).

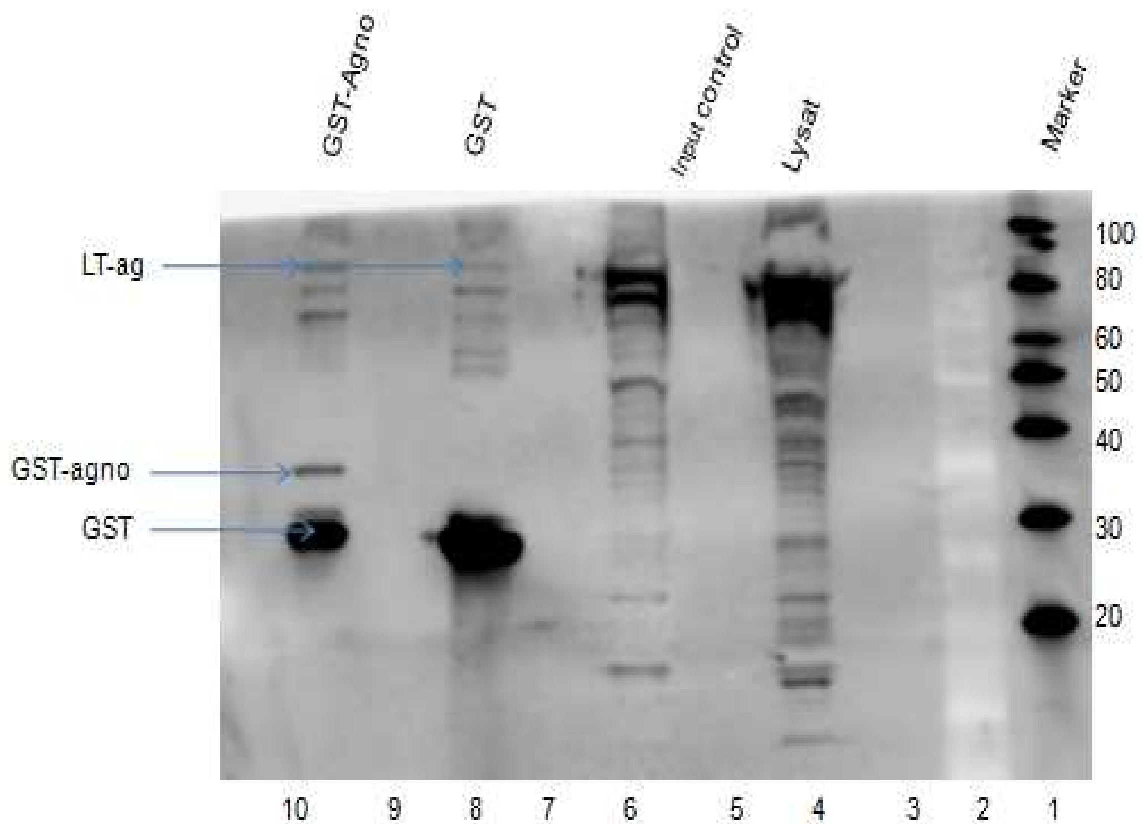


Figure 18: GST pulldown may suggest an interaction between GST-agno and LT-ag**.** HEK 293 cells were transfected with expression plasmid encoding LT-ag, and lysed one day post transfection. GST and GST-agnoprotein were added to the cell lysates, and thereafter immobilized on gluthatione-S-Transferase beads. Bound complexes were washed intensively and resolved on SDS-PAGE and analyzed by Western blotting for the presence of LT-ag. Lane 1: Magic Marker, lane 2: See blue, lane 3:empty well, lane 4: lysat, lane 5: empty well, lane 6: input control, lane 7: empty well, lane 8: GST, lane 9: empty well, lane 10: GST-agno+ LT-ag. The figure is a representative of 3 experiments.

4.4 Interaction between agnoprotein and Large T-antigen by co-immunoprecipitation

To further examine the association of agnoprotein with LT-ag *in vivo* and *in vitro* co-immunoprecipitations were performed.

4.4.1 *In vivo* co-immunoprecipitation between agnoprotein and Large T-antigen

HEK 293 cells were cotransfected with 8 µg expression plasmids encoding agnoprotein, and LT-ag or the empty vector pRcCMV. One day post transfection, agnoprotein was immunoprecipitated with antibody against agnoprotein. The antibody containing protein complexes were immobilized on sepharose beads. Bound immunocomplexes were washed intensively and resolved on SDS-PAGE and analyzed by Western blotting for the presence of agnoprotein and LT-ag using the appropriate antibodies. As seen in Figure 19 agnoprotein is efficiently immunoprecipitated by the agnoprotein antibody (compare input control lane 4 and 8 with immunoprecipitated proteins in lane 6 and 10). Unfortunately, it is not possible to evaluate the presence of LT-ag and we cannot tell whether LT-ag has co-immunoprecipitated with agnoprotein. The lanes 3, 5, 7 and 9 were supposed to be empty, but unfortunately a smear is seen in them due to poor loading.

