The role of the large T antigen in the i*n* *vitro* replication of BPyV

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A Thesis Submitted to the Veterinary Medicine Faculty of the University of Glasgow for the degree of Master of Science

February 2009

Abstract

Bovine Polyomavirus (BPyV) is a member of Polyomaviridae, a family of double stranded DNA viruses. When the virus was initially discovered it was thought to be of simian origin (Wognum *et al.*, 1984), but further research indicated that the virus was of bovine origin and that primate cultures had been contaminated through the use of foetal bovine serum (Parry *et al*., 1983). Studies have also shown that cattle populations world-wide have been exposed to the virus, which does not cause significant pathogenicity (Nairn *et al*., 2003). In addition, 71% of veterinary surgeons, 50% of cattle farmers and 40% of abattoir workers tested positive for the presence of antibodies to BPyV (Parry *et al.,* 1986). BPyV

Very little investigative work has been carried out on bovine polyomavirus but the entire sequence has been published and shows similarities in genome organization to the non-rodent polyoma viruses, such as SV-40 (Schuurman *et al.,* 1990). In contrast to other polyomaviruses, BPyV has a relatively small genome although the significance of this has not been determined. A reliable *in vitro* cultivation assay for BPyV has been established; however the mode of replication is significantly different to reported polyomaviruses. BPyV requires 6-8 weeks of culture before virus replication is detected, even by sensitive methods such as quantitative polymerase chain reaction (QPCR) (Nairn *et al.,* 2003).

The main reasons for the concern surrounding BPyV are the exposure risk, the ability of the virus to jump species and its cell transforming capacity described by Schuurman *et al* (1992) in rodent cells transformed with BPyV. To investigate these observations, the aim of this thesis was to understand the role of BPyV large T antigen, which is known to be directly involved in the replication of BPyV.

During this thesis levels of BPyV viral genome and large T mRNA were assessed by QPCR and Reverse Transcriptase-QPCR (RT-QPCR) during an 8 week cultivation period. High titres of BPyV were only detected in cells supplemented with 1% FCS. BPyV was unable to grow in cells supplemented with 10% FCS, with only low titres of virus being detected within the first 3 weeks. A direct correlation between the large T mRNA and viral replication in cells supplemented with 1% FCS was determined, with large T mRNA being detected immediately prior to exponential viral replication. In addition, cell cultures were assessed using SDS-PAGE and Western Blot techniques to detect proteins specific to large T mRNA and cyclin A (an S-Phase promoting factor). Results indicate that the large T antigen is able to force the cell to enter S-Phase, when both cellular and viral DNA are replicated together.

Detection of large T antigen was inconclusive during the cultivation period, although later experiments attempting to over-express the large T antigen did show the presence of this protein. During this experiment, the over-expression of the large T antigen was shown to be toxic to cells, with cells showing vacuolation and death. In addition, an individual clone of BPyV was isolated and sequenced. The consensus sequence was translated into amino acid, and several differences observed, one in particular that could affect the replication initiation and efficiency.

Contents

Tables

Abbreviations

BPyV	Bovine Polyomavirus
DNA	Deoxyribonucleic acid
cDNA	Complementary Deoxyribonucleic acid
ssDNA	Single stranded Deoxyribonucleic acid
dsDNA	Double stranded Deoxyribonucleic acid
RNA	Ribonucleic acid
mRNA	Messenger Ribonucleic acid
tRNA	Transfer Ribonucleic acid
SV-40	Simian Virus 40
QPCR	Quantitative Polymerase Chain Reaction
PCR	Polymerase Chain Reaction
RT-QPCR	Reverse Transcriptase Quantitative Polymerase Chain Reaction
Small T Antigen	Small Tumour antigen
Middle T Antigen	Middle Tumour antigen
Large T mRNA/Antigen	Large Tumour mRNA/Antigen
FCS	Foetal Calf Serum
SDS-PAGE	Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
S-Phase	Synthesis Phase
MuLV	Murine Leukemia virus
MOI	Multiplication f Infection
nm	Nanometers
kb	Kilobases
bp	Basepairs
NS	Non structural
B19	Parvovirus B19

AAV-2	Adeno-associated virus type 2
BKV	BK Virus
JCV	JC Virus
PyV	Murine Polyomavirus
HaPV	Hamster Papovavirus
LPV	Lymphotropic Papovavirus
BFDV	Budgerigar Fledgling Disease Virus
KV	Murine Polyomavirus (Kilham virus)
BPV-2	Baboon Polyomavirus 2
RKV	Rabbit Kidney Vaculating Virus
SA-12	Simian Virus Agent 12
RPV	Rat Polyomavirus
ORI	Origin of replication
VP1, 2 and 3	Viral Capsid Proteins 1, 2 and 3
PP2A	Protein phosphatase 2A
MHC	Major histocompatibility complex
MDBK	Madin Darby Bovine Kidney
p53	Deregulating protein 53
α	Alpha
cr1	Conserved region 1
cr2	Conserved region 2
HPDKGG	Hexapeptide region
GITC	Guanidine isothiocyanate
TBE	Tris-borate buffer
ABI	Applied Biosystems
DMEM	Dulbecco’s Modified Eagle’s Medium
HBSS	Hanks’ Balanced Salt Solution
EDTA	Ethylenediamine tetraacetic acid
HI	Heat Inactivated
PBS	Phosphate Buffered Saline

Taq	Thermus Aquaticus
SDS	Sequence detection system
C_T	Threshold cycle
UNG	Uracil-N-Glycosylase
RT	Reverse Transcriptase
IPC	Internal Positive Control
RIPA	Radio Immuno Precipitation Assay
PVDF	Polyvinylidene fluoride
DAB	3,3'-Diaminobenzidine
r^2	Squared correlation coefficient
kD	kilodaltons
RC	Reverse compliment
AA	Amino Acid
I	Isoleucine
V	Valine
K	Lysine
R	Arginine
L	Leucine
S	Serine

Chapter 1 – Introduction

Viruses are obligate intracellular parasites that can only replicate by infecting host cells and usurping the cellular machinery. Viruses in their simplest form contain a single molecule of nucleic acid and a protein coat, sometimes enclosed by an envelope composed of lipids, proteins, and carbohydrates. The nucleic acid of viruses can be present in one of two forms – Ribonucleic acid (RNA) and Deoxyribonucleic acid (DNA).

There are many thousands of viruses that come in a variety of shapes and sizes. They are found to replicate in just about every cell type from the simplest bacteria to complex mammalian systems. Most viruses only infect specific types of cells in one host species, not generally crossing species barriers. The host range (which refers to the spectrum of host cells in which a virus can multiply) is usually determined by the specific attachment of viral proteins to the host cell's surface. Thus, if a host lacks the receptor for a virus or if the host lacks some component necessary for the replication of a virus, the host will inherently be resistant to the virus. For example, mice lack receptors for *Polioviruses* and thus are resistant. Similarly, humans are resistant to plant and many animal viruses. The host range of a virus can also be determined by penetration, which unlike viral attachment and adsorption, is an energy dependent process (i.e. the cells must be metabolically active). Three main mechanisms are thought to be involved in this process: Translocation of the entire virus across the cell membrane, Endocytosis of the virus into intracellular vacuoles, and Fusion of the viral envelope with the cell membrane (requires the presence of a fusion protein).

However, some viruses are able to cross this species barrier. Direct interactions between infected host species and recipient species (contact with saliva, nasal secretions and faeces) enables some viruses to be transferred to a new host. In cell culture it is possible to infect human kidney cells with the RNA virus *Murine*

Leukemia virus (MuLV) by direct cell to cell contact and a high multiplication of infection (MOI) (Byrne *et al.*, unpublished data). As the MuLV buds from the human cells the tropism (i.e. the cell type that the virus is most likely to infect) will be changed. The ability of viruses to cross species barrier has been well documented recently, especially concerning the RNA virus Avian Influenza virus H5N1.

1.1 DNA Viruses

1.1.1 Structure

DNA viruses range in size from 20 nanometers (e.g. Parvoviruses) to several hundred nanometers (e.g. Poxviruses). The nucleic acid genome may be single stranded (ssDNA) or double stranded (dsDNA) and the number of genes encoded can be limited to 2-3 genes (e.g. Parvoviruses) up to a number of hundreds (e.g. Poxviruses). All DNA viruses contain a protein shell enclosing the genetic material called a capsid, composed of subunits or capsomeres, which can be made from a single type or several types of protein. The capsid and genomic material is referred to as the nucleocapsid - which may have icosahedral, helical or complex symmetry (figures 1.1, 1.2 and 1.3).

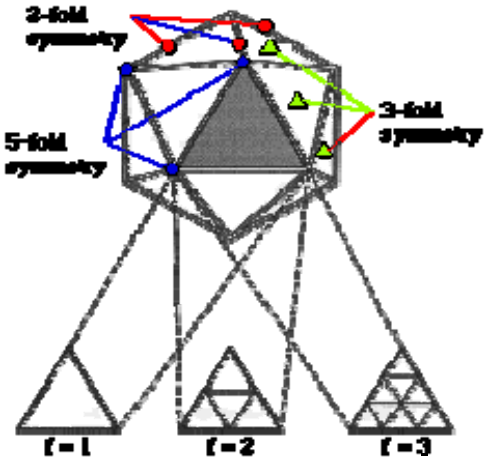


Figure 1.1 – Example of icosahedral symmetry showing the subunits of the capsid. Polyomaviruses are examples of viruses with this type of symmetry.

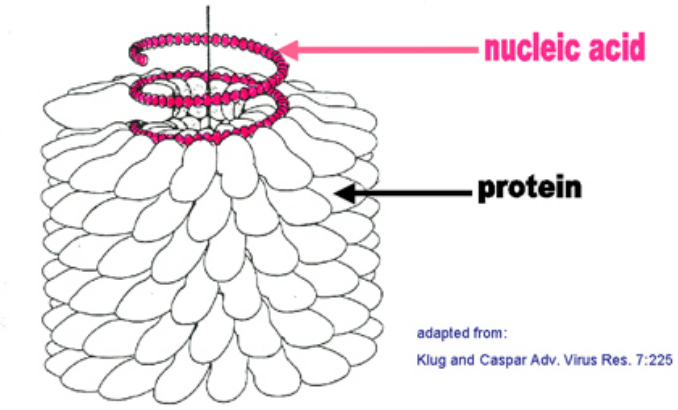


Figure 1.2 – Example of helical symmetry showing arrangement of the proteins and nucleic acid. Tobacco Mosaic Virus is a virus exhibiting this type of symmetry.

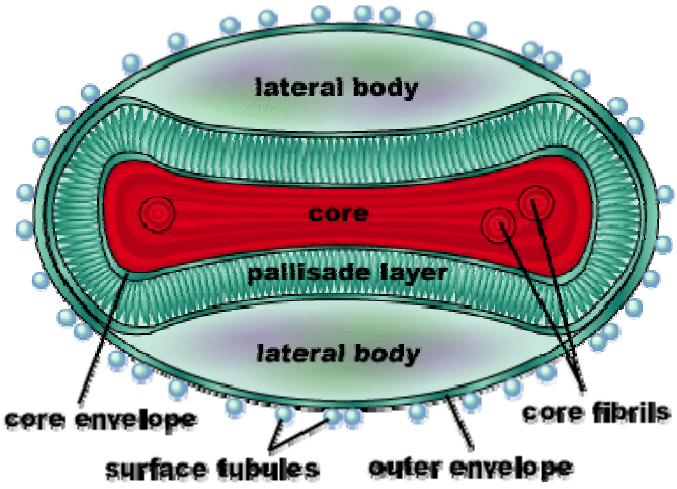


Figure 1.3 – Example of complex symmetry. This structure can not defined by a mathematical equation as can a simple helix or icosahedron. Poxviruses are examples of viruses with this type of symmetry.

The capsid of some DNA viruses is enclosed by an envelope consisting of lipids, proteins, and carbohydrates, usually obtained by budding through the membranes of host cells. In other cases the envelope may be derived from other membranes such as those of the Golgi body or the nucleus.

1.1.2 DNA viral infection

The first step in the infection of a cell by a DNA virus is the attachment of the virus to the cell surface. Although DNA viruses have evolved varying methods, in general the viral attachment protein (or antitreceptor) recognizes specific receptors on the outside of the cell, which may be protein, carbohydrate or lipid.

Once attached the virus enters the cell, uncoats to releases the genomic material and express their genes using specific host cell proteins. The viruses are then

assembled and exit the cells. In some cases viral DNA can become incorporated in the host cell genome (provirus). The provirus is passively replicated along with the host genome and passed onto the cell offspring, with all descendents of the infected cell containing the provirus in their genomes. Under some circumstances such as changes in the host's environmental conditions or health, the provirus can be activated and begins massive transcription of the viral genome. This can lead to the destruction of its host as the protein synthesis machinery is hijacked to produce more viruses.

Viral infection of cells does not always result in viral replication; this may be due to infection with defective virus, or other mechanisms which are a feature of the host cell. Infection with viruses which can form latent infections such as herpes or polyomaviruses latency results from either infection of non-replicating cells or replication in conjunction with the host cell so that the cell cycle is not interrupted. The viral genome persists intact and at some later time may enter a productive acute infection phase (possibly due to immunosupression of the host) to ensure a spread of its progeny to a new host.

1.1.3 Modes/Diversity of Replication

The structure, function, host range, pathogenesis and particularly the replication strategy of DNA viruses varies dramatically from virus to virus, even within viruses of the same family.

Parvoviridae are among the smallest and simplest of viruses. They have few functional genes, relying on the mechanisms and enzymes present in the cells to facilitate replication. A member of the parvoviridae, the dependoviruses, also requires a "helper" virus (adenovirus or herpesviruses) to replicate (figure 2). All parvoviridae are small (20-25 nanometers (nm)), single stranded non-enveloped DNA viruses approx. 5 kilobases (kb) in length containing only 4-5 genes (two

non structural (NS1 and 2) and 2-3 structural proteins depending on the virus) that replicate and assemble in the nucleus.

In contrast, poxviruses are large (200-400nm), complex double stranded DNA viruses of 130-300kb in length, containing approximately 250 genes that replicate and assemble in the cytoplasm by expressing their own DNA dependant RNA polymerase, allowing these viruses to replicate with minimal cellular resources (figure 3). The progeny of both types of virus are released by host cell disintegration, although some poxvirus particles may bud from the cell acquiring an extra membrane.

These two examples highlight the contrast between size, complexity and modes of replication between DNA viruses. With the exception of the poxviruses, most viruses are heavily dependent on the host cellular mechanisms, with viral replication only possible when cells are replicating. However, all DNA viruses do share a common requirement and that is to generate mRNA that can be translated into proteins in order to replicate their genomes.

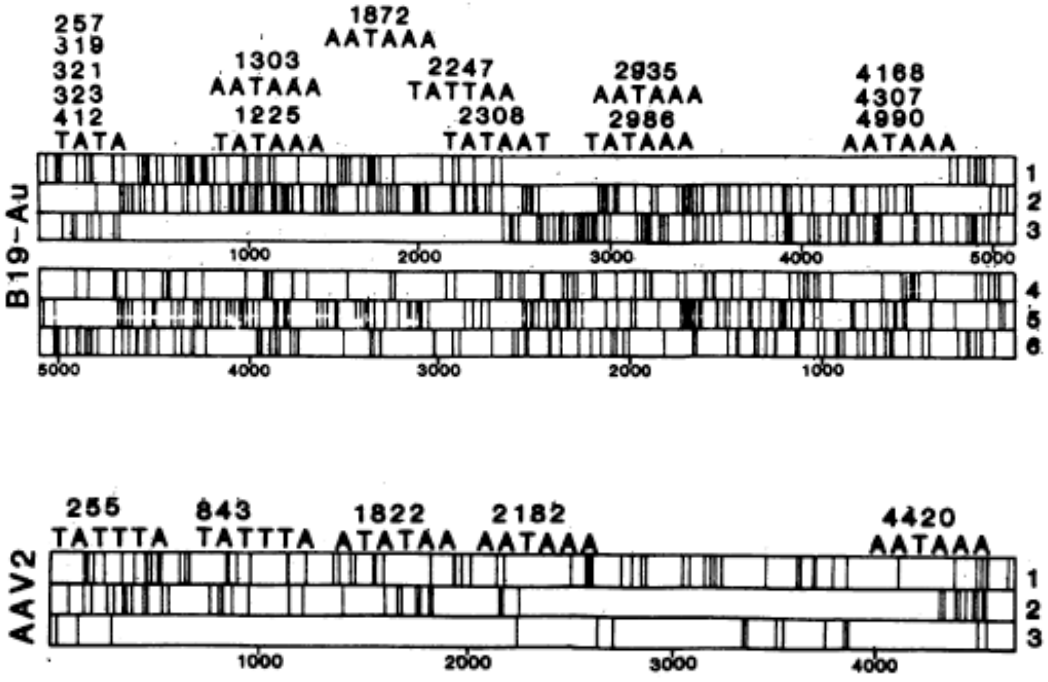


Figure 2 - Comparison of the genome organization of the *Parvoviridae* virus Human Parvovirus B19 and adeno-associated virus AAV-2 (*Dependovirus*). Note the difference in complexity between 2 viruses of the same family. (Adapted from J. Virol. Shade *et al*., 58 (3): 921).

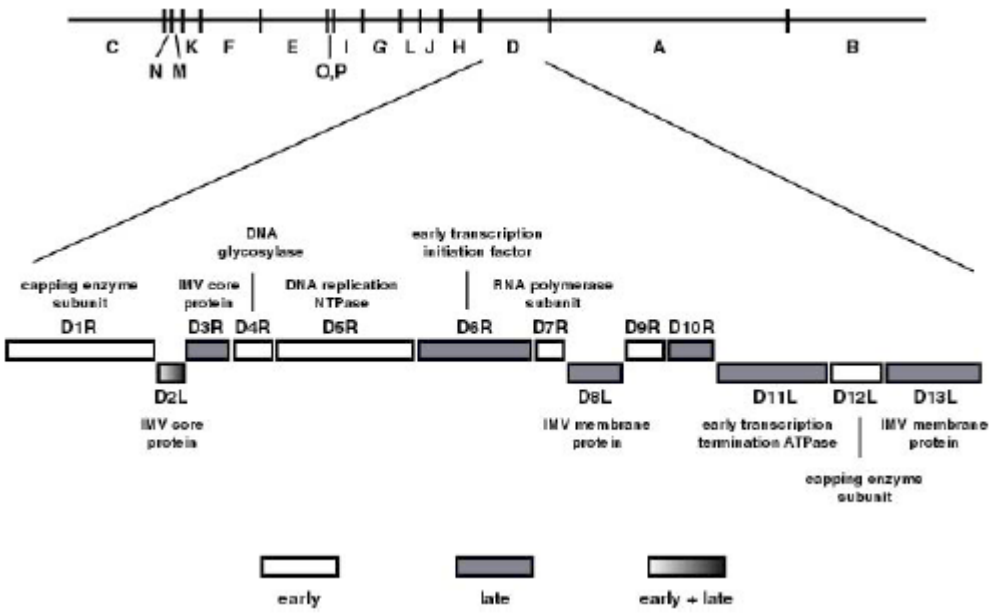


Figure 3 - Example of poxvirus genome organization and some of the numerous genes that allow these viruses to virtually replicate independently of the cell. This figure highlights 12 functional genes in this short section of the viral genome, compared to the 3-5 functional genes present in the entire genome of parvoviruses.

1.2 Polyomaviruses

Polyomaviruses are the sole genus of virus within the family Polyomaviridae, which is a subfamily of the Papovaviridae family. They are widely distributed in nature and have been described in many mammals and birds. Polyomaviruses cause inapparent infections in most hosts, and although largely species specific, the primate polyomavirus SV-40 and BPyV have been shown to infect non-primate and non-bovine cells, respectively (Wognum *et al*., 1984. Parry *et al*., 1983). Polyomaviruses are small (40-50nm), non-enveloped, icosahedral in shape and contain single molecules of covalently closed, superhelical dsDNA. Table 1 illustrates the known polyomaviruses and the variety of primary host species.

Table 1 –Polyomaviruses

Virus	Abbreviation	Host
BK Virus	BKV	Human
JC Virus	JCV	Human
Simian Virus 40	SV40	Rhesus monkey
Murine Polyomavirus	PyV	Mouse
Hamster Papovavirus	HaPV	Hamster
Lymphotropic Papovavirus	LPV	African Green Monkey
Budgerigar Fledgling Disease Virus	BFDV	Parakeet
Bovine Polyomavirus	BPyV	Cattle
Murine Polyomavirus (Kilham virus)	KV	Mouse
Baboon Polyomavirus 2	BPV-2	Baboon
Rabbit Kidney Vaculating Virus	RKV	Rabbit
Simian Virus Agent 12	SA12	Baboon
Rat Polyomavirus	RPV	Rat

1.2.1 Polyomaviruses and disease

In the majority of cases Polyomaviruses only cause clinical symptoms in immunodeficient hosts (Barbanti-Brodano *et al.,* 1998). Human polyomavirus (JCV and BKV) infection in humans is common with seroprevalence rates in the population of 65-90% being reached by the age of ten years (Polyomaviruses and Human Diseases, Nasimul Ahsan, 2006). The kidney is where the virus lies dormant for many years although in the majority of patients only a subclinical infection may result. However, in immunocompromised patients, the virus may reactivate and cause significant disease, especially in conditions that bring about T cell deficiencies (Knowles *et al.,* 2006). The human polyomaviruses are known to cause hemorrhagic cystitis in recipients of bone marrow transplantation (BKV) and progressive multifocal leukoencephalopathy (JCV) in immunocompromised

patients, for example, with *Human Immunodeficiency virus* infection. The current antiviral agents are unsuccessful in treating polyomavirus-related illnesses, with current strategies concentrating on the modification and/or improvement in the patients' immune system.

