The Development of Penguinpox Virus (PEPV) as a Vaccine Vector:

Transfer Vector Construction and Rescue of Virus Growth in Rabbit Kidney Cells (RK-13) by Vaccinia Virus K1L Gene.

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General Table of Contents

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Lists of Figures

List of Tables

List of Abbreviations

ABPV	Albatrosspox virus
AIDS	Acquired immune deficiency syndrome
β-gal	β-gaactosidase
BHK-21	Syrian baby hamser kidney fibroblast cells
bp	Base pair
CAM	Chorioallantoic membrane
CEF	Chicken embryo fibroblast cells
CNPV	Canarypox virus
CPE	Cytopathic effects
DMEM	Dulbecco's Modified Eagle medium
DNA	Deoxyribonucleic acid
EEV	Extracellular enveloped virus
FCS	Foetal calf serum
FLPV	Falconpox virus
FWPV	Fowlpox virus
Gpt	Guanine-hypoxanthine phosphoribosyltransferase
Grttn	Gag, reverse transcriptase, Tat and Nef polyprotein
GAG	Glycosaminoglycan
GC	Guanine and Cytosine
GUS	β-glucuronidase
HIV	Human immunodeficiency virus
HOPV	Houbarapox virus
hr	Host range
IEV	Intracellular enveloped virus
IMV	Intracellular mature virus
ITR	Inverted terminal repeats
kb	Kilobase
LSDV	Lumpy skin disease virus
m.o.i	Multiplicity of infection
MDBK	Madin-Darby bovine kidney epithelial cells
µg	Microgram
µl	Microlitre
mg	Milligram
ml	Millilitre
MLPV	Macawpox virus
mM	Milimolar
MVA	Modified Vaccinia Ankara
ng	Nanogram

nm	nanometer
^{o}C	degree Celsius
PBS	Phosphate buffered saline buffer
PCR	Polymerase chain reaction
PEPV	Penguinpox virus
Pfu	Plaque/Pocks forming unit
PGPV	Pigeonpox virus
PRPV	Parrotpox virus
RK-13	Rabbit kidney fibroblast cells
rpm	revolution per minute
RT	Reverse Transcriptase
SLPV	Starlingpox virus
SRPV	Sparrowpox virus
TBE	Tris-Boric-EDTA
TK	Thymidine Kinase
TKPV	Turkeypox virus
U	Units
UCT	University of Cape Town
UV	Ultraviolet light
VACV	Vaccinia virus
WHO	World Health Organization
wt	Wild type
wtPEPV	Wild type Penguinpox virus
YT	Yeast-Tryptone

Abstract

Worldwide, there are an estimated 33.4 million people infected with Human Immunodeficiency Virus (HIV-1), of which 67% live in sub-Saharan Africa (UNAIDS, 2009). Even though many drugs have been developed which are effective at reducing the viral load to undetectable levels (UNAIDS, 2009), because of the integrative nature of the viral genome and its ability to evade elimination in nonreplicating cells, the ultimate solution for long-term control of HIV infection would be to have an effective prophylactic vaccine. Of the many vaccine trials which have taken place, the most promising results have been obtained from the recent phase 3 clinical trial which tested the ability of a dual protein and Canarypox virus recombinant to protect humans against HIV-1 infections (Reks-Ngarm *et al*., 2009). Because poxviruses are being developed as vaccine vectors against a number of diseases, it is important to continue the search for novel poxvirus vectors, in particular, those that do not cross-neutralize one another.

This thesis describes the preliminary work performed on the development of Penguinpox virus (PEPV) as a vaccine vector. No cells are known to be permissive for the growth of PEPV; hence this virus was grown and titrated on chorioallantoic membranes (CAMs) of 10-12 day old chick embryos. Growth curve analysis confirmed that rabbit kidney fibroblast cells (RK-13) are not permissive for PEPV and also that PEPV does not adapt to growth in these cells upon multiple passage. This meant that RK-13 cells could potentially be used for the selection of recombinant PEPV which has the Vaccinia virus (VACV) K1L host range gene recombinantly integrated into its genome. The K1L gene has been used to rescue growth of MVA in RK-13 cells and so used as a selective marker gene in the construction of recombinant virus (Staib *et al*., 2000). The aim of this project was to establish whether the K1L gene could be used as a

selection marker for the construction of recombinant PEPV.

In order to make a recombinant poxvirus it is necessary to construct a transfer vector, which is used to transfect poxvirus-infected cells, and then to isolate the recombinant virus from these cells. After a series of cloning procedures a transfer vector was constructed. It contained the selective host range gene K1L, the marker gene GUS, direct repeat sequences flanking these two genes, a unique *Sma* I restriction enzyme site outside of the direct repeats and, flanking this cassette, the two ends of the PEPV thymidine kinase (TK) gene. This transfer vector was shown to rescue both early and late gene expression in RK-13 cells. However, no recombinant PEPV could be recovered.

This is the first attempt to rescue PEPV growth in a non-permissive cell line with a VV host range gene. This work provides a foundation for future development of PEPV into a novel vaccine vector.

Chapter 1: Literature Review

1.1 ***Poxviridae***

1.1.1 Classification and Phylogeny

The *Poxviridae* is divided into two subfamilies which are highly divergent: the *Entomopoxvirinae* and the *Chordopoxvirinae,* which infect insects and vertebrates respectively (Gubser *et al*., 2004). The *Entomopoxvirinae* subfamily is divided into 3 genera (a, b and c) while the *Chordopoxvirinae*, the focus of this review, are divided into 8 genera, (*Avipoxvirus*, *Molluscipoxvirus*, *Orthopoxvirus*, *Capripoxvirus*, *Suipoxvirus*, *Leporipoxvirus*, *Yatapoxvirus* and *Parapoxvirus*) (Babkin *et al*., 2005).

The genome sequence of at least one member of each *Chordopoxvirus* genus is available in the genbank for analysis. Analysis of sequenced *Chordopoxvirus* genomes was conducted based on comparison of the sizes of these genomes, the number of unique genes, gene arrangement and phylogenetic analysis of the amino acid sequences of 17 conserved proteins among *Chordopoxviruses* (Gubser *et al*., 2004; Babkin *et al*., 2005). This analysis showed *Avipoxviruses* to be the most divergent amongst the *Chordopoxvirus* genera followed by *Molluscipoxvirus*. *Avipoxvirus* is the only *Chordopoxvirus* genus which infects avian species whereas *Molluscipoxvirus* is a strict human pathogen. Both of these viruses have evolved unique immunomodulatory proteins to evade or counteract their species specific (human and avian species) immune response. The remainder of the *Chordopoxvirus* sub-family can be categorised into two clusters. The *Leporipoxvirus*, *Yatapoxvirus*, *Capripoxvirus*, *Suipoxvirus* and possibly *Parapoxvirus* genera were grouped together due to the relatively small size of their genomes (range from ~120kb to ~160kb) and the few unique genes found in the central conserved regions of their genomes. Members of the other group, *Orthopoxvirus*, were

found to possess relatively larger genomes (larger than 160kb) and to have unique genes present at the N-terminal region of the genome (Gubser *et al*., 2004). However, it has also been suggested that *Parapoxvirus* is more closely related to *Molluscipoxvirus* as both genera possess higher GC content (64 ~ 64.5%) compared to the rest of the *Chordopoxviruses* (GC contents of 25% ~ 43.6%). It was suggested that *Avipoxviruses* diverged from the mammalian poxviruses which possess low GC contents. However, due to the progressive adaptation of the *Avipoxvirus* genus to avian species, it has become highly diverged from the rest of the mammalian *Chordopoxviruses* (Babkin *et al*., 2005).

1.1.2 Structure of Poxvirus

1.1.2.1 Genome Structure

Poxviruses are large double-stranded DNA viruses, with genomes ranging from 130 to 300kb (Moss *et al*., 2007). Comparisons of sequenced poxvirus genomes showed there are 90 genes conserved in all *Chordopoxviruses* and 49 genes in all poxviruses. These conserved genes are required for transcription, translation, DNA replication, repair and viral structural components (Gubser *et al*., 2004). Conserved genes are generally located in the central region of the genome with gene conservation decreasing towards the termini (Afonso *et al*., 2000). The non-conserved genes, many of which are not essential for replication in cultured cells, are involved in host range and pathogenicity. At the ends of the poxvirus genome, the two strands of DNA are covalently-linked by terminal hair-pin loop structure (Baroudy *et al*., 1982). Inverted terminal repeat sequences (ITR) are found adjacent to the hair-pin loop structures. The lengths of these sequences vary amongst different poxviruses, however a highly

conserved concatemer resolution motif is found within the ITR as this motif is essential for DNA replication of poxviruses (Moss, 2007).

1.1.2.2 Virion Structure

Figure 1.1 shows the structure a typical poxvirus virion. Poxviruses are characterised by a complex, enveloped, brick-shaped virion, but can also assume a pleomorphic shape. The brick-shaped virion is generally ~260 nm by 220-450 nm while the pleomorphic virion is 160-190 nm by 250-330 nm. The core can display itself in a tubular unit, a globular unit or as spiral filaments (Condit *et al*., 2006). Virions may possess one or two lateral bodies accompanying the core, nested between the core membrane and the surface membrane. Generally Poxviruses are found in two distinct, infectious virus particle types, the intracellular mature virus (IMV, with a single outer membrane) and the extracellular enveloped virus (EEV, with a double outer membrane) with both particles capable of initiating the infectious cycle. These two virion forms differ in their surface glycoproteins and in the number of membranes that enclose the virion. However, there are two other forms of virion present in virus infected cells. Once IMV particles are produced following viral replication, they migrate via microtubule mediated trafficking and are wrapped with Golgi-derived membranes to form intracellular enveloped virus (IEV). The IEV sheds one of its outer membranes as it fuses with the cell membrane, forming cell-associated enveloped virus (CEV). CEV is either propelled toward neighbouring cells (McFadden, 2005) or released directly as free EEV particles. In general, CEV and EEV forms are particularly important for cell-cell spread whereas IMV is disseminated late in the infectious cycle at the stage of cell death (McFadden, 2005).

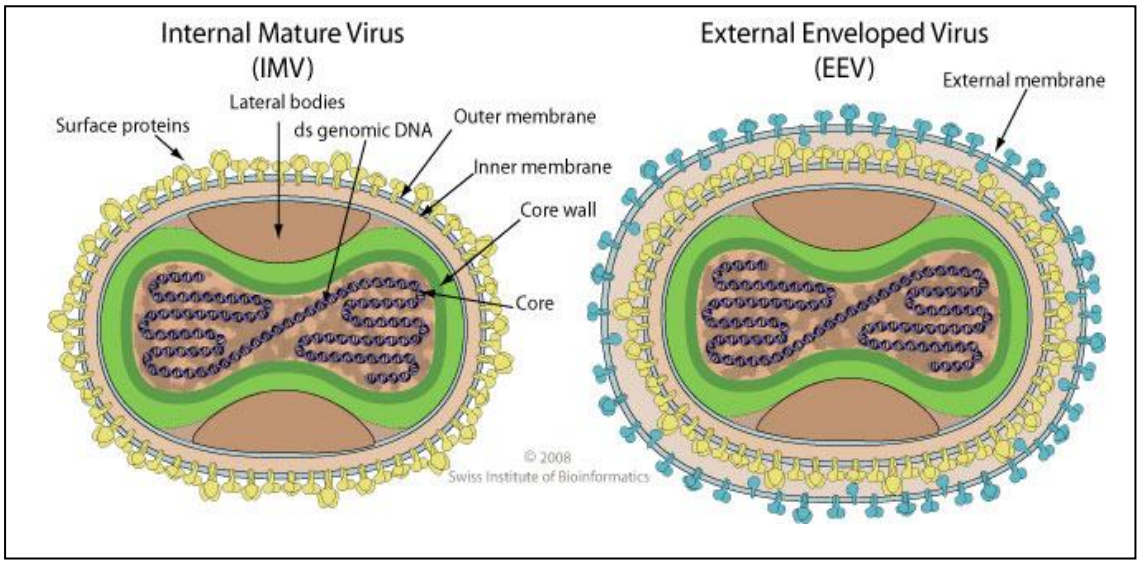

Figure 1.1. Diagram showing the virion structure of a typical poxvirus (http://expasy.org/viralzone/all_by_species/174.html).

1.1.3 Virus Entry, Replication, Transcription and Morphogenesis

Virus replication involves several stages. Poxviruses are efficient at binding and entering mammalian cells, regardless of whether the cell is nonpermissive or permissive. Binding of the virus to the cell surface and subsequent fusion of the virus and the cell membranes is well studied, however, the specific host receptors involved in poxvirus infection and the fusion event between the virion and the host cell membrane are still poorly understood. To date, it has been shown that several virion proteins are crucial for binding of the virion to the cell surface (McFadden, 2005). Poxvirus proteins A27, H3, D8 and A26 were found to interact with host cell surface proteins (Chiu *et al*., 2007) such as the ubiquitously expressed Glycosaminoglycans (GAG), heparin sulfate proteoglycan (HSPG), chondroitin sulfate proteoglycan (CSPG) and extracellular matrix protein laminin. However, none of these viral-cell protein interactions alone are essential for virus attachment to the cells, therefore virus may enter the cells through alternative mechanisms (Chiu *et al*., 2007).

For IMV, attachment of the virus is facilitated by the virus transmembrane, which is made up of several virus proteins and host cell surface protein. Once the virus binds to the host cell surface receptor, plasma membrane fusion occurs, releasing the viral core into the host cell cytoplasm (Moss, 2007). The initial fusion event is poorly understood, however, several conserved viral proteins have been identified that play a role in facilitating viral entry. Proteins such as VACV A28, A21 have been linked to the fusion-entry event and virions that have deletion of or mutation in these proteins are still capable of binding to the cell surface but unable to enter the cells (Moss, 2007). Because EEV has an extra membrane, the penetration mechanism is divided into two stages. The first stage involves removal of the outer membrane of EEV. Once EEV surface proteins interact with the host cell surface proteins, a serial ligand-induced, non-fusogenic mechanism is induced, resulting in disruption of the outer membrane (Law *et al*., 2006) and release of the inner virion (IMV). The second stage involves fusion of the IMV with the host cell surface membrane. The released viral core is then transported via microtubules into the host cell cytoplasm.

Recent studies have shown Vaccinia virus could enter cells through two distinct pathways. Vaccinia virus is capable of entering the cells through direct fusion with the plasma membrane at neutral pH, and recent studies have shown that VACV is capable of using a low-pH endosomal entry pathway. The choice of two distinct pathways may contribute to the ability of VACV to enter a large variety of cells (Townsley *et al*., 2006). Initially, the WR strain of VACV was shown to preferentially use the low-pH endosomal entry pathway, and addition of endosomal acidification inhibitor drastically reduced viral entry. Mercer *et al* (2010) later discovered that the IHD-J VACV strain utilized distinct forms of macropinocytosis for host-cell entry. The IHD-J strain is more

dependent on glycosaminoglycan binding for infection and less dependent on acidification of the endosome (Mercer *et al*., 2010). Recently, it has been discovered that the VACV A25 and A26 surface proteins are fusion suppressors for mature virions and determine strain-specific virus entry pathways into HeLa, CHO-K1 and L cells (Chang *et al*., 2010). Overall, both Vaccinia virus strains WR and IHD-J employed an entry strategy based on apoptotic mimicry, whereby they were disguised as apoptotic debris to enter host cells (Mercer *et al*., 2008). The ability of VACV to enter the host cell in this manner may be attributed to its relatively large size. VACV is comparable in size to apoptotic bodies that are macropinocytosed by professional phagocytes and other cell types (Mercer *et al*., 2008). This strategy has several advantages for the entry of virus: 1) it permits endocytic internalization of particles too big for other endocytic pathways. 2) it allows the virus to enter different cell types, as intake of apoptotic debris is common to most cells, and 3) this mechanism of entry prevents detection by the immune response (Mercer *et al*., 2008).

The large genome of poxviruses allows replication to take place in the cytoplasm of infected cells as it encodes all of its own replication machinery. Poxvirus gene expression is divided into three phases: early, intermediate and late. The early genes encode factors needed for intermediate gene expression, while the intermediated genes encode factors required for late gene expression and the late genes encode structural proteins (for the packaging of the virus) and factors needed for early gene expression. Hence components required for transcription of early genes are pre-packaged inside the viral core, and expression of early genes begins as soon as the core is released into the cytoplasm. Early genes also encode proteins that are involved in viral DNA synthesis and defense against host cell immune responses. These immune defense molecules include proteins which alter the host cellular environment and inactivate cellular

immune responses in order to allow productive viral replication. Following early gene expression, replication of the genome begins with a nick in the covalent bond linking the two DNA strands of the Poxvirus genome (the double stranded DNA is now opened up and allows replication to occur). As replication occurs in the cytoplasm (away from the host cell replication machinery, in the nucleus) poxviruses express their own transcription factors and therefore, unsurprisingly, promoters of poxviruses can only be recognised by poxvirus RNA polymerase. Poxvirus replication is self-priming, producing large concatamers which are subsequently cleaved and repaired to produce intact viral genomes. Assembly of the virus occurs in the cytoskeleton of host cells but the assembly mechanism is not well understood and maturation of the virus may depend on the host cell (Moss, 2007). Once replication has been completed and the late genes have been expressed, viral crescents (composed of viral proteins and a host derived single lipid membrane) are formed. The viral genome and components required for initiation of early gene expression are packed into the viral crescents to form immature virions. Proteolysis of core proteins transforms the immature virion into intracellular mature virions (IMV) (Moss, 2007). Figure 1.2 shows a schematic diagram of the poxvirus replication cycle.