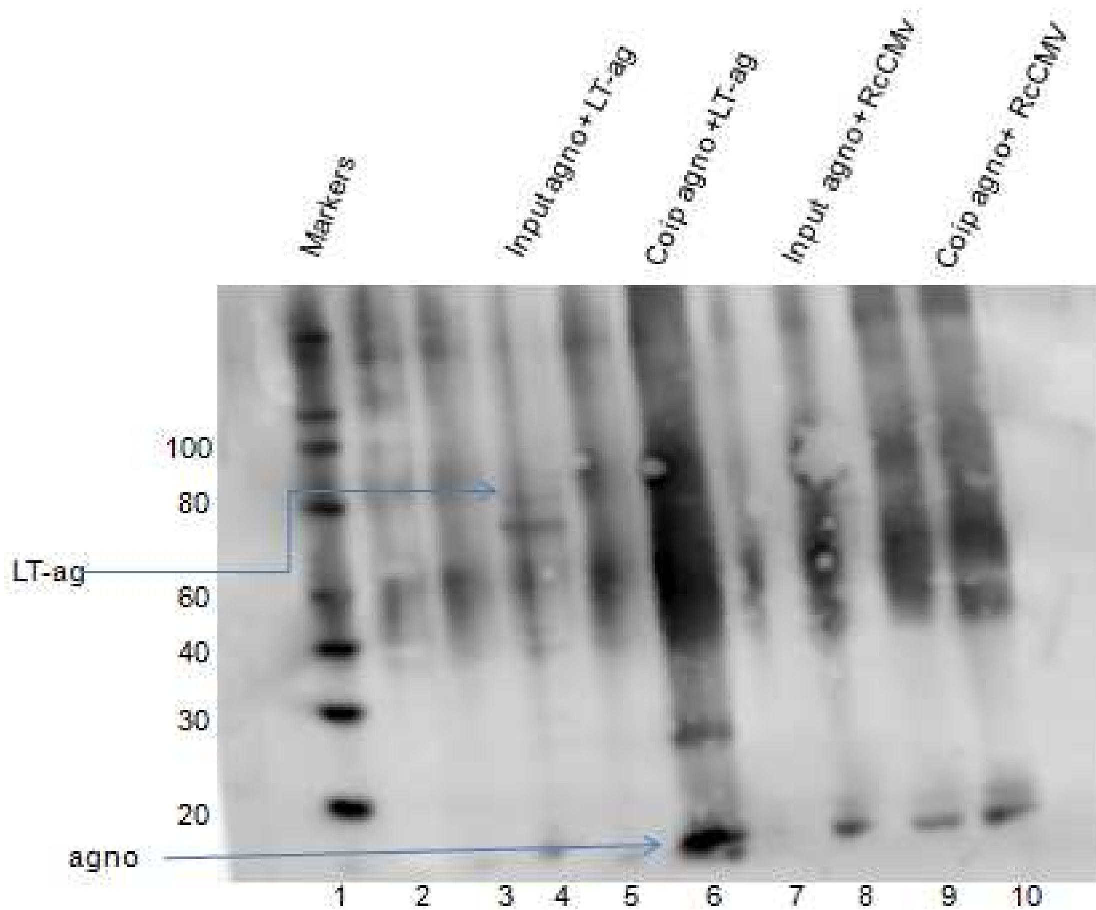


Figure 19: Immunoprecipitation by use of antibodies against BKV agnoprotein. HEK293 cells were transfected with expression plasmids encoding BKV agnoprotein and LT-ag or the empty vector pRcCMV. Agnoprotein was precipitated by use of antibodies specific for the viral protein, and immunoblot were performed using antibodies against LT-ag and agnoprtoein. Lane 1: Magic Marker. lane 2: emtpy well, lane 3: empty well, lane 4: 5% of cell lysate (input control) of cells transfected with expression plasmids encoding BKV agnoprotein and LT-ag, lane 5: Empty well, lane 6: immunoprecipitated BKV agnoprotein + LT-ag, lane 7: empty well, lane 8: 5% cell lysate (input control) of cells transfected with expression plasmid encoding BKV agnoprotein and pRcCMV, lane 9: empty well, lane 10: immunoprecipitated agnoprotein + pRcCMV.

We then did the reciprocal experiment and transfected HEK293 cells with expression plasmids encoding LT-ag and agnoprotein or empty vector. LT-ag is hardly seen in the input controls (lane 4 and 8, Figure 20), but clearly co-immunoprecipitated agnoprotein (compare lane 4 and 6). Unfortunately a band of size of agnoprotein is found in lane 10, which may be due to unspecific binding of the antibody or mistakes during preparation of the transfection mixtures.