The ability of the human polyomaviruses (JCV and BKV) to cross species has been documented in Syrian hamsters (Corallini *et al*., 1982. Stoner *et al*., 1986). Tumors were induced following intravenous inoculation of BKV in Syrian golden hamsters. The types of induced tumors, or neoplasms, included ependymomas, carcinoma of pancreatic islets, lymphoma, osteosarcomas, undifferentiated sarcoma, kidney and renal pelvis carcinoma (Corallini *et al*., 1982). Intracerebral inoculation of newborn Golden Syrian hamsters with JCV induced a broad range of tumors including medulloblastoma, astrocytoma, glioblastoma, primitive neuroectodermal tumors and peripheral neruoblasoma in more than 85% of inoculated animals (Del Valle *et al*., 2001). PCR analysis in the appropriate tissues of human cancer patients has suggested that JCV may be associated with colon cancer (Enam *et al*., 2002).

The simian polyomavirus SV-40 was introduced into the human population in the mid 1950's from contaminated poliovaccines, and evidence now indicates that SV40 may have been transmitted in humans, independent of the earlier administration of vaccine. SV40 infections of humans are associated with inflammatory kidney diseases and with specific tumor types: mesothelioma, lymphoma, brain, and bone (Barbanti-Brodano *et al*., 2004).

Species tropisms of BPyV have been studied and growth in non-bovine cells has been documented. Studies have also shown that 60% of veterinarians in the Netherlands have antibodies to BPyV, although the significance of this at his point is unknown (Osterhaus *et al*., 1993).The sequence homology shared among several polyomaviruses (JC, BK, SA12, and SV40) (Pipas, 1992) may indicate a

shared ancestry, enabling polyomaviruses to infect several species, including humans.

Approximately 60% of bovine sera (Schuurman *et al*., 1991), which is used in the production of biopharmaceuticals, have been shown to contain bovine polyomavirus sequences. However, it was not until recently that it was thought to be of concern to humans, and it is now recommended by regulatory authorities that any therapeutic that has been in contact with bovine derived reagents be screened (EMEA guidelines, 2002).

1.2.2. Polyomavirus Genome Organisation

The polyomavirus genome is divided into early and late regions, corresponding to the portions of the genome that are expressed during the early and late stages of infection. The viral genomes of polyomaviruses consist of circular DNA molecules of approx. 5000 nucleotides in length (figure 4).

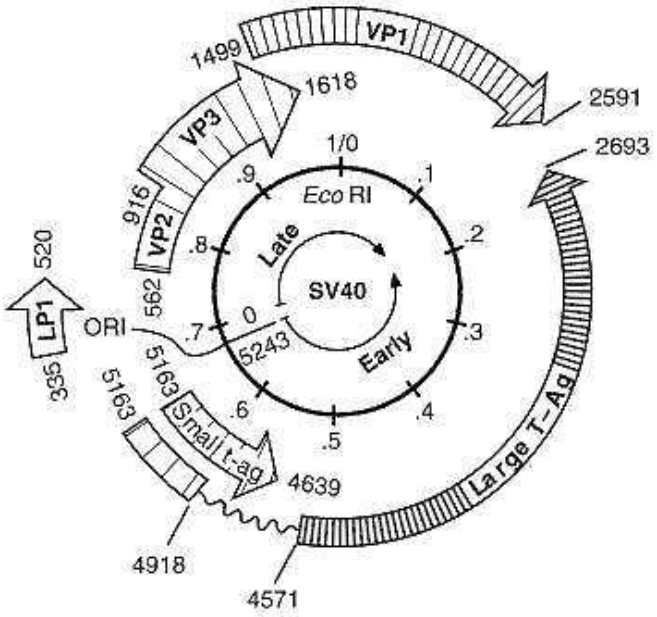


Figure 4 – Genome organisation of the Polyomavirus SV-40. The centre of the figure illustrates the direction of the early and late phases, proceeding in different directions. Both phases are initiated at the origin of replication (ORI). Small and large T antigens are expressed during the early phase. The viral capsid proteins VP1, 2 and 3 are expressed during the late phase. The small agnoprotein LP1 is also expressed during the late phase and is thought to be involved in VP1 localization and viral capsid assembly (Modified from Butel JS, Jarvis DL: The plasma-membrane-associated form of SV40 large tumor antigen: biochemical and biological properties. Biochim Biophys Acta 865:171, 1986).

The non-coding region of polyomaviruses contains the origin of replication and the early and late promoter-enhancer sequences. Viral transcription proceeds clockwise and anti-clockwise and two phases of transcription are present - early and late. The early phase is initiated prior to viral DNA replication; the late phase being initiated following viral DNA replication.

Early RNA transcripts are processed by splicing to form the mature mRNAs for the early proteins (the T (tumour) antigens). There are 3 types – Small, Middle (not all polyomaviruses express this antigen) and large T antigens. Little is known regarding the function of the small T antigen although it does increase the efficiency of viral replication (Cicala *et al*., 1994). Recent studies have provided more information concerning the small T antigen, also suggesting that the small T antigen modifies the viral chromatin by altering the temporal coordination of virus growth and the cell cycle (Dahl *et al*., 2007), possibly by interactions with protein phosphatase 2A (PP2A plays a critical role in the cellular processes of protein synthesis, DNA replication, transcription, and metabolism) that leads to the transactivation of several genes involved in S-phase induction (Kluckly *et al*., 2007). Little is also known about the middle T antigen although it is thought to be important for cell transformations (Asselin *et al*., 1983).

The large T antigen has been researched in more detail and functions directly in initiating viral replication. The large T antigen is also responsible for recruiting specific host cell proteins, blocking cellular anti-viral mechanism, and initiating viral replication (Brodsky *et al*., 1998). Studies have also shown that the large T antigen of JCV modulates the Wnt-signaling pathway via interaction with beta-catenin, one of the most important components of the canonical Wnt pathway (Bhattacharyya *et al*., 2007). The canonical Wnt pathway describes a series of events that occur when Wnt proteins (known for their roles in cancer) bind to cell-surface receptors.

Viral DNA replication occurs in the nucleus during the late phase, where the viral capsids proteins VP1, VP2, and VP3 are translated.

1.2.3 Replication / Life Cycle of polyomaviruses (sourced from Fields Virology unless otherwise stated)

1.2.3.1 Attachment

Using SV40 as a model, the polyomavirus receptors appear to be major histocompatibility complex (MHC) class I antigens (Norkin, 1999). Host receptors for some polyomaviruses are not known, but most appear to contain sialic acid (haemagglutinin) and are widespread in many tissue/species. The VP1 antigen is thought to be responsible for receptor binding as anti-VP1 antibodies (Goldmann *et al*., 1999) and VP1 point mutations (Nakanishi *et al*., 2007) prevents binding of the virus.

1.2.3.2 Entry and Uncoating

The viral capsid proteins VP2 and 3 are myristylated and are believed to interact with cellular membranes to facilitate entry. Virions are taken up by endocytosis and are transported to the nucleus by the interaction of endocytic vacuoles with the cytoskeleton.

Virus particles enter the nucleus by the nuclear pores where uncoating occurs. The remaining stages of the replication cycle all occur in the nucleus. It has been proposed that VP2 and VP3 antigens mediate the nuclear entry of viral DNA, and may also be involved in the uncoating process, although the details are unknown (figure 5) (Nakanishi *et al*., 2007).

1.2.3.3 Functional Gene Expression and Genome Replication

Inside the nucleus, the virus mini-chromosome (genome-histone complex) is transcribed by the host cell RNA polymerase II to produce early mRNAs. Because of the relative simplicity of the viral genome (and small number of functional genes), polyomaviruses are heavily dependent on the cell for transcription and genome replication. However, the viral genome contains cis-acting regulatory signals (surrounding the origin of replication) which enable the virus to direct transcription, replication and the expression of the trans-regulatory proteins. These trans-regulatory proteins are produced by alternative splicing of the early T-antigens.

The early gene promoter contains strong enhancer elements which cause it to be active in newly infected cells. It is thought that the small T-antigen is not essential for virus replication, but indirectly (i.e. it interacts with cellular proteins but does not bind directly to the virus genome) enhancing transcription from the late promoter.

Following expression of the large T antigen in the nucleus, transcription of the early genes is repressed by direct binding of the protein to the origin of replication, preventing transcription from the early promoter and causing the switch to the late phase of infection. After viral replication has occurred, transcription of the late phase genes is initiated from the late promoter and results in the synthesis of the structural proteins.

For efficient viral replication, it is imperative for the host cell to enter the synthesis phase (S phase), when both cellular and viral DNA are replicated together. The S phase is a period in the cell cycle during interphase, between the

G1 and G2 phases. At the beginning of the S phase, each chromosome is composed of one coiled DNA double helix molecule, which is called a chromatid. At the end of this stage, each chromosome has two identical DNA double helix molecules, and therefore is composed of two sister chromatids. During S phase, the centrosome is also duplicated. These two events are unconnected, but require many of the same factors to progress. The end result is the existence of duplicated genetic material in the cell (and viral genomic material), which will eventually be divided into two. ‘Protein to protein’ interactions between T-antigen and DNA polymerase-alpha directly stimulate replication of the virus genome, also inactivating the tumour suppressor proteins causing G1-arrested cells to enter S phase.

Adherent cells that are grown in flask systems will often become ‘contact inhibited’ and will stop growing (e.g. Madin Darby Bovine Kidney (MDBK) cells), while other cell types will overgrow and die (e.g. Vero). In the case of MDBK cells, the cells will not enter S-phase, remaining in G1 phase until they are passaged. If cells are in G1 phase, then polyomaviruses will be unable to replicate. Therefore, in addition to increasing transcription, another important function of the T-antigen is to alter the cellular environment by inducing S-phase.

1.2.3.4 Assembly/Maturation and Release

Polyomavirus structural proteins contain 'nuclear localization signals' which results in their accumulation in the nucleus, where they migrate after being synthesized in the cytoplasm. Assembly occurs in the nucleus, and since the structure of the virus is relatively simple, assembly & maturation of the particle are simultaneous.

Some virus particles are exported to the cell surface in cytoplasmic vacuoles, with the remaining virus particles being released when the cell undergoes lysis.

Mechanisms of cell injury are not clear, but cell death is not uncommon due to the severe interference with normal cellular functions. The complete replication cycle for most polyomaviruses takes 48-72hours (depending on MOI).

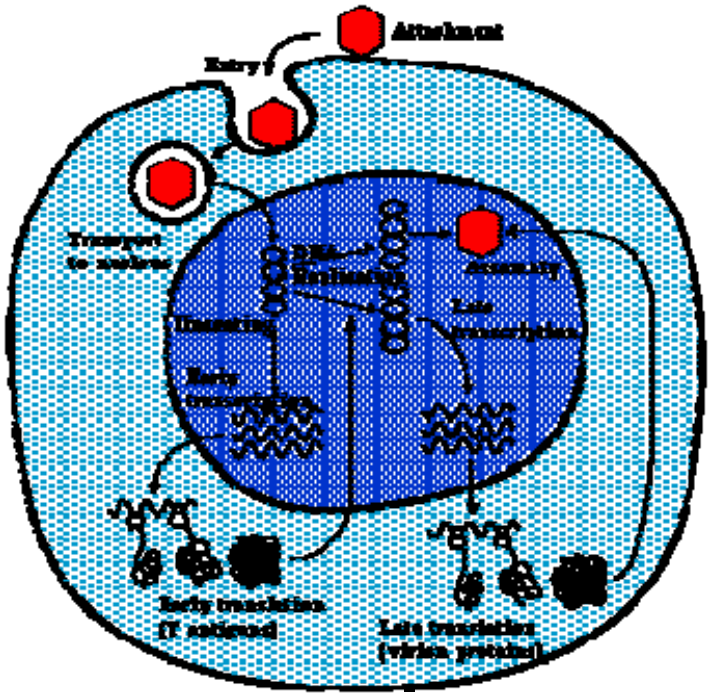


Figure 5 – The life cycle of SV-40. Figure highlights the attachment, entry, uncoating, replication and assembly of SV-40. Also note the interaction of the early and late translation products (T antigen and viral capsid proteins) (Adapted from Fields Virology).

1.3 Bovine Polyomavirus

Bovine polyomavirus was originally known as *Stump tailed macaque virus* (STMV) (Reissig *et al*., 1976) which, along with some closely related polyomavirus isolates, was originally isolated from monkey kidney cell cultures.

It was first thought that the monkeys from which the virus was isolated from were the natural hosts, but subsequent work by Parry *et al* in 1983 and Wognum *et al* in 1984 showed that antibodies against BPyV could not be detected in Cynomolgus Macaques (high levels were detected in cattle). In addition, the isolation of the virus from bovine kidney cell cultures (Coackley *et al*., 1980), and assessments of viral morphology strongly suggested that all the viruses mentioned were of bovine origin (Schuurman *et al*., 1991). It is now thought that bovine polyomavirus is probably the most ancient of all mammalian polyomaviruses (Gottlieb *at al*., 2001). BPyV does not cause significant pathogenicity in cattle and no disease has yet been ascribed to the agent (Nairn *et al*., 2003).

The genome of BPyV has been shown to be similar in organisation (Schuurman *et al.,* 1990) (but not sequence homology – only 15%) (Pipas, 1992) to the non rodent polyoma viruses, such as SV-40, and consists of a circular DNA molecule of 4697 nucleotides which makes it the smallest polyomavirus genome sequenced to date (figure 6). Like all polyomaviruses viral replication occurs in two distinct phases; early and late gene expression, separated by genome replication. However, the early region of BPyV seems to have an uncommon organisation. In contrast to the characteristic presence of only one intron of 250 to 400 nucleotides, two small introns separated by 80bp have been identified in the early region encoding the large T antigen (Schuurman *et al*., 1992). It has been suggested that one of the small introns identified in the large T antigen was involved in the formation of the putative small T antigen (Schuurman *et al*., 1990), however, no evidence has been found to confirm this and even to this day the ability of BPyV to code for the small T antigen is questionable. No evidence of middle t has been found so far, and it seems that only rodent polyomaviruses encode for this antigen (Gottlieb *et al*., 2001).

In BPyV the large T antigen may be able to carry out the functions of a small T antigen, notably the interaction with PP2A, similar to that of the middle T antigen function of *murine polyomavirus* (Gottlieb *et al*., 2001).

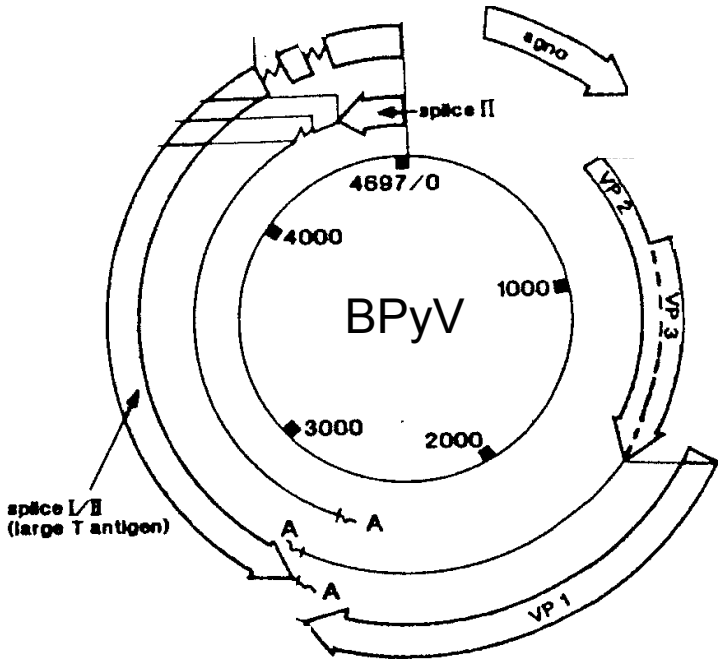


Figure 6 – Genome Organisation of *Bovine Polyomavirus*. Genome organisation is similar to SV-40 with an early and late phase, although not marked as clearly on this figure. The origin of replication is at the top of the diagram with the early phase proceeding anti-clockwise (T antigens), and late phase clockwise (viral capsids). Note the splice site present in the early phase for the expression of the large T antigen (Source – Schuurman *et al*., 1990).

1.4 BPyV large T antigen

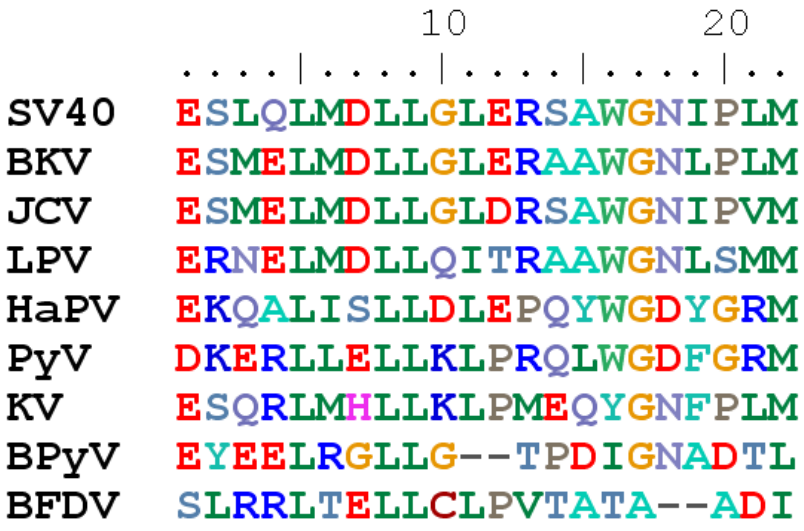
Of the early phase antigens that are produced during BPyV replication, the most critical is the large T antigen, which regulates both viral genome and host cell cycle replication. The large T antigen functions by acting directly on cellular proteins. Viral replication is initiated by the binding of the large T antigen to the origin of replication (ori) region of the BPyV genome; a process controlled by phosphorylation (Brodsky *et al*., 1998). As well as being responsible for initiating replication, the binding of the large T antigen is also thought to cause the cells to leave G1 phase and enter S phase, promoting both cellular and viral replication by deregulating protein 53 (p53) and members of the retinoblastoma family (Moens *et al*., 2007)).

During viral replication the large T antigen has several other important functions. Following binding to the *ori* region, the large T antigen then recruits cellular proteins such as polymerase α, primase, topoisomerase, and the DNA replication factor RPA through direct physical contact, at the same time also inhibiting the host cells anti-viral functions. The large T antigen also regulates transcription control, initiating the transcription of the viral capsid and cellular target genes (VP1, 2 & 3) (Brodsky *et al*., 1998).

With the exception of BPyV, Polyomaviruses during the first 20 hrs after infection are spent driving the host cells into the S phase so that cellular proteins and mechanisms are available to initiate viral replication. It can be hypothesized that replication would not be possible if the large T antigen wasn't present, or was in some way defective. A number of questions arise concerning the function of large T - would replication be possible if the levels of the large T antigen were too low? Would replication only be initiated when a sufficient amount of large T antigen (or defective large T antigen) had accumulated? Could this explain the delay of the *in vitro* amplification of BPyV?

When the sequences of several large T antigens are aligned (using pairwise alignment), the overall amino acid identity varies between 15 and 80%. JCV, BKV, and SV40 are all closely related, with 75% homology. In contrast, the avian *budgerigar fledgling disease virus* (BFDV) and BPyV show the least identity (15 to 35%) with other members of the group (figure 7). T antigens encoded by the remaining viruses, *Lymphotropic polyomavirus* (LPV), *hamster polyomavirus* (HaPV), *K virus* (KV), and PyV, show an intermediate level of homology (45 to 55%) both with each other and with the SV40 large T antigen. One possible explanation for this is the evolution of the different viruses to interact with different hosts (Pipas, 1992).

A. large T antigen conserved region 1 (cr1)



B. The highly conserved hexapeptide (HPDKGG) region

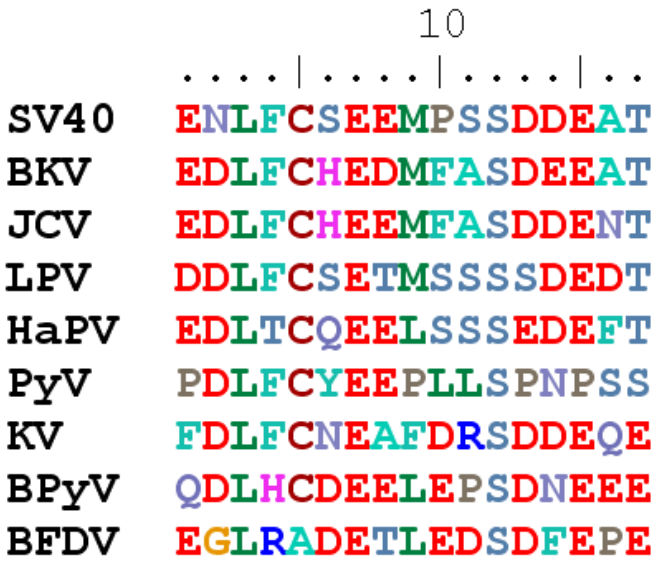


Figure 7 – Alignment of polyomavirus large T amino acid regions. In SV40, the amino-terminal domain plays an essential role in both viral replication and transformation. Three sequence elements within the amino-terminal domain are conserved among the T antigens - conserved region 1 (cr1), conserved region 2 (cr2) and the hexapeptide HPDKGG. Sequence homology between cr1 and HPDKGG are highlighted. BPyV and BFDV show the least degree of identity with other polyomaviruses, with between 15 and 35% homology (Adapted from Pipas, 1992).