1.2 Host Range

1.2.1 Poxvirus Tropism

Poxviruses show species specificities that range from narrow to broad. To date, the fundamental mechanisms that mediate the host tropism of individual poxviruses is still poorly understood. One of the challenges in identifying the specific poxvirus host tropism determinants is that at least 3 levels of tropism are exhibited, cellular tropism,

viral replication and pathogenesis. Each of these levels involves different aspects of virus-host interaction (McFadden, 2005).

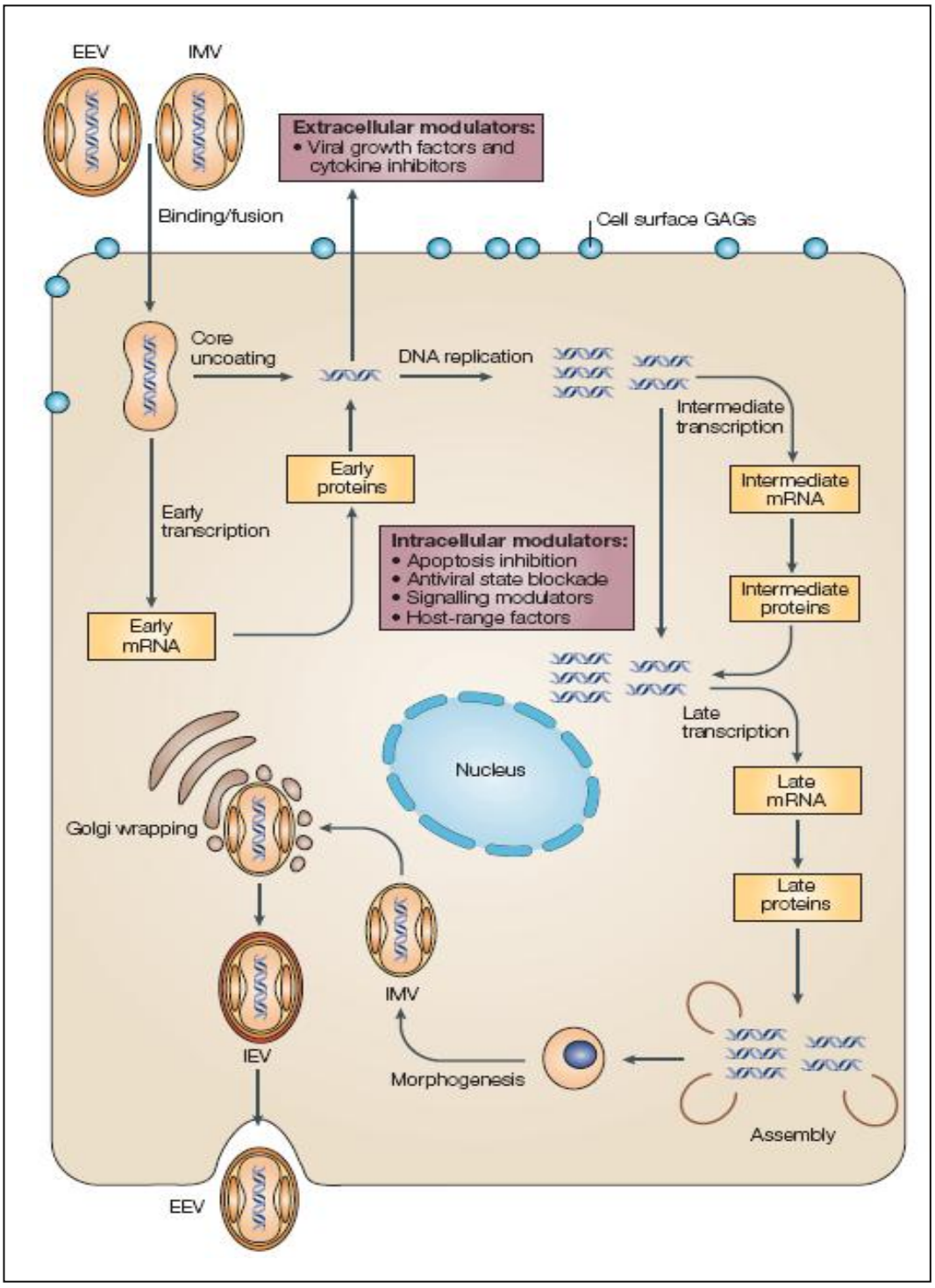

Figure 1.2. Poxvirus replication cycle (McFadden, 2005).

Cellular tropism. In vitro studies have shown that most Poxviruses can bind a broad range of host cells and initiate infection but whether the infection is permissive (production of infectious viral progeny) or non-permissive (abortive replication) depends on specific poxvirus-cell pairing. In non-permissive cells, abortive replication may be due to the inability of the virus to circumvent innate intracellular barriers (McFadden, 2005). At least four categories of intracellular factors have been identified as potential restriction points for regulating whether a poxvirus is able to complete its replication cycle (McFadden, 2005). The first one is the status of the cell cycle as studies have shown that poxviruses encode growth factors that are capable of manipulating the cell cycle. The second one is the lineage and differentiation state of the infected cells, as some poxviruses are dependent on the precise differentiation state of the host cell. The third one is the presence/absence of complementing host factors that are required by the virus to complete its replication cycle. The last one is the signal transduction pathways used by the host cells to defend against virus infection. Many poxviruses have evolved to counteract the host cell immune response (McFadden, 2005).

The second level of tropism refers to the levels of virus replication in specific host organs or tissues. This tropism can be influenced by the factors that mediate the cellular tropism as well as the host tissue-specific antiviral responses.

The third level of tropism refers to the pathogenesis and symptoms of disease in the infected organism, which is influenced by the first and second level of tropism as well as the overall host immune response.

In general poxvirus genes that are nonessential for the replication of the virus in tissue-culture are called virulence genes. Following gene-knockout experiments, certain

poxvirus mutants were unable to replicate in previously permissive animal cell lines. These discoveries led to the investigation of specific virus/host interactions (Chung *et al*., 1998). As previously described, early genes are expressed by the prepackaged elements and are required for transcribing the later elements. Mutants have been identified which are capable of producing certain early proteins but do not express intermediate and late genes. This finding suggested that these mutations may have played an important role in the early stages of viral replication (Guerra *et al*., 2005). Among these mutants, several genes were found to be responsible for host range restriction in specific cell lines, and became known as the host range genes (*hr* genes) (Bradley *et al*., 2005).

All poxviruses are predicted to encode a unique collection of host range genes. However, discovery of each host range gene is a result of targeted gene-knockout analysis, making identification and functional analysis of these genes complex and time consuming. For example, VACV mutated in a gene-knockout experiment was unable to grow in previously permissive RK-13 cells; this discovery of the VACV K1L gene, which is described more fully in chapter 3, section 3.1. Cowpox CP77 was discovered in a similar manner (Ramsey-Ewing *et al*., 1996). Most species within the *Orthopoxvirus* genus have a broad host range and are capable of full replication in a wide range of host cells (in vitro studies). This can probably be explained by the large coding capacity of poxviruses which allows them to encode a wide range of specific proteins to break the species barrier (Alcami *et al*., 2000). Most of the better characterised host range genes have been found to be conserved amongst the *Orthopoxvirus* genus, suggesting the importance of these genes in modulating host cell immune responses. Vaccinia virus (VACV), one of the most extensively studied *Orthopoxvirus* (as well as Poxvirus), has provided researchers ample data for studying host range genes.

1.3 Avipoxviruses

1.3.1 Classification and Phylogeny

As previously discussed, further classification of the *Chordopoxvirinae* is determined by the host organism of the virus. Members of the *Avipoxvirus* genus cause disease in poultry and wild birds. To date, poxvirus infection has been identified in more than 230 of the known avian species. The following viruses have been identified as official members of the genus: Fowlpoxvirus (FWPV), Canarypoxvirus (CNPV), Pigeonpoxvirus (PGPV), Parrotpoxvirus (PRPV), Turkeypoxvirus (TKPV), Sparrowpoxvirus (SRPV), Starlingpoxvirus (SLPV), Houbarapoxvirus (HOPV), Macawpoxvirus (MCPV), Falconpoxvirus (FLPV), Albatrosspoxvirus (ABPV) (Jarmin *et al*., 2005). Phylogenetic analysis based on alignment of partial P4b nucleotide sequences of various isolates from different species showed that they group into 3 clades: A, B, C. (Jarmin *et al*., 2005).

Two types of disease are seen following *Avipoxvirus* infection. The most common is the cutaneous form characterised by the formation of small lesions on the unfeathered region of the avian host. *Avipoxvirus* infection can also result in the diptheric form of disease characterised by formation of lesions in the mucous membranes of the respiratory tract. In poultry, the cutaneous form is often assoicated with low mortality whereas the diptheric form is associated with high mortality due to the occlusion of the oropharynx. Signs of *Avipoxvirus* infection have been noted in migratory birds such as quail and finch, which likely contribute to the distribution of the *Avipoxvirus* genus throughout the world (Kow, 1992).

In contrast to other poxvirus genera, the *Avipoxvirus* genus shows a restricted host-range and can only replicate in specific avian species and cell lines of avian origin. Most mammalian cell lines are non-permissive for avianpoxviruses. The only known mammalian cell line reported to be permissive was derived from syrian baby hamster kidney fibroblasts (BHK-21) suggesting that this cell line may lack factors/pathways that inhibit viral replication (Weli *et al*., 2005). As previously mentioned, Avian poxviruses do not share similarity with the other *Chordopoxvirus* genera regarding gene arrangement and host restriction. Most members of the *Chordopoxvirinae* show similar gene order within the conserved central region of the genome, whereas rearrangement of these conserved genes is seen in the *Avipoxviruses*. This suggests that the *Avipoxvirus* genus is the most divergent genus within the *Chordopoxvirus* sub-family (Gubser *et al*., 2004).

1.3.2 Penguinpoxvirus (PEPV)

In 1988, marine birds along the coast of South Africa were reported ill following an oil spill. African penguins brought into The Southern African Foundation for the Conservation of Coastal Birds (SANCCOB) were found to have lesions around the eyes. Virus isolated from these lesions was characterised using restriction enzyme analysis and electron microscopy, and the results were compared to other known avian poxviruses (Kow, 1992). Electron micrograph images of the isolated virus showed that it was a poxvirus and the morphology of virus was similar to that of FWPV. Restriction enzyme analysis and comparison to other known avian poxviruses including CNPV, FWPV and Parrotpoxvirus, confirmed that it was a novel avian poxvirus (Kow, 1992). Phylogenetic analysis revealed that the virus groups with *Avipoxviruses* in clade A, subclade A2 (Carulei *et al*., 2009).

Further characterization involved cell culture studies to investigate growth characteristics of PEPV in mammalian and avian cell lines (Stannard *et al*., 1998). Various cell lines, which included (CV-1 (monkey kidney), Vero (African green monkey kidney), HeLa (human cervical carcinoma), MDBK (bovine kidney), RK-13 (rabbit kidney), HEF (human embryo fibroblasts) and CEF (primary chick embryo fibroblasts)), were infected with PEPV. No increase in viral titre was observed and no infectious virus was obtained after a single passaging event. Visualisation of infected cells using electron microscopy revealed the presence of immature viral particles, indicating that virus maturation was blocked at a late stage of viral replication. Transient expression analyses were performed to determine the degree of virus maturation. Mammalian and avian cells, CV-1 (monkey kidney), Vero (African green monkey kidney), HeLa (human cervical carcinoma), MDBK (bovine kidney), RK-13 (rabbit kidney), HEF (human embryo fibroblasts) and CEF (primary chick embryo fibroblasts), were infected with PEPV and transfected with plasmid containing a reporter gene under the control of the Vaccinia virus P11 late promoter. The results showed variation in the level of reporter gene expression, suggesting that PEPV is more adapted to certain cell lines. A high level of reporter gene expression was detected in CEF and CV-1 cells suggesting a late block to viral replication in these cell lines whereas low levels of reporter gene expression was detected in HeLa, MDBK, HEF and RK-13 cells, suggesting an earlier block to viral replication in these cell lines (Stannard *et al*., 1996). This also indicated that PEPV is capable of expressing foreign antigens in mammalian cells which raised the possibility of using PEPV as a vaccine vector. The genome of PEPV has recently been sequenced and will provide a foundation for further investigation of host range specificity of PEPV (Carulei, unpublished data).

1.4 Poxviruses as Vaccine Vectors

With the advent of the Human Immunodeficiency Virus (HIV) pandemic and reassorted influenza virus, novel vaccine vectors are continuously required in this vaccine versus virus battle, especially against these rapidly mutating viruses. Poxviruses have been used as vectors for the expression of antigenic proteins and the success of poxviruses as vaccine vectors is due to their ability to accommodate large foreign gene inserts as well as their unique immunological properties in eliciting long-term, protective, humoral and cell-mediated immune responses. Vaccinia virus vaccine was used successfully to eradicate smallpox (Fenner *et al*., 1988). However the VACV vaccine was found to cause adverse effects such as eczema vaccinatum, myocarditis and vaccinial encephalitis in immunosuppressed individuals and young children (Remichkova, 2010). These effects sometimes resulted in fatal complications and substantial morbidity causing concern about the safety of using VACV as a vaccine. These adverse effects led to the development of highly attenuated, replication deficient strains, Modified Vaccinia Ankara (MVA) and a highly attenuated Vaccinia virus strain NYVAC as well as the host range restricted Canarypoxvirus strain ALVAC. These vectors have good safety profiles and elicit strong immune responses against vectored antigens.

1.4.1 Modified Vaccinia Ankara (MVA)

MVA was derived from the Ankara strain of VACV by >500 passages in chicken embryo fibroblasts (CEFs). DNA sequencing and analysis revealed that 15% (30kb) of the parental viral genome was deleted from the termini and central region. Multiple genetic deletions and mutations resulted in loss of the ability to perform complete replication in most mammalian cells. To date, only two cell lines (BHK-21 and CEFs)

have been found to be permissive to MVA infection. In the late 20th century, MVA was inoculated into more than 120 000 individuals in Germany during the Smallpox eradication campaign, with no reported adverse effects. Thus MVA is now considered to be a suitable platform for the next generation of safer recombinant poxvirus vectors and has been clinically tested against infectious diseases such as AIDS, malaria and human papillomavirus-associated cancer (Nájera *et al*., 2006).

In nonpermissive cells, the MVA replication cycle is abolished during the late stage. Also, cytopathic effect (CPE) (cell function, metabolism and morphology) induced by MVA is minor compared with the wild type VACV strain. This may be attributed to the fact that MVA has lost 15% of its parental genome and may relate to the absence of the early proteins, for example the VACV K1L gene (see Figure 1.3). Many deleted or mutated genes are implicated in the modulation of the host response. It was assumed that these deletions and mutations are responsible for the replication-deficient phenotype of MVA. Comparing MVA to the Copenhagen strain of VACV, the left terminal region of the MVA genome contains 4 large deletions and many fragmented open reading frames. However the central conserved region remains intact with the exception of F5L, F11L and O1L genes, the function of these genes are still not well studied. The right terminal region contains 3 large deletions as well as many disruptions of open reading frames (see Figure 1.3) (Gómez *et al*., 2008). Despite its restricted replication capacity, MVA elicits a strong immune response, possibly due to the absence of the host range genes. The majority of these host range gene contribute to immunomodulation. Loss of these immunomodulatory genes result in a strong immune response being elicited by the host.

1.4.2 NYVAC

NYVAC, another attenuated strain of VACV, well-known for its safety profile, was originally derived from the VACV Copenhagen strain, and attenuated by targeted deletion of 18 open reading frames involved in host range, virulence and pathogenesis of the virus (Gómez *et al*., 2008). Thus NYVAC is replication deficient in cells of human, murine and equine origin, however CEFs and Vero cells are permissive to NYVAC infection.

Figure 1.3 shows a comparison of the deleted genes of MVA and NYVAC. Certain genes are deleted in either NYVAC or MVA and some are absent from both viruses. In NYVAC infected cells, CPE was observed early after infection in a range of permissive and nonpermissive cell lines (Gómez *et al*., 2008). This indicates that the presence of CPE is independent of host range restriction. Rounding of the cells is dependant on the synthesis of early proteins, indicating that NYVAC is capable of producing early proteins in both permissive and nonpermissive cells. In nonpermissive cells, for NYVAC, there is a block in the virus life cycle prior to the formation of immature virions, whereas for MVA, the bock occurs after the formation of immature virions (Nájera *et al*., 2006). While MVA produces both early and late viral proteins, certain late viral proteins are not detected in NYVAC infected cells, however the absence of the late viral proteins is not due to a blockage in the transcription of these late genes, but rather at the level of translation. This feature has contributed to the safety of NYVAC, since it is unable to synthesize the late viral proteins needed for the assembly and formation of infectious viral particles under nonpermissive conditions.

Apoptosis plays a key role in cellular defense against viral infection and poxviruses

encode certain proteins to counteract programmed cell death. Apoptosis is observed in nonpermissive cells infected with MVA or NYVAC that were previously permissive to the wild type VACV. This may reflect the loss of specific viral genes, for example, K1L, as will be discussed in Chapter 3, section 3.1. Deactivation of NF-κB is one of the mechanisms utilized by poxviruses to counteract apoptosis, and this mechanism may be mediated by the K1L protein (Nájera *et al*., 2006). However, the percentage of apoptotic cells following MVA infection is significantly lower than that following NYVAC infection. This may be due to the expression of different host range genes in MVA and NYVAC. This may be due to MVA expressing a certain late protein which inhibits apoptosis, or an early protein (possibly C7L) that plays a role in inhibiting apoptosis; which NYVAC does not have. C7L was deleted from NYVAC but remains intact in MVA (see Figure 1.3), and may therefore contribute to the differences in induction of apoptosis (Nájera *et al*., 2006).