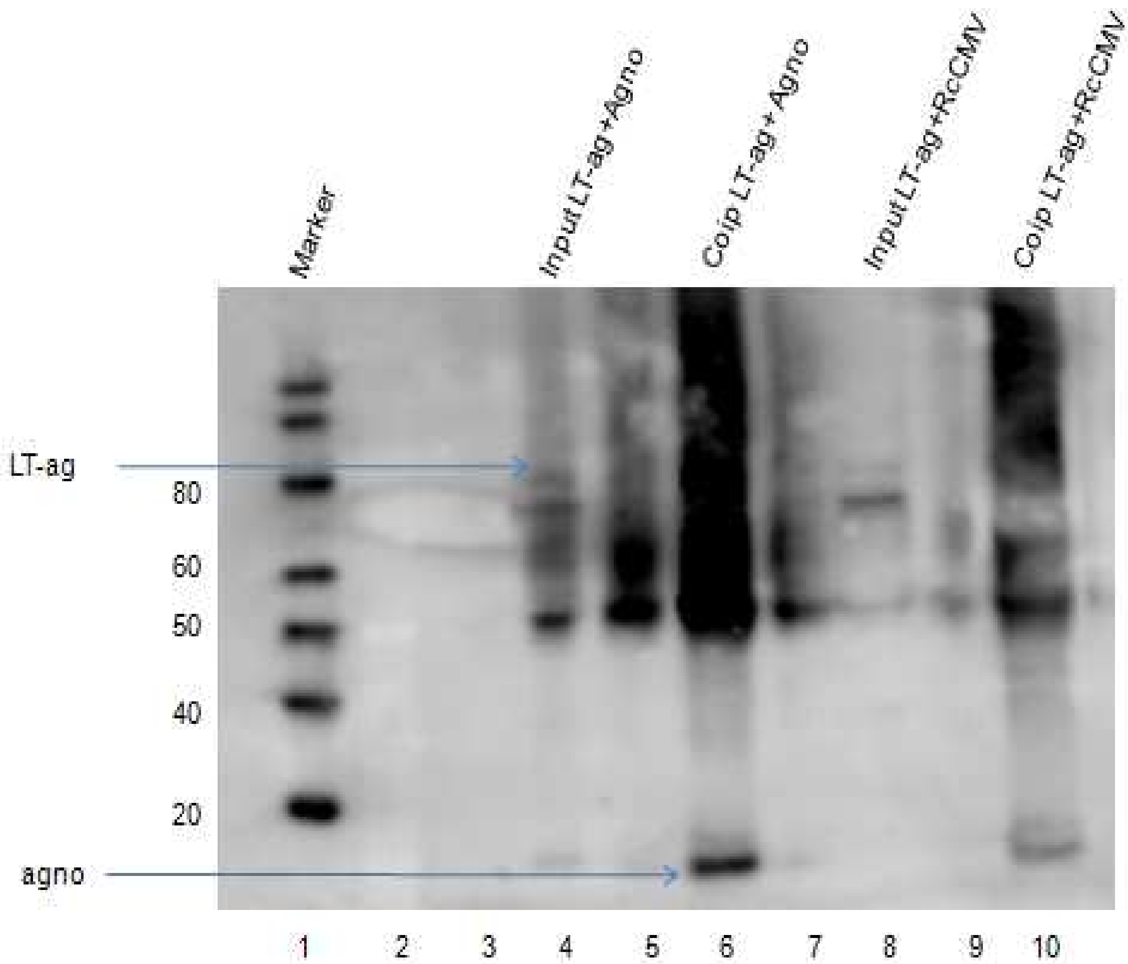


Figure 20: BKV LT-ag co-immunoprecipitates with BKV agnoproteins from HEK cell lysates. HEK 293 cells were transfected with expression plasmids encoding BKV LT-ag and BKV agnoprotein or empty vector pRcCMV. Antibodies against LT-ag were used to immunoprecipitate LT-ag. The immunocomplexes were separated and evaluated by immunoblot using antibodies against LT-ag and agnoprotein. Lane 1: Magic Marker, lane 2: empty well, lane 3: empty well, lane 4: 5% of cell lysate (input control) of cells transfected with expression plasmids encoding BKV LT-ag and agnoprotein, lane 5: Empty well, lane 6: immunoprecitated LT-ag+agno, lane 7: empty well, lane 8: 5% cell lysate (input control) of cells transfected with expression plasmid encoding BKV LT-ag+ pRcCMV, lane 9: empty well, lane 10: immunoprecipitated LT-ag+pRcCMV.

4.4.2 *In vitro* co-immunoprecipitation

Next, we wanted to evaluate whether the interaction between BKV agnoprotein and LT-ag is direct. Thereto, we decided to do *in vitro* co-immunopreciptitation between purified proteins.

In order to perform the *in vitro* co-immunoprecipitation, three different batches of purified LT-ag prepared by members from the research group were evaluated by western blot in order

to evaluate the purity of LT-ag. As shown in the Figure (21), LT-ag is detectable in all purifications and the quality of all batches of LT-ag seems similar.