For polyomaviruses the large T antigen is critical for replication, being responsible for many functions. The majority of polyomavirus research has been carried out on the simian polyomavirus SV-40. Does BPyV (and the BPyV large T antigen) also function in the same manner as SV-40?

1.5 Aims

The use of media supplemented with bovine serum is widespread in biological and medical laboratories. The frequent detection of BPyV in commercial batches reported by Schuurman *et al* in 1991 indicates that there is a serious risk of not only contaminating cell cultures used for research, but also cell cultures used for the production of biopharmaceuticals. The downstream applications of these products are an obvious concern, especially since the link with SV-40 and cancers in humans were made, although the role of SV40 in carcinogenesis (in transplanted patients) may be minimal (Paracchini *et al*., 2006). With the possibility that SV-40 is involved in carcinogenesis the presence of another non-human polyomavirus (BPyV) in serum used for the production of biopharmaceuticals is a concern.

A reliable *in vitro* cultivation of BPyV (Nairn *et al*., 2003) has been established; however the mode of replication is different in comparison to other polyomaviruses. In this study BPyV required at least 5 weeks of continual culture in cells fed with media supplemented with 1% Foetal Calf Serum (FCS) before virus replication was detected. These results contradict the other models of polyomavirus replication which has shown that viral replication occurs in cells doubling at a high rate in the first 1-2 weeks.

The aim of this thesis is to assess the levels of BPyV and large T antigen expression during an 8 week infectivity experiment (and to assess large T mRNA levels prior to exponential viral amplification) using cells fed with media supplemented in 1% and 10% FCS. The aim is also to understand what is happening within the cells during this period, in particular following exponential amplification.

1.6 Objective

The objective of this project is to understand by what mechanism(s) the virus is maintained during the period of limited or no replication, and to investigate the role of the large T antigen in the initiation of replication observed 5-8 weeks after inoculation.

Part A

What levels of BPyV genome replication and large T mRNA expression are observed during the culture of virus on cells grown in 1% and 10% FCS? Indication of expression levels may explain why the virus requires 5-8 weeks before exponential replication is observed. This will be assessed by initiating an in vitro assay with BPyV and MDBK cells and quantitating the expression of both BPyV viral genomes and large T mRNA. Novel Quantitative Polymerase Chain Reaction (QPCR) and Reverse Transcriptase QPCR (RT-QPCR) assays will be designed and validated prior to assessment.

Part B

Does the infection of cells that are growing rapidly result in eventual disappearance of the virus from the culture system? If so, would this be of any use to the biotechnology industry, reducing the need for expensive and time consuming pre-clinical safety testing? This will also be assessed during the 8-week infectivity experiments.

Part C

Most polyomaviruses replicate between 48 and 72 hours, and the increased concentration of the large T antigen has already been established in the replication of other polyoma viruses. Why is BPyV different? The objective of this experiment is to assess large T mRNA levels prior to the onset of viral replication. If the large T antigen is as critical as first thought, could a more efficient BPyV infectivity assay be utilised either for the detection of large T mRNA, or inoculation of material onto cells expressing high levels of large T antigen? This will be achieved by cloning BPyV large T mRNA into an appropriate vector for expression in MDBK cells.

Part D

The ability of other polyomaviruses to move the infected cell into S-phase to necessitate viral replication has been determined, but it is the same for cells infected with BPyV? If so, why does it take so long for this to be initiated? By using infectivity results, along with sequencing information, SDS-PAGE and western blot analysis this will be determined.

Chapter 2
Materials and Methods

2.1 Reagents

2.1.1 Bacterial Strains and Plasmids

The Eukaryotic TA Expression Kit Bidirectional (Invitrogen) was used for the cloning of large T PCR products. This kit utilized the *Taq* polymerase non template-dependent activity that adds a single deoxyadenosine (A) to the 3' ends of the duplex molecules. The pCR3.1 linearized vector supplied with this kit has single 3' deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector. After ligation, One Shot (TOP10F') competent cells are transformed and individual recombinant plasmids analysed for orientation by sequencing. The recombinant plasmid with the correct orientation was purified for transfection into MDBK cells (ATCC # CCL-22).

2.1.2 Oligonucleotides

Primers and probes for Q-PCR analysis were synthesized by Applied Biosystems. TAGN Newcastle synthesized sequencing and conventional PCR primers. The orientation of the primers with respect to the target sequence is indicated below in tables 2, 3 and 4, with "f" representing sense orientation and "r" the antisense orientation. "p" represents probe.

Table 2 – Conventional PCR Primers

Name	Primer Sequence	Target Sequence	
BPyVF	AAGTCATGGAATTAACATCTG	BPyV LargeT	f
BPyVR	CTCATCATTTTTCCAGAATAT	BPyV LargeT	r

Table 3 – Sequencing primers

Name	Primer Sequence	Target Sequence	
LargeT1F	ATGGAATTAACATCTGAGGAATATGAGG	BPyV LargeT	f
LargeT251F	TCAATCAAGGCTTTGATGAGCA	BPyV LargeT	f
LargeT501F	TATCCAGGCAGCCTACAAATGTAC	BPyV LargeT	f
LargeT751F	GTGCAAGAAGAAGAAAAGTCTGTAAATT	BPyV LargeT	f
LargeT1001F	CTGTGTTAGCAAAAAGGAGATTAGATATG	BPyV LargeT	f
LargeT1251F	GCCTGTAAACTGTGGGAAAACTACA	BPyV LargeT	f
LargeT1509F	ACATCAAAATAAGGTGACTCAGATCTTC	BPyV LargeT	f
LargeT1751F	AGGAATGTGTAGTCAAGTGGAAAGAGA	BPyV LargeT	f
LargeT1852R	CCAGAATATTTTTTCCTTGTTCAATGT	BPyV LargeT	r
LargeT1607R	ACTTTTTGATACATACGGATTTTGACTG	BPyV LargeT	r
LargeT1360R	TTCCTAATTCAAATGCCAGTCTTTC	BPyV LargeT	r
LargeT1110R	CCGAGCGCCAAACAAATC	BPyV LargeT	r
LargeT608R	TTTTCCATTGCATTTACCCGAT	BPyV LargeT	r
LargeT360R	GGTGGCTTGACTTCCCGG	BPyV LargeT	r
LargeT110R	TGCACCTTGCATGCCTTC	BPyV LargeT	r

Table 4 – Q-PCR Primers and Probes

Q-PCR Primers and Probes			
Name	Primer Sequence	Target Sequence	
BPyV large T-945F	TGGTATGGTGTCTTCTATGCTCAAG	BPyV LargeT	f
BPyV large T-1016R	TTACAACTGCACCTGAAGCATGT	BPyV LargeT	r
BPyV large T-971T (MGB)	AGTATGTGACAATCCCAGACG	BPyV LargeT	p
SpliceLargeT-60F	TGGCAATGCAGATACTTTGAAAA	BPyV LargeT mRNA	f
SpliceLargeT-137R	TCTTCATTCCCTCCTTTATCTGGAT	BPyV LargeT mRNA	r
SpliceLargeT-86T	CATTCCTGAAGGCATGCAAGGTGCA	BPyV LargeT mRNA	p
VP1 100F	Propriety Information	BPyV VP1	f
VP1 120R	Propriety Information	BPyV VP1	r
VP1 85 T	Propriety Information	BPyV VP1	p

2.2 Preparation and Manipulation of Nucleic Acids

2.2.1 General

Experiments involving the handling of nucleic acids were performed in accordance with standard practices to avoid cross contamination. Separate air-spaces were used for reagent preparation, sample processing, manipulation and the amplification and analysis of PCR/QPCR/RT-QPCR products. A one-way system was in operation and no amplified products were taken into the air-spaces used for sample preparation. Sentinel controls for airborne contamination consisting of open tubes containing all of the reagents and primers for amplification were present at all stages of sample preparation. These were processed along with the test samples.

2.2.2 DNA Extractions

DNA extractions were carried out using a Qiagen QIAamp DNA Mini Kit (Qiagen) following Appendix A – 'Protocol for Cultured Cells'. The Qiagen QIAamp DNA Mini Kit works on the principle that nucleic acid is adsorbed onto a silica gel membrane after appropriate adjustment to allow optimal binding conditions (in the presence of carrier nucleic acid where required). Residual contaminants were removed during several washes and purified nucleic acid eluted in elution buffer.

2.2.3 Viral RNA Extractions from cells

Viral RNA extractions from cells were carried out using a Qiagen RNeasy Mini Kit (Qiagen), following the protocol for animal cells. The RNA extraction principle combines the selective binding properties of a silica-gel-based membrane with the speed of microspin technology. Samples were lysed and homogenized in the presence of a highly denaturing guanidine isothiocyanate (GITC)-containing buffer, which immediately inactivated RNases. Ethanol was

added to provide appropriate binding conditions, and the sample applied to an RNeasy mini column for binding to the membrane and clearing of contaminants. RNA was then eluted in water.

2.2.4 Viral RNA extraction from supernatants

Viral extractions from supernatants were carried out using a QIAamp Viral RNA Mini Kit (Qiagen), following the QIAamp Viral RNA Mini Spin Protocol. The QIAamp Viral RNA Mini Kits principle again combines the selective binding properties of a silicagel-based membrane with the speed of microspin technology. Samples were lysed under highly denaturing conditions to inactivate RNases. Buffering conditions were adjusted to provide optimum binding of the RNA to the QIAamp membrane, and samples loaded onto the QIAamp Mini spin column. The RNA bound to the membrane, and contaminants were efficiently washed away in two steps using two different wash buffers. RNA was eluted in RNase-free buffer.

2.2.5 Plasmid DNA extraction

Plasmid DNA was extracted using a Qiagen Plasmid Mini Kit (Qiagen), following the manufacturers instructions. QIAGEN plasmid purification principles are based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to QIAGEN Anion-Exchange Resin under appropriate low-salt and pH conditions. RNA, proteins, dyes, and low-molecular-weight impurities were removed by a medium-salt wash. Plasmid DNA was then eluted in a high-salt buffer and concentrated/desalted by isopropanol precipitation.

2.2.6 Agarose Gel Electrophoresis

PCR amplification products were visualised by gel electrophoresis using agarose gels prepared with Analytical Grade Agarose (Sigma) using flat bed electrophoresis apparatus. Gels were made with 1% w/v agarose in pre-made 1xTBE buffer (Sigma), and 0.1% GelStar Nucleic Acid Stain (Cambrex). PCR products were prepared in 10x loading buffer (Blue Juice - Invitrogen), 1xTBE and electrophoresed at approx 100v for approx 2 hours. Gels were observed using an Ultra Violet imaging system and photographs taken.

2.2.7 Determination of Nucleic Acid Concentrations

The concentration of nucleic acids was determined by spectrophotometry at 260nm. An absorbance of $1.0cm^{-1}$ was taken to be equivalent to 50mg/ml for DNA and 40mg/ml for RNA. For sequencing, the concentration of DNA (e.g. PCR products) was estimated by electrophoresing an aliquot of the DNA solution in an agarose gel (see 2.2.6) and comparing band intensities with that of known standards.

2.2.8 Automated DNA Sequencing

DNA sequencing was performed using an Applied Biosystems (ABI) ABI310 Genetic Analyzer utilising the dideoxy method of DNA sequencing developed by Sanger et al 1977. BigDye Terminator v1.1 reagents were supplied by Applied Biosystems.

2.2.9 Nucleotide and Protein Sequence Analysis

Computer aided analysis of nucleotide sequences were performed using the DNASTAR and BioEdit suite of programs.

2.3 POLYMERASE CHAIN REACTION

2.3.1 General

Polymerase chain reactions (PCR's) were carried out using Roche *Taq* Polymerase (Roche Diagnostics). PCR's were performed using a Perkin Elmer Thermal Cycler (Applied Biosystems). In general, reaction conditions consisted of a 3 minute denaturation step at 95^0C, followed by 30-35 cycles of 95^0C for 30 secs (denaturation), a 30 secs annealing step (5-10^0C below the estimated melting temperature of the primer), and 72^0C for 30-60 secs (extension/polymerisation). A final extension/polymerisation step of 72^0C for 10 minutes was carried out.

2.3.2 Prevention of Contamination

Separate air-spaces were used for reagent preparation, sample processing, manipulation of any positive control material and the amplification and analysis of PCR products. A one-way system was followed and no amplified products were removed into the air-spaces used for sample preparation. Sentinel controls for airborne contamination consisting of open tubes containing all of the reagents and primers for amplification were present at all stages of sample preparation. These are processed along with the test samples.

2.3.3 cDNA Preparation

cDNA was prepared from total RNA. The Superscript III First-Strand Synthesis for RT-PCR Kit (Invitrogen) was used to produce cDNA for a two-step PCR process using Oligo$(dT)_{20}$ to prime the reaction. cDNA was stored at -20^0C.

2.4 Eukaryotic Cell Culture

2.4.1 Growth Medium and Cell Lines

MDBK (ATCC # CCL-22) cells were used in this study. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) containing either 1% or 10% foetal calf serum (FCS) (Invitrogen) previously screened for the presence of BPyV by QPCR and shown to be negative. All cells were grown at 37^0C and were either fed and gassed with 5% CO_2 (for cells grown in 1% FCS) or passaged, fed and gassed with 5% CO_2 (for cell grown in 10% FCS) weekly.

2.4.2 Passaging of Cells

Cells grown in T-75 flasks DMEM supplemented with 1% FCS were re-fed every 7 days. Supernatants (s/n) were harvested and the cell monolayer washed with 5ml of HBSS (Invitrogen). Cells were fed with fresh media and gassed with 5% CO_2. Cells grown in DMEM supplemented with 10% FCS were passaged every 7 days. Supernatant was harvested and the cell monolayer washed with 5ml of HBSS and Trypsin-EDTA (Invitrogen), before incubating with 2ml Trypsin-EDTA at 37^0C. Once cells had detached from the monolayer 8ml PBS (Sigma) was added and 1ml returned to flask (1/10 split). Cells fed with fresh media and gassed with 5% CO_2. Cells were incubated at 37^0C.

2.4.3 Infection of Cell Lines

4ml of BPyV positive supernatant ($2.5x10^6$ ge/ml – see section 2.5.6) was prepared including polybrene (final conc. – 10ug/ml) (Sigma). Media was removed from cells (80-90% confluent) and inoculum added and incubated for 2-3 hours at 37^oC. Inoculum removed, cells fed with fresh media and gassed with 5% CO_2 and incubated at 37^oC.

Negative control flasks were 'inoculated' with 2ml media plus polybrene (final conc. 10ug/ml), and incubated for 2-3 hours at 37^{o}C. Inoculum removed, and cells fed and gassed – incubated at 37^{o}C.

Heat Inactivated (HI) flasks were inoculated with 4ml of heat inactivated BPyV (2.5x10^{6} ge/ml – incubated at 95^{o}C overnight) plus polybrene (final conc. 10ug/ml), and incubated for 2-3 hours at 37^{o}C. Inoculum removed, and cells fed and gassed – incubated at 37^{o}C. Heat Inactivated controls are included in order to show that any positive signals in cells were due to infectious virus particles, and not from residual inoculum.

2.4.4 Harvest of Culture Samples

Flasks of cells grown in 1% FCS were harvested each week – s/n and cells (1% cells are not passaged further to initial findings that BPyV replicates on cells growing at a very low doubling rate). S/n from 10% cells was harvested and cells split 1/10, retaining the remaining 9ml cells for testing (approx 1 x 10^{7} cells). 140ul aliquots of s/n will be taken and stored at –20^{o}C with remaining s/n being stored at –80^{o}C. Cells were spun down and re-suspended in 1mls PBS and frozen at -80^{0}C.

2.4.5 Cell counts

Harvested cells were counted by adding 0.5ml of harvested cells (in 9ml for 10% flasks and 10ml for 1% flasks) to 0.5ml of trypan-blue (Sigma). Cells from each flask were aspirated and added to a hemocytometer and counted x 4, also recording viability (percentage of live cells compared to total cells)

2.5 Q-PCR (Quantitative Polymerase Chain Reaction)

2.5.1 General

The use of real time polymerase chain reaction (PCR) detection allows the quantitative determination of the copy number of a nucleic acid target molecule. TaqMan® PCR detection utilises the 5' exonuclease activity of *Taq* polymerase to hydrolyse an internal TaqMan® probe labeled with a 5' fluorescent reporter dye and a 3' quencher molecule. As amplification of the target molecule proceeds, the reporter dye is released from the 5' end of the probe, and an increase in fluorescence proportional to the increase in PCR product observed. Calculation of the target copy number is based on when amplification of the target is first detected on an ABI 7900HT SDS. The 7900HT system determines the threshold cycle (C_T), defined as the fractional cycle number at which fluorescence passes above a calculated threshold baseline. Absolute quantitation is performed by comparing the C_T values obtained for the unknown samples against the standard curve generated by known numbers of target molecules.

2.5.2 Primer Design

The area of interest (intron/exon junction of large T region (SpliceLargeT-60F, 137R & 86T), Large T DNA (BPyV large T-945F, 1016R & 971T), VP1 region (VP1 100F, 120R & 85T) was inputted into ABI Primer Design software and appropriate primers and probe selected (see 2.1.2).

2.5.3 Q-PCR/RT-QPCR Conditions

Q-PCR reactions plates were cycled as follows.

PCR conditions - DNA targets

Stage 1: 50^{o}C for 2 minutes (AmpErase UNG incubation)
Stage 2: 95^{o}C for 10 minutes (AmpliTaq Gold™ activation)

40 cycles consisting of:

Stage 3: 95^{o}C for 15 seconds (denaturation)
Stage 4: 60^{o}C for 1 minute (annealing/extension)

RT-PCR conditions – RNA targets

Stage 1: 48^{o}C for 30 minutes (reverse transcription)
Stage 2: 95^{o}C for 10 minutes (AmpliTaq Gold™ activation)

40 cycles consisting of:

Stage 3: 95^{o}C for 15 seconds (denaturation)
Stage 4: 60^{o}C for 1 minute (annealing/extension)

2.5.4 Reagents

The relevant primers and probes were added to TaqMan Universal PCR Master Mix, or TaqMan Universal RT-PCR Mix (including 40X superscript RT), and exogenous internal positive control (IPC) (see below). This mix is then aliquoted and the relevant amount of water (for sentinel), negative DNA (for Negative), extracted BPyV material (for test samples), and synthetic oligonuceotides (for positives) added.

2.5.5 Internal Control

Exogenous Internal Positive Control (Applied Biosystems) is an exogenous internal positive control (IPC) reagent included in all QPCR (and RT-QPCR) master mixes. The IPC reagents are included into the target reaction mix to establish that all negative PCR results are truly negative and not due to failed amplification.

2.5.6 Positive Controls

Positive controls (DNA and RNA) were manufactured as synthetic oligonucleotides (Eurogentec) that were designed to span the amplicon of each primer set, with an additional 5 bases in the event of any degradation to the control. Positive controls were supplied lyophilised and resuspended in the recommended volume by the supplier to produce the required concentration. This data was used along with Avagadro's number to calculate the molecules/µl, which is equivalent to genome copies per/µl (or genome equivalents).

2.6 Transfection of MDBK cells with large T mRNA

2.6.1 Growth Medium and Cell Lines

MDBK (ATCC # CCL-22) cells were grown in DMEM containing horse serum (Invitrogen). All cells were grown at 37^0C in vented flasks in a 5% CO_2 incubator.

2.6.2 Kill Curve

Cells were seeded in 6 well plates at $1x10^5$ cells/ml (2ml per well) and incubated at 37^0C in a 5% CO_2 incubator 24 hours prior to transfection. Cells were seeded in medium without antibiotics. 24 hours following seeding, cells

were re-fed with 10% DMEM supplemented with G418 sulphate (Sigma) at concentrations between 5mg/ml and 0.1mg/ml. Cells were then incubated and observed for cell death. Ten days following seeding the concentration of G418 that had killed approx 95% was chosen as the selection concentration to be used following transfection (plasmid used for cloning and transfection contains resistance to G418 – only cells containing this insert will survive). The concentration that was deemed to have killed approx 95% cells was 800µg/ml of G418.