Even with these differences, NYVAC induces similar immune responses to MVA. In general MVA induces specific CD8+ T cell and CD4+ responses whereas NYVAC induces greater levels of CD4+ T cells. Thus for vaccination purposes, by using both vectors in prime/boost strategies it may be possible to produce broader cellular and humoral immune responses, which are required for vaccination strategies against HIV. Prime/boost strategies employing heterologous vectors, especially with recombinant poxviruses as the boost, have proven to be excellent regimens to elicit antigen-specific cellular immune responses. Despite the above mentioned advantages of MVA and NYVAC, pre-existing anti-VACV immunity in VACV vaccinated individuals (vaccinated during the global eradication of smallpox) may reduce the effectiveness of attenuated VACV strains as vaccine delivery vectors (Gómez *et al*., 2008).

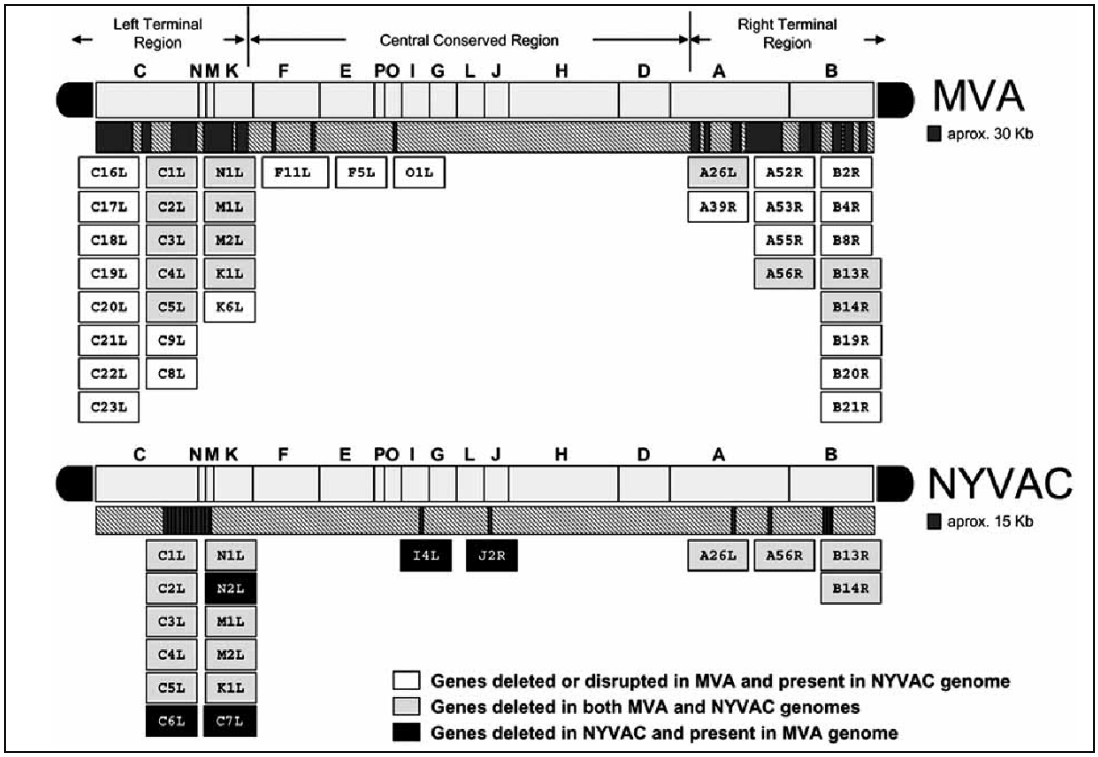

Figure 1.3. Scheme of deleted genes in MVA and NYVAC genomes (Gómez *et al*., 2008).

1.4.3 ALVAC

Due to the possibility of pre-existing anti VACV immunity, vectors based on poxviruses other than VACV have been extensively researched. *Avipoxviruses* are naturally replication deficient in most mammalian cells (BHK-21 cells are permissive to some *Avipoxviruses*), giving members of this genus a significant biosafety advantage in terms of vector development. Currently the two most well known vectors in the *Avipoxvirus* genus are Fowlpoxvirus (TROVAC) and Canarypoxvirus (ALVAC) (Tulman *et al*., 2004).

ALVAC was derived from a Canarypoxvirus isolated from a single pox lesion on an infected canary. The virus was attenuated by 200 serial passage in CEF cells. Like the attenuated VACV strains, ALVAC is capable of expressing foreign antigens in non-permissive cells and eliciting strong immune responses. Initially ALVAC and TROVAC

were evaluated and developed to vaccinate birds against avian diseases. Use of these vectors has expanded to other veterinary and human vaccine applications. ALVAC vectors have been developed to express genes from rabies virus, canine distemper virus, feline leukemia virus, human immunodeficiency virus, simian immunodeficiency virus and Japanese encephalitis virus (Yu *et al*., 2006).

During the development of HIV vaccines, studies showed ALVAC is capable of inducing early inflammatory responses in mice and guinea-pigs, in both the infectious and heat-inactivated forms, and is not influenced by the route of virus inoculation (intramuscular, subcutaneous or intravenous). This suggests that inactivated poxviruses are capable of retaining immunostimulatory properties (Boudet *et al*., 2001). The safety of an ALVAC-HIV vaccine consisting of 5 different ALVAC constructs was tested on 1497 volunteers, of which less that 3% vaccinees showed severe local responses and less than 1% experienced severe pain or tenderness. Reactogenicity was not related to the inserted genes and did not vary between different constructs (de Bruyn *et al*., 2003). Various HIV subtype B Canarypox-HIV vector primes and boosters containing subunit glycoprotein 120 or 160 (gp120 or gp160) were evaluated in phase I and II clinical trials, and were shown to be safe and simultaneously elicit strong immune responses (Russell *et al*., 2007). In 2003, a phase 3 trial of an ALVAC-HIV (vCP1521) prime with a VaxGen bivalent gp120 AIDSVAX B/E boost took place in Thailand. 16402 healthy men and women between the ages of 18 and 30 were randomly chosen to participate in the trial. Preliminary results showed a statistically though mild protective efficacy of 31% in the vaccinated group. The results showed a modest benefit in reducing the risk of HIV infection but further investigation on whether the efficacy decreased over the first year of vaccination or whether the efficacy may be greater in individuals at lower risk is still to be examined (Reks-Ngarm *et al*., 2009).

1.5 Project Motivation

According to the latest World Health Organisation report, 33.4 million people currently live with HIV/AIDS worldwide of which 67% of the total number of people infected with HIV lives in sub-saharan Africa (UNAIDS, 2009). Therefore, there is an urgent need for a HIV vaccine. Although existing poxvirus-based vaccine vectors have shown promising results in clinical trials (Russell *et al*., 2007; Reks-Ngarm *et al*., 2009), pre-existing VACV immunity and past Orthopoxvirus-based complications have stimulated the search for safer, novel poxvirus vectors.

PEPV, a novel, local avian poxvirus has the potential for development as a vaccine vector due to its host range restriction and non cross-reactivity with *Orthopoxviruses* (Stannard *et al*., 1996). However, to construct a recombinant poxvirus one cannot simply clone the foreign gene of interest directly into the viral genome; as poxvirus DNA is large (180~350kb) and noninfectious. The standard method of producing a recombinant virus is to infect cells with the virus and transfect these same cells with a transfer vector. The transfer vector would contain the gene of interest together with a selection marker, flanked by sequences that are homologous to a nonessential gene of the virus. Once both virus and transfer vector are inside the cell homologous recombination can take place between the flanking sequences of the transfer vector and the viral genome and the foreign gene of interest is transferred from the transfer vector into the nonessential region of the virus (shown in Chapter 4, Figure 4.1). The purpose of the selection marker is to select the recombinant virus from the wild type virus.

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As discussed in section 1.3.2, PEPV is unable to complete its replication cycle in any of

the cell lines tested. This characteristic could be used in the construction of recombinant PEPV. VACV K1L protein is capable of rescuing the growth of mutant VACV and wild type MVA in RK-13 cells (see Chapter 3, section 3.1). It is possible that transient expression of VACV K1L gene could rescue the growth of PEPV in RK-13 cells. The aim of this study was to rescue the growth of PEPV in RK13 cells and hence utilise K1L as a selective marker for the construction of recombinant PEPV. The first objective was to confirm that RK-13 cells are non-permissive for the growth of PEPV by constructing a standard growth curve.

The second objective was to construct a transfer vector capable of transient expression of VACV K1L protein in RK-13 cells infected with PEPV. The TK gene was used as a site of insertion. TK sequences were PCR amplified from PEPV and cloned into the plasmid pUC21. K1L and the reporter gene GUS were inserted between these flanking sequences in subsequent cloning steps.

The third objective was to rescue PEPV growth in RK-13 cells which were infected with PEPV and transfected with the transfer vector. If homologous recombination were to take place, all recombinant virus containing K1L would complete its replication cycle in RK-13 cells while remaining wtPEPV will be unable to complete its replication cycle.

The final objective was to isolate a recombinant PEPV containing K1L and GUS. The long term goal of this project is to construct a recombinant PEPV which expresses HIV-1 genes and could be used as a HIV-1 vaccine.

Chapter 2: Growth and titration of PEPV on Chick Chorioallantoic Membranes (CAMs)

2.1 Introduction

The long term goal of this project is to construct a recombinant PEPV. For this purpose it is necessary to prepare PEPV stocks of high titre and known concentration. PEPV was required for growth curve experiments to determine whether it is able to complete its replication cycle in RK-13 cells (Chapter 3) and for the construction of recombinant PEPV (Chapter 5). Although a number of cell lines (CV-1 (monkey kidney), Vero (African green monkey kidney), HeLa (human cervical carcinoma), MDBK (bovine kidney), RK-13 (rabbit kidney), HEF (human embryo fibroblasts) and CEF (primary chick embryo fibroblasts)) have been tested for permissively to PEPV growth, a suitable cell-line that can be used to grow PEPV is yet to be identified (Stannard *et al*., 1998). At present the only known method of growing up and harvesting PEPV is to grow the virus on chorioallantoic membranes (CAM) of embryonated hens' eggs that are 9-10 days old.

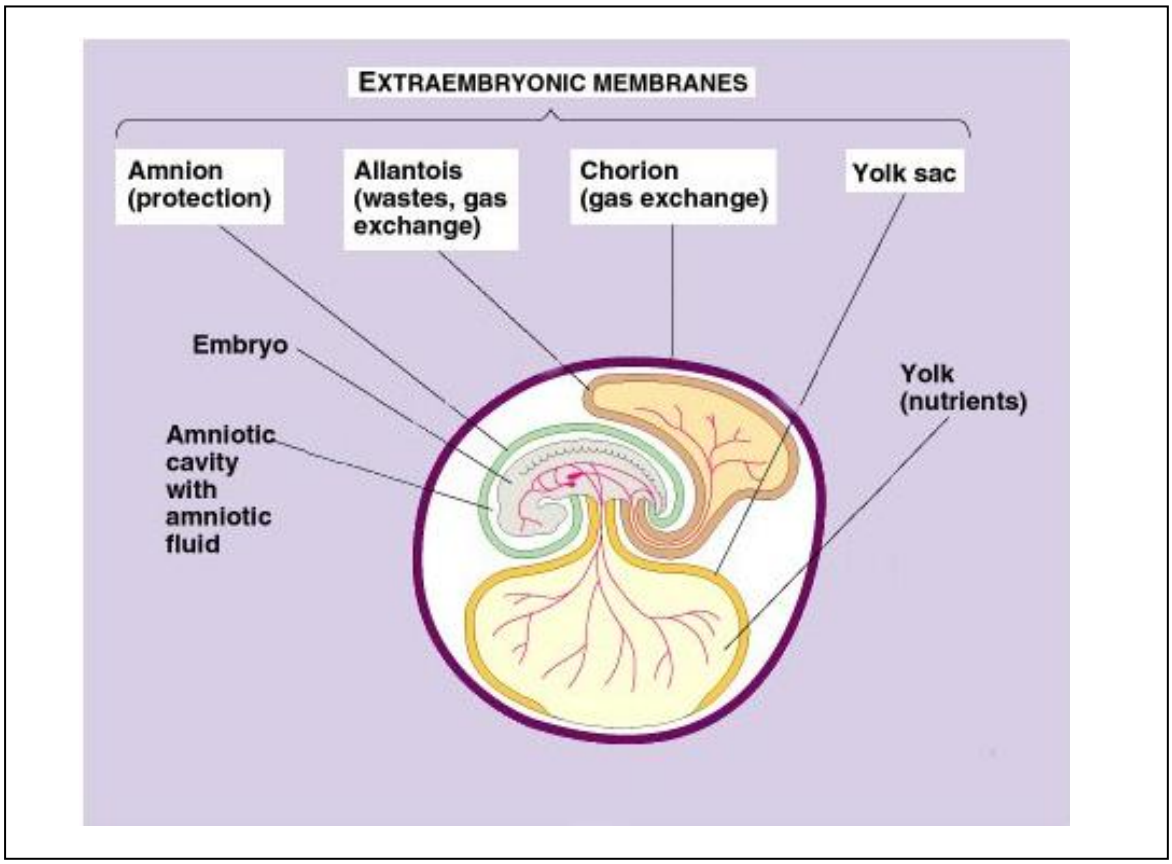

Figure 2.1. Diagram showing the contents of a typical fertilized egg.

Figure 2.1 shows a diagram of a typical fertilized egg. The chorioallantoic membrane consist of an outer layer of stratified epithelium, the respiratory surface of the egg and an inner layer of endoderm (lining the allantoic cavity). The use of chick embryos for poxvirus diagnosis was first described in 1937, and has been used in poxvirus diagnostics (Joklik, 1962). Some poxviruses such as *Parapoxviruses*, *Yatapoxviruses* and *Molluscipoxviruses* do not form pocks on CAM whereas *Orthopoxviruses* (including Variola virus, Monkeypox virus, Cowpox virus and Vaccinia virus), *Avipoxviruses*, *Leporipoxviruses* and *Capripoxviruses* produce pocks on CAMs (Mercer *et al*., 2007).

Different species of poxvirus show different characteristics of pocks produced on the chick CAM (Fenner *et al*., 1989). Most of the *Orthopoxvirus* strains, (Cowpox virus, Monkeypox virus, various strains of Vaccinia virus and Elephantpox virus) produce red pocks on CAMs (mutant strains of these poxviruses produce white pocks), whereas Variola *virus* produces typical white pocks on chick CAMs (Baxby, 1969; Baxby *et al*., 1977). The *Avipoxviruses* produce typical white pocks and the infected CAMs are generally thickened at the area of inoculation (Tripathy, 2009). Interestingly, the pock morphology can be influenced by changing the incubation temperature of the egg, positioning of CAM during incubation and the source and age of the chick embryo. The fact that there is a particular age of embryo (generally 10~12 days) which produces a high proportion of a typical pock suggests the need for a particular developmental stage in the embryo, however the precise physiological mechanism being affected by the environmental change is not well understood (Baxby, 1969).

In this study, the method used for the isolation of PEPV was a modification of the method used by Joklik (1962) and is described in sections 2.2.2 and 2.2.3.

2.2 Materials and Methods

2.2.1 Inoculation of Chick Chorioallantoic Membranes (CAMs)

Fertilized eggs that were 7 ~ 12 days old were ordered through the UCT Medical School Animal Unit (Ethis Approval No. AEC 007/020). Eggs were incubated at 37^{o}C for the duration of viral growth; this provided favorable conditions for the growth and development of the chick embryo, and hence, good nutrition for PEPV, which grows on the chorioallantoic membranes (CAM).

Eggs were first observed with an egg-candler to locate suitable regions for PEPV infection. Infection must not take place directly on the blood capillary but nearby. A pencil mark was made at this position and also on the central region of the air sack. An egg pricker was used to puncture the shell on the marked regions. A drop of phosphate buffered saline (PBS) containing penicillin and streptomycin [20ml of 1xPBS (137mM NaCl, 2.7mM KCl, 100mM Na_2HPO4, 2mM KH_2PO4, pH 7.2) with 200µl of P/S (1000U/ml Penicillin, 10mg/ml Streptomycin)] was dropped at the dorsal opening and allowed to enter the egg by creating a suction force, using a rubber bulb, at the air sack opening. This resulted in the dropping of the CAM. PBS is a water based salt solution, which is used to maintain a constant pH. It is isotonic and non-toxic to cells. Therefore it is used as a diluent or to rinse flasks of cells. The eggs were left at 37^{o}C for 1.5 hours.

A PEPV viral stock (obtained from the Department of Clinical Laboratory Sciences, Virology Division, UCT) was diluted using PBS as the pH value of PBS (pH 7.2) is the same as that of living tissue. It was essential to first dilute the viral stock prior to infection, as infection with concentrated viral suspension onto the CAM could result in

killing of the chicken embryo and hence reduction in growth of the virus. 100µl of the diluted viral stock was injected onto the CAM, using a syringe with surgical needle, at the marked dorsal opening. To prevent contamination of the CAM through the dorsal opening, melted candle wax was placed next to the dorsal opening. As soon as the wax cooled down, a wooden tongue compressor was used to spread the wax across the dorsal opening. If heated wax was immediately placed across the dorsal opening, the liquid wax would damage the infected CAMs.