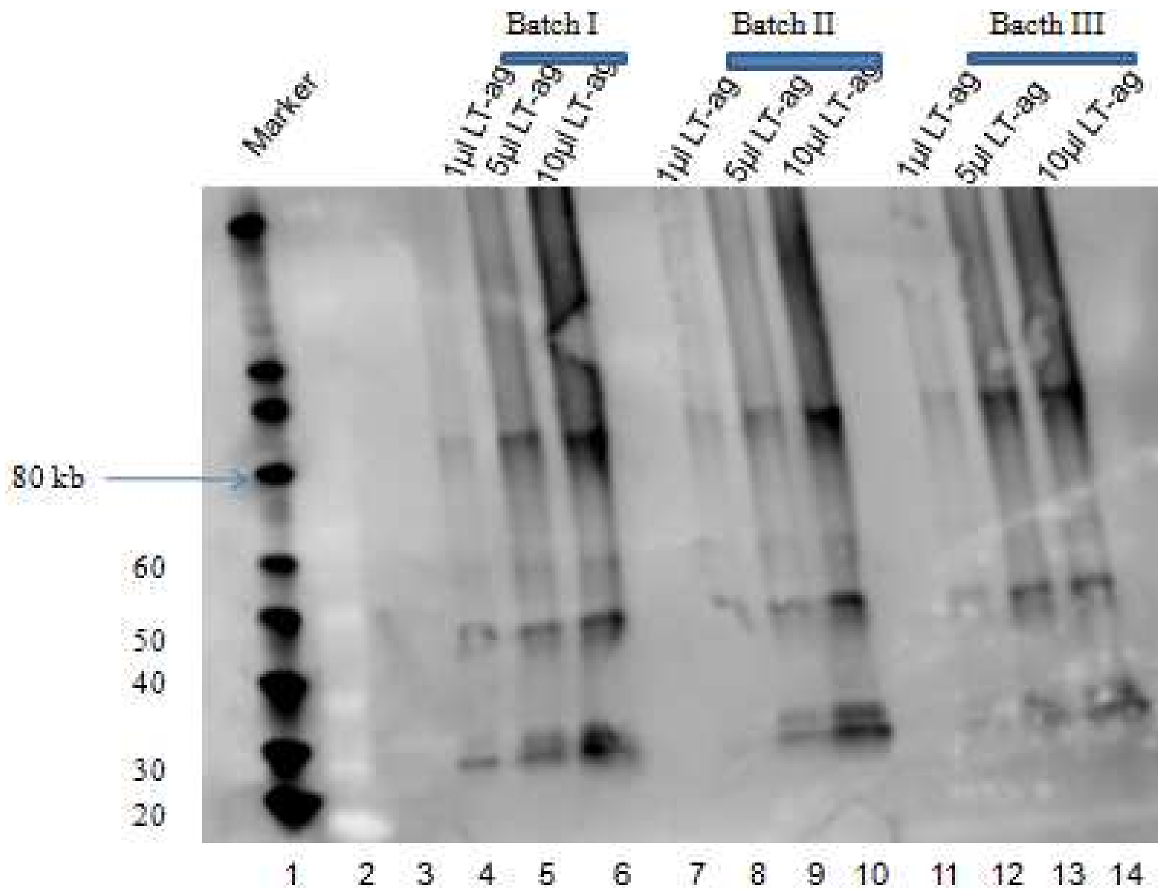


Figure 21: Different batches of purified LT-antigen protein. Lane1: Magic marker, lanes 4-6: LT-ag batch I, lanes 8-10: LT-ag batch II, lanes 12-14: LT-ag batch III.

In vitro co-immunoprecipitation experiments using purified BKV agnoprotein (from the group) and LT-ag were then performed. Thereto, both proteins were incubated in an eppendorf tube for 30minutes and then antibodies against agnoprotein were used to immunoprecipitate agnoprotein. The presence of (co-) immunoprecipitated proteins was evaluated by immunoblot using antibodies against LT-ag and agnoprotein. As demonstrated in Figure 22, agnoprotein is present in the input controls (lane 3 and 4) and agnoprotein is efficiently immunoprecipitated (lane 6 and 8). LT-ag is seen as a weak band in the input control (lane 4), and a weak band of approximately 80 kD is found in co-immunoprecipitated together with agnoprotein as well (lane 8)

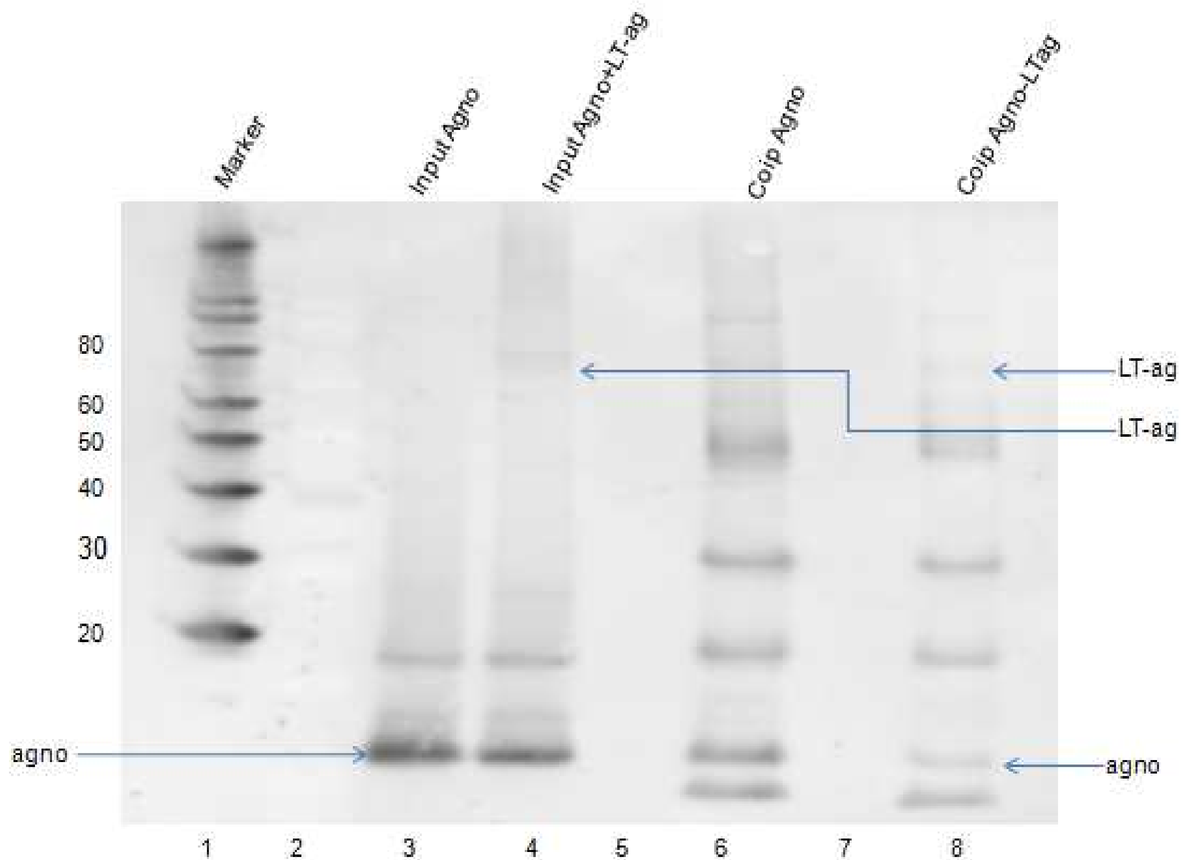


Figure 22: *In vitro* co-immunoprecipitation by use of antibodies against BKV agnoprotein**.** Purified agnoprotein alone or together with LT-ag protein were added into immunoprecipitation buffer. Agnoprotein was immunoprecipitated using antibody against agnoprotein. The precipitated complexes were resolved on SDS-PAGE and analyzed by Western blotting using antibodies agnoprotein and against LT-ag. Lane 1: Magic Marker, lane 3: input control agnoprotein, lane 4: input agnoprotein plus LT-ag, lane 6: co-immunoprecipitated agnoprotein, lane 8: coimmunoprecipitated agnoprotein plus LT-ag. Lanes 2, 5 and 7 are empty wells.