2.6.3 Seeding and Transfection of Cells

Cells were seeded in 6 well plates at $1x10^5$ cells/ml (2ml per well) and incubated at 37^0C in a 5% CO_2 incubator. Cells were seeded in media without antibiotics. Approx 24 hours following seeding the transfection reactions were prepared by diluting large T vector (1-2µg) and Lipofectamine 2000 reagent (Invitrogen) in OptiMEM (Invitrogen). A no-DNA reaction was also set up. Complexes were combined and incubated for 20 mins. Following incubation, reactions were added to the cells (80-95% confluent) containing media. Plates were incubated at 37^0C in a 5% CO_2 incubator. 24 hours following transfection cells were re-fed with media supplemented with 800µg/ml of G418 for selection. Cells were returned to an incubator and observed daily.

2.6.4 Ring cloning

Ten days following transfection, when all no-DNA cells had been killed, colonies of cells were ‘ring cloned’ and transferred to fresh plates. Selection was added and cells incubated. Cells were split to larger flasks upon reaching confluency. 3 weeks following transfection, when cells had been bulked up to T-175 flasks, cells were harvested and tested for the presence of large T mRNA.

2.7 Protein and Western Blot analysis

2.7.1 General

Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot analysis was carried out on cells harvested during the infectivity and transfection assays for the presence of the large T antigen and the cell cycle protein cyclin A involved in cell division.

2.7.2 Preparation of protein for SDS-PAGE and Western Blotting

500µl of each cell suspension was centrifuged at 450g for 5 mins and supernatant removed. Pellets resuspended in 200µl of RIPA buffer (Sigma), vortexed and incubated on ice for 5 minutes. Tubes were vortexed to resuspend and pelleted cells and quick frozen in liquid nitrogen. Samples stored at –80°C.

2.7.3 Quantitation of protein

Protein concentration was carried out using the EZQ Protein Quantitation Kit (Invitrogen). Approximately 6µg of protein was used during SDS-Page and Western Blotting.

2.7.4 SDS-PAGE Gel Staining

Samples were analyses using reducing SDS-PAGE and stained using GelCode Blue Stain Reagent (Pierce) the resulting banding patterns analysed, scanned and stored.

2.7.5 Western Blotting

Samples were analysed using reducing SDS-PAGE combined with Western blotting. The components to be stained are resolved during gel

electrophoresis according to their molecular weight. The gel is then blotted on to a PVDF (Polyvinylidene fluoride) (Millipore) membrane where it is then either washed with SV40 large T Antigen (BD Biosciences) or Anti-cyclin A (AB-3) Mouse mAb (Calbiochem) primary antibodies and Anti-Mouse IgG (Fc specific) Peroxidase Antibody (Sigma) secondary antibody. The membranes were developed using 3,3'-Diaminobenzidine (DAB) Enhanced Liquid Substrate System tetrahydrochloride (Sigma). The developed film was scanned and stored.

Table 4 – Reagents

Reagent	Supplier
Eukaryotic TA Expression Kit Bidirectional	Invitrogen
MDBK cells	ATCC
QPCR/RT-QPCR Primers	Applied Biosystems
PCR Primers	TAGN Newcastle
QIAamp DNA Mini Kit	Qiagen
RNeasy Mini Kit	Qiagen
QIAamp Viral RNA Mini Kit	Qiagen
Plasmid Mini Kit	Qiagen
Analytical Grade Agarose	Sigma
1xTBE buffer	Sigma
GelStar Nucleic Acid Stain	Cambrex
Blue Juice	Invitrogen
BigDye Terminator v1.1 Reagents	Applied Biosystems
Taq Polymerase	Roche Diagnostics
Superscript III First-Strand Synthesis Kit	Invitrogen
Dulbecco's Modified Eagle's Medium	Invitrogen
Foetal Calf Serum	Invitrogen
HBSS	Invitrogen
Trypsin-EDTA	Invitrogen
PBS	Sigma
Polybrene	Sigma
Trypan-Blue	Sigma
TaqMan Reagents	Invitrogen

Synthetic oligonucleotides	Eurogentec
Horse serum	Invitrogen
G418 sulphate	Sigma
Lipofectamine 2000	Invitrogen
OptiMEM	Invitrogen
SDS-PAGE gels	Invitrogen
RIPA buffer	Sigma
EZQ Protein Quantitation Kit	Invitrogen
GelCode Blue Stain Reagent	Pierce
PVDF membranes	Millipore
SV40 large T Antigen	BD Biosciences
Anti-cyclin A (AB-3) Mouse mAb	Calbiochem
Anti-Mouse IgG (Fc specific) Peroxidase Antibody	Sigma
3,3'-Diaminobenzidine	Sigma

Chapter 3 Assay Validation

3.1 Principles of QPCR and RT-QPCR

QPCR and RT-QPCR assays allow highly sensitive and specific detection of DNA and RNA sequences as well as offering the ability to quantify the target sequence. Specific instruments (ABI Sequence Detection Systems (SDS) 7700/7900) are able to detect fluorescence during QPCR, allowing the 'real-time' detection of PCR product accumulation via the hydrolysis of probes labeled with both a fluorescent reporter and a quencher. Quantitative data is derived from a determination of the cycle at which the amplification product signal crosses a pre-set detection threshold (threshold cycle, C_T value), which is proportional to the amount of starting material.

3.2 Assay Validation

It is necessary that analytical procedures are appropriately validated to demonstrate that they are suitable for the intended use. The intention of the QPCR (and RT-QPCR) assays described in this thesis is to measure the amount of viral targets present in cell and supernatant harvests. In light of this information, the following validation characteristics have been evaluated for all QPCR and RT-QPCR assays utilised during this project. Assay validation was carried out in accordance with the ICH Topic Q2A and Q2B.

3.2.1 Accuracy and Precision

The accuracy of the assay was assessed by spiking known amounts of BPyV synthetic control into various substances such as water, MDBK DNA, and contaminants expected to be present in the testing products (trypsin and protein). The precision (and repeatability) of the QPCR's was assessed by carrying out multiple runs with different operators and machines.

3.2.2 Specificity

Specificity of the assay was assessed by testing each QPCR assay in the presence of components most likely to be present. For these QPCR's DNA from various species was considered the most appropriate. Bovine (MDBK), Human (Human Placental DNA), and Rodent (Chinese Hamster Ovary) DNA were all used to asses specificity. Other polyoma viruses were unavailable during validation to determine specificity.

3.2.3 Quantitation Limit, Linearity and Range

The quantitation limit was assessed by analysing each positive control in replicates of 8, and determining the lowest concentration to show precise and accurate results. Linearity and range was assessed by analysing each standard in triplicate and determining the level that had a suitable level of precision, accuracy and linearity. The r^2 data generated refers to the squared coefficient of correlation and should be above 0.98 (recommended by the manufacturers of the TaqMan system). For the slope, an efficiency of 100% would relate to a value of -3.3. An acceptable reaction should have an efficiency between 90% and 110%, which corresponds to a slope between -3.6 and -3.0.

3.2.4 Primer/Probe Optimisation

The primer and probe concentrations were optimised prior to validation to ensure optimal reaction concentrations. This is achieved by varying the concentrations of firstly the primers, and then the probe.

CHAPTER 4 - Results

What levels of large T mRNA expression are observed with BPyV in comparison to other polyomaviruses such as SV-40, and does infection of rapidly growing cells with BPyV result in eventual disappearance of the virus from the culture system?

What are the levels of large T mRNA prior to the onset of viral replication, and could a more efficient infectivity assay be developed by inoculating material onto cells expressing high levels of the large T antigen? The ability of other polyomavirus to move the infected cell into S-phase has been determined, but it is the same for cells infected with BPyV?

4.1 Validation Results

Primer and probe optimisation studies demonstrated the optimal primer probe combination to be 900:900:225μM (Forward Primer: Reverse Primer: Probe) for all assays. All assays were shown to be precise, specific, accurate and robust (assay sensitivity or linearity consistent using different batches of reagent on different days). The range was determined to be $1x10^1$ to $1x10^6$ copies (or genome equivalents) per reaction for both QPCR's, and $1x10^2$ to $1x10^6$ copies/reaction for the RT-QPCR. The limit of quantitation for both QPCR's was determined to be $1x10^1$ copies per reaction, the lowest level at where precision and accuracy where observed. The limit of quantitation of the RT-QPCR's was determined to be $1x10^2$ copies per reaction. Quantities above $1x10^6$ will still be reported for all assays but it should be noted that these results may not be accurate.

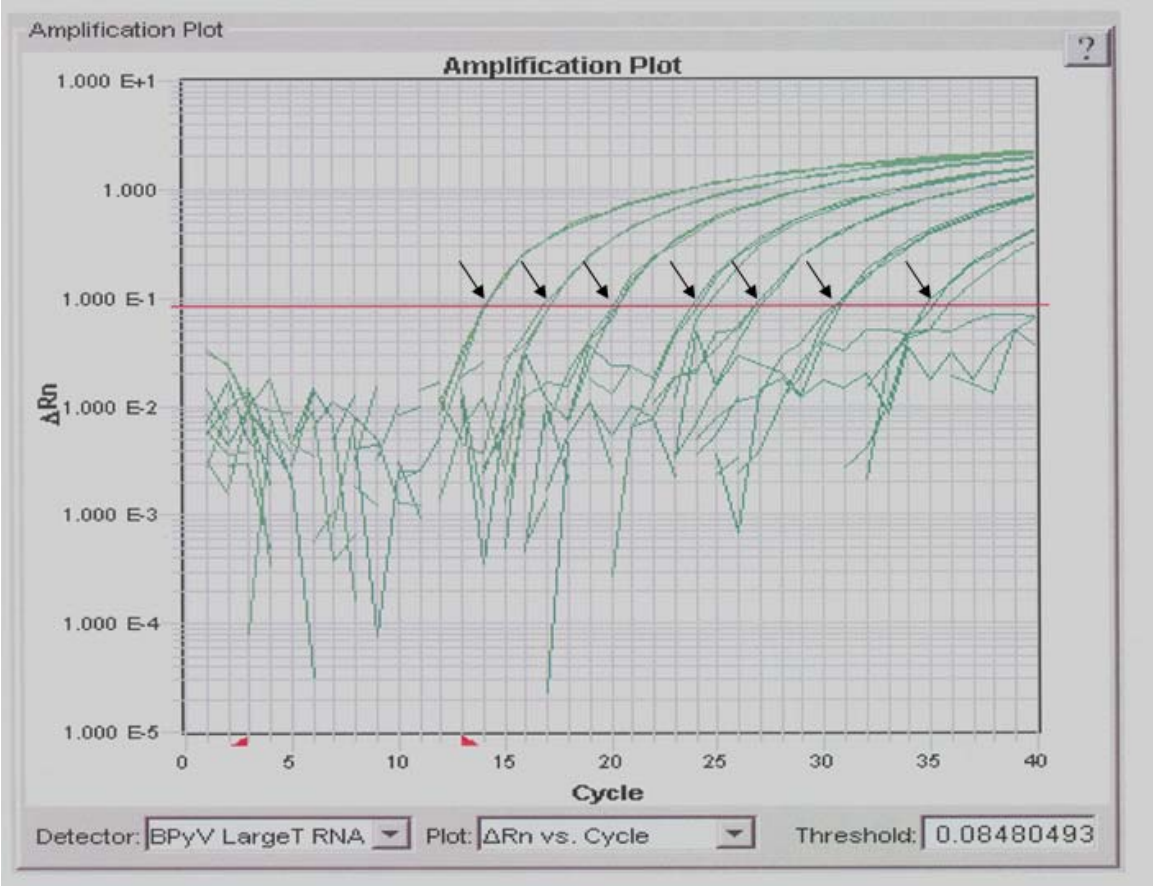


Figure 8 - Example of amplification plots (using large T mRNA primers and probes). Amplification plots for serial dilutions of large T RNA positive controls (tested in triplicate) are shown in green (marked with arrows). The highest concentration ($1x10^8$) is shown at the left of the plot, and the lowest concentration ($1x10^2$) is shown at the right of the plot. The red line is referred to as the 'threshold' and is set as close to the exponential phase of amplification as possible. The red arrows are referred to as the 'baseline' and are set to minimize background signals. The point at which each dilution crosses the threshold is plotted in a standard curve (see figure 9) and quantities of unknowns calculated (Source – MSc experimental data).

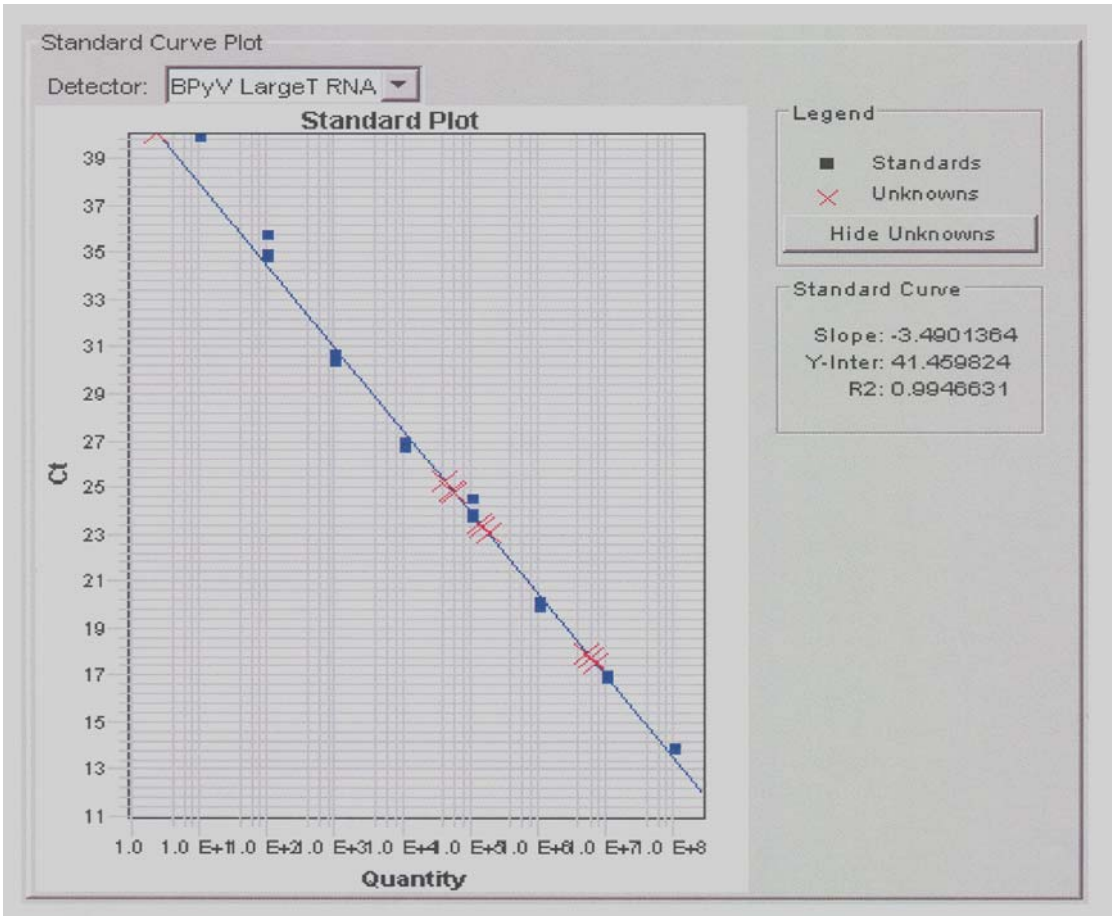


Figure 9 - Example of a Standard Curve. The data from the amplification plot detailed in figure 8 was plotted in this standard curve. The C_T numbers refers to the threshold cycle, which is the cycle that the amplification plot passes through the red threshold line (see figure 8). The C_T values of unknown samples are plotted against the curve (as shown with the red x's) and quantities calculated. The statistical analysis of the slope indicates how linear and therefore quantitative the assay has been. The optimal slope is -3.3, Y-Inter 40 (as this assay was run over 40 cycles) and R^2 1.00. Slope values of -3.0 and -3.6, and R^2 values above 0.98 are indicative of a linear assay.

4.2 Are there differences between BPyV cultured on cells supplemented with 1 and 10% FCS?

Nairn et al documented in 2003 that the mode of replication for BPyV was unusual when compared to other polyomaviruses. It was observed that viral replication was only detected after 5-8 weeks of continuous culture (with no passaging) on cells doubling at a low rate in media supplemented in 1% Foetal Calf Serum (FCS) (Personal Reference, Nairn *et al.,* 2003). What is

the mechanism of replication, and is the inoculation and subsequent growth of BPyV on cells supplemented with 10% FCS different?

To assess this an 8-week infectivity assay was initiated as detailed in section 2.4 of the Methods and Materials (Eukaryotic Cell Culture). Supernatant and cells were harvested weekly from MDBK cells inoculated with BPyV and cultured in DMEM supplemented with 1% and 10% FCS. A heat inactivated (HI) control (which was inactivated by incubation at 95ºC for 24 hours) was also included in order to show that positive signals in cells were due to infectious virus particles, and not from residual inoculum. During preliminary studies (Personal Reference, Nairn *et al.,* 2003), amplification signals were detected in cells not able to support BPyV viral replication. Inoculation of these cultures with HI inactivated BPyV generated similar results, proving that signals detected were due to residual inoculum, and not true amplification.

Supernatants were harvested from flasks and nucleic acid extracted using a Qiagen DNA Mini kit (Section 2.2.2 of Materials and Methods) and tested by Q-PCR with primers and probes (BPyV LargeT-945F, 1016R and 971T) designed to the large T region of the BPyV viral genome. Extracted material was tested along with a synthetic DNA oligonucleotide diluted to represent 10^1-10^6 genome equivalents in order to quantitate any viral genomes present.

During the first 3 weeks of the infectivity assay BPyV viral genomes were detected in cells supplemented with both 1% and 10% FCS, although signals detected in 1% FCS samples were several logs higher following week 1. From week #'s 4 to 8 significant increases in BPyV viral genomes was only detected in cells grown in 1% FCS, with no viral sequences detected following week 3 in cells grown in 10% FCS (results illustrated in figure 10).

In summary, infection of rapidly growing cells with BPyV (being passaged and fed with media supplemented with 10% FCS) resulted in the eventual disappearance of the virus from the culture system. In contrast it takes 4-5 weeks of continuous culture in cells at a very low doubling rate, only being fed

weekly with media supplemented with 1% FCS and not passaged, before exponential virus replication is detected. This is uncharacteristic among polyomaviruses in which the complete replication cycle takes 48-72 hours. Polyomaviruses only have 4-5 functional genes so rely heavily on cellular mechanism for replication. Therefore BPyV inoculated onto cells that are constantly multiplying (as they would be if grown in media supplemented in 10% FCS and passaged regularly) should be replicating. In this period no viral replication was observed in the negative or HI controls (figure 11).

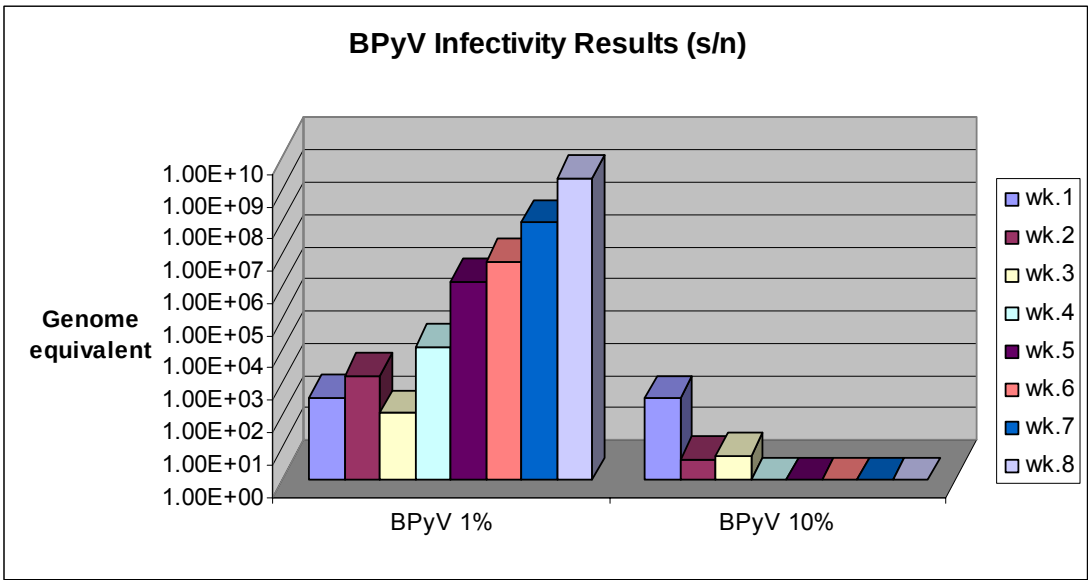


Figure 10 - Comparison of BPyV viral genomes in infected MDBK cells grown in DMEM supplemented with 1% and 10% FCS. Exponential amplification can be observed from wk 4 in MDBK cells infected with BPyV and grown in media supplemented with 1% FCS. No amplification is observed from week # 4 in the 10% cultures. Data shown is from duplicate flasks tested and mean quantities calculated.