Lastly, eggs were rotated by hand to allow the viral suspension to spread evenly across the CAM. Eggs were then incubated at 37^{o}C for 96 hours.

2.2.2 Viral Harvest

The following procedure was carried out, as described by Daria Kow (Kow, 1992).
Sterile, sharp scissors were used to cut along the sides of each PEPV infected egg from the air sac opening. The dropped CAM was removed from the egg shell using sterile forceps. The infected CAM was placed in a physiological saline (0.85% w/v sodium chloride) solution immediately after it had been detached from the shell. A series of washes in saline solution removed blood and other contaminants from the membranes. A few CAMs were spread out onto Petri dishes and examined for the presence of pocks. Washed CAMs on the Petri dishes were photographed.

Four to five infected CAMs were transferred to a sterile McCartney bottle with glass beads. 5ml of ice-cold McIlvain's solution (0.1M citric acid, 0.2M disodium hydrogen orthophosphate, pH7.4) was added into each bottle. McIlvain's solution was used to create a hypotonic environment for suspension of the viral particles (Fenner, 1989).

Bottles were then balanced via the addition of ice-cold McIlvain's solution and shaken vigorously by hand for two minutes. Membranes could be placed at 4^{o}C overnight or immediately processed as described below.

The bottles were centrifuged at 800 rpm for 10 minutes. The purpose of centrifugation was to pellet the debris and contaminants and release virus into the McIlvain's solution. Supernatants were recovered and transfered into another sterile ice-cold McCartney bottle. The bottles with recovered supernatants were kept on ice to maintain virus viability. 5ml of ice-cold McIlvain's solution was added again to the bottle with CAMs and glass beads. The bottles were shaken vigorously by hand for two minutes and centrifuged again at 800 rpm for 10 minutes. The supernatants were recovered and added to the previously recovered supernatants. These steps were repeated 3~4 times to extract the maximum amount of virus possible from the CAMs. The bottles containing recovered supernatants were balanced with ice-cold McIlvain's solution and centrifuged at 1500 rpm for 30 minutes. This was to pellet any remaining CAM tissue debris. The supernatants were then transferred to sterile Beckman JA20 centrifuge tubes.

Centrifuge tubes were balanced and 5ml of 36% (w/v) sucrose solution was carefully pipetted to the bottom of the centrifuge tubes. The high density of the sucrose solution created a cushion, which trapped any excess CAM debris during centrifugation through the cushion. Centrifuge tubes were centrifuged for 1 hour at 11000 rpm at 4^{o}C in a Beckman J2-21 centrifuge. After centrifugation, supernatants were discarded and the pellets were resuspended in 0.5ml of Tris-EDTA buffer (10mM Tris, 1mM EDTA, pH 9). Sucrose dextran gradients were used to further purify the viral extract. 10ml of 10% (w/v) dextran was first pipetted into each centrifuge tube, 5ml of 36% (w/v) sucrose solutions was then carefully pipetted beneath the dextran solution and, finally, the viral

suspension was placed on top of the dextran layer. The tubes were centrifuged at 11000 rpm for 1 hour at 4^{o}C. The supernatants were removed and pellets were resuspended in 0.5ml Tris-EDTA buffer (pH 9). The viral suspension was aliquotted into several 1.5ml microfuge tubes (100µl per tube) and stored at -80^{o}C to maintain virus viability.

2.2.3 Titration of PEPV

Titration was carried out to quantify the viable virus. Viral titres are standardly expressed as pock (or plaque) forming units per milliliter (pfu/ml).

One of the stock PEPV aliquots (100µl) was retrieved from the -80^{o}C freezer, thawed and diluted with 900µl PBS solution (10 fold dilution). A dilution series of 10^{-1} to 10^{-5} was set up and dilutions of 10^{-2} to 10^{-5} were used for the titration. One membrane was inoculated with PBS (uninfected).

A total of 12 eggs were infected, two eggs for the 10^{-2} dilution, three eggs for each dilution of the 10^{-3} to 10^{-5} dilutions and one egg inoculate with PBS only. Inoculation was performed as described in section 2.2.1 and the CAMs were harvested as described in section 2.2.2. Membranes infected with a dilution which gave countable pocks were used to determine the virus titre. The pocks were counted on each membrane and the average was determined.

The virus titre was calculated as:

Virus titre (pfu/ml) = Number of pocks formed on CAM x dilution factor x 10 (as 100µl was used to inoculate each egg and titre is expressed in pfu/ml)

2.3 Results

A PEPV stock was made from starting material (10^7 pfu/ml) obtained from the Division of Medical Virology, Department of Clinical Sciences. The PEPV stock was diluted down to 10^{-3} (10^4 pfu/ml) and 10^{-4} (10^3 pfu/ml) with 1X PBS and each dilution was used to inoculate 20 chick CAMs as described in section 2.2.1. Four days post inoculation of the CAMs, PEPV was harvested from all 40 CAMs as described in section 2.2.2, aliquotted into 1.5ml microfuge tubes (100µl per tube) and stored at -80°C. For the quantification of the virus, titration was carried out on one of the frozen aliquots as described in section 2.2.3. Figure 2.2 shows representative membranes from this titration.

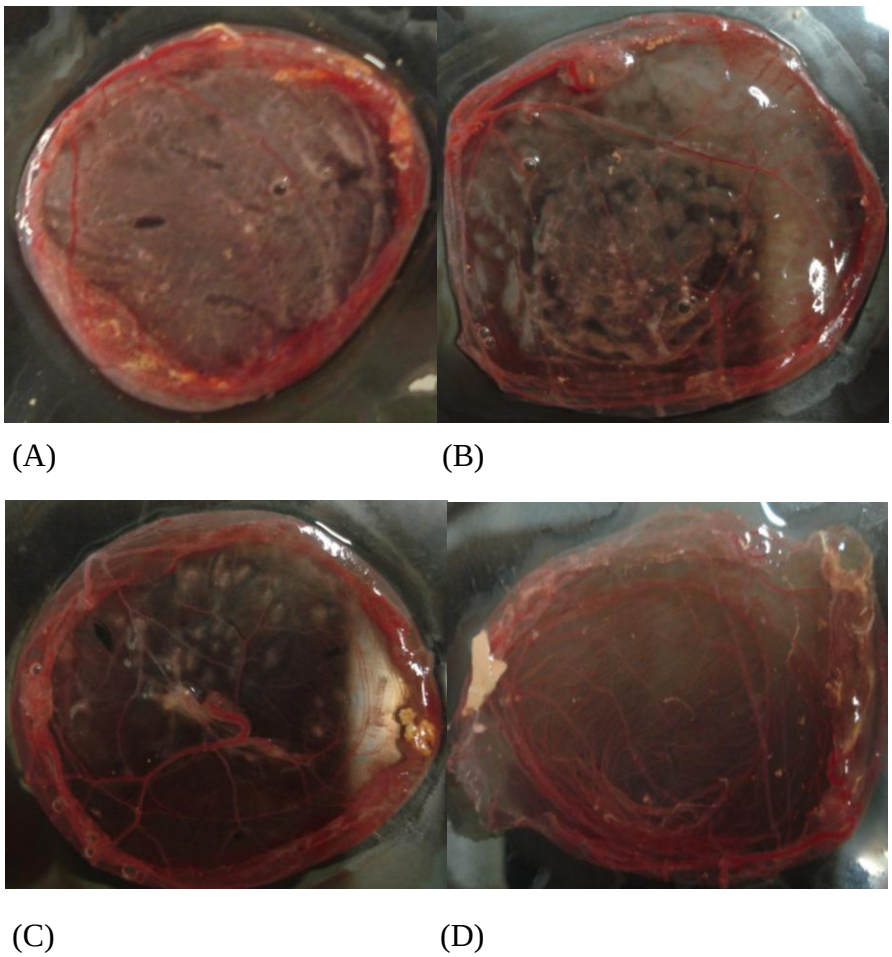

Figure 2.2. Titration of PEPV on chick CAMs. CAMs were infected with dilutions of A) 10^{-3}; B) 10^{-4} and C) 10^{-5} or left uninfected D).

A clear membrane was seen for the uninfected CAM (Figure 2.2D). Individual pocks were seen on the CAM infected with the highest dilution of PEPV (Figure 2.2C). As the concentration increased the number of pocks increased and gradually merged with one another (Figure 2.2B) and in the end formed a confluent layer of pocks (Figure 2.2A). Membranes infected with the 10^{-5} dilution were used to determine the virus titre. Pocks were counted and the average determined as listed in Table 2.1. An average of 30.5 pocks (per 100µl) were counted from the eggs infected with the 10^{-5} dilution (see Table 2.1). The titre was calculated by multiplying the number of pocks (30.5) by the dilution factor (10^5) to give a value of $3.05x10^6$ pfu (pocks forming unit)/100µl. This was converted to pfu/ml by multiplying by 10. The final titre obtained was $3.05x10^7$ pfu/ml. The final viral volume obtained from the initial viral stock preparation was 1.5ml, therefore the total yield of virus from the 40 eggs was $4.6x10^7$ pfu.

Table 2.1. Table showing numbers of pocks on PEPV infected CAMs and subsequent determination of viral titre (pfu/ml) (Stock 1)

Dilution	No. of Pocks (per 100µl)			Average No. Of Pocks per 100µl	Average Titre (pfu/ml)
	1	2	3		
10^{-2}	Over confluent	Over confluent	Unfertilized egg	Unable to calculate	Unable to calculate
10^{-3}	Over confluent	Over confluent	Over confluent	Unable to calculate	Unable to calculate
10^{-4}	>200	>200	>200	>200	$>2x10^7$
10^{-5}	30	31	unfertilized	30.5	$3.05x10^7$

Later in the project a second viral stock was prepared in the same manner and titre was determined as shown in Table 2.2. However, when determining the titre of the second viral stock, the dilution series was extended to 10^{-6}. This is because, in the first titration, the only dilution factor that one could use for determining the titre of PEPV was 10^{-5}, which produced 10~100 pocks on the surface of PEPV infected CAMs. All the other dilution factors produced too many pocks on the surface of the CAM for accurate

counting of pocks. Therefore, further dilution would ensure single pocks, for accurate determination of the titre of PEPV.

Table 2.2. Table showing numbers of pocks on PEPV infected CAMs and subsequent determination of viral titre (pfu/ml) (Stock 2)

Dilution	No. of Pocks (per 100µl)			Average No. Of Pocks per 100µl	Average Titre (pfu/ml)
	1	2	3		
10^{-3}	Over confluent	Over confluent	Over confluent	Unable to calculate	Unable to calculate
10^{-4}	>100	>100	>100	>100	$>1x10^{7}$
10^{-5}	14	21	Egg rotten	17.5	$1.75x10^{7}$
10^{-6}	3	10*	3	3	$3.0x10^{7}$

*outlier, therefore not considered in the average No. of Pocks.

This stock gave an average number of 17.5 pocks (per 100µl) on 10^{-5} dilution and 3 pocks (per 100µl) on 10^{-6} dilution. The titre was thought to be in between $1.75x10^{7}$ and $3.0x10^{7}$ pfu/ml. The dilution of 10^{-5} gave >10 pocks/membrane, which would be more accurate than using figures <10 pocks / membrane. However, one egg was rotten and only two values could be used. Therefore for future work, one would suggest to use more than 3 CAMs per dilution. For the purpose of this project, the viral titre of stock 2 was estimated to be $2x10^{7}$ pfu/ml. The final viral volume obtained from the initial viral stock preparation was 1.5ml, therefore the total yield of virus from the 40 eggs was $3x10^{7}$ pfu.

2.4 Discussion

The pocks produced by PEPV in this study, showed a typical white pock of 1~2mm in diameter. This is similar to the appearance of pocks produced by Fowlpox virus (Kow, 1992), and is consistent with previous work done on PEPV (Kow, 1992).

The two preparations of PEPV from 40 chick CAMs each resulted in viral titres of > 10^{7} pfu/ml ($3.05x10^{7}$ pfu/ml and $2x10^{7}$ pfu/ml). During the titration of the

harvested PEPV stock, for both experiments, 2 of the 3 CAMs had to be discarded. It would therefore be advisable to use more than 3 CAMs in the future for a more precise determination of viral titre. To accurately determine the titre of PEPV on CAMs, one is required to count individual pocks, which is only possible if there are less than 100 pocks per CAM. It is therefore important to use a large dilution series in the titration of a large virus stock preparation, as inoculation of CAMs with less diluted PEPV will result in formation of more than 100 pocks per CAM, which cannot be considered for the determination of the titre, as a large number of pocks on a single CAM will result in the merging of individual pocks which cannot be accurately counted (shown in Figure 2.2A and B).

In this study, the amount of virus harvested from CAMs infected with PEPV is relevantly low compared to those infected with *Orthopoxviruses*. Vaccinia virus, virus and Cowpoxvius were shown to produce 10^8 pfu/CAM (Joklik, 1962), whereas in this study, 40 CAMs produces 10^7 pfu in total ($4.6x10^7$ pfu and $3.0x10^7$ pfu respectively). However, in the study done by Joklik (1962), 10^5 to 10^6 pfu of virus was inoculated onto the CAM whereas in this study, only 10^2 to 10^3 pfu of virus was inoculated onto the CAM. Inoculation with higher concentrations of PEPV was attempted, however, it ultimately killed the chick embryos and no pocks could be harvested from the infected CAMs (result not shown). In several poxvirus-based vaccine trials, subjects are inoculated with 10^5~10^7 pfu of recombinant virus (Coupar *et al*., 2006; Nájera *et al*., 2006; Elahi *et al*., 1998; Zheng *et al*., 2006). Hence if one intends to construct a recombinant PEPV as a potential vaccine candidate, growing on chick CAMs may not produce sufficient virus for a clinical trial. If a recombinant PEPV could be constructed which grows in RK-13 cells, it may be possible to generate stocks if higher titre in these cells.

Chapter 3: Growth Curve of PEPV in RK-13 Cells

3.1 Introduction

3.1.1 Discovery of VACV K1L gene

Host range genes were discovered by gene-knockout analysis, as described in section 1.2.1. Mutants display restricted ability to grow in cell lines permissive to wildtype virus infection. By comparing the mutant genomes to the wild type virus genome, one could identify the gene or genes responsible for viral tropism in specific cell lines. A mutant strain of Vaccinia virus (wild type strain: Copenhagen) was discovered to be non-permissive in rabbit fibroblast (RK-13) cells and certain human cell lines (Perkus *et al*., 1990). Transient expression of a certain VACV gene was found to be capable of rescuing VACV infection in the above mentioned cells. This led to the discovery of the host range gene K1L (Rosel *et al.*, 1986).

3.1.2 Action of the VACV K1L Gene Product

K1L protein is expressed during the early phase of viral replication and in the absence of K1L protein, viral replication is abolished. Furthermore, studies have shown that both viral and host protein synthesis is rapidly shut down in $K1L^{-ve}$ Poxvirus infected cells. However early viral mRNAs and proteins are still detected in the host cytoplasm (Ramsey-Ewing *et al.*, 1996).

K1L has been demonstrated to inhibit host cell immune responses by inhibiting activation of the classical NF-κB pathway (Figure 3.1). This pathway regulates the expression of genes involved in immune responses, inflammation, apoptosis and proliferation. Activation of the NF-κB pathway is initiated through many signals

including pathogen infection, proinflammatory cytokines, and exposure to mutagen. Activation of the pathway begins with the phosphorylation of IκB kinase (IKK) which subsequently phosphorylates IκBα. Phosphorylated IκBα is then polyubiquitinated and targeted by the 26S proteosome. Degradation of IκBα by the proteosome eventually releases the NF-κB transcription factor. A Key step in the NF-κB pathway is the phosphorylation of IκBα by IKK. By comparing the K1L knock-out mutant with wild type virus in a previously permissive cell line, it was deduced that K1L has the ability to inhibit the activation of the NF-κB pathway by preventing the degradation of IκBα. However, inhibition of host NF-κB activation does not seem to be related to the host range function of K1L as inhibition of NF-κB activation was observed during infection of both permissive and nonpermissive cell lines (Shisler *et al.*, 2004).