We also did the reciprocal experiment by using purified BKV LT-ag and agnoprotein. LT-ag in presence or absence of agnoprotein were added to immunoprecipitation buffer and incubated for 30 minutes. Then antibodies against LT-ag were added to immunoprecitate LT-ag from the buffer. The presences of immunoprecipitated and co-immunoprecipitated proteins were evaluated by immunoblot using antibodies against LT-ag and agnoprotein. As shown in the Figure 23, LT-ag is hardly seen in the input controls (lane 3 and 4), while it might slightly be seen in the immunoprecipitate (lane 6 and 8). However agnoprotein was efficiently co-immunoprecipitated by LT-ag (lane 8).

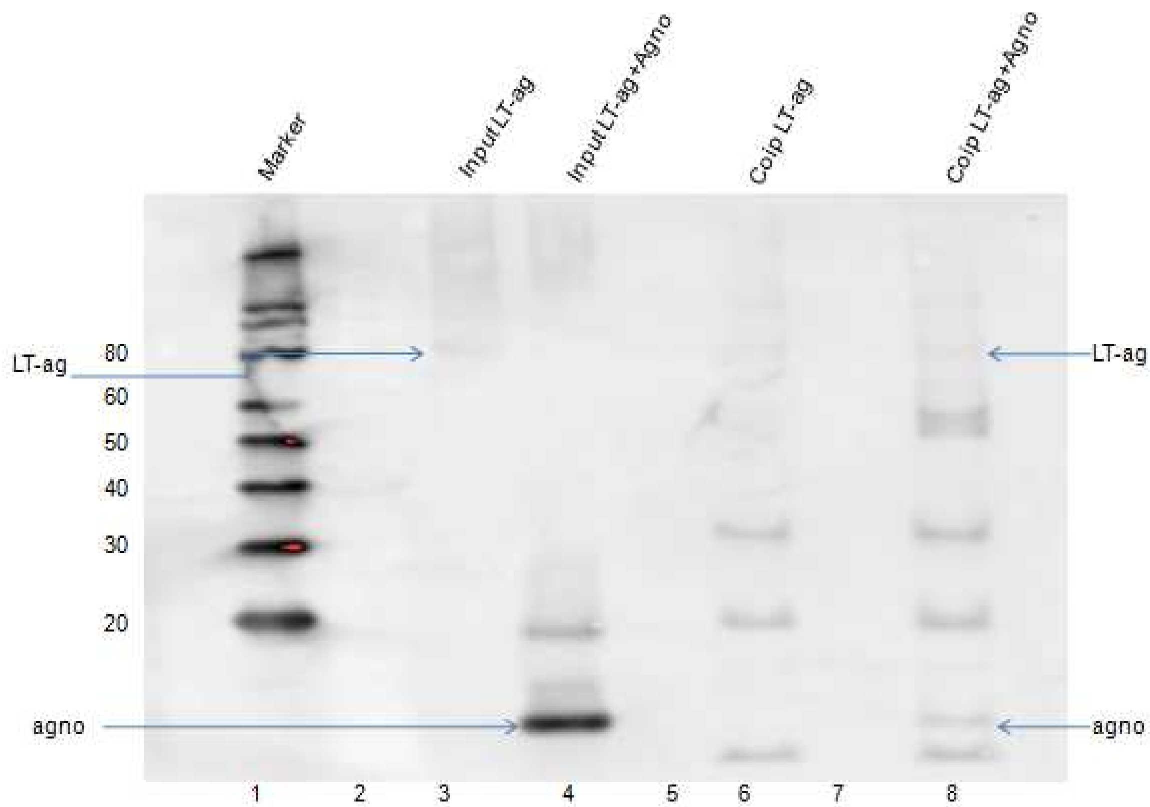


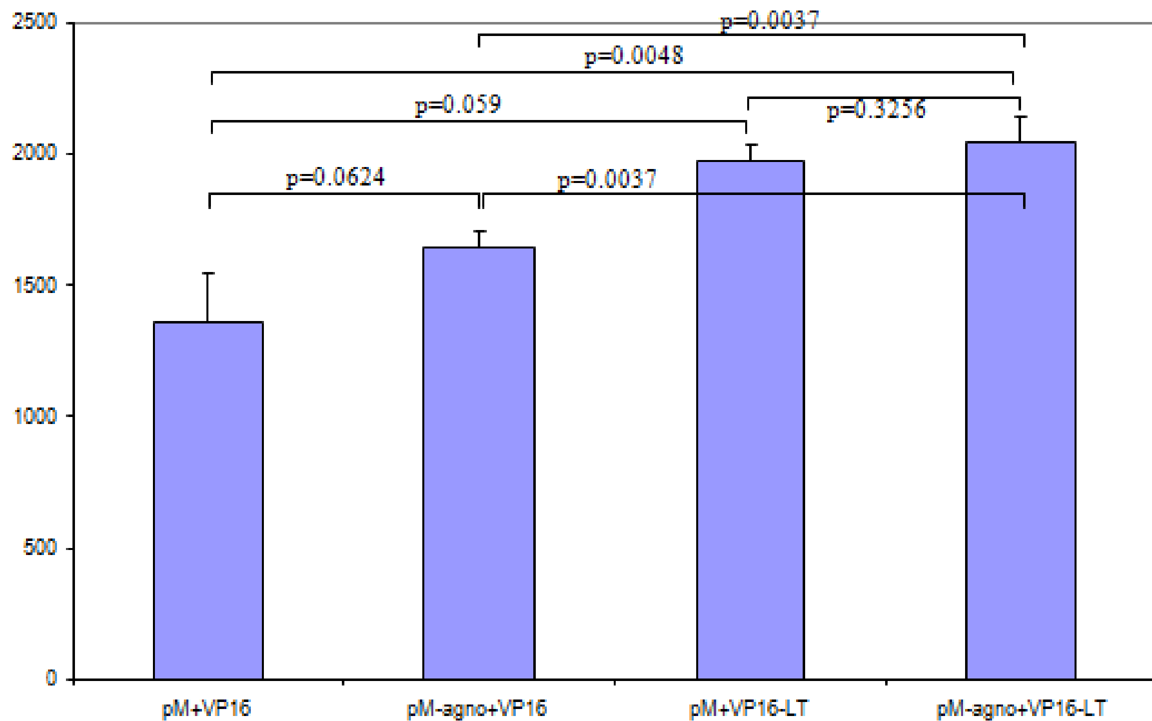
Figure 23: *In vitro* co-immunoprecipitation by use of antibodies against BKVLT-ag**.** Purified proteins of LT-ag alone or together with agnoprotein was added into immunoprecipitation buffer. LT-ag was immunoprecipitated using antibody against LT-ag. The precipitated complexes were resolved on SDS-PAGE and analyzed by Western blotting using antibodies agnoprotein and against LT-ag. Lane 1: Magic Marker, lane 3: input control LT-ag, lane 4: input LT-ag plus agnoprotein, lane 6: co-immunoprecipitated LT-ag, lane 8: coimmunoprecipitated LT-ag plus agnoprotein.

4.5 Mammalian Two Hybrid

Finally, we decided to evaluate whether LT-ag and agnoprotein could interact by use of mammalian two hybrid system.

We performed co-transfection experiment in HEK293 cells by using a reporter construct (p(GAL4)$_5$-E1b-LUC) with various amounts of expression plasmids for VP16-LT-ag, pM-agno, and empty vector. As illustrated in Figure 24, agnoprotein alone has no effect on the promoter activity of the reporter plasmid (compare pM+VP16 (lane 1) with pM-Agno+ VP16 (lane 2); difference is not significant with P=0.0624). LT-ag alone has also no effect on the promoter (compare pM+VP16 (lane1 with pM+VP16-LT-ag (lane 4); with p=0.059, which is not significant). However, co-expression of agnoprotein plus LT-ag has a significant effect (P=0.0048) (compare pM+VP16 (lane 1) with pM-Agno + VP16-LT-ag (lane 4)). This may

suggest that agnoprotein and LT-ag interact. Whether this interaction is direct or mediated by another protein cannot be determined with this assay.



P values >0.05 are not significant!

Figure 24: Mammalian two hybrid. A reporter plasmid (p(Gal4)$_5$-E1b-LUC) (0.5µg) was introduced in HEK 293 in combination with plasmids expressing the GAL4 DNA-binding domain (pM), the transactivation domain of VP16 (VP16) or fusion proteins expressing VP16-LT-ag or GAL4-agno (pM- Agno). At 24h post-transfection luciferase enzyme activity was determined. The luciferase activity is expressed as relative luciferase units. Each value is the average of three independent parallels. The figure represents the results of one experiment and similar results were obtained in 3 other independent experiments.