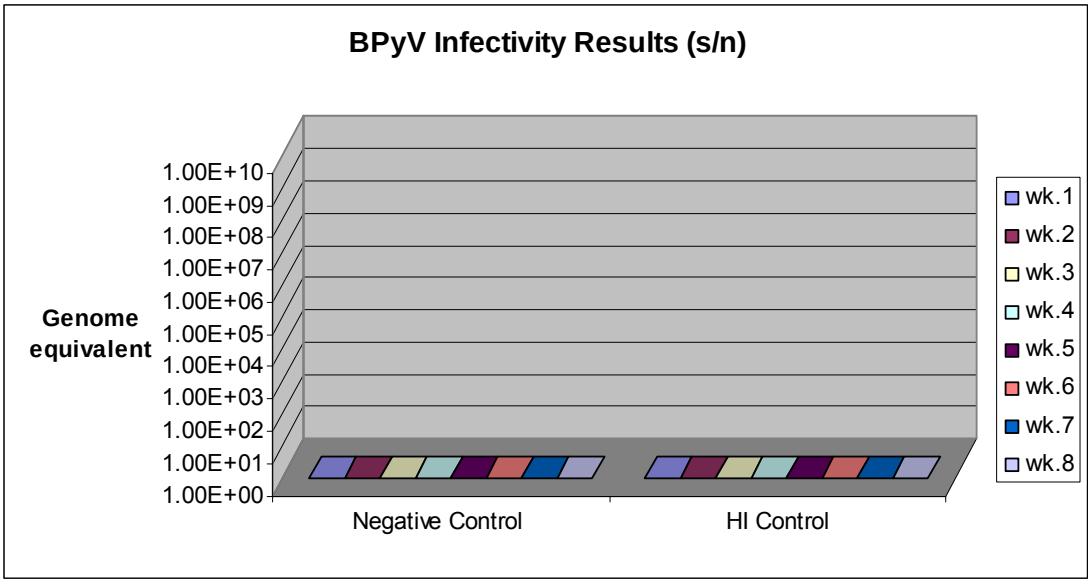


Figure 11 - Assessment of BPyV viral genomes (DNA) in negative and heat inactivated (HI) flasks. No amplification is observed in any of the controls. Data shown is from duplicate flasks tested.

4.3 At what stage is large T mRNA expressed?

During the same 8 week period that supernatant was harvested as detailed in section 4.2, cells were also harvested and total RNA extracted (as detailed in section 2.2.3) in an attempt to quantitate BPyV large T mRNA.

Of the early phase antigens that are expressed during BPyV replication, the most critical is the large T antigen, which is thought to be directly responsible in the initiation of viral replication. Extracting RNA from cells harvested each week would indicate when large T mRNA was expressed but also show if there was any correlation between large T mRNA expression and BPyV viral genome replication. Extracted RNA was tested by RT-QPCR with primers and probes (SpliceLargeT-60F, 137R and 86T) designed to the large T region (intron-exon region in an attempt to only detect mRNA). RT-QPCR's were analysed using a synthetic RNA oligonucleotide in the range of 10^1 – 10^6 genome equivalents in order to quantitate mRNA targets. Samples were tested with and without RT (which reverse transcribes RNA to DNA) in order to distinguish between viral mRNA and viral genomic material. Primers and

probe are designed in an intron-exon region, which should only amplify reverse transcribed RNA, but there is always a possibility that viral genomic material will also be amplified. Analyzing samples plus and minus RT allows determination of mRNA levels by subtracting the levels of minus RT reactions from plus RT reactions.

Large T mRNA was not detected until weeks 4-5 of the culture period, when exponential replication of BPyV was also detected in the supernatant. The expression of mRNA from both flasks is similar, although mRNA expression was only detected week 4 in one of the duplicate flasks (results illustrated in figure 12).

Virus inoculated onto cells grown in media supplemented with 10% FCS showed no production of large T mRNA, although detection of replicating virus in these cells during wk #'s 1-3 indicates that large T mRNA must be present, but potentially at a level lower than the limit of quantitation for the RT-QPCR assay (which is $1x10^2$ copies per reaction) (results illustrated in figure 13). This may also be the case for cells grown in media supplemented with 1% FCS (figure 10). No mRNA expression was detected in the negative and heat inactivated cells (figure 14).

It is possible that large T mRNA is expressed in the first 3-4 weeks of culture, but errors during transcription is resulting in large T mRNA being unable to be detected by the current primer/probe design. To determine if large T antigen was present in these initial harvests, cells were prepared for protein analysis by SDS-PAGE as detailed in section 2.7 (figure 15).

Gels were set up with kD protein markers (lane 1 and 12), week 1 and 4 1% FCS MDBK negative controls from the 8-week infectivity assay (lane 2 and 3) and wks 1 - 8 1% FCS A MDBK samples from the 8-week infectivity assay (lanes 4-11). There is no protein band present in the samples that can correlated to the large T antigen (approx 65kD). It may be noted that the production of the large T antigen is heavily regulated, and therefore may not

be at a level that would allow visualization. There is also the potential that some cellular proteins are masking the appearance of the large T antigen. Can these large T antigens be detected by western blot analysis with antibodies specific to large T?

Western blots were prepared (section 2.7 of the methods and materials) using a large T specific primary antibody in an attempt to detect the large T antigen. Western blots were set up with a kD protein marker (lane 1), week 1, 4 and 8 1% FCS MDBK negative controls from the 8-week infectivity assay (lane 2 - 4), wks 2 - 8 1% FCS A MDBK samples from the 8-week infectivity assay (lanes 5-11), and a positive control (SV-40 infected Vero cells - lane 12). No bands were observed in lanes 2 (week 1, 1% negative control) and 6 (wk 4 1% FCS A sample). No bands of the excepted size (approx. 65kD) were present in the remaining lanes. Faint bands are present above the 60kD marker for week 4 and 8 1% FCS negative control, and weeks 2, 3 and 5 – 8 1% FCS A sample, however these must be non-specific due to observations in the negative controls (lanes 3 and 4) (figure 16). No cells were available for assessment due to poor cell growth for week 1 (1% FCS A or B samples) inoculated flasks. The only commercial large T antibody that was available was produced to the large T antigen of SV-40, and it was unclear if this antibody would also be specific to the large T antigen of BPyV. The SV-40 large T antigen is approx 79kD. Confirmation of large T mRNA expression was shown by RT-QPCR, but subsequent experiments proved inconclusive, although several factors (low levels of expression, unspecific primary antibodies) may have been responsible for this.

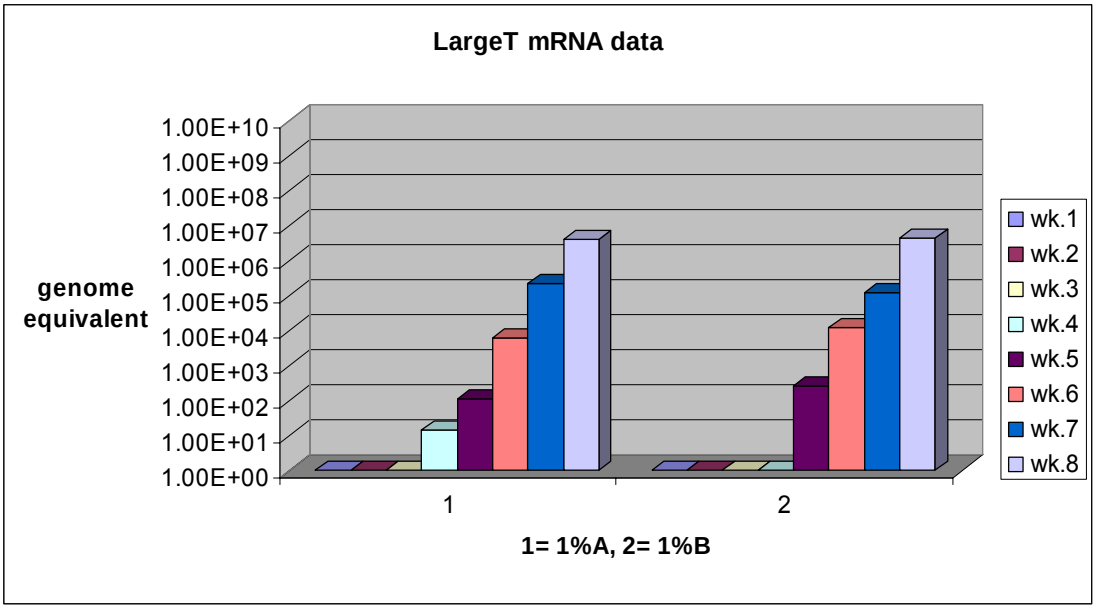


Figure 12 - Data from duplicate flasks of MDBK cells grown in media supplemented with 1% FCS and inoculated with BPyV. Expression of large T mRNA can be observed wk 4 for the 1%A harvests, and wk 5 for the 1%B harvests.

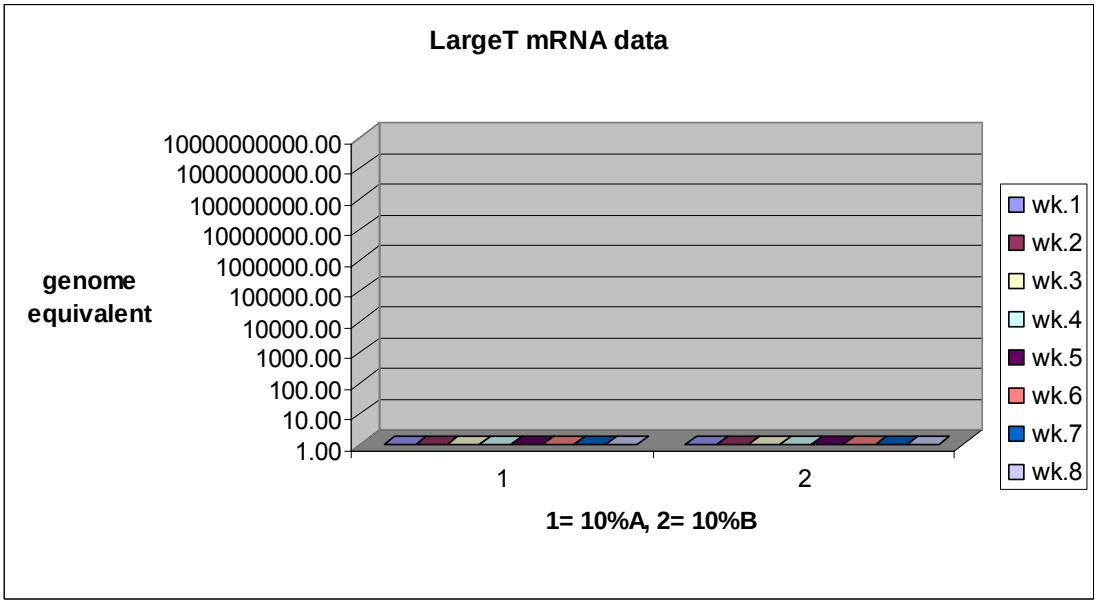


Figure 13 - Data from duplicate flask of MDBK cells grown in media supplemented with 10% FCS and inoculated with BPyV. Expression of large T mRNA was not detected in any of the harvests, although the detection of viral genomes at the start of the infectivity assay indicates that large T mRNA may have been present in the first 3 weeks.

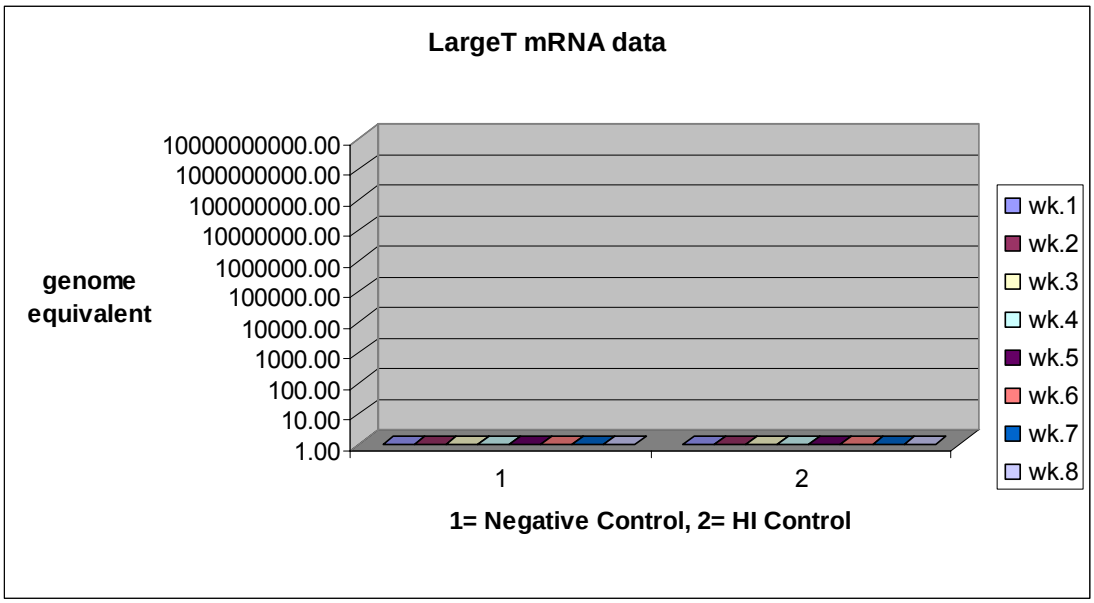


Figure 14 - Data from negative and heat inactivated (HI) control flasks. No amplification is observed in any of the controls. Data shown is from duplicate flasks tested.

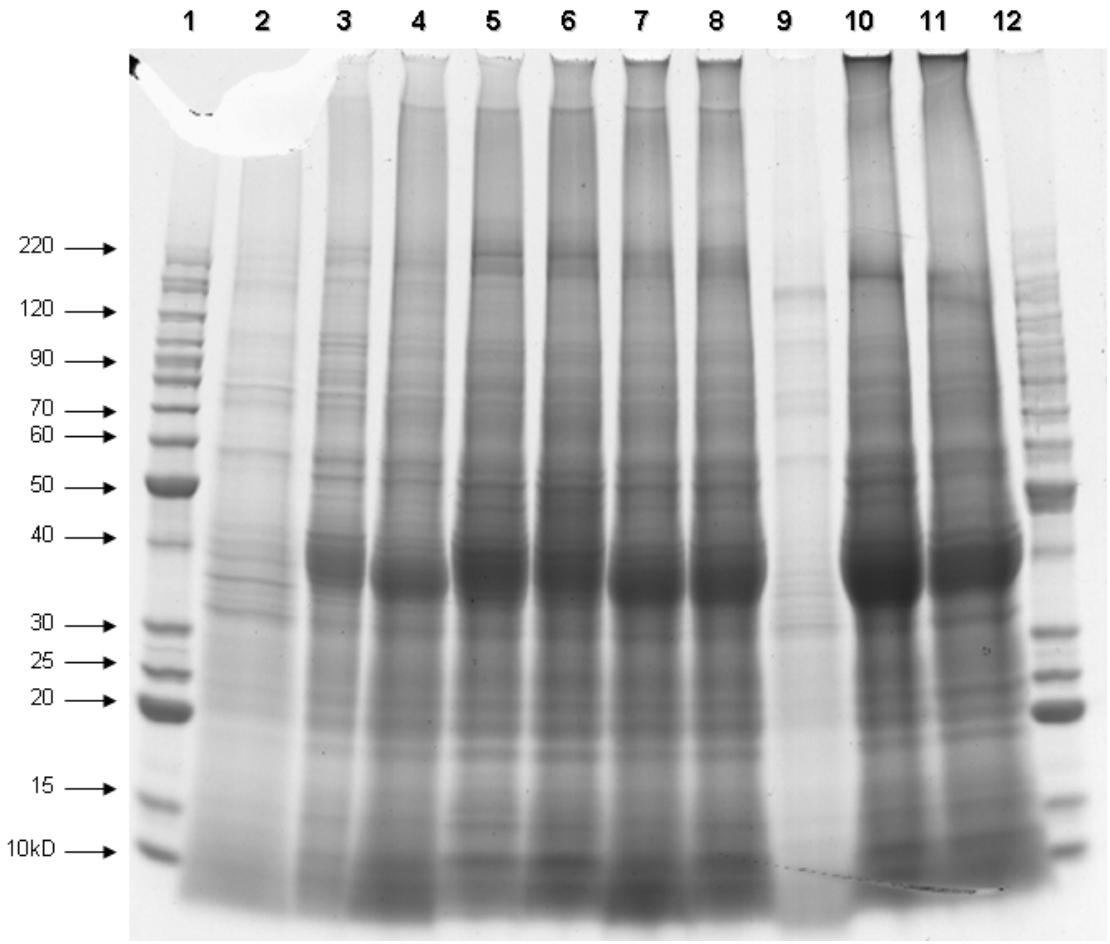


Figure 15 – SDS-PAGE using 8-week infectivity cell cultures (stained with GelCode Blue Stain). Approximately 6µg of protein was assessed. No bands are present that would indicate the presence of the large T antigen (65kD). Gels were run with kD protein markers (lane 1 and 12), infectivity negative controls (MDBK) cultured in 1% FCS and harvested week 1 and 4 (lane 2 and 3) and infectivity samples (MDBK) inoculated with BPyV, cultured with 1% FCS, and harvested week 1 to week 8 (lanes 4-11). Large T mRNA was detected by RT-QPCR in infectivity samples inoculated with BPyV and harvested week 4 to week 8 (lanes 7-11) (figure 12).

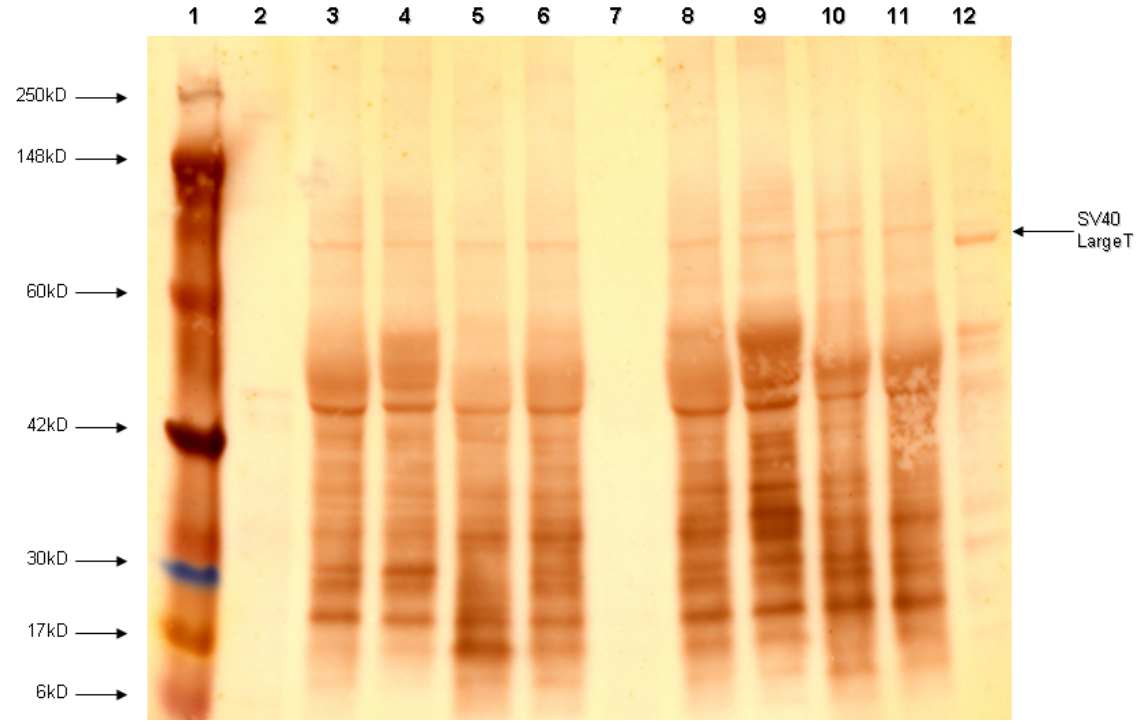


Figure 16 – Western Blot analysis of 8-week infectivity cell cultures using SV40 large T primary antibody and Anti-Mouse IgG Peroxidase secondary antibody. Membranes were developed using the DAB Substrate system. Approximately 6µg of protein was assessed. No large T antigen bands are present (approx. 65kD). Faint bands are present just above the 60kD marker in lanes 3-6 and 8-11, however must be non-specific due to observations in the negative controls (lanes 3 and 4). The large T antibody was raised against SV-40 large T antigen – it is unclear if this antibody will also be specific to BPyV. Western Blots were set up with a kD protein marker (lane 1), infectivity negative controls (MDBK) cultured in 1% FCS and harvested week's 1, 4 and 8 (lane 2 - 4), infectivity samples (MDBK) inoculated with BPyV, cultured with 1% FCS, and harvested week 2 to week 8 (lanes 5-11), and a positive control (SV-40 infected Vero cells - lane 12).

4.4 Are infected cells in S phase?

During the same 8-week infectivity period that BPyV and large T mRNA sequences were assessed (sections 4.2 and 4.3), cells concentrations, viability and cyclin A expression were also assessed.

If polyomaviruses require cellular mechanisms in order to replicate, and one of the functions of the large T antigen is to move cells into S phase to support viral replication, then performing cell counts at each harvest could potentially

explain why we are only detecting high titres of virus in cells grown in media supplemented with 1% FCS. Assessing viability of cells during this 8-week infectivity period will also be of interest, to determine if viral infection is causing any cell death. Of particular interest are the cells grown in media supplemented with 1% FCS as they are not passaged for the entire 8 week experiment, and only fed with fresh media weekly.