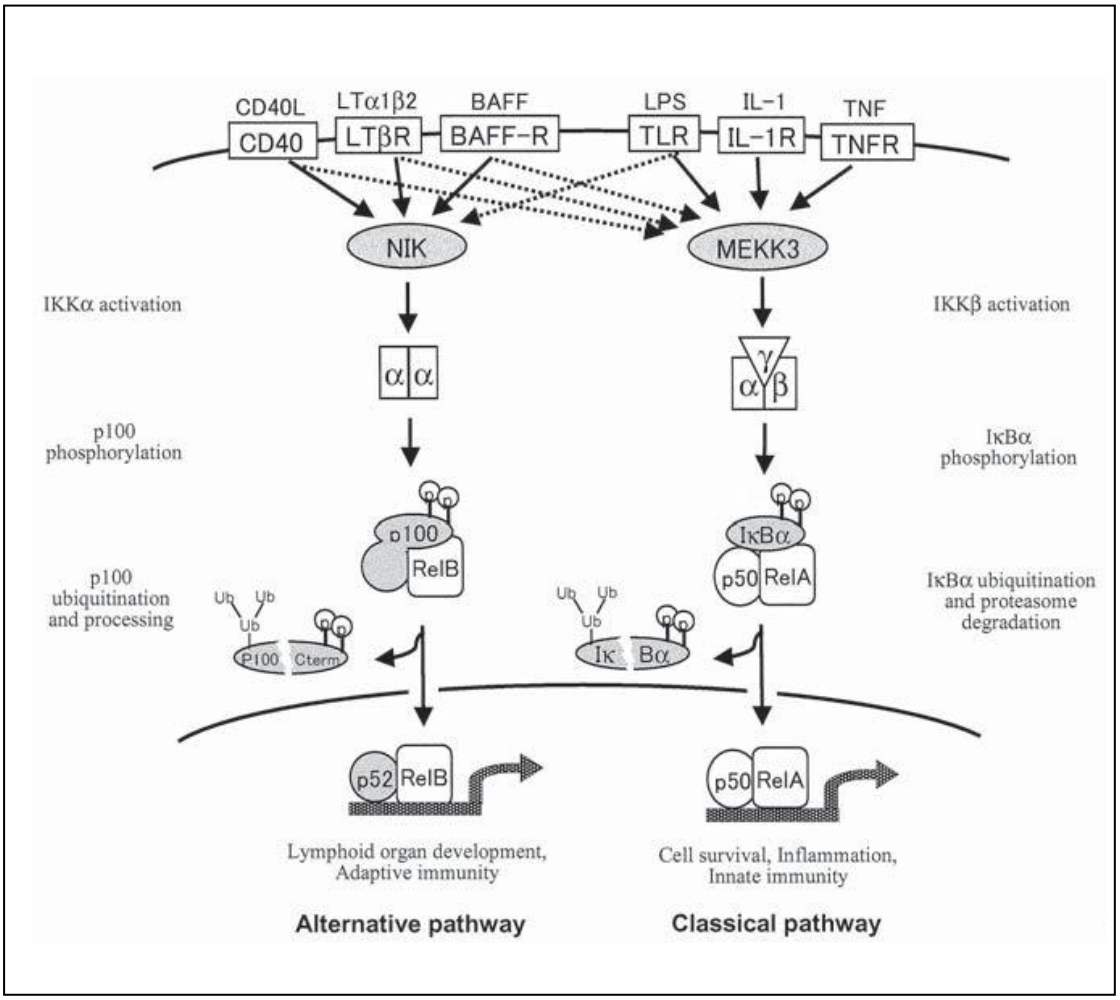

Figure 3.1. The classical and alternative pathways of NF-kB activation (Nishikori 2005).

Interestingly, the K1L gene shows functional correlation with another host range gene, CP77 found in Cowpox virus. The replication defect of K1L-deleted Vaccinia virus mutants in most human cells and RK-13 cells was complemented by the CP77 gene in growth rescue experiments (Meng *et al.*, 2006). However, limited sequence homology was observed between these 2 host range genes. The only homology seen between the two genes is that both genes consist of multiple ankyrin repeats (ANKs), a common 33-residue protein motif, which is involved in mediating protein-protein interactions. This motif can be found in proteins responsible for cytoskeletal organisation, cyclin-dependent kinase inhibition, signal transduction and transcriptional regulation. The structures of the ANK-containing proteins are well conserved, however each of these proteins interacts specifically with its binding partner. Binding specificity is a result of a variable surface residue within the ankyrin repeats and of the stacking of different numbers of repeats in one protein (Meng *et al.*, 2006). Thus considering the only homology of K1L protein and CP77 protein are these ANK sequences, the variable surface residues on the repeats may play a key role in the specific host range function of K1L protein.

K1L protein was found to interact with ACAP2, a human GTPase activating protein. This protein is a homolog of a rabbit protein previously identified to bind to K1L (Bradley *et al.*, 2005). Therefore interaction with ACAP2 was hypothesized to be the host range determinants of K1L. An investigation was then conducted to determine which of the ANKs are essential for K1L to maintain its function (Meng *et al.*, 2006). Through deletion and base substitution, it was found that ANK6 and the C-terminal of non-ANK residues are clearly not required, as deletions in these regions do not affect VACV replication in HeLa cells. ANK4 and ANK5 are required for maintaining the

structure of K1L protein as deletion of either one of these two ANK sequences abolished VACV replication while substitution with functionally unrelated ANKs restored the VACV replication. Residues in ANK1 and the C-terminal regions of ANK2 and ANK3 were found to be essential for the function of VACV replication in HeLa cells. These ANK sequences are located in different regions of the K1L gene, but the overall protein structure of K1L showed that the tertiary structure of the product of these ANKs sequences were positioned at the same region in the K1L protein. Substitution at these regions resulted in disruption of the binding surface and reduction in replication of VACV. Although substitution at these regions reduced VACV replication, it seems these substitutions do not participate in the host range function of K1L, as substitution reduced replication in both permissive and non permissive cells. However, two vital residues were identified: phenylalanine 82 and serine 83. Substitution of either residue reduced the growth rate but substitution of both residues completely abolished viral replication in HeLa cells (Meng *et al.*, 2006).

Interestingly, deletion or substitution at certain ANK sequences may not affect VACV replication in HeLa cells (cells of human origin) but results in a loss of replication in RK-13 cells (rabbit kidney fibroblast cells). The N-terminal region of ANK2 and ANK3 as well as the entire ANK5 and c-terminals of non-ANK sequences are not essential for viral replication in HeLa cells, but substitution or deletion in these regions results in termination or reduced viral replication in RK-13 cells. In addition, only substitution of both phenylalanine 82 and serine 83 will result in termination of viral replication in HeLa cells, but substitution of either residue will terminate replication in RK-13 cells. These results indicate that host restriction in RK-13 and human cell lines are different. The mechanism of restriction has not yet been elucidated. Interestingly, ACAP2 binding is not sufficient for the host-range function as previously hypothesized. Substitution of

the phenylalanine 82 and serine 83 residues of the ankyrin repeats resulted in abolishing the host-range function but still allowing the K1L protein to bind to ACAP2. Thus there must be other mechanisms that determine the K1L host range function (Meng *et al.*, 2006).

It was recently discovered (Willis *et al*., 2009) that, K1L, CP77 and C7L host range proteins play a role in the PKR-elF2α pathway (see Figure 3.2). Both CP77 and C7L, capable of complementing K1L deleted Vaccinia mutants, have been shown to inhibit PKR (protein kinase receptor) activation (Willis *et al*., 2009). The PKR-elF2α pathway is involved in down regulating protein synthesis and hence preventing virus replication. The key protein that mediates this pathway is the eukaryotic translation initiation factor 2 (elF2α). In uninfected cells, elF2α remains in the inactive unphosphorylated state. Activated (phosphorylated) elF2α inhibits protein synthesis by preventing translation initiation. Various proteins have been identified which phosphorylate elF2α, including PKR. In virus infected cells, PKR is commonly found to mediate the phosphorylation of elF2α and hence prevent viral transcription. Poxvirus proteins including K1L have been found to inhibit the PKR- elF2α pathway by binding to PKR itself or its DNA sequence and prevent its from being transcribed in order to prevent PKR activation. K1L was found to inhibit PKR protein activation by binding to PKR (Willis *et al*., 2009). Further investigation showed that a substitution mutation at the ANK2 surface residue resulted in a block in viral replication in cell lines permissive to wild-type virus infection.

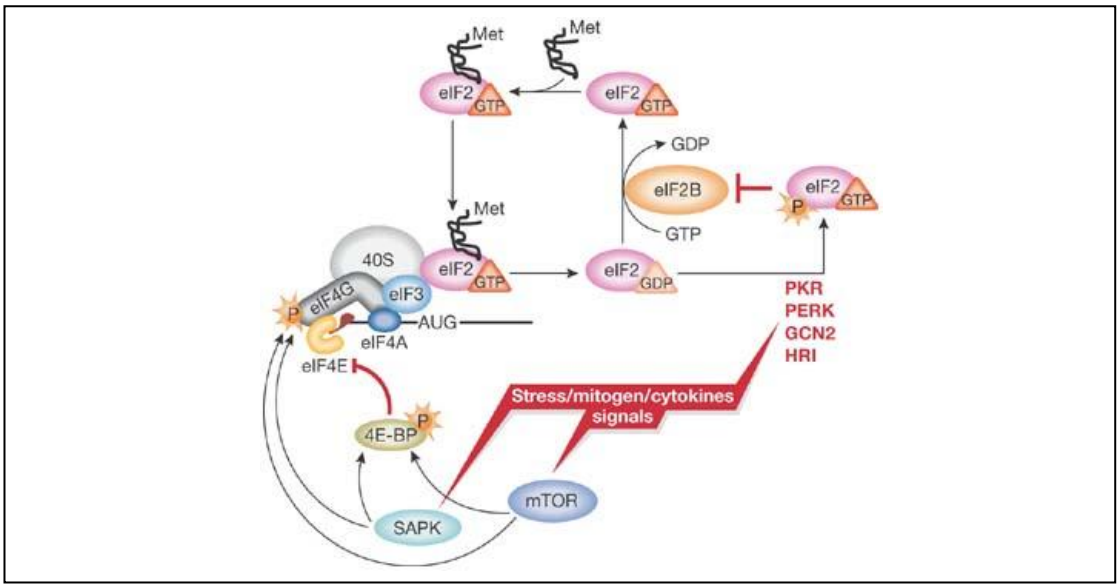

Figure 3.2. The classical PKR- eIF2α pathway (Holcik and Pestova, 2007)

The CP77, K1L and C7L proteins complement each other to allow for Vaccinia virus replication in HeLa cells, but only K1L and CP77 are capable of complementing each other to allow for replication in RK-13 cells. All 3 proteins play a role in inhibiting PKR activation, although only CP77 and K1L possess ankyrin repeats. One could hypothesize that these two proteins utilise a similar molecular mechanism to alter PKR activation, whereas C7L may utilise an alternative pathway. One can speculate that the specificity of the surface residue in the ankyrin repeat of K1L and CP77 may play an important role in host range function in RK-13 cells, due to specific protein-protein interaction with PKR (Willis *et al*., 2009).

As reported previously, PEPV is unable to complete its replication in any of the cell lines tested (Stannard *et al*., 1998). However, studies have shown expression of VACV K1L gene can rescue the growth of certain Poxviruses in RK-13 cells and hence been used as a selective marker for selecting recombinant plasmid (Sutter *et al.*, 1994; Staib *et al*., 2000; Meng *et al*., 2006). The hypothesis in this thesis is that transient expression of VACV K1L can rescue the growth of PEPV in RK-13 cells. VACV K1L, cloned into

a transfer vector, can be used both to rescue the growth of PEPV in RK-13 cells and also as a selective marker for selecting recombinant PEPV. However, growth curves have not been generated to confirm that RK-13 cells are not permissive for PEPV. Thus, for the purpose of this project, it is important to clarify that RK-13 cells are not permissive for PEPV so that VACV K1L can be used to rescue the growth of PEPV in RK-13 cells and hence select for the recombinant PEPV.

Growth curves have been used widely to study the growth of virus under different conditions such as determining the growth of Poxviruses in different cell lines (Drexler *et al*., 1998). Moreover, growth curves have been used in determining the function of Poxvirus host range genes by examining the effects of partial gene knockouts or by replacing certain region of the host range genes with homologous repeats on the growth of Vaccinia virus (Meng *et al*., 2006). This chapter describes experiments carried out to investigate whether or not RK-13 cells are permissive for PEPV. To achieve this, a standard growth curve was generated to monitor the change in titre of PEPV in RK-13 infected cells over a period of 6 days. A second growth curve was generated in this study to examine if PEPV is capable of adapting to RK-13 cells after multiple passages.

3.2 Materials and Methods

3.2.1 Cells

Rabbit Kidney cells (RK-13) were supplied by American Type Culture Collection (ATCC). An aliquot of cells was seeded into 10ml of DMEM with 10% FCS (foetal calf serum), 1% P/S (1000U/ml Penicillin, 10mg/ml Streptomycin) and 0.1% Fungin, in a $75cm^3$ flask. Flasks were then incubated at 37^oC, 5% CO_2.

For cell passaging, the medium was first discarded and the cells rinsed with PBS solution to remove any trace of serum which may contain trypsin inhibitor. 2ml of 1x Trypsin was added to the flask, which was incubated at 37^{o}C for 10 minutes. The purpose of the trypsin was to release the seeded cells from the flask. After 10 minutes, 3ml of DMEM + 10% FCS was added to the contents in the flask to neutralize the trypsin. The contents were then transferred to a sterile 15ml tube.

From the 15ml tube, 1µl was added to 9µl of trypan blue, carefully mixed and transferred to a gridded slide, to be observed using a microscope. The purpose of this action was to count the number of cells present in the 15ml tube. It was vital to know the number of cells harvested, as known numbers of cells were required to be seeded into fresh plates or flasks, depending on the requirement of the experiment.

Known numbers of cells were then transferred to the flask or plates, and the required amount of medium + FCS + P/S was added.

3.2.2 Viral Growth in RK-13 Cells

3.2.2.1 Poxvirus Infection of RK-13 Cells

Infectious virus was diluted with DMEM containing PS and Fungin (presence of FCS would inhibit virus adsorption). The medium in a 25cm^{3} flask or in the wells of a 6-well plate was removed, the cells were rinsed with 1XPBS and DMEM with P/S and fungin was added to each well or flask. Cells were infected with pox virus using different MOIs, (multiplicity of infection = ratio of infectious virus particles to cells).

Plates or flasks were incubated at 37^{o}C for 1.5hrs~2hrs for the virus to be adsorbed. Once the virus had been adsorbed, the medium was removed and the cells were rinsed with 1xPBS gently. DMEM containing 2%FCS was added (1 ml per well and 2.5 ml per 25cm^{3} flask) and the cells were incubated at 37^{o}C at 5% CO_2

3.2.2.2 Harvesting of Poxvirus

To harvest virus, plates or flasks with infected cells were frozen at -20^{o}C. Once all the cells were frozen, they were then thawed at room temperature. This procedure was performed 3 times to lyse the cells and release the virus. The lysates were transferred to sterile 15ml tubes or 1.5ml microfuge tubes and stored at -20^{o}C. To passage the harvested virus, harvested virus was used to infect fresh cells as described in section 3.2.2.1.

3.2.2.3 Growth Curve of PEPV in RK13 Cells

The growth curve experiments were performed, firstly to confirm Stannard's observation that RK-13 cells are not permissive for PEPV (Stannard *et al*., 1998) (see section 3.2.2.3.1) and, secondly, to determine whether PEPV can adapt and complete its replication cycle in RK-13 cells after multiple passages (see section 3.2.2.3.2).

3.2.2.3.1 Standard Growth Curve of PEPV in RK-13 Cells

RK-13 cells were seeded into seven 25cm^{3} sterile tissue culture flasks. Each of the flasks was infected with 3x10^{5} wtPEPV. Infection procedures were as described in 3.2.2.1. Once DMEM with 2%FCS, P/S and Fungin was added to the flasks, one of

the flasks was immediately stored at -20^{o}C; this flask was labeled T_0. The rest were incubated at 37^{o}C. Each day one of the flasks was removed from the incubator and stored at -20^{o}C. These flasks were labeled as T_1, T_2 and so forth. Once all the flasks had been removed from the 37^{o}C incubator, virus was harvested as mentioned in 3.2.2.2. Harvested virus was titrated on chick CAMs, as described in section 2.2.3. A growth curve was generated from the titre of virus found at each time interval to determine if wtPEPV was able to grow in RK-13 cells.

3.2.2.3.2 Growth Curve to Examine whether wtPEPV Adapts to Growth in RK-13 Cells Upon Multiple Passages

RK-13 cells were seeded into two 25cm^3 sterile tissue culture flasks. Both flasks were infected with wtPEPV of known titre (3x10^5 pfu). Infection procedures were as described in 3.2.2.1. Once DMEM with 2%FCS, P/S and Fungin was added to the flasks, one of the flasks was immediately stored at -20^{o}C, this flask was labeled as P_0. The other flask, labeled as P_1 was incubated at 37^{o}C for 3 days. After 3 days, virus was harvested as described in section 3.2.2.2. One fifth of the harvested virus was used to infect RK-13 cells in another freshly prepared 25cm^3 flask. The remaining contents were stored at -20^{o}C. This procedure was repeated until the virus had been passaged for 5 times.

Once the virus had been passaged in RK-13 cells for 5 times, the harvested virus was titrated on chick CAMs, as described in section 2.2.3. A growth curve was then generated from the titre of virus found in each passage to determine if wtPEPV was able to adapt and grow in RK-13 cells after several passages.

3.3 Results

3.3.1 Growth Curve of PEPV in RK-13 cells

A growth curve was generated as described in section 3.2.2.3. The growth of PEPV was monitored from Day 0 to Day 6. Each 25cm^3 flask was infected with 3x10^5 pfu of PEPV. Once the virus was harvested from the infected flasks, virus from each time point was diluted 10 fold and 100 fold, i.e. 3 different viral dilutions. A total of 63 eggs were infected, three eggs for each dilution and 7 time points. Four days post inoculation, infected CAMs were spread onto petri dishes for examination of pock formation. The virus titre was calculated for each time point and a growth curve was drawn up from this data (see Figure 3.3).