5. DISCUSSION

The ubiquitous human polyomavirus BK (BKV) efficiently infects man all over the world. The infection occurs in 70% of the human population during childhood and remains in the latent state throughout life [104]. Recent studies showed that mutant JCV and BKV lacking agnoprotein expression have a different growth cycle compared to the wild-type virus, suggesting the importance of agnoprotein in the viral lytic cycle [108]. The exact functions of BKV agnoprotein remain partially unknown. To elucidate the role of agnoprotein several studies have been done. Studies of the function of JCV agnoprotein show that agnoprotein suppresses both LT-ag mediated transcription of the viral late gene promoter and LT-ag induced replication of viral DNA also co-immunoprecipitation assays demonstrated that agnoprotein and LT-ag physically interact with each other [62].

In this thesis, we found that BKV LT-ag interferes with early viral transcription in a concentration-dependent manner. BKV agnoprotein interferes with LT-ag regulated early transcription, and the two proteins were found to physically interact with each other.

Previous studies with SV40 revealed that LT-ag regulates early and late transcription depending on the concentration of the viral protein [87]. Our result on early transcription is in agreement with this, as the effect of LT-ag on transcriptional activity of BKV early promoter depended on the concentration of the expressing plasmids (Figure 4.1).

In the presence of agnoprotein, a substantial decrease in LT-ag induced viral DNA replication was observed (Figure 16). The observed effect of agnoprotein on LT-ag mediated transactivation of the BKV early promoter suggests that agnoprotein may exert a negative regulatory effect on transactivation by LT-ag. LT-ag is expressed early in viral life cycle, and controls early and late transcription in addition to viral replication. The expression of agnoprotein occurs after 24-33 hours in Vero cells [64]. (results not shown), and at this time point it may be that there is a reduced need of expression of early proteins perhaps due to increased expression of the late genes. At later stages in the life cycle, DNA replication ceases and viral genomes are assembled with the capsid protein into virions. So there is no need for LT-ag at this stage. Agnoprotein may therefore form a negative feedback loop that helps to terminate viral expression and viral DNA replication initiated by LT-ag.