When exponential amplification is observed (4-5 weeks), all cell counts decreased gradually, with the exception of the cells grown in media supplemented with 1% FCS, which are the cells in which BPyV amplification is being detected (figure 17). Counts for cells grown in the 10% FCS B flask increased after this period, but from week 6 the cell counts gradually decline, unlike the cells grown in media supplemented in 1% FCS which remain constant (figure 18). There were no differences in cell viability which remained between 95% and 100% throughout the experiment (figure 19). Does the large T antigen induce S-phase allowing cellular and viral replication, as reported for other polyomaviruses?

If BPyV does have the ability to move cells into S-Phase, which has been indicated by counting viable cells, then would it be possible to detect any S-Phase promoting factors such as cyclin A or the absence of proteins such as cyclin E, which are destroyed during this phase? In order to determine if the cells were indeed moving into S-Phase towards the end of the infectivity period, samples that were previously analysed (section 4.2 and 4.3) for the presence of BPyV and large T mRNA were also assessed by western blot analysis (as detailed in section 2.7) with an antibody specific to cyclin A (figure 20).

Western blots were set up with a kD marker (lane 1), week 1, 4 and 8 1% FCS negative MDBK control samples from the 8-week infectivity assay (lane 2 - 4), wks 2 - 8 1% FCS A MDBK samples from the 8-week infectivity assay (lanes 5-11), and a positive control (HeLa cells - lane 12). Lanes 2 (week 1, 1% negative control) and 7 (wk 4 1% FCS A sample) did not show any bands.

However, in lanes 9 – 11 (wks 6, 7 & 8 1% FCS A samples), bands can be observed that are the size expected from cyclin A (approx. 65kD). These bands do not appear to be present in earlier harvests or negatives. Closer examination of the gels does show bands in other samples, but this is to be expected as there will be some growth or turnover of cells even in fully confluent cultures. These results confirm the theory that BPyV is able to move the cells into S-Phase to necessitate replication. FACS (fluorescence-activated cell sorter) analysis would have been a useful tool is confirming these observations, but unfortunately this technology was not available during this experiment.

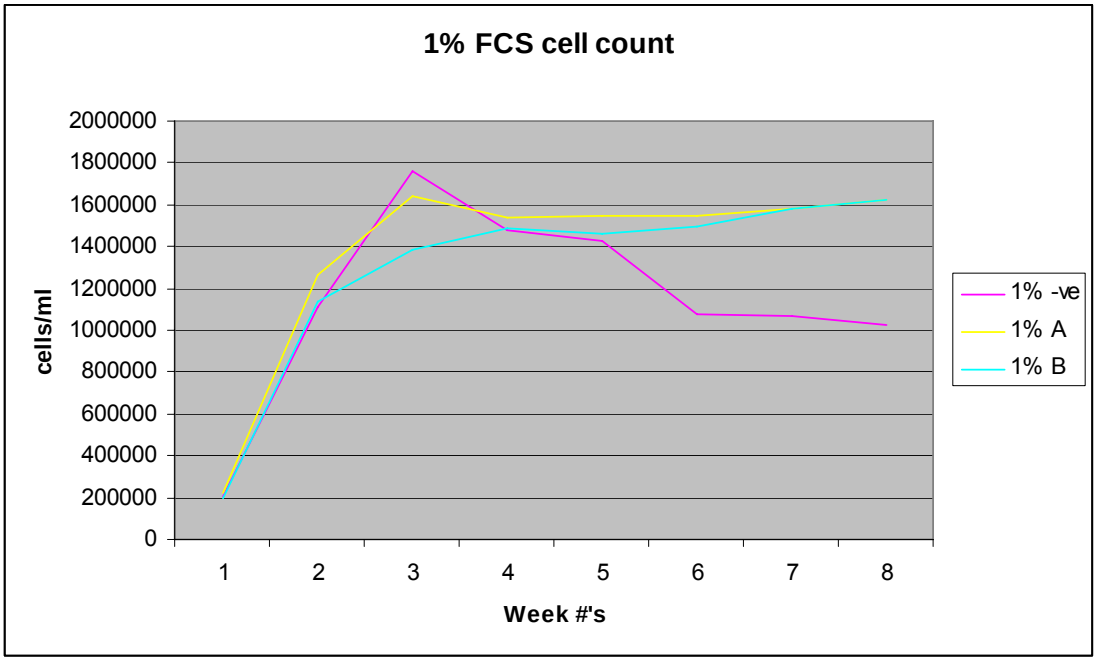


Figure 17 - Cell counts for MBK cells inoculated with BPyV and maintained with media supplemented with 1% FCS. With the exception of the –ve cells, counts from the A and B flasks remained constant from wk's 4-8, at the same time that exponential amplification was observed.

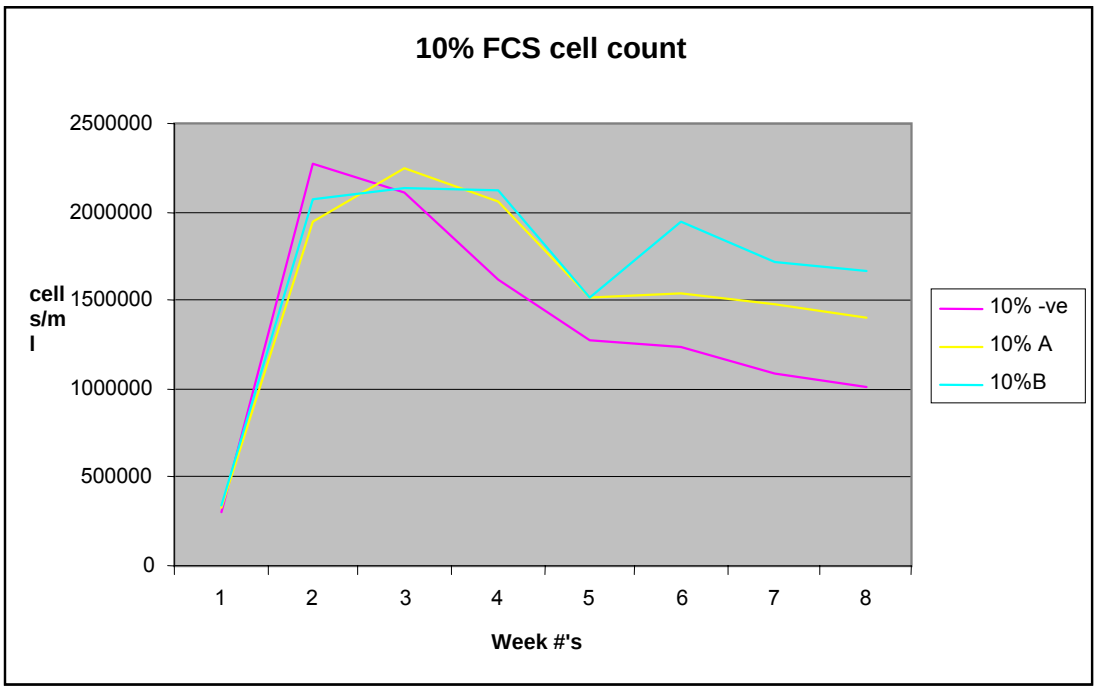


Figure 18 - Cell counts for MDBK cells inoculated with BPyV and maintained with media supplemented with 10% FCS. With the exception of counts carried out during week 6 of the 10% B cells, counts gradually decreases from week's 3-4. No viral amplification is detected from this time.

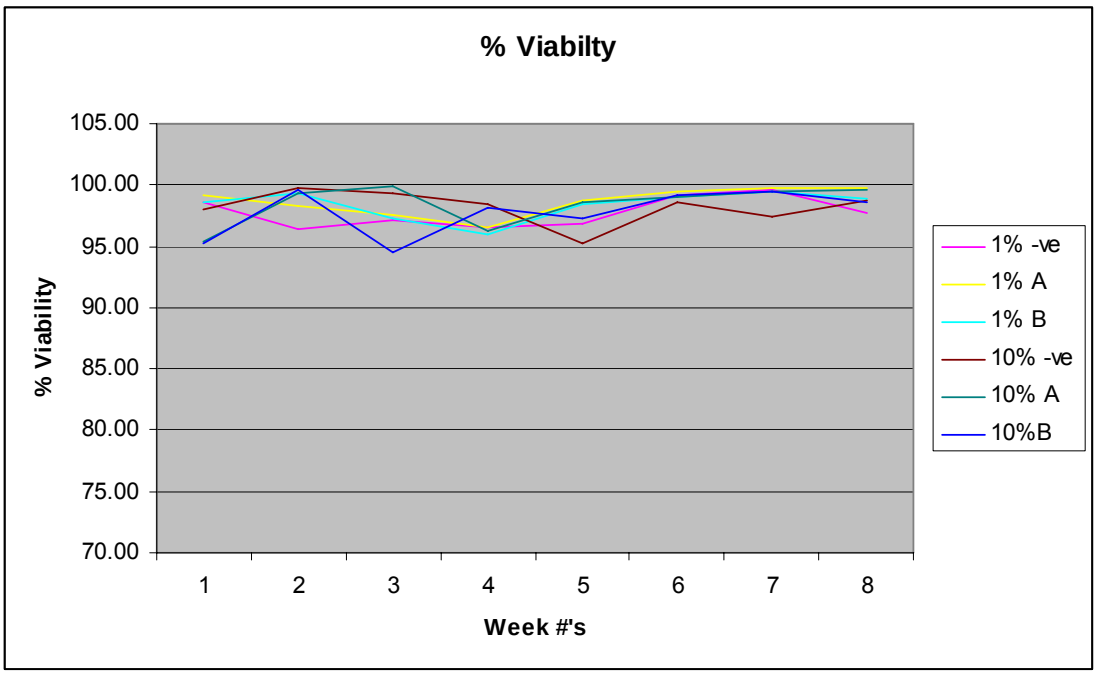


Figure 19 - Percentage viability for MDBK cells inoculated with BPyV and maintained with media supplemented with 1% and 10% FCS. % viabilities vary little over the 8 week infectivity assay.

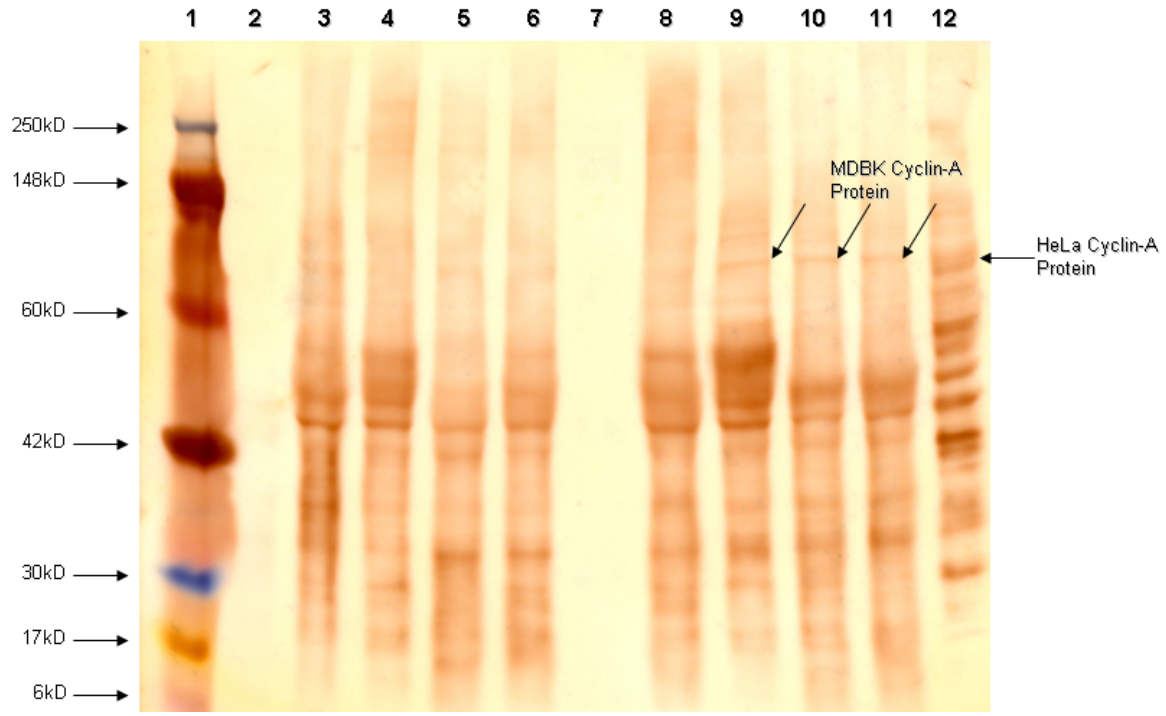


Figure 20 – Western Blot analysis of 8-week infectivity cell cultures using Anti-cyclin A primary antibodies and Anti-Mouse IgG Peroxidase secondary antibody. Membranes were developed using the DAB Substrate system. Approximately 6µg of protein was assessed. Bands are present at the size expected for cyclin A (65kD) in week 6, 7 & 8 harvests. Bands at this intensity are not observed in any other harvests. Western blots were set up with a kD protein marker (lane 1), infectivity negative controls (MDBK) cultured in 1% FCS and harvested week's 1, 4 and 8 (lane 2 - 4), infectivity samples (MDBK) inoculated with BPyV, cultured with 1% FCS, and harvested week 2 to 8 (lanes 5-11), and a positive control (HeLa cells - lane 12). The difference in size between the MDBK and HeLa cyclin A protein is expected.

4.4.1 Statistical analysis of data presented in section 4.2 to 4.4

An attempt to apply statistical analysis to the data generated within these sections was unsuccessful due to low numbers of replicates and high variability within the data set. The student (paired) t-test could have provided meaningful data, but one of the preconditions for doing this test was that 'there should not be significant differences in the Standard Deviations from the data set' (Wardlaw, 1985). It should be noted that if differences can

biologically confirmed, which has been clearly demonstrated, then statistical analysis has limited use.

4.5 Does sequencing of the large T antigen isolated from cultures differ from published sequences?

As part of the experiment that was initiated to clone the large T mRNA for expression in MDBK cells (as detailed in section 4.6), a clone of large T mRNA was sequenced to confirm correct coding and orientation.

During experiments detailed in sections 4.3, it was suggested that the large T antigen may have been defective, or differed in sequence from published data. Sequencing of this clone could potentially clarify this. The sequence of the large T mRNA (as detailed in section 2.2.8 of the Materials and Methods section) obtained from the clone was compared with the large T mRNA sequence published by Schuurman *et al* (figure 21) and subsequent sequences differences highlighted in grey.

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                  ....|....| ....|....| ....|....| ....|....| ....|....|
                          10         20         30         40         50
BPyV(Schur)RC     ATGGAATTAA CATCTGAGGA ATATGAGGAG CTTAGGGGGC TCTTAGGAAC
LargeT            ATGGAATTAA CATCTGAGGA ATATGAGGAG CTTAGGGGGC TCTTAGGAAC

                  ....|....| ....|....| ....|....| ....|....| ....|....|
                          60         70         80         90        100
BPyV(Schur)RC     CCCTGATATT GGCAATGCAG ATACTTTGAA AAAGGCATTC CTGAAGGCAT
LargeT            CCCTGATATT GGCAATGCAG ATACTTCGAA AAAGGCATTC CTGAAGGCAT

                  ....|....| ....|....| ....|....| ....|....| ....|....|
                         110        120        130        140        150
BPyV(Schur)RC     GCAAGGTGCA TCATCCAGAT AAAGGTAAAT ATATTTAGTA TTGATCTGTA
LargeT            GCAAGGTGCA TCATCCAGAT AAAGGTAAAT ATTTTTAGTA CTGATCTGTA

                  ....|....| ....|....| ....|....| ....|....| ....|....|
                         160        170        180        190        200
BPyV(Schur)RC     CATGCAAATC TTGTTTACAG GCAGTAAATC TGATTTTATT TTTAGGAGGG
LargeT            CATGCAAATC TTGTTTACAG GCAGTAAATC TGATTTTATT TTTAGGAGGG

                  ....|....| ....|....| ....|....| ....|....| ....|....|
                         210        220        230        240        250
BPyV(Schur)RC     AATGAAGAAG CAATGAAAAG ACTTCTGTAT TTGTATAATA AAGCAAAAAT
LargeT            AATGAAGAAG CAATGAAAAG ACTTCTGTAT TTGTATAATA AAGCAAAAAT
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                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         260        270        280        290        300
BPyV(Schur)RC    TGCTGCAAGT GCCACTACTA GCCAGGTATG GTATTTCTTA ATTATTGGAT
LargeT           TGCTGCAAGT GCCACTACTA GCCAGGTATG GTATTTCTTA ATTATTGGAT

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         310        320        330        340        350
BPyV(Schur)RC    ATATAAGCTT AAAAAATAAA AATATATACT TACCTAAGAT TTTCTGGTTA
LargeT           ATATAAGCTT AAAAAATAAA AATATATACT TACCTAAGAT TTTCTGGTTT

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         360        370        380        390        400
BPyV(Schur)RC    AGGTTCCAGA ATATGGCACC TCACAGTGGG AACAGTGGTG GGAAGAATTC
LargeT           AGGTTCCAGA ATATGGCACC TCACAGTGGG AACAGTGGTG GGAAGAATTC

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         410        420        430        440        450
BPyV(Schur)RC    AATCAAGGCT TTGATGAGCA AGATCTGCAT TGTGATGAGG AATTAGAGCC
LargeT           AATCAAGGCT TTGATGAGCA AGATCTGCAT TGTGATGAGG AATTAGAGCC

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         460        470        480        490        500
BPyV(Schur)RC    ATCAGATAAT GAGGAAGAAA ATCCGGCGGG AAGCCAGGCC CCGGGAAGTC
LargeT           ATCAGATAAT GAGGAAGAAA ATCCGGCGGG AAGCCAGGCC CCGGGAAGTC

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         510        520        530        540        550
BPyV(Schur)RC    AAGCCACCCC ACCCAAGAAA CCTAGAACAT CACCTGACTT TCCTGAGGTT
LargeT           AAGCCACCCC ACCCAAGAAA CCTAGAACAT CACCTGACTT TCCTGAGGTT

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         560        570        580        590        600
BPyV(Schur)RC    TTGAAAGAAT ATGTAAGCAA TGCTCTGTTC ACAAATAGAA CATATAATTG
LargeT           TTGAAAGAAT ATGTAAGCAA TGCTCTGTTC ACAAATAGAA CATATAATTG

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         610        620        630        640        650
BPyV(Schur)RC    CTTTATAATA TTTACTACTG CTGAGAAAGG AAAAGAATTA TATCCTTGTA
LargeT           CTTTATAATA TTTACTACTG CTGAGAAAGG CAAAGAATTA TATCCTTGTA

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         660        670        680        690        700
BPyV(Schur)RC    TCCAGGCAGC CTACAAATGT ACATTTATTG CCTTATATAT GTATAATGGT
LargeT           TCCAGGCAGC CTACAAATGT ACATTTATTG CCTTATATAT GTATAATGGT

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         710        720        730        740        750
BPyV(Schur)RC    GATTCTGTTC TGTATATTAT CACTGTTGGA AAACATCGGG TAAATGCAAT
LargeT           GATTCTGTTC TGTATGTTAT CACTGTTGGA AAACATCGGG TAAATGCAAT

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         760        770        780        790        800
BPyV(Schur)RC    GGAAAATCTG TGTAGTAAAA AATGTACTGT GAGCTTCCTA CAAGCCAAGG
LargeT           GGAAAATCTG TGTAGTAAAA AATGTACTGT GAGCTTCCTA CAAGCCAAGG

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         810        820        830        840        850
BPyV(Schur)RC    GAGTTCTAAA ACCTCAGGAG GCCTATAATG TTTGCTGCAC ATTTGAACTT
LargeT           GAGTTCTAAA ACCTCAGGAG GCCTATAATG TTTGCTGCAC ATTTGAACTT

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         860        870        880        890        900
BPyV(Schur)RC    ATCTCCCAAA ACATACAAGG AGGTCTGCCA AGTAGTTTTT TTAACCCTGT
LargeT           ATCTCCCAAA ACATACAAGG AGGTCTGCCA AGTAGTTTTT TTAACCCTGT
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                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         910        920        930        940        950
BPyV(Schur)RC    GCAAGAAGAA GAAAAGTCTG TAAATTGGAA ACTCATTTCT GAATTTGCTT
LargeT           GCAAGAAGAA GAAAAGTCTG TAAATTGGAA ACTCATTTCT GAATTTGCTT

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         960        970        980        990       1000
BPyV(Schur)RC    GCAGCATTAA ATGTACAGAC CCCCTTTTAT TAATGGCATT ATATTTAGAA
LargeT           GCAGTATTAA ATGTACAGAC CCTCTTTTAT TAATGGCATT ATATTTAGAA

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        1010       1020       1030       1040       1050
BPyV(Schur)RC    TTTACAACTG CACCTGAAGC ATGTAAAGTA TGTGACAATC CCAGACGCCT
LargeT           TTTACAACTG CACCTGAAGC ATGTAAAGTA TGTGACAATC CCAGACGCCT