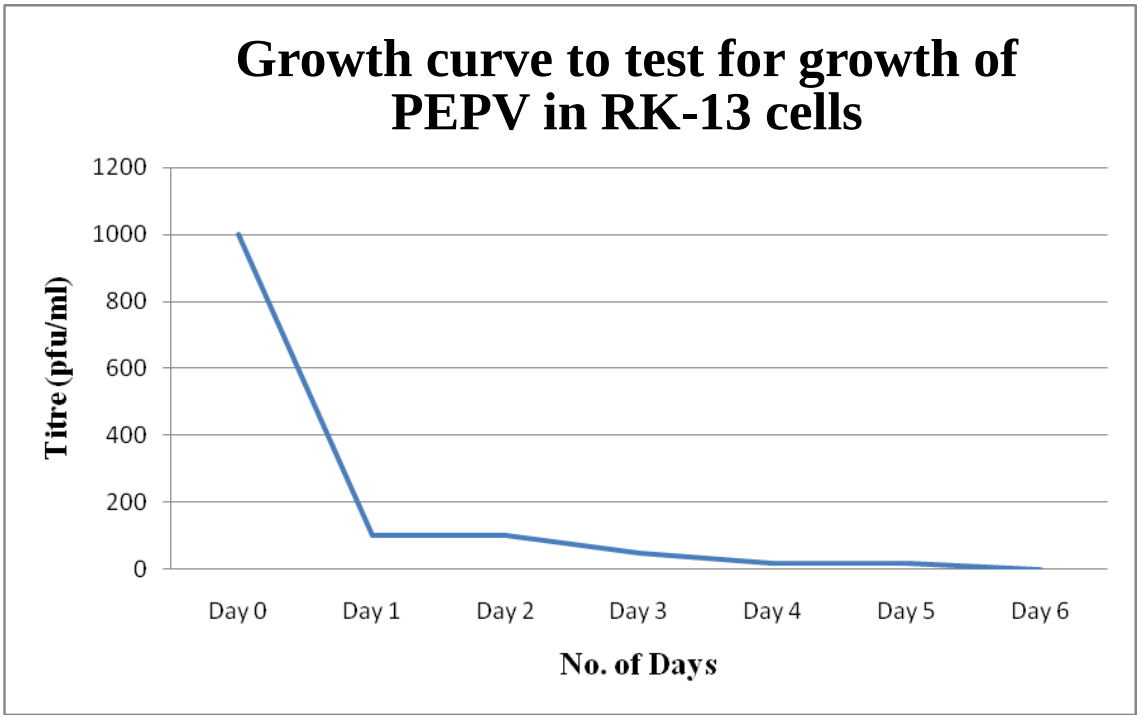

Figure 3.3. Growth curve to examine the growth of PEPV in RK-13 cells. Seven flasks of RK-13 cells were infected with 3x10^5 pfu/flask of PEPV. At each time point, a flask was frozen. At Day 6, the virus from all flasks was titrated on chick CAMs (expressed as pfu/ml).

Figure 3.3 shows a 100-fold difference in amount of virus recovered on Day 0 compared to what was added ($3x10^5$ pfu/flask). A dramatic decrease in viral concentration from 1000 pfu/ml at day 0 to 100 pfu/ml at day 1 was observed. The viral concentration gradually decreased and eventually was undetectable on Day 6.

3.3.2 Growth Curve to Examine Whether wtPEPV Adapts to Growth in RK-13 Cells Upon Passage in RK-13 Cells

It was of importance to investigate whether PEPV is able to adapt to growth in RK-13 cells after multiple passages. Hence an experiment was set out as described in section 3.2.2.3.2. Wild type PEPV was passaged 5 times in $25cm^3$ sterile tissue culture flasks. The first $25cm^3$ flask was infected with $3X10^5$ pfu of PEPV. After 3 days the virus lysate was used to infect another flask. This was repeated until the virus had been passaged 5 times.

Once all the virus was harvested from the infected flasks, virus from each time point was diluted 10 fold and 100 fold, i.e. 3 different viral dilutions. A total of 54 eggs were infected, three eggs for each dilution, 3 dilutions and 6 passages including P_0. 4 days post inoculation infected CAMs were spread onto petri dishes and the pocks were counted. The virus titre was calculated for each passage and a growth curve was generated from this data (see Figure 3.4).

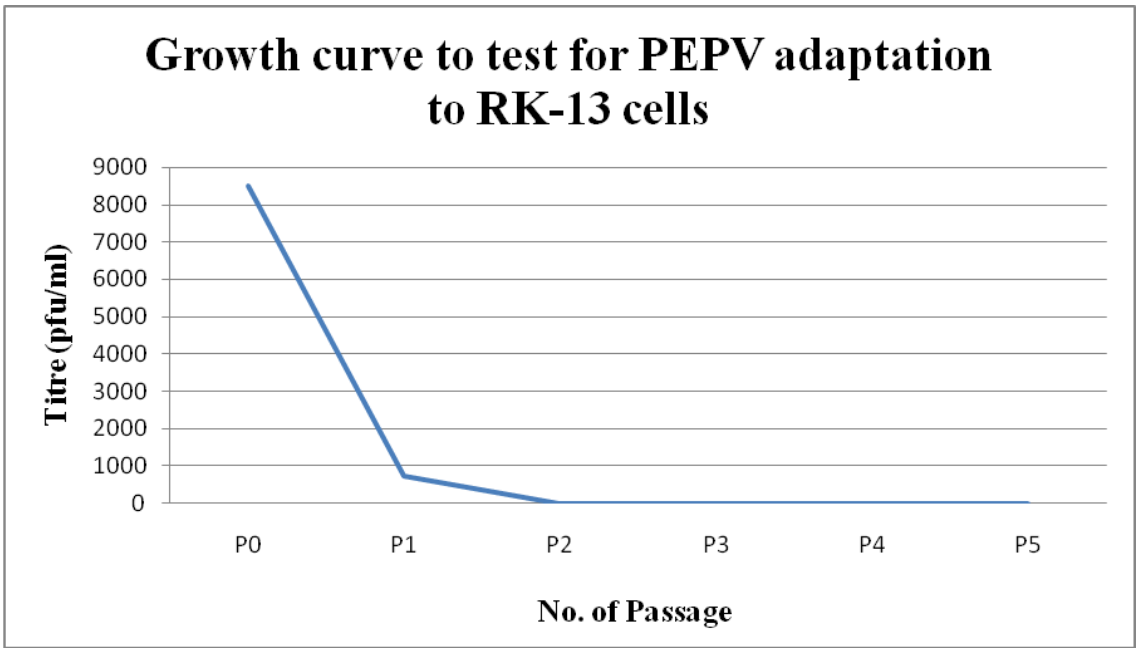

Figure 3.4. Growth curve to examine if wtPEPV adaptes to growth in RK-13 cells upon multiple passages. 2 flasks of RK-13 cells were infected with $3x10^5$ pfu of PEPV. After infection, a flask was frozen. The second flask was left for 3 days, then frozen (P_1), P1 was freeze/thawed 3 times, then used to infect another flask of RK-13 cells. After 3 days this flask was freeze/thawed 3 times (P_2) and the procedure repeated until the virus had been passaged 5 times. Virus from all the flasks was titrated on chick CAMs and expressed as pfu/ml.

The data (Figure 3.4) shows a dramatic decrease in viral titre after the first passage (from 8500pfu/ml to 750pfu/ml). No virus was seen after the second passage. This indicated that PEPV was unable to adapt to grow in RK-13 cells at the titres which were being used;

(An observation: there is 8x more virus in the second growth curve than the first at P_0 and Day 0 respectively)

3.4 Discussion

Stannard and her group tested whether PEPV would recognize late promoter sequences derived from VACV (Stannard *et al*., 1998). A plasmid containing the reporter gene β-gal under the control of the VACV late promoter, P11, was transfected into RK-13 cells infected with PEPV. No expression was detected. Expression was, however, detected in other cells, showing that PEPV does indeed recognize the P11 promoter (Stannard *et al*., 1998). This experiment further showed that RK-13 cells are not permissive for the growth of PEPV and that the block occurs before DNA replication as replication takes place before late gene expression can take place. The transcriptase responsible for recognizing the late promoter is synthesized during the intermediate phase of the Poxvirus replication cycle (Moss, 2007). No late gene expression took place due to the fact that no transcriptase was present to recognize the P11 promoter and hence transcribe the β-Gal gene (Stannard *et al*., 1998). This may indicate that after PEPV enters RK-13 cells, replication is blocked during or before the intermediate phase (Moss, 2007).

One can presume that most of the PEPV was capable of infecting and entering the host cells, as studies have shown that the binding and entry of Poxviruses into mammalian cells is an efficient process, and restriction only occurs after the virus has entered the cell (McFadden, 2005). These results show that after PEPV enters the host cell and becomes uncoated, the virus is unable to complete its replication cycle and hence unable to produce infectious viral progeny. The virus detected on Day 1 was probably the PEPV that remained on the host cell surface, before it under-went uncoating. This growth curve experiment showed that RK-13 cells are non-permissive for PEPV growth, which was important to establish before using VACV K1L host range gene as a selective

marker for recombinant virus construction, to ensure that the only virus which grows in RK-13 cells is that with the VACV K1L gene present.

During the project an attempt was made to passage the wtPEPV in RK-13 cells, in order to determine if PEPV is capable of adapting itself to grow in RK-13 cells (see section 3.3.2). This was the first attempt to passage PEPV in a known cell line. During the process, the viral load reduced dramatically upon the first passage and no virus was detected after the second passage. Despite $3X10^5$ pfu of PEPV being used to infect the cells, none of it was capable of adapting to RK13 cells. One can argue that an increase in concentration of PEPV can raise the possibility of finding mutated PEPV to adapt to RK13 cells. However, our aim was not to adapt the virus to grow in RK-13 cells, but rather to show that the virus does not adapt to grow in RK-13 cells when used at the same titre as that used for infection and transfection experiments (Chapter 5). Previous work done by Stannard *et al* (1998) and tissue culture work done in this project showed that PEPV alone was unable to advance its replication cycle to the intermediate phase of the replication cycle in RK-13 cells. It is not known whether all early phase proteins are expressed. However it is unlikely for PEPV to adapt to growth in RK13 cells with the block in its replication cycle occurring at such an early stage.

Chapter 4: Construction of Transfer Vector pNCH-3, Containing VACV K1L and GUS Flanked by PEPV TK Sequence

4.1 Introduction

The long term goal of this project is to develop a recombinant PEPV for use as a vaccine. The noninfectious nature of poxvirus DNA and its large genome prevent one from direct manipulation of the viral DNA. However, the viral DNA can be manipulated by means of homologous recombination between the viral genome and a transfer vector (Wyatt *et al*., 1998; Panicali *et al*., 1982; Hahn *et al*., 2000).

Insertion of foreign DNA into poxviruses involves the construction of a recombinant plasmid containing the foreign DNA/genes, flanked by poxviruses genomic sequences. This transfer vector is introduced into poxvirus infected cells and recombination takes place between the flanking poxvirus sequences and the homologous sequences of the poxvirus genome during replication within the cells (see Figure 4.1). Hence the foreign gene inserts within the transfer vector will subsequently be packaged into the infectious virus (Panicali *et al*., 1982).

Insertion of the foreign gene into the poxvirus can be lethal to the virus if the foreign gene is inserted into an essential region of the genome; therefore it is important to allow the recombination to take place within a nonessential region. Many genes are critical for replication and completion of the virus life cycle. However, certain genes are not essential to the virus and are suitable sites for the insertion of foreign genes. One of the most commonly used nonessential genes in the construction of recombinant viruses is the thymidine kinase (TK) gene. The function of thymidine kinase is to phosphorylate thymidine, which is important for synthesizing DNA and thereby plays an important role in cell division, however such kinases exist in most living cells, and TK is not essential for the growth of poxviruses. It has been widely used as the locus of insertion

for foreign genes (Kit, 1985; Hahn *et al*., 2000; Perkus *et al*., 1989). It has been used in the construction of recombinant swinepox viruses expressing classical swine fever virus protein (Hahn *et al*., 2000). It also has been used in the construction of a recombinant Fowlpox virus, expressing the protein of bovine viral diarrhea virus (Elahi *et al*., 1999). Homologous recombination is a rare event and can be influenced by the length of the homologous flanking sequence (Yao *et al*., 2001). Sequences of at least 50bp are required for homologous recombination and the longer the flanking sequences, the greater the probability of recombination (Yao *et al*., 2001).

For the construction of an ideal transfer vector, there are two other elements that would optimize the construction of a recombinant poxvirus, a selective marker gene and a reporter gene. The purpose of the positive selection marker is to enable one to identify and select the recombinant virus from the rest of the non-recombinants.

The presence of a reporter gene such as β-Galactosidase, β-glucuronidase or luciferase allows one to visualize plaques produced by recombinant virus. In the construction of a transfer vector, the reporter gene is required to be under the control of a poxvirus promoter that can be recognized by the poxvirus. During the selection of recombinant virus, the infected and transfected cells are incubated with the colourless substrate of the reporter gene product. The expressed reporter gene will subsequently convert its colourless substrate and into a coloured product. This enables one to identify cells which are expressing the reporter gene. The reporter gene used in this project was β-glucuronidase (GUS), an enzyme isolated from the bacterium *Escherichia coli*. This enzyme was first identified by Jefferson (1989); since then, it has become one of the most frequently used reporter genes. For the detection of transfected cells or recombinant virus in a GUS reporter gene system, the infected and transfected cells are

incubated with the colourless substrate, 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc). Cells expressing GUS enzyme will convert the substrate into a blue product and the cells will appear blue.

The rest of the elements for constructing an ideal transfer vector include a pair of direct repeat sequences flanking these two foreign genes, which, ultimately could remove the selective marker and reporter genes (this is to ensure safety of the final recombinant virus), and lastly restriction enzyme sites for the future insertion of vaccine antigen genes, which are located outside of the direct repeats, so that the required gene is not deleted from the final recombinant virus. The restriction enzyme sites need to be located downstream of a poxvirus promoter so that transcription of the vaccine antigen is under the control of a poxvirus promoter (see Figure 4.1).

In this study, a transfer vector (pNCH-3) was constructed based on the backbone of the pLW51 plasmid (see Appendix I for plasmid map). The plasmid vector pLW51 (Wang *et al*., 2004) contains a β-lactamase gene (amp^R) which confers ampicillin resistance amp^R, which acts as a selection marker for transformation in bacteria. The vector also contains a β-glucuronidase (GUS) gene under the control of a VACV p11 promoter. The function of this reporter gene was described above and its presence will allows one to detect cells which have been both infected with poxvirus and transfected with plasmid. This is because the p11 promoter is a poxvirus late promoter, which can only be recognized by poxvirus transcription factors. However, pLW51 does not possess a selective marker for the selection of recombinant PEPV from the non recombinants, hence in this study the Vaccinia virus K1L host range gene was cloned into the pLW51 vector to determine if it could be used for selection of recombinant virus. The previous chapter (section 3.1) describes the use of K1L as a selection marker. Once homologous recombination takes

place, recombinant virus with VACV K1L will grow in RK-13 cells whereas the wild-type PEPV will remain replication deficient, thus enabling one to select for the recombinant.

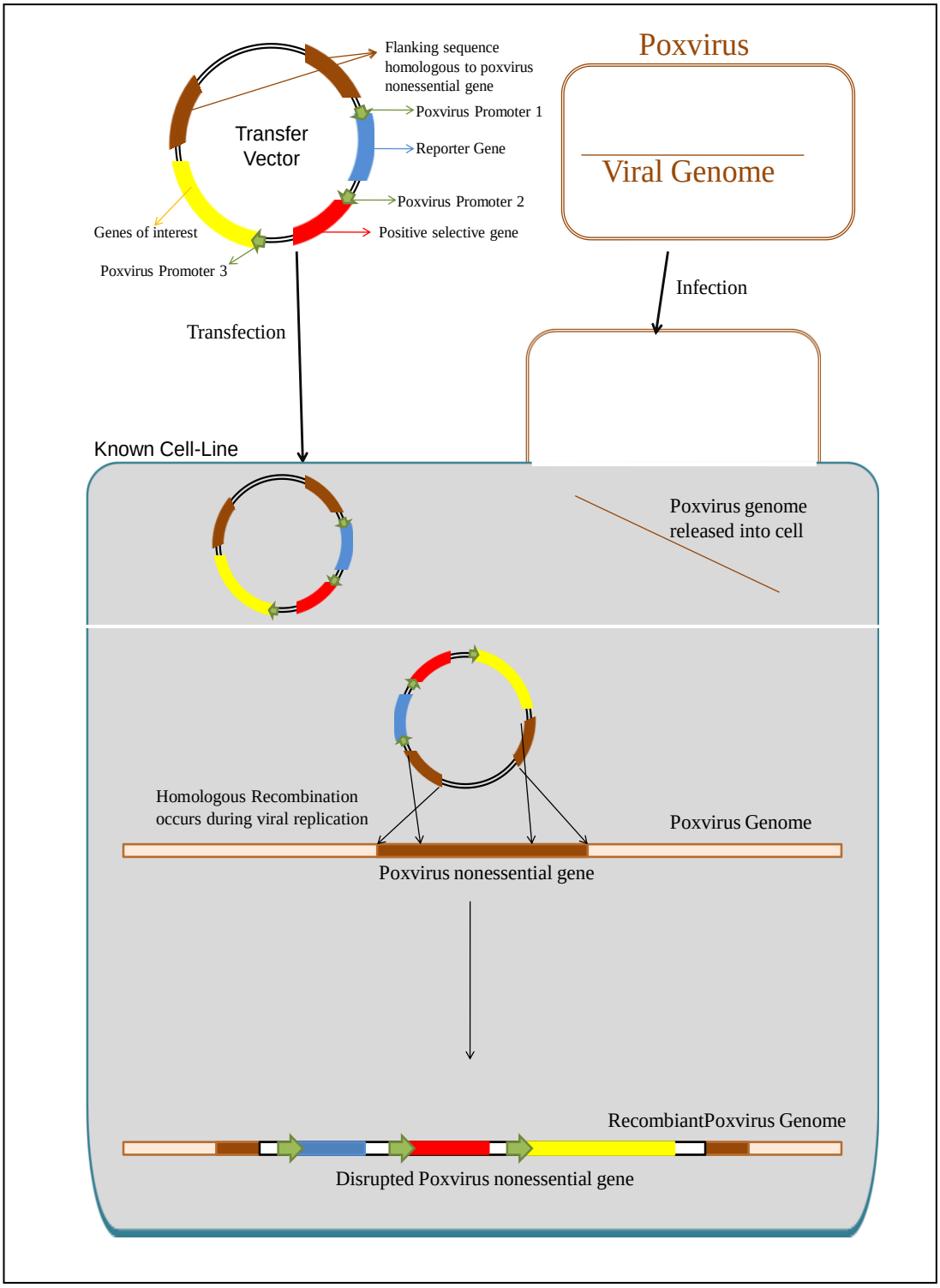

Figure 4.1. Schematic diagram (not to scale) of the construction of recombinant Poxvirus (adaptation from Byrd *et al.*, 2008). Cells are infected with virus, then transfected with a transfer vector. Homologous recombination takes place in the cytoplasm of the cell.

pLW51 also contains multiple cloning sites for future construction of a vaccine vector situated downstream of two VACV promoters, PsynII promoter and pmH5 promoter. One can then decide to either clone downstream of the PsynII promoter for strong expression or downstream of the pmH5 promoter for weaker expression. As previously discussed, a pair of direct repeats is essential to ensure the safety of the vector. In pLW51, a pair of direct repeat sequences are located on each side of GUS, the one repeat being just upstream of the p11 promoter, so that ultimately, GUS can be removed from the desired reombinant pox virion genome.