Results from the GST pulldown experiment show that agnoprotein and LT-ag are interacting physically (Figure 18) LT-ag was retained on the gluthatione beads containing GST- agno.

However, an interaction between GST beads and LT-ag was also found. This may be due to unspecific binding between LT-ag and GST. Another possibility is that the antiserum against LT-ag contains antibodies against GST, which could be possible if for instance GST-LT ag was used as antigen during preparation of the serum. Finally another option for this interaction may be due to poor handling of the samples during the experiments.

We detected interaction between BKV agnoprotein and LT-ag in the mammalian two hybrid assay, as well as some of the in vivo and in vitro co-immunoprecipitations. In the *in vivo* co-immunoprecipitation in the figure (19) agnoprotein was efficiently immunoprecipitated by the agnoprotein antibody. Unfortunately, it is difficult to evaluate whether LT-ag co-immunoprecipitated and we cannot tell for sure if LT-ag has co-immunoprecipitated with agnoprotein. The reciprocal result yielded that agnoprotein was co-immunoprecipitated together with LT-ag (Figure 20). Similar results were obtained from the *in vitro* co-immunoprecipitations, and the mammalian two hybrid assay also suggested an interaction between the viral proteins.

During this thesis, we had problems with the antibody against LT-ag, as it was difficult to detect LT-ag bands. The antibody immunoprecipitated LT-ag and co-immunoprecipitated agnoprotein (Figure 23), but LT-ag is hardly seen on the Western Blot (Figure 23 and Figure 22). We also tried to confirm presence of LT-ag by doing immunoblot of lysates used in the reporter assays and mammalian two hybrid assays, but failed probably due to the antibody.

In conclusion, the studies presented in this thesis provide some evidence that the human polyomavirus (BKV) agnoprotein interacts physically with the viral regulatory protein, LT-ag and can modulate the transregulatory activity of this protein in a dose-dependent manner. The transactivation study gave encouraging results and it is reasonable to believe that agnoprotein exerts negative regulatory effect on transcription of LT-ag by slowing the rate of LT-ag expression which leads to late gene expression and switching-off viral DNA replication.
The importance of this regulatory event in the pathogenesis of PyVAN and PyVHC remains to be elucidated. As such, one may hypothesize that the negative regulatory effect of agnoprotein on viral replication may alter the course of disease progression. It is of interest to investigate the interaction of these two proteins further. This interaction could be used as

therapeutic strategy to prevent viral release in the transplanted kidney and thereby avoid nephropathy and hemorrhagic cystitis.

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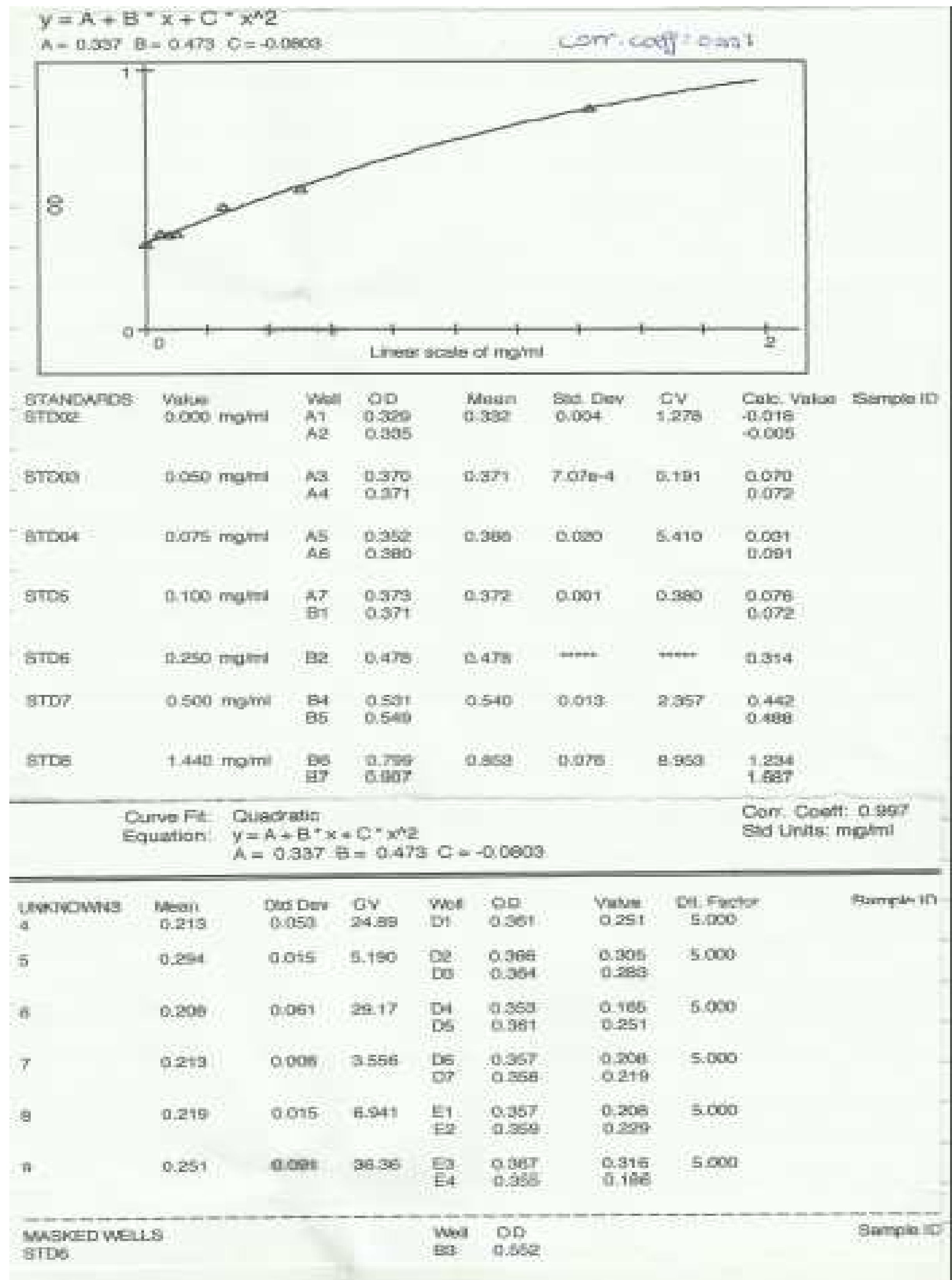
Appendix