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        1060       1070       1080       1090       1100
BPyV(Schur)RC    TGAGCATAGA AGACACCATA CCAAAGACCA CACCTTAAAT GCTTTACTGT
LargeT           TGAGCATAGA AGACACCATA CCAAAGACCA CACCTTAAAT GCTTTACTGT

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        1110       1120       1130       1140       1150
BPyV(Schur)RC    TTCAGGACAG CAAAACTCAG AAAACCATAT GTAATCAGGC CTGTGATACT
LargeT           TTCAGGACAG CAAAACTCAG AAAACTATAT GTAATCAGGC CTGTGATACT

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        1160       1170       1180       1190       1200
BPyV(Schur)RC    GTGTTAGCAA AAAGGAGATT AGATATGAAA ACCTTAACTA GAAATGAATT
LargeT           GTGTTAGCCA AAAGGAGATT GGATATGAAA ACATTAACTA GGAATGAATT

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        1210       1220       1230       1240       1250
BPyV(Schur)RC    ATTAGTGCAA AGGTGGCAGG GCCTTTTTCA AGAAATGGAG GATTTGTTTG
LargeT           ATTAGTGCAA AGGTGGCAGG GCCTTTTTCA AGAAATGGAG GATTTGTTTG

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        1260       1270       1280       1290       1300
BPyV(Schur)RC    GCGCTCGGGG GGAGGAGCAT TTAGCCCACC GAATGGCGGC AGTTATGTGG
LargeT           GCGCTCGGGG GGAGGAGCAT TTAGCCCACC GAATGGCGGC AGTTATGTGG

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        1310       1320       1330       1340       1350
BPyV(Schur)RC    CTCAATGCCC TACATCCCAA TATGCCAGAT GTTATCTTTA ATTATATCAA
LargeT           CTCAATGCCC TACATCCCAA TATGCCAGAT GTTATCTTTA ATTATATCAA

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        1360       1370       1380       1390       1400
BPyV(Schur)RC    GATGGTGGTT GAAAATAAAC CAAAACAGAG ATATCTCCTC TTAAAGGGGC
LargeT           GATGGTGGTT GAAAATAAAC CAAAACAGAG ATATCTCCTC TTAAAGGGGC

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        1410       1420       1430       1440       1450
BPyV(Schur)RC    CTGTAAACTG TGGGAAAACT ACAGTTGCTG CAGGCCTCAT AGGCCTGTGT
LargeT           CTGTAAACTG TGGGAAAACT ACAGTTGCTG CAGGCCTCAT AGGCCTGTGT

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        1460       1470       1480       1490       1500
BPyV(Schur)RC    GGGGGAGCTT ATTTAAATAT AAATTGTCCC CCTGAAAGAC TGGCATTTGA
LargeT           GGGGGAGCTT ATTTAAATAT AAATTGTCCT CCCGAAAGAC TGGCATTTGA

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        1510       1520       1530       1540       1550
BPyV(Schur)RC    ATTAGGAATG GCAATTGATC AATTTACAGT TGTGTTTGAA GATGTTAAAG
LargeT           ATTAGGAATG GCAATTGATC AATTTACAGT TGTGTTTGAA GATGTCAAAG
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                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1560       1570       1580       1590       1600
BPyV(Schur)RC       GTAAGAAATC CAGTAAATCC TCCCTTCAGA CTGGAATTGG TTTTGAAAAT
LargeT              GTAAGAAATC CAGTAAATCC TCCCTTCAGA CTGGCATTGG TTTTGAAAAT

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1610       1620       1630       1640       1650
BPyV(Schur)RC       TTAGATAATT TAAGAGATCA TTTAGATGGT GCAGTCCCAG TAAACCTTGA
LargeT              TTAGATAATT TAAGAGATCA TTTAGATGGT GCAGTCCCAG TAAACCTTGA

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1660       1670       1680       1690       1700
BPyV(Schur)RC       AAGAAAACAT CAAAATAAGG TGACTCAGAT CTTCCCACCT GGAATTGTTA
LargeT              AAGAAAACAT CAAAATAAGG TGACTCAGAT CTTCCCACCT GGAATTGTTA

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1710       1720       1730       1740       1750
BPyV(Schur)RC       CCTGTAATGA ATATGACATA CCCTTAACAG TCAAAATCCG TATGTATCAA
LargeT              CCTGTAATGA GTATGACATA CCCTTAACAG TCAAAATCCG TATGTATCAA

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1760       1770       1780       1790       1800
BPyV(Schur)RC       AAAGTAGAAC TACTGCATAA TTATAACTTA TATAAAAGTC TTAAAAATAC
LargeT              AGAGTAGAAC TATTGCATAA TTATAACTTA TATAAAAGTC TTAAGAATAC

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1810       1820       1830       1840       1850
BPyV(Schur)RC       TGAGGAAGTG GGCAAAAAAA GATATTTACA GAGTGGAATC ACCTGGCTCT
LargeT              TGAGGAAGTG GGCAAAAAAA GATATTTACA GAGTGGAATC ACCTGGCTCT

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1860       1870       1880       1890       1900
BPyV(Schur)RC       TATTACTCAT CTACTTTAGG TCTGTAGATG ATTTCACAGA AAAACTGCAG
LargeT              TATTACTCAT CTACTTTAGG TCTGTAGATG ATTTCACAGA AAAACTGCAG

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1910       1920       1930       1940       1950
BPyV(Schur)RC       GAATGTGTAG TCAAGTGGAA AGAGAGAATT GAGACAGAGG TGGGAGATAT
LargeT              GAATGTGTAG TCAAGTGGAA AGAGAGAATT GAGACAGAGG TGGGAGATAT

                    ....|....| ....|....| ....|....| ....|....| ....|....|
                            1960       1970       1980       1990       2000
BPyV(Schur)RC       GTGGTTGTTA ACCATGAAAG AAAACATTGA ACAAGGAAAA AATATTCTGG
LargeT              GTGGTTGTTA ACCATGAAAG AAAACATTGA ACAAGGAAAA AATATTCTGG

                    ....|....|
                            2010
BPyV(Schur)RC       AAAAATGA--
LargeT              AAAAATGA–
```

Figure 21 – Reverse compliment (RC) sequence comparison of large T mRNA (cloned from supernatant harvested week 8 of an infectivity assay (not part of this project)) and the published large T mRNA sequence (Schuurman *et al*., 1990). Differences in nucleotide sequences are highlighted in grey.

Several differences were observed between the 2 sequences, but to fully understand the impact of these differences both sequences were translated to

the relevant amino acid sequence (using the spliced mRNA coding sequence) for further comparison.

The translation highlighted 3 amino acid changes between the cloned large T mRNA and the amino acid translated from Schuurman *et al*'s published sequence. The change detailed at position 190 - 'I' to 'V' (Isoleucine to Valine) is a conservative change with both amino acids having nonpolar side chains that should not affect the functioning on the large T antigen. The change detailed at position 535 - 'K' to 'R' (Lysine to Arginine) – are both basic amino acids that again should not affect the functioning of the large T antigen. However, the change detailed at position 26 from 'L' to 'S' (Leucine to Serine), could potentially affect the functioning and structure of the large T antigen (results illustrated in figure 22). Leucine has a nonpolar hydrophobic side chain, whereas Serine has a polar hydrophilic neutral side chain. The sequence of amino acids in a protein and the chemical nature of their side chain allow proteins to do their function, and this Leucine to Serine change may affect the functioning of the large T antigen. Previous studies with yeast (Santos *et al*., 1996) and pBBR host range plasmids (Toa L *et al*., 2005) have reported that the Leucine to Serine change decreases the decoding efficiency of the transfer RNA (tRNA), and increases the plasmid copy number, respectively. The amino acid change observed with the cloned large T mRNA sequence has yet to be fully understood, although it should be noted that the Schuurman sequence was isolated from the wild.

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       10         20         30         40         50         60
LargeTschu   MELTSEEYEE LRGLLGTPDI GNADTLKKAF LKCKVHHPDK GGNEFAMKRL LYLYNKAKIA
LargeTpb     MELTSEEYEE LRGLLGTPDI GNADTSKKAF LKCKVHHPDK GGNEFAMKRL LYLYNKAKIA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                      70         80         90        100        110        120
LargeTschu   ASATTSQVPE YGTSQWEQWW EEFNGFDEQD LHCDEELEPS DNNEEENPAG SQAPGSQATP
LargeTpb     ASATTSQVPE YGTSQWEQWW EEFNGFDEQD LHCDEELEPS DNNEEENPAG SQAPGSQATP

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     130        140        150        160        170        180
LargeTschu   PKKPRTSPDF PEVLKEYVSN ALFTNRTYNC FIIFTTAEKG KELYPCIQAA YKCTFIALYM
LargeTpb     PKKPRTSPDF PEVLKEYVSN ALFTNRTYNC FIIFTTAEKG KELYPCIQAA YKCTFIALYM
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            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    190        200        210        220        230        240
LargeTschu  YNGDVLYIIT VGKHRVNAME NLCSKKCTVS FLQAKGVLKP QEAYNVCCTE ELISQNIQGG
LargeTpb    YNGDVLYVIT VGKHRVNAME NLCSKKCTVS FLQAKGVLKP QEAYNVCCTE ELISQNIQGG

            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    250        260        270        280        290        300
LargeTschu  LPSSFFNPVQ EEEKSVNWKL ISEFACSIKC TDPLLIMALY LEFTTAPEAC KVCDNPRRLE
LargeTpb    LPSSFFNPVQ EEEKSVNWKL ISEFACSIKC TDPLLIMALY LEFTTAPEAC KVCDNPRRLE

            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    310        320        330        340        350        360
LargeTschu  HRRHHTKDHT LNALLFQDSK TQKTICNCAC DTVIAKRRLD MKTLTRNELL VQRWQGLFQE
LargeTpb    HRRHHTKDHT LNALLFQDSK TQKTICNCAC DTVIAKRRLD MKTLTRNELL VQRWQGLFQE

            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    370        380        390        400        410        420
LargeTschu  MEDLFGARGE EHLAHRMAAV MWLNALHPNM PDVIFNYIKM VVENKPKQRY LLLKGPVNCG
LargeTpb    MEDLFGARGE EHLAHRMAAV MWLNALHPNM PDVIFNYIKM VVENKPKQRY LLLKGPVNCG

            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    430        440        450        460        470        480
LargeTschu  KTTVAAGLIG LCGGAYLNIN CPPERLAFEI GMAIDQFTVV FEDVKGKKSS KSSLLQTGIG
LargeTpb    KTTVAAGLIG LCGGAYLNIN CPPERLAFEI GMAIDQFTVV FEDVKGKKSS KSSLLQTGIG

            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    490        500        510        520        530        540
LargeTschu  FENLDNNLRD HLDGAVPVNL ERKHQNKVTQ IFPPGIVTCN EYDIPLTVKI RMYQKVELLH
LargeTpb    FENLDNNLRD HLDGAVPVNL ERKHQNKVTQ IFPPGIVTCN EYDIPLTVKI RMYQRVELLH

            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    550        560        570        580        590        600
LargeTschu  NYNLYKSLKN TEEVGKKRYL QSGITWLLLL IYFRSVDDFT EKLQECVVKW KERIETEVGD
LargeTpb    NYNLYKSLKN TEEVGKKRYL QSGITWLLLL IYFRSVDDFT EKLQECVVKW KERIETEVGD

            ....|....| ....|....
                    610
LargeTschu  MWLLTMKENI EQGKNILEK
LargeTpb    MWLLTMKENI EQGKNILEK
```

Figure 22 – Comparison of cloned large T amino acid (AA) sequence with Schuurman *et al* (1990) published sequence. Differences are highlighted in grey.

4.6 – Does over-expression of the large T antigen result in high titres of virus?

The experiment detailed in section 4.3 suggested that accumulation of the large T antigen may be a factor in the initiation of exponential viral replication. In theory, the inoculation of BPyV (or samples containing infectious BPyV) onto MDBK cells expressing high levels of the large T antigen may results in earlier detection of BPyV. To assess the effect of over expression, large T

mRNA was cloned into a linearized plasmid vector and transfected onto MDBK cells as detailed in methods section 2.6.1 to 2.6.4

Three weeks following transfection, cells began to detach from flasks, with remaining cells showing extensive vacuolation (figure 23). In an attempt to recover the experiment, cells were passaged and seeded in media with varying amounts of FCS - 1%, 5% & 10%. Cells were observed regularly and 3-4 days following passage all cells showed signs of vacuolation. 6-7 days following passage cells started to detach in large sheets. It wasn't apparent what was causing the cells to react this way, so remaining cells were harvested for RT-QPCR and protein analysis. Interestingly, vacuolation observed within the transfected cells is a classic sign of SV-40 cytopathic effect (figure 23).

Total RNA was extracted from these cells and tested for the presence of large T mRNA using primers and probe (SpliceLargeT-60F, 137R and 86T) designed to the large T region of BPyV. All harvests were negative for the presence of large T mRNA, however the multi-components of the reactions (that show the different dye fluorescence levels during the assay) indicated that factors within the reactions were inhibitory. It is not unusual for samples containing high levels of 'target' to show inhibition and appear to be negative (with the assumption that large quantities of large T mRNA were present). Subsequent dilutions of the extracted samples did not detect large T mRNA and still indicated inhibition.

To determine if the large T antigen was present in these cultures, and the mRNA potentially undetectable by RT-QPCR methods, samples were prepared for SDS-PAGE analysis as detailed in section 2.7 (figure 24). Protein was prepared and gels set up with kD protein markers (lane 1 and 10), 8-week infectivity negative MDBK control cells cultured in 1% FCS (lane 2), non transfected MDBK cells (lane 3) and transfected MDBK cell cultures (4 – 9).

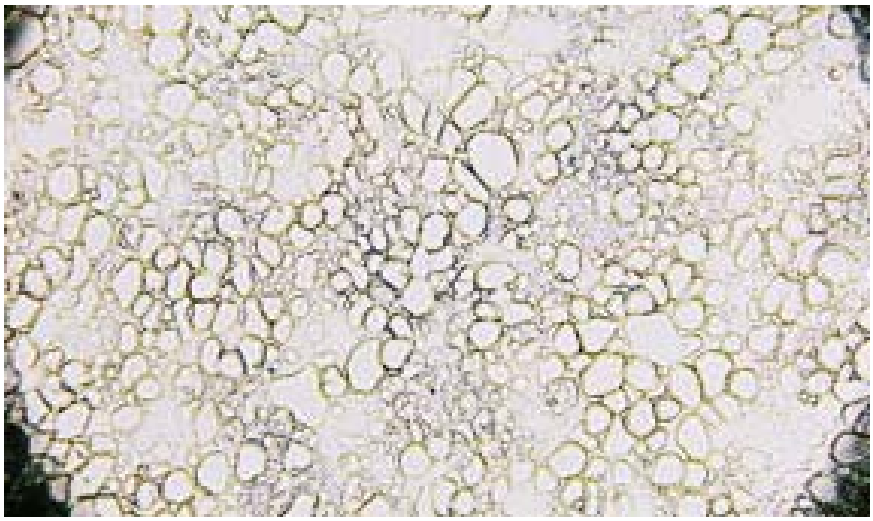
The banding patterns obtained from the infectivity assay (lane 2) were weak and direct comparison to the other lanes was not possible. However, a band of the expected size for the large T antigen (65kD) was present in lanes 4-9 (MDBK cells transfected with BPyV) but not present in lane 3 (non transfected MDBK cells), suggesting that the transfection experiment was a success. It should be noted that expression of the large T antigen within the virus system is regulated, while the expression of the large T antigen within the vector system is highly promoted, potentially explaining vacuolation and inhibition observed during RT-QPCR.

In addition to data obtained from RT-QPCR and SDS-PAGE analysis, western blot analysis (see section 2.7) was employed in an attempted to detect the expression of the large T antigen and cyclin A protein using specific antibodies (figures 25 and 26).

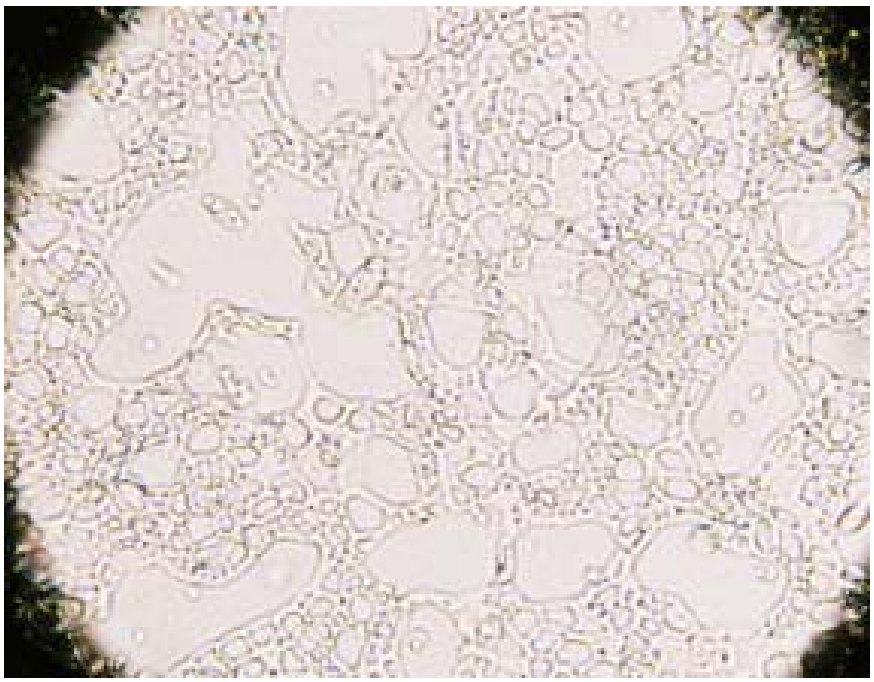
The first western blot was prepared using the large T specific primary antibody in an attempt to detect large T protein expression. Western blots were set up with protein from the transfection experiments as detailed previously. Westerns were set up with a kD protein marker (lane 1), non transfected MDBK cells (lane 5), transfected cell cultures (6 – 11), and a positive control (SV-40 infected Vero cells - lane 12). Blanks were set up in lanes 2-4. Bands were present at a size expected for large T antigen in all samples, including the negatives. As detailed previously, the large T antibody is specific to SV-40, and specificity to BPyV is unknown.

The second western blot was also prepared using a cyclin A specific primary antibody in an attempt to determine if transfected cells were in S-Phase. Western blots were set up as before (see above). In all samples, similar banding was observed, but no bands of the expected size for cyclin A (65kD), and no obvious banding differences between non-transfected and transfected cells. From this evidence it appears that the transfected cells were not in S-Phase.

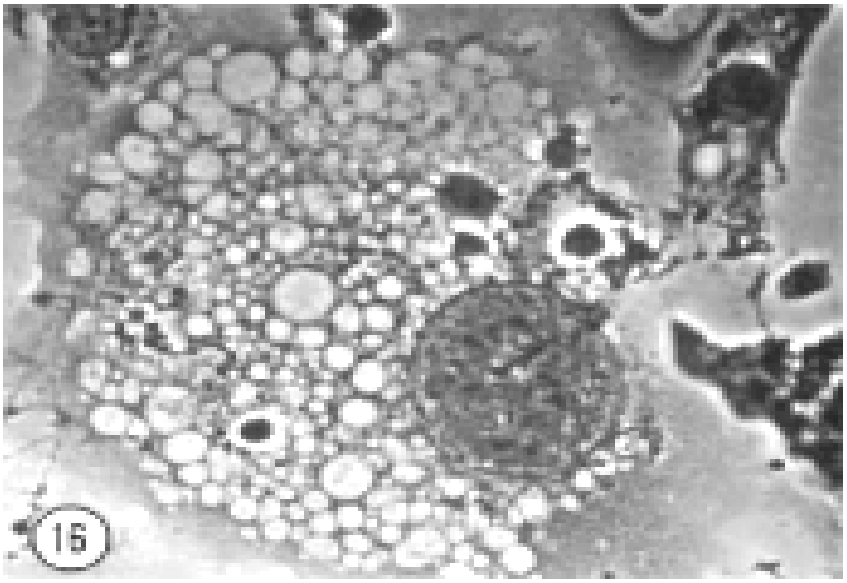
A)



B)



C)



D)

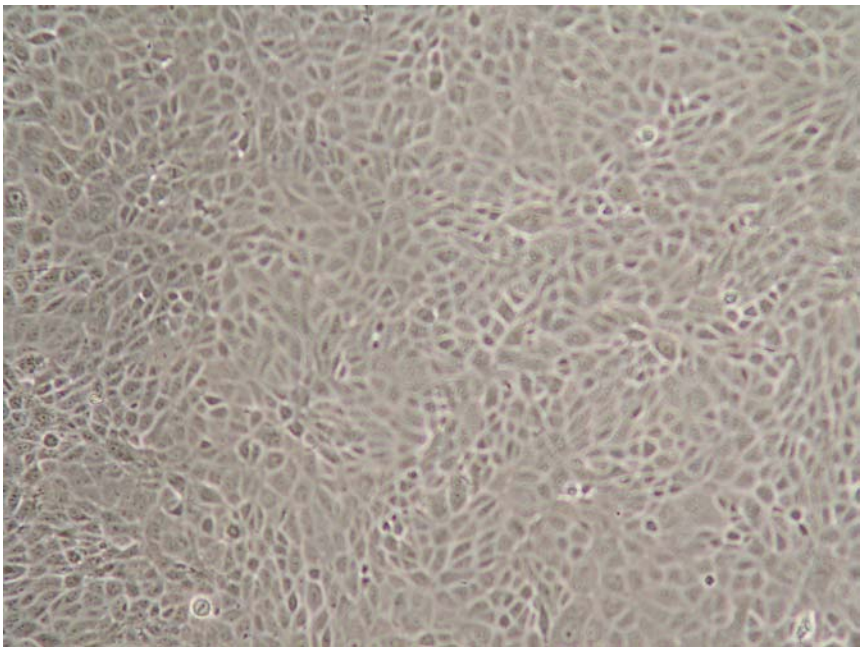


Figure 23 – MDBK cells transfected with large T mRNA (A) and (B) showing vacuolation. (C) Vacuolation of African green monkey cells inoculated with SV40 (Picture taken 72 h after inoculation) (Source - Melnick *et al*., 1963). (D) Non-transfected healthy cells.