The final transfer vector construct (pNCH-3) was designed to contain a multiple cloning site downstream of the pmH5 promoter, a GUS reporter gene, a VACV K1L gene, a pair of direct repeat sequence flanking the GUS and K1L gene and lastly PEPV TK sequences flanking the cassette. Figure 4.2 shows a schematic diagram of the transfer vector used in the construction of recombinant PEPV and Figure 4.3A-D shows schematic diagrams of the cloning procedures followed for the construction of the transfer vector pNCH-3.

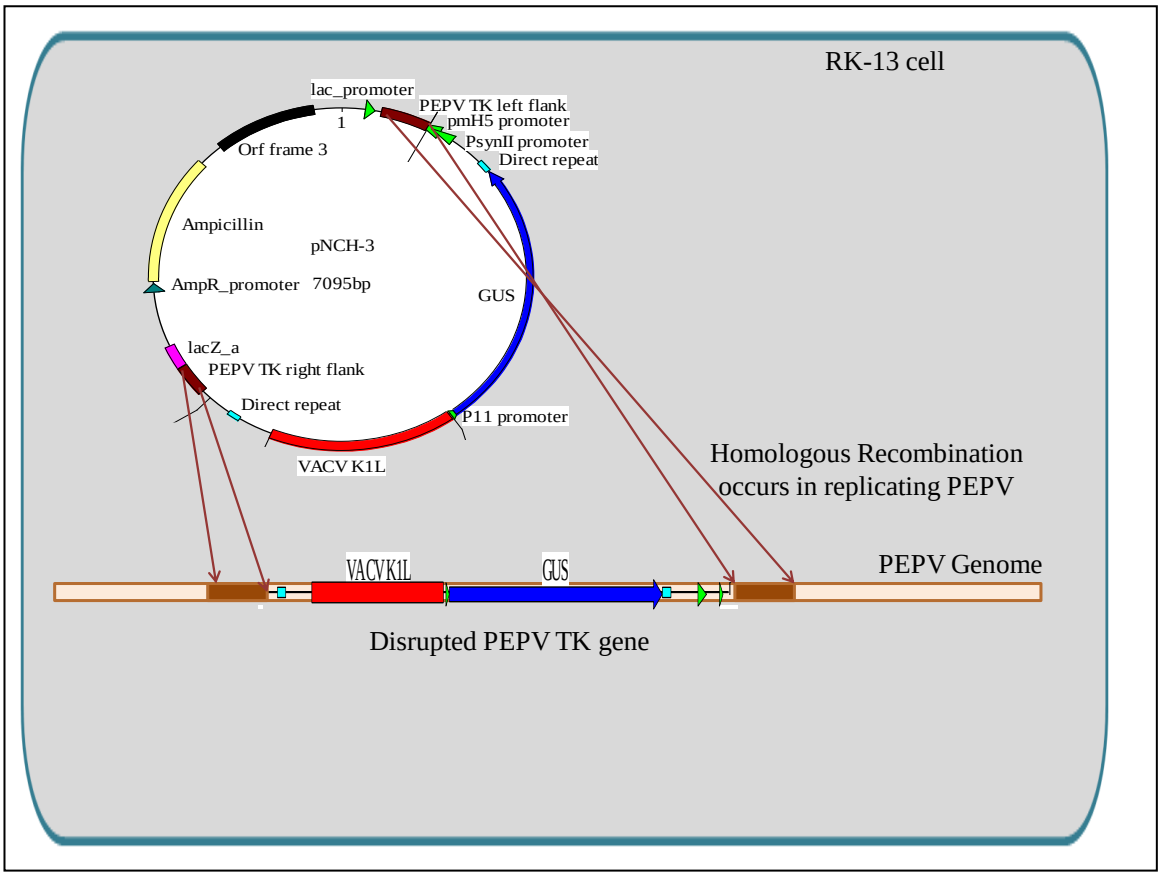

Figure 4.2. Schematic diagram (not to scale) of the transfer vector used in the construction of recombinant PEPV. Foreign genes were inserted between the PEPV TK flanking sequences (brown). The VACV K1L selection gene (red) and E.Coli GUS reporter genes (dark blue) were flanked by direct repeat (light blue). Outside of the direct repeat and within the TK flanks are 2 promoters, psynII and pmH5 (green arrows). A single S*ma* I restriction enzyme site is located immediately downstream of the pmH5 promoter.

4.2 Materials and Methods

Figure 4.3 shows the procedure employed in the construction of the transfer vector pNCH-3. The first step was PCR amplification of the PEPV flanking sequences and the VACV K1L gene from PEPV and VACV genomic DNA respectively. In this study, amplified PCR products were first cloned into pGEM T Easy vector (Appendix I), thereafter several cloning procedures were performed before the final transfer vector pNCH-3 was constructed.

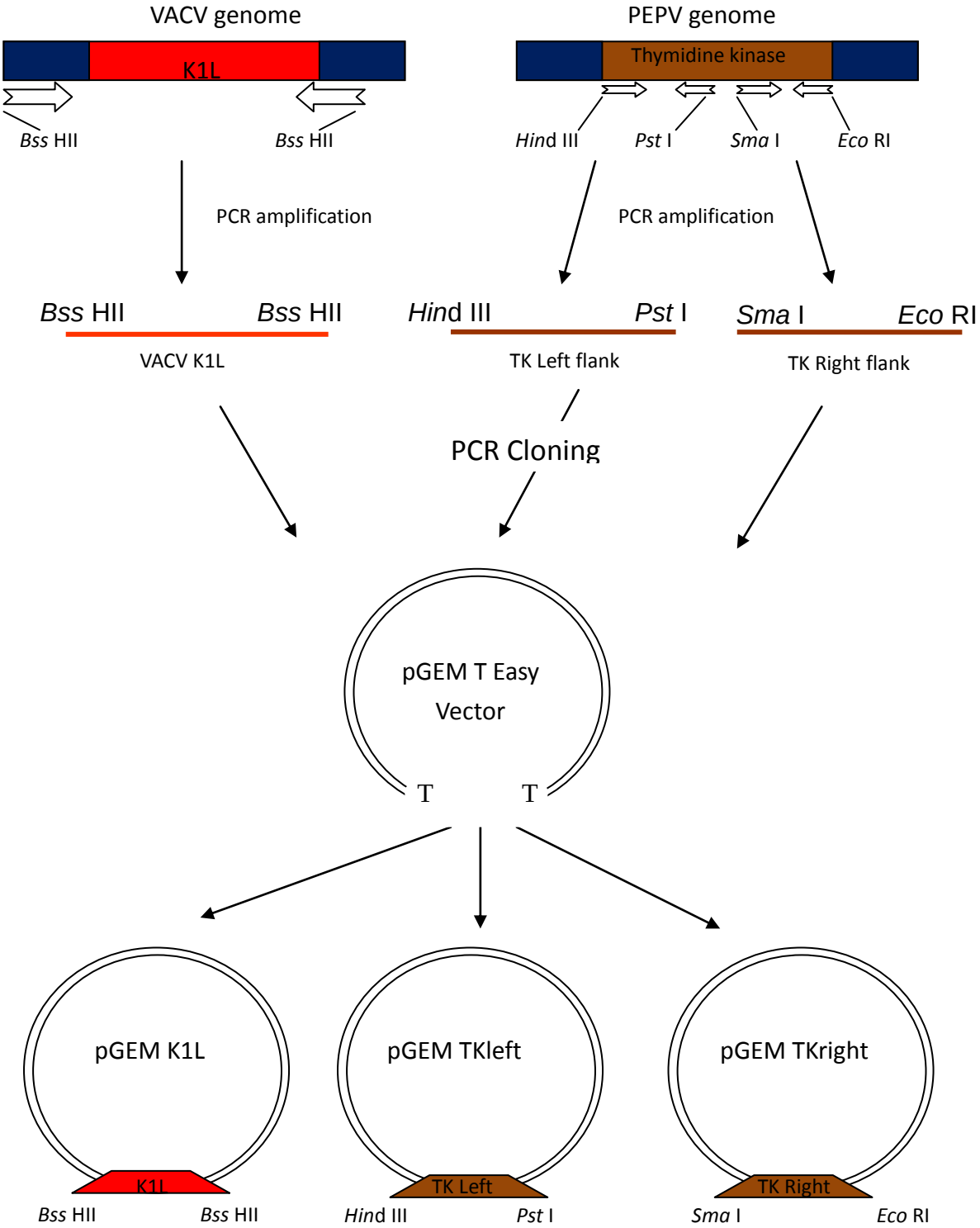

Figure 4.3A. Schematic diagram (not to scale) of the first part of the cloning procedures followed in the construction of pNCH-3 transfer vector. VACV K1L host range gene, PEPV flanking sequences, TK left and TK right, were amplified from VACV and PEPV genome respectively. The amplified DNA fragments were separately cloned into pGEM-T Easy.

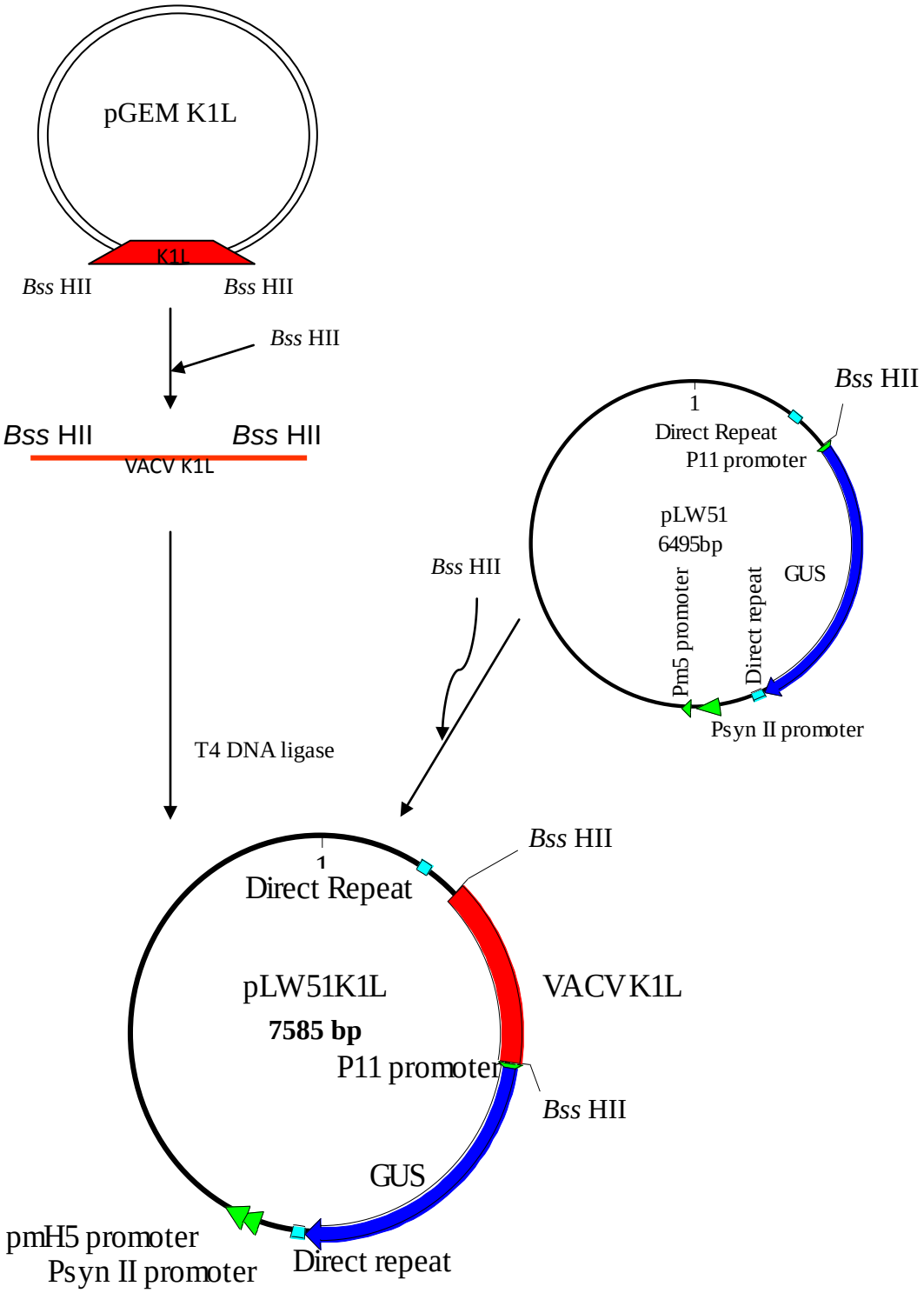

Figure 4.3B. Schematic diagram (not to scale) of the second part of the cloning procedures followed in the construction of pNCH-3 transfer vector. VACV K1L gene was extracted as a *Bss* HII fragment from pGEM K1L (Figure 4.3A) and cloned into the *Bss* HII site of pLW51.

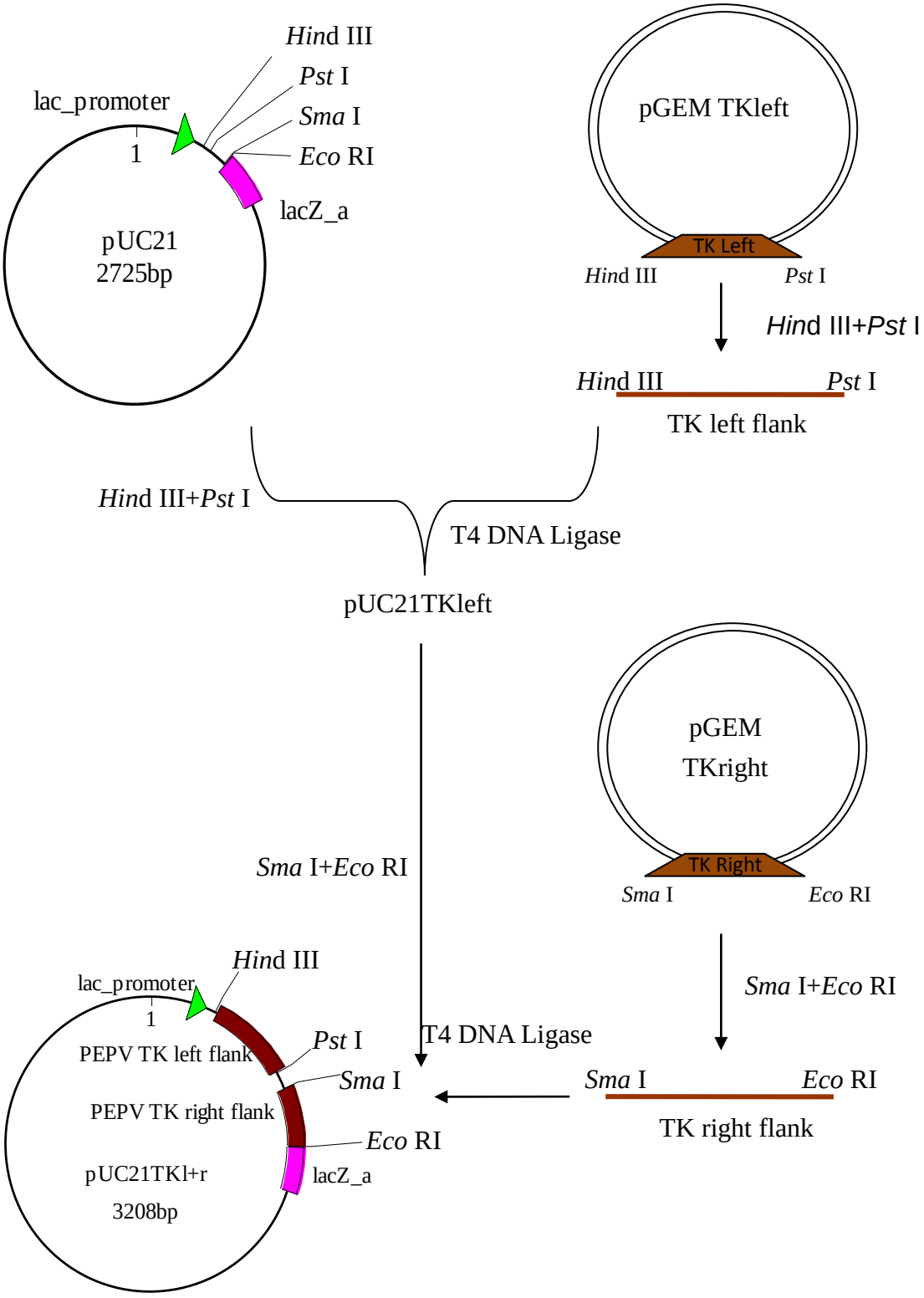

Figure 4.3C. Schematic diagram (not to scale) of the third part of the cloning procedures followed in the construction of pNCH-3 transfer vector. Gel extracted and purified PEPV TK flanks were cloned into pUC21 vector sequentially. The TK left flank was first cloned into the *Hind* III and *Pst* I cloning sites of pUC21. The TK right flank was then cloned into the recombinant pUC21TKleft plasmid into the *Sma* I and *Eco* RI cloning sites.