GST and GST-agno concentration measurement.



STANDARDS	Value	Well	O.D	Mean	Std. Dev	CV	Calc. Value	Sample ID
STD02	0.000 mg/ml	A1	0.329	0.332	0.004	1.278	-0.016	
		A2	0.335				-0.005	
STD03	0.050 mg/ml	A3	0.370	0.371	7.07e-4	0.191	0.070	
		A4	0.371				0.072	
STD04	0.075 mg/ml	A5	0.352	0.366	0.020	5.410	0.031	
		A6	0.380				0.091	
STD5	0.100 mg/ml	A7	0.373	0.372	0.001	0.380	0.076	
		B1	0.371				0.072	
STD6	0.250 mg/ml	B2	0.478	0.478	*****	*****	0.314	
STD7	0.500 mg/ml	B4	0.531	0.540	0.013	2.357	0.442	
		B5	0.549				0.488	
STD8	1.440 mg/ml	B6	0.799	0.853	0.076	8.953	1.234	
		B7	0.907				1.687	

Curve Fit: Quadratic
Equation: y = A + B * x + C * x^2
A = 0.337 B = 0.473 C = -0.0803

Corr. Coeff: 0.997
Std Units: mg/ml

UNKNOWNS	Mean	Std Dev	CV	Well	O.D	Value	Dil. Factor	Sample ID
4	0.213	0.053	24.89	D1	0.361	0.251	5.000	
5	0.294	0.015	5.190	D2	0.366	0.305	5.000	
				D3	0.364	0.283		
6	0.208	0.061	29.17	D4	0.353	0.165	5.000	
				D5	0.361	0.251		
7	0.213	0.008	3.556	D6	0.357	0.208	5.000	
				D7	0.358	0.219		
8	0.219	0.015	6.941	E1	0.357	0.208	5.000	
				E2	0.359	0.229		
9	0.251	0.091	36.36	E3	0.367	0.316	5.000	
				E4	0.355	0.186		

MASKED WELLS	Well	O.D	Sample ID
STD6	B3	0.552	

DATA FILE:	2010-12-10-sophie		PAGE: 1
DESCRIPTION:			
PROTOCOL:			PRINTED: 10-12-10
DESCRIPTION:			
MODE:	Endpoint	AUTOMIX: OFF	
WAVELENGTH:	590		CALIBRATION: ON
MEAN TEMP:	24.40°C		SET TEMP: OFF

Curve Fit: Quadratic   Corr. Coeff: 0.997

Equation: y = A + B * x + C * x^2   Std Units: mg/ml

A = 0.337 B = 0.473 C = -0.0803

STANDARDS	Value	Well	OD	Mean	Std Dev	CV	Sample ID
STD02	0.000 mg/ml	A1	0.329	0.332	0.004	1.278	
		A2	0.335				
STD03	0.050 mg/ml	A3	0.370	0.371	7.07e-4	0.191	
		A4	0.371				
STD04	0.075 mg/ml	A5	0.352	0.366	0.020	5.410	
		A6	0.380				
STD5	0.100 mg/ml	A7	0.373	0.372	0.001	0.380	
		B1	0.371				
STD6	0.250 mg/ml	B2	0.478	0.470	*****	*****	
STD7	0.500 mg/ml	B4	0.531	0.540	0.013	2.357	
		B5	0.549				
STD8	1.440 mg/ml	B6	0.799	0.853	0.076	8.953	
		B7	0.907				

UNKNOWNS	Mean	Std Dev	CV	Well	OD	Value	Dil. Factor	Sample ID
1	0.451	0.039	8.546	C1	0.382	0.479	5.000	
				C2	0.377	0.424		
10	0.064	0.053	82.23	E5	0.340	0.027	5.000	
				E6	0.347	0.101		
11	0.181	0.098	54.27	E7	0.361	0.251	5.000	
				F1	0.348	0.112		
12	0.294	0.046	15.57	F2	0.362	0.262	5.000	
				F3	0.368	0.326		
13	1.457	0.008	0.569	F4	0.468	1.451	5.000	
				F5	0.469	1.463		
14	0.627	0.117	18.68	F6	0.388	0.544	5.000	
				F7	0.403	0.710		
15	8.270	1.158	14.01	G1	0.932 &	9.089	5.000	
				G2	0.864 &	7.451		
16	2.762	0.672	24.32	G3	0.610	3.237	5.000	
				G4	0.537	2.287		
2	0.337	0.015	4.5[illegible]	C3	0.370	0.348	5.000	
				C4	0.368	0.326		
3	0.326	0.046	14.05	C5	0.371	0.359	5.000	
				C6	0.365	0.294		
4	0.213	0.053	24.88	C7	0.354	0.178	5.000	