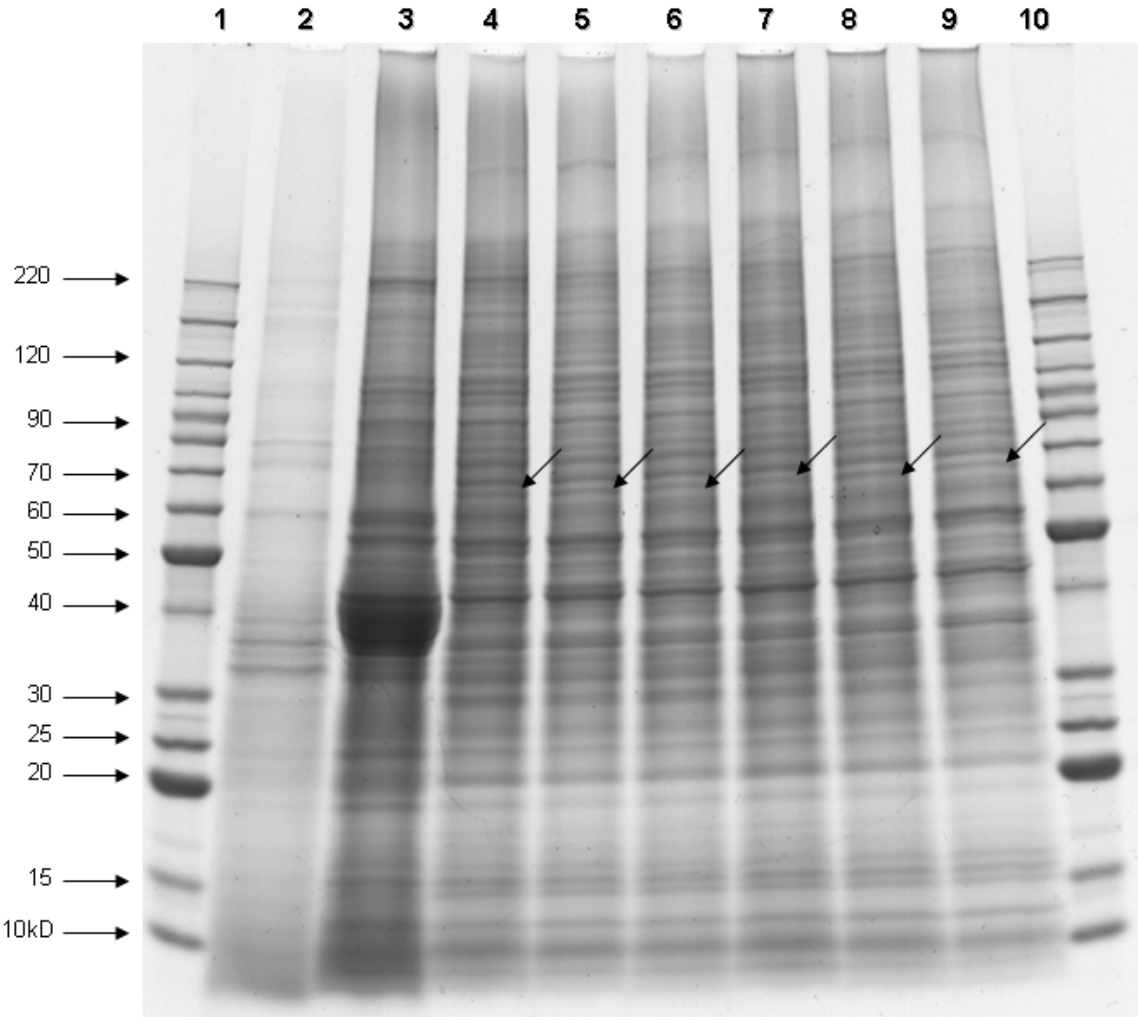


Figure 24 – SDS-PAGE of transfected cell cultures (stained with GelCode Blue Stain). Approximately 6µg of protein was assessed. Bands are present at the expected size that would indicate the presence of the large T antigen (approx. 65kD) in lanes 4-9 (marked with arrows). Gels were set up with kD protein markers (lane 1 and 10); infectivity negative control (MDBK) cells cultured in 1% FCS (lane 2), non-transfected MDBK cells (lane 3) and MDBK cells transfected with BPyV (lanes 4 – 9).

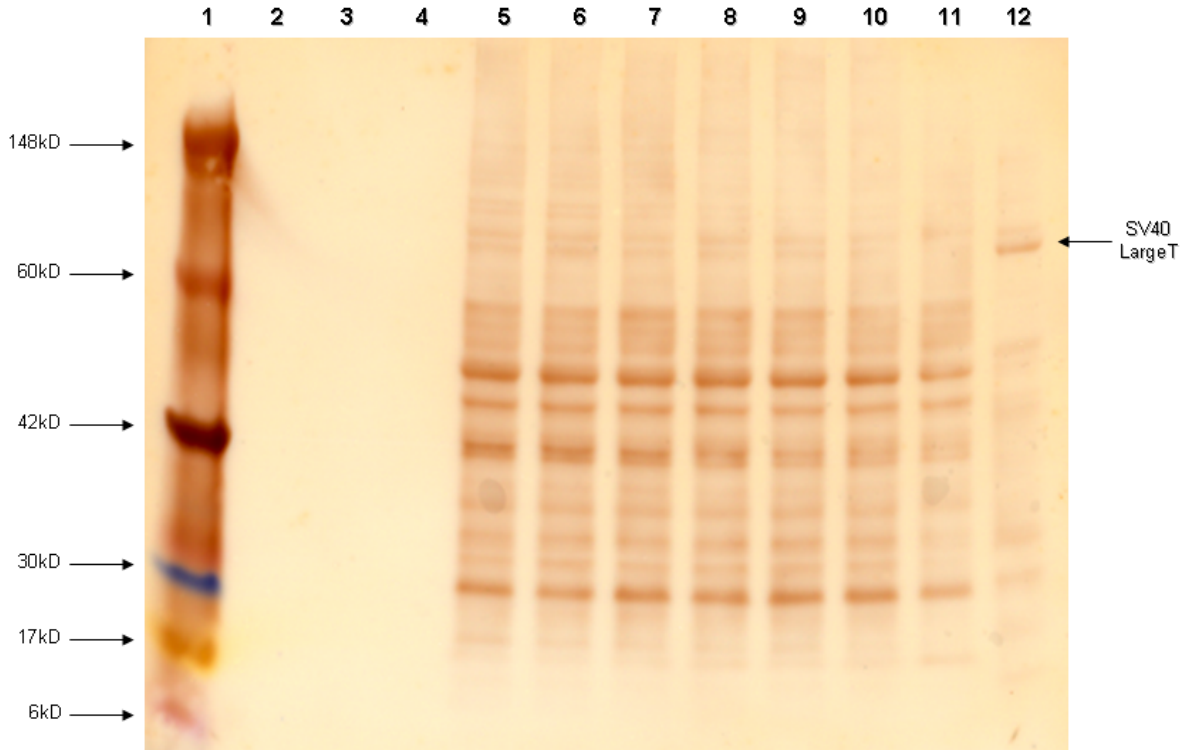


Figure 25 – Western Blot analysis of transfection experiment using SV40 large T primary antibody and Anti-Mouse IgG Peroxidase secondary antibody. Membranes were developed using the DAB Substrate system. Approximately 6µg of protein was assessed. No bands are present that would suggest the presence of BPyV large T antigens. Westerns were set up with a kD protein marker (lane 1), non transfected MDBK cells (lane 5), MDBK cells transfected with BPyV (6 – 11), and a positive control (SV-40 infected Vero cells - lane 12). Blanks were set up in lanes 2-4.

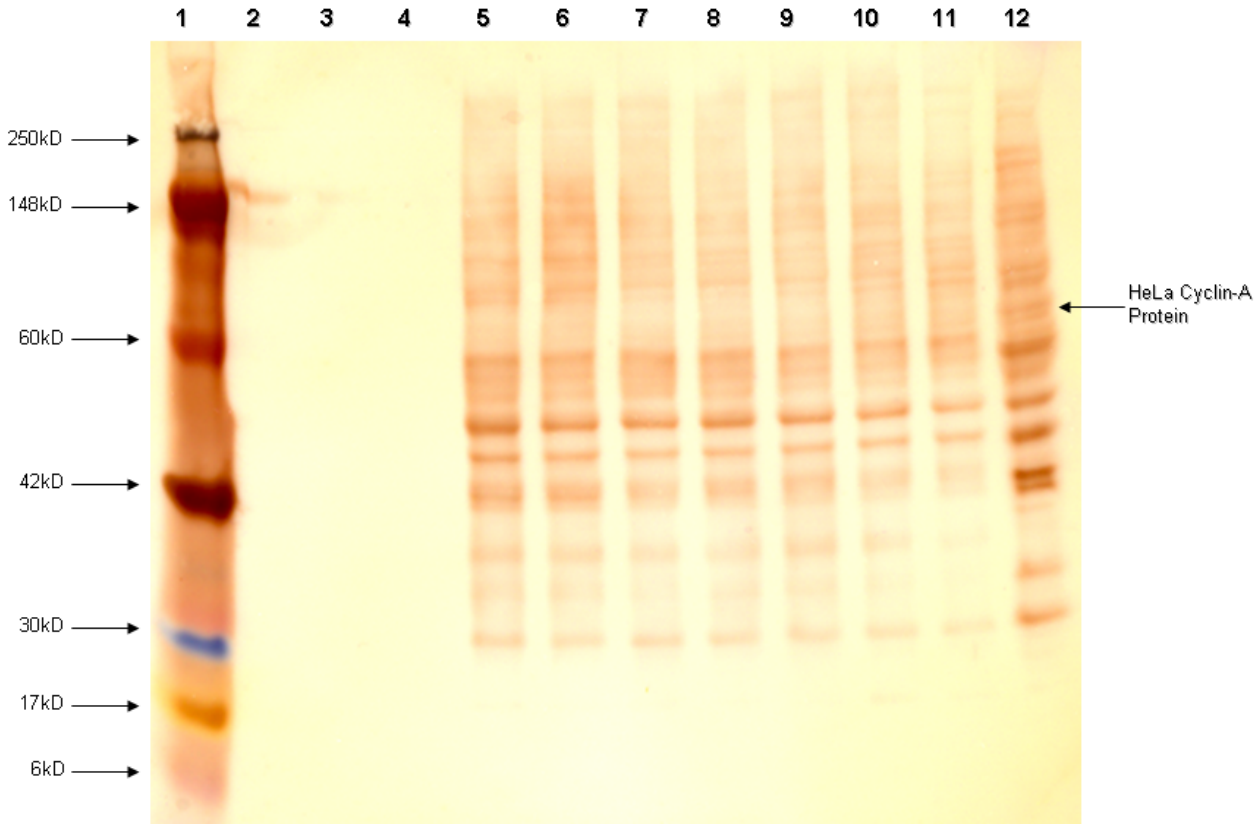


Figure 26 – Western Blot analysis of transfection experiment using Anti-cyclin A primary antibodies and Anti-Mouse IgG Peroxidase secondary antibody. Membranes were developed using the DAB Substrate system. Approximately 6µg of protein was assessed. No bands are present that would suggest the presence of cyclin A protein (65kD). Westerns were set up with a protein marker (lane 1), non transfected MDBK cells (lane 5), MDBK cells transfected with BPyV (6 – 11), and a positive control (HeLa cells - lane 12). Blanks were set up in lanes 2-4.

Chapter 5 - Discussion

The results of these experiments confirm that the large T antigen has an important role in the *in vitro* replication of Bovine Polyomavirus (BPyV). Not only does an increase in large T mRNA correlate to an exponential increase in viral genomes, results also indicate that the large T is involved in moving the MDBK cells to S phase. It was demonstrated that high levels of BPyV were detected in cells supplemented with 1% FCS; with large T mRNA expression being detected immediately prior to exponential viral replication. Low levels of viral replication were only detected in the first 3 weeks in cells supplemented with 10% FCS. No large T mRNA expression was detected in these cells. An attempt to generate cells over-expressing the large T antigen was inconclusive, due to cell pathology.

Analysis of DNA extracted from cells inoculated with BPyV and cultured for 8 weeks (with 1% and 10% FCS) showed that exponential viral replication was only detected 5 weeks following inoculation onto cells grown in 1% FCS. BPyV was initially detected in cells grown in media supplemented with 10% FCS, but the titre of virus dropped each week until it was undetectable after 4 weeks. It is unclear what caused the eventual disappearance of the virus from the culture system. In contrast, titres of virus obtained from cells grown in media supplemented with 1% FCS showed consistent growth over the first 3-4 weeks before exponential replication was observed. This is uncharacteristic among polyomaviruses, in which the complete replication cycle takes between 48 and 72 hours. BPyV only has 4 known functional genes so relies heavily on cellular mechanisms for replication. Why is replication observed in cells that are only doubling at a low rate? What is the function of the large T antigen during this initial period of replication?

BPyV may be removed from the culture system when grown in 10% FCS due to the cellular machinery replicating at a rate that does not allow the virus to effectively recruit the necessary cellular proteins to allow efficient viral replication. This scenario may also allow the cell to effectively combat BPyV viral infection, a mechanism which may be possible *in vitro* but is unlikely *in*

vivo. Cells growing at a low doubling rate in 1% FCS (which is more similar to that likely in the in vivo situation) may allow the virus to recruit cellular proteins and allow expression of the necessary viral antigens. However, would the use of a culture system that is proven not to support the replication of BPyV be of any use to the biotechnology industry? The testing of biopharmaceuticals is costly and time consuming, and the culture of pharmaceuticals grown in a manner that would reduce the risk of BPyV contaminants would be beneficial, although the need to test for contaminants, especially BPyV, may always be required.

To assess the possible roles of the large T antigen, mRNA was extracted from cells harvested during the 8 week infectivity assay. This analysis showed that large T mRNA was not detected until wk. 4 (in the A flask only – large T mRNA was not detected until wk. 5 in the B flask), a week before true exponential replication was observed, suggesting that the presence, if not the accumulation of large T mRNA was an important factor in the exponential replication of BPyV. No large T mRNA was detected in the cells grown in media supplemented with 10% FCS, although large T mRNA may have been present during this time but at levels too low for detection (signals suggesting viral replication were observed wk's 1-3).

If replication is being observed during these initial weeks, and viral replication is not possible without this antigen, then why is large T mRNA not being detected? The quantitation limit of the RT-QPCR assay is 10^2 large T mRNA molecules per reaction, and approximately 10^3 to 10^4 viral genomes per reaction (for cells supplemented with 1% FCS) are detected in the first 4 weeks of the infectivity assay. This equates to a maximum of 0.1 - 0.01 molecules of large T mRNA per virus particle. Is viral replication possible with such a small ratio of large T to virus? It is possible that the majority of large T mRNA generated during this time differs in sequence and is therefore not detectable by RT-QPCR (but still functional)? It may take several weeks and therefore multiple passages before non-defective large T antigens are produced resulting in the exponential replication observed. SDS-PAGE and western blot analysis was utilised at this point to determine if the large T

antigen was indeed present in these initial weeks, but undetectable by RT-QPCR. Results obtained were inconclusive, possible due to the non-specificity of the large T antibody (raised against SV40). The expression of the large T antigen is regulated by the virus - is it maintained at low levels for a reason? The rate of large T antigen production in the initial infection period may be regulated in order to allow the virus (and therefore the large T antigen) to recruit the necessary cellular proteins and efficiently inhibit the antiviral mechanisms. We can hypothesize that accumulation of the large T antigen is critical for production of high titres of virus, but the antigen may also be regulated or latent during initial infection. If the virus is latent, then what is the switch to replication? Latent polyoma viruses have been shown to cause tumors in patients, but only when they become immunocompromised – how could this process be replicated in cell culture? The virus may remain latent until the cells are confluent and doubling at a low rate before controlling the replication machinery (including the anti-viral mechanisms) of the cell. Results obtained suggest that the accumulation or regulation of the virus takes approximately 3-4 weeks. However, we can only hypothesize on the level and roles of large T antigen in these initial weeks, as results obtained from these experiments were inconclusive.

To determine if the accumulation of large T antigen was a factor in exponential viral replication, MDBK cells were transfected in an attempt to generate cells continuously expressing high levels of large T antigen. The procedure appeared to be toxic to the cells, although SDS-PAGE analysis indicated a band of the expected size for the large T (65kD) antigen in all transfected cell cultures (no bands present in negative controls). Western Blot analysis was inconclusive. It is possible that large T mRNA is not being translated or folded incorrectly, therefore not being able to function efficiently. Interestingly, the vacuolation observed in the cells is a classic sign of SV-40 cytopathic effect, never reported with BPyV. This may indicate that BPyV has a tighter control over the large T antigen than SV-40. Again, we can only hypothesize concerning the success of this experiment, but it should be noted that the vector is designed to heavily promote the inserted large T mRNA,

which may be toxic to the cells. Production of large T antigen may need to be regulated to keep the infected cells alive, explaining why levels of large T mRNA are not detected in the early stages of the infectivity assay (but not why cell death is not observed in the later stages of the infectivity experiment). Cell death could have been due to the transfection procedure, but the vector used is specifically designed for expression in mammalian systems.

How is BPyV able to replicate and produce such high titres of virus in cells supplemented with 1% FCS and doubling at a low rate? Does the large T antigen have a role in altering the state of the cell? It has been shown with SV-40 that the large T antigen is involved in moving the cells into S-Phase - is this also the case with BPyV?

Counts from cells harvested during the 8-week infectivity assay indicated that following the point when exponential replication of BPyV was detected, all cell counts decreased gradually, with the exception of cells infected and grown in 1% FCS. Was the BPyV large T antigen moving these cells into S-Phase? SDS-PAGE analysis was inconclusive, but western blot analysis of these cells using an antibody specific to cyclin A showed a band of the expected size (65kD) present in harvests from week #'s 6, 7 and 8. On closer examination, faint bands of the same size can be observed in other harvests, but this is not unusual as some semblance of cell growth must be taking place. However, why does it take 6 weeks before we see the cells entering S-Phase? Does the large T antigen need to be at a level that allows it to alter the cells replication phases? Does the cell have to become confluent before large T antigen can be effectively expressed?

In the process of generating a vector for expression in cells, a sample of BPyV virus, which had been cultured for 8 weeks and harvested, was cloned. As part of the cloning procedure, sequencing was carried out to confirm the correct sequence and orientation. This sequence analysis highlighted several base pair differences between the cloned large T mRNA and the published large T mRNA sequence (isolated from the wild). One of these differences

resulted in an amino acid change from hydrophobic side chain to polar hydrophilic neutral side chain. This change could potentially affect the folding and functioning of the antigen. Studies have shown that this amino acid change can result in both the decreasing and increasing of replication efficiency. If this is an example of the type of large T antigen being expressed after 8 weeks of culture, then a high percentage of large T antigen mutants may explain why it takes 4-5 weeks to see exponential amplification of viral genomes.

Since the start of this project, only 2 papers have been published on Bovine Polyomavirus. In February 2005 a paper was published concerning the 'Detection and molecular characterisation of bovine polyomavirus in bovine sera in New Zealand' (Wang *et al*., 2005). This paper documents the detection of BPyV in 46 (70%) batches of FBS, 11 (32%) batches of calf sera and two (5%) batches of ABS/plasma, although similar results have been documented previously. The paper concludes that BPyV is a frequent contaminant (showing genomic variations) of commercial bovine serum in New Zealand, and that the incidence of BPyV in adult bovine serum products is much lower than in FBS and calf serum. The significance of the high prevalence of BPyV DNA in bovine serum products has been discussed previously.

In December 2006 another paper was published concerning the 'Identification of human and animal adenoviruses and polyomaviruses for determination of sources of fecal contamination in the environment' (Hundesa *et al*., 2006). This paper discusses the detection of BPyV in 'samples potentially affected by either porcine or bovine fecal contamination', and documents that this is the 'first time that….BPyV have been reported to occur in environmental samples'. The significance of this study to the MSc is unclear, although the number of papers published highlights the lack of research carried out on BPyV.

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