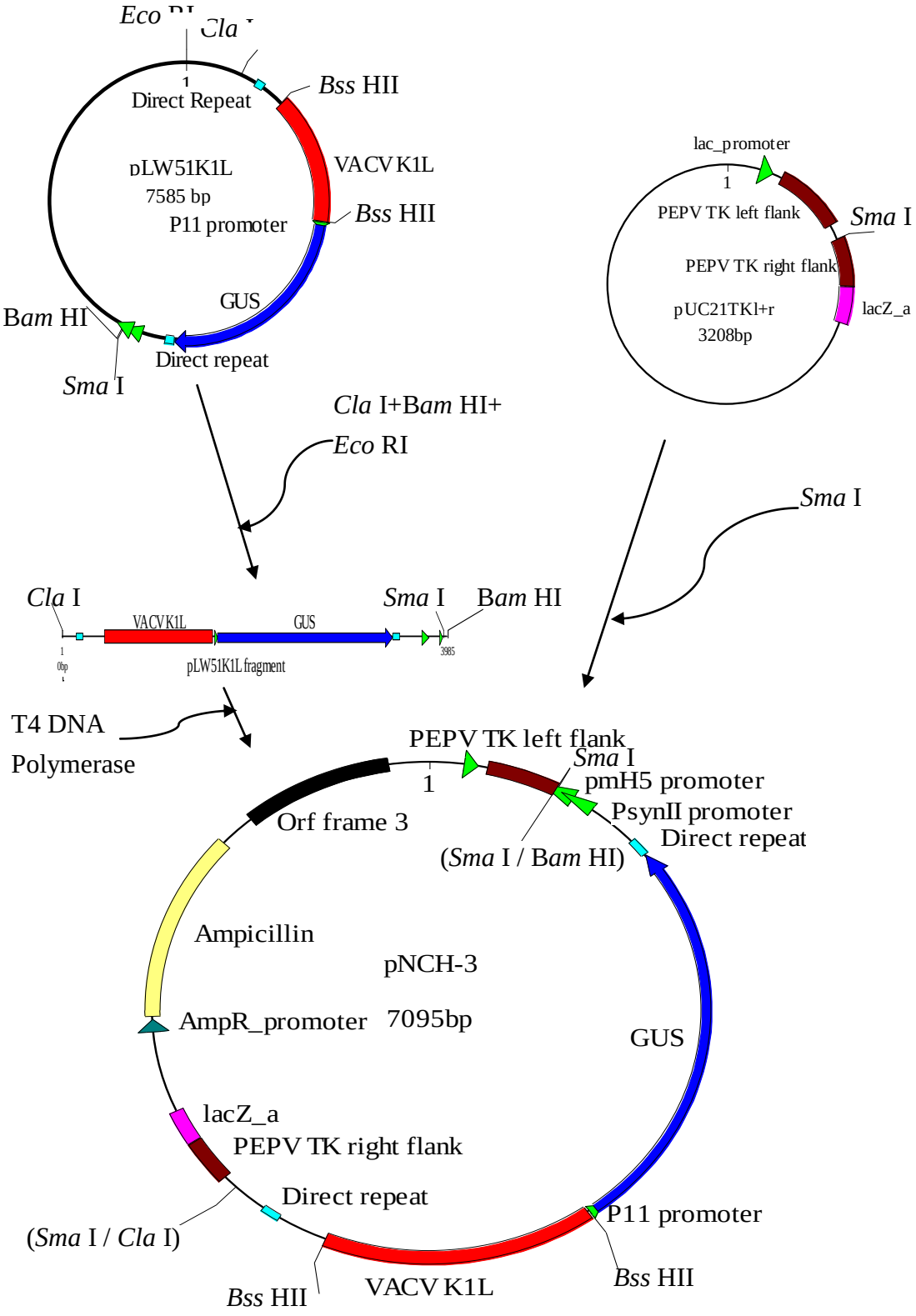

Figure 4.3D. Schematic diagram (not to scale) of the last part of the cloning procedures followed in the construction of pNCH-3 transfer vector. pLW51/K1L plasmid was digested with C*la* I, B*am* HI and *Eco* RI, producing a 3.8kb fragment comprised of many of the essential elements for the transfer vector. This fragment was first blunted-ended then cloned into pUC21tkL+R vector into the *Sma* I site. *Eco* RI was used to aid in the purification of the 3.8kb fragment from the vector fragment.

4.2.1 PEPV and VACV DNA Extraction

PEPV and VACV DNA was required as template in the PCR reactions for the generation of PCR products which were to be used in the construction of a transfer vector, required for the generation of recombinant PEPV (see Figure 4.3A).

Poxvirus DNA extraction was performed as described by Sambrook (1989) with a modification to the lysis buffer. The virions were lysed using a lysis buffer of 10% Na-N-Lauryl Sarcosinate, 54% (w/v) sucrose solution and 1M Tris-HCl (pH 7.8). The solution was autoclaved and stored at 4^{o}C. β-mercaptoethanol was added to the lysis buffer to a final concentration of 200mM prior to DNA extraction.

An equal volume of lysis buffer was added to the viral suspension and mixed via inversion. The mixture was then incubated at room temperature for 15 minutes to allow the lysis buffer to lyse the viral membrane completely. Proteinase K (final concentration was 2mg/ml) was added, followed by RNase A (final concentration was 100µg/ml). Lysates were incubated at 56^{o}C for 1.5 hours. The purpose of the step was to denature and degrade all the proteins present in the solution including nucleases. This is to prevent the nucleases from degrading the DNA. RNase A was used to degrade the RNA.

After lysis and Proteinase K digestion an equal volume of Phenol solution was added to the contents and mixed via inversion. Mixed solutions were then centrifuged at 13000rpm for 5 minutes at room temperature, in an Eppendorf 5417C benchtop centrifuge. Two layers were seen after centrifugation. The top aqueous layer, containing viral DNA, was carefully transferred into a sterile 1.5ml microfuge tube and the bottom layer, containing phenol, lipid and protein, was discarded. This procedure was repeated

to ensure the removal of all impurities. Next, an equal volume of chloroform:isoamyl alcohol (24:1) was added to the recovered viral DNA. Mixtures were mixed via inversion and subjected to centrifugation at 13000rpm (Gene Company, Eppendorf Centrifuge 5417C) for 5 minutes at room temperature. Again two layers were seen after centrifugation. The top layer, consisting of the purified DNA, was transferred to a new sterile 1.5ml microfuge tube. This step was repeated to ensure all traces of phenol were removed. One-tenth of the final volume of ice cold 3M sodium acetate was added to the DNA followed by 2.5 times the volume of ice cold 95% ethanol. This was to precipitate the DNA. The solution was first mixed by inversion and then incubated at -80^{o}C for 1 hour or at -20^{o}C overnight.

The microfuge tubes were centrifuged at 13000rpm for 20 minutes at 4^{o}C (Gene company, Eppendorf Centrifuge 5415R). This procedure was to pellet the precipitated DNA. After centrifugation, the supernatants were removed and DNA pellets were washed with 200µl ice-cold 70% ethanol and centrifuged again. The ethanol was carefully removed to avoid the DNA pellet being discarded. DNA pellets were resuspended in 50µl Tris-EDTA buffer (pH 7.5) or HPLC water and stored at -20^{o}C.
The DNA concentration was determined by nanodrop (NanoDrop® Spectrophotometer ND-1000) and the DNA integrity was checked by standard agarose gel electrophoresis.

4.2.2 Polymerase Chain Reaction (PCR)

PCR was performed for the initial steps in the construction of the transfer vector (see Figure 4.3A). There were three primer sets designed for polymerase chain reaction (PCR) amplification of the required DNA fragments (shown in Table 4.1).

Table 4.1. Primers used to amplify TK left, TK right and VACV K1L fragments

Primers	Orientation	Usage	Sequence
TK Left	Forward	Amplify TK left fragment	5' AAGCTTATGGCTTCCGGAAGTATCCATGTT 3' *Hin*d III
TK Left	Reverse	Amplify TK left fragment	5' CTGCAGACTATATCTAGGAAGAATTGACCTTC 3' *Pst* I
TK Right	Forward	Amplify TK right fragment	5' CCCGGGAGAATTTAGTGAATCTATGGCTAA 3' *Sma* I
TK Right	Reverse	Amplify TK right fragment	5' GAATTCATCCATTACTTCTTTACTCTCTG 3' *Eco* RI
VACV K1L	Forward	Amplify VACV K1L fragment	5' GCGCGCTATGGGTACGGTGTAAGGAATCAT 3' *Bss* HII
VACV K1L	Reverse	Amplify VACV K1L fragment	5' GCGCGCGTGGGAGAATCTAATTAGTTTTTC 3' *Bss* HII

ATGGCTTCCGGAAGTATCCATGTTATTACAGGTCCTATGTTTTCAGGTAAA
ACATCAGAACTAGTAAGAAGAATAAAAAGATTTATGCTATCTAACTTTAA
ATGTATTATTATTAAACATTGTGGAGATAATAGATATAACGAAGATGATATA
AATAAAGTATGTACACACGATAGATTGTTTATGGAAGCTATAGCATCTTCT
AATCTATCTGTATTGGTACCTAAGATATTAAAGGATGGAATTGAAGTAATA
GGTATAGACGAAGGTCAATTCTTCCTAGATATAGTAGAATTTAGTGAATCT
ATGGCTAATTTAGGTAAAATAGTTATTATAGCCGCGCTTAATGGTGATTTT
AAACGCGAATTATTTGGAAACGTGTGTAAGCTATTACCATTAGCAGAGAC
AGTTTCCAGTTTAACAGCTATTTGTGTGAAATGCTATCGCGAAGCTTCGT
TTTCAAAACGCATTACAGAGAGTAAAGAAGTAATGGATATAGGTGGTAA
AGATAAATACATGGCTGTGTGTAGGAAATGCTTTTTTAGCAAGTAA

Figure 4.4. Sequence of PEPV Thymidine Kinase gene. The red underlined sequences indicate the position of primer sequences used to amplify a fragment from the left end (TKleft). The dark blue underlined sequence indicates the primer sequence used to amplify a fragment from the right end (TKright).

Two primer sets were designed, as described in section 4.2.2.1, based on the sequence from the thymidine kinase (TK) gene of PEPV. This gene sequence was determined by Olivia Carulei, Division of Medical Virology, Department of Clinical Laboratory Sciences, UCT (sequence shown in Figure 4.4). Primer sets were designed to amplify two PEPV TK fragments, one from the left end (TKleft), flanked with *Hin*d III and *Pst* I

(underlined) and one from the right end (TKright), flanked with *Eco* RI and *Sma* I (underlined).

GTGGGAGAATCTAATTAGTTTTTCTTTACACAATTGACGTACATGAGTCTGAGTTC
CTTGTTTTTGCTAATTATTTCATCCAATTTATTATTCTTGACTATATCGAGATCTT
TTGTATAGGAGTCAGACTTGTATTCAACATGCTTTTCTATAATCATTTTAGCTATT
TCGGCATCATCCAATAGTACATTTTCCAGATTAGCAGAATAGATATTAATGTCGTA
TTTGAACAGAGCCTGTAACATCTCAATGTCTTTATTATCTATAGCCAATTTAATGT
CCGGAATGAAGAGAAGGGAATTATTGGTGTTTGTCGACGTCATATAGTCGAGCAAG
AGAATCATCATATCCACGTGTCCATTTTTTATAGTGATGTGAATACAACTAAGGAG
AATAGCCAGATCAAAAGTAGATGGTATCTCTGAAAGAAAGTAGGAAACAATACTTA
CATCATTAAGCATGACGGCATGATAAAATGAAGTTTTCCATCCAGTTTTCCCATAG
AACATCAGTCTCCAATTTTTCTTAACAAACAGTTTTACCGTTTGCATGTTACCACT
ATCAACCGCATAATACAATGCGGTGTTTCCCTTGTCATCAAATTGTGAATCATCCA
GTCCACTGAATAGCAAAATCTTTACTATTTTGGTATCTTCCAATGTGGCTGCCTGA
TGTAATGGAAATTCATTCTCTAGAAGATTTTTCAATGCTCCAGCGTTCAACAACGT
ACATACTAGACGCACGTTATTATCAGCTATTGCATAATACAAGGCACTATGTCCAT
GGACATCCGCCTTAAATGCATCTTTGCTAGAGAGAAAGCTTTTCAGCTGCTTAGAC
TTCCAAGTATTAATTCGTGACAGATCCATGTCTGAAACGAGACGCTAATTAGTGTA
TATTTTTTCATTTTTTATAATTTTGTCATATTGCACCAGAATTAATAATATCTCTA
ATAGATCTGATTAGTAGATACATGGCTATCGCAAAACAACATATACACATTTAATA
AAAATAATATTTATTAAGAAAATTCAGATTTCACGTACCCATCAATATAAATAAAA
TAATGATTCCTTACACCGTACCCATA

Figure 4.5. Sequence of VACV K1L gene. The red underlined sequences indicate the primer sequences used to amplify VACV K1L together with its promoter element.

The DNA sequence of a Vaccinia virus host range gene, K1L, sequence was retrieved from Genbank (Accession No. NC_006998, see Figure 4.5). A primer set was designed such that both the VACV K1L gene and its promoter would be amplified by PCR. A *Bss* HII restriction enzyme site was included at the 5' end of each primer (see Table 4.1) so that this K1L gene could easily be cloned into the *Bss* HII site of the transfer vector pLW51.

4.2.2.1 Primer Design

When designing PCR primers the following two factors are taken into consideration:

1) The melting temperature of the primers (T_m). It is essential that the melting temperature of the forward and the reverse primers are approximately the same. This is to ensure that both the forward and reverse primers can anneal to the template DNA at the same temperature. The PCR reaction will not take place if both primers do not anneal at the same time. The T_m was determined using the following formula:

T_m = 4 x (no. of Guanosine and Cytosine) + 2 x (no. of Adenine and Thymine)

2) The base composition of the primer. During the primer designing process, it is essential to consider the specificity of the primers, i.e. does the primer only anneal to the template DNA at the desired location. There is a 25% probability for each base in a primer to bind randomly to the template DNA. Therefore the probability of a designed primer, with X number of bases, to bind randomly to the template DNA is equal to $(25\%)^{x}$. Thus the longer the primer, the lower the possibility of the primer binding nonspecifically. It is also important to ensure that the primer pairs do not have palindromic sequences which could bind to one another and that the two primers do not have reverse complementary sequences which would allow the primers to bind to one another.

Primers were synthesized by UCT synthetic DNA laboratory, using a Beckman Oligo 1000M DNA synthesizer.

4.2.2.2 PCR Amplification

PCR master mix reagents were supplied by Bioline, United Kingdom. The reagents consisted of PCR buffer with $MgCl_2$, dNTPs and *Taq* DNA polymerase for the PCR reaction.

PCR amplification was performed as described by Sambrook (1989). The reaction conditions were set as follows:

PEPV DNA was first denatured at 95^{o}C for 10 minutes prior to the main PCR cycle. This was to ensure all double stranded DNA was denatured and formed single stranded DNA. In the main PCR reaction cycle, DNA was first denatured at 95^{o}C for 30 seconds; then primer annealing to the single stranded template was allowed to take place at 5^{o}C below the melting temperature of the primers, for 30 seconds; and thirdly, extension of the DNA from the primers took place at 72^{o}C, the length of time for extension depending on the length of DNA to be amplified. 1kb of DNA fragment required 1 minute of extension time. For this study, in total, 30 cycles of denaturation, annealing and extension were performed. Finally, to ensure complete extension of DNA, the PCR reaction was left at 72^{o}C for 7 minutes. Products were stored at 4^{o}C.

Amplified fragments were detected by agarose gel electrophoresis, using 1% agarose gels.

4.2.3 DNA Purification

PCR products and Plasmid DNA were purified before being cloned. A specific volume of amplified DNA was measured. A half volume of phenol was added to the PCR

products, the contents were vortexed, a half volume of chloroform:isoamylalcohol (24:1) was added, the mixture was vortexed again and then centrifuged for 2 minutes at 13000rpm (Gene Company, Eppendorf Centrifuge 5417C), at room temperature. Two layers were seen, a bottom layer consisting of protein trapped by phenol and a top layer consisting of the DNA products, which was transferred to a sterile 1.5ml microfuge tube. This procedure was repeated for 1~2 times to ensure the DNA was pure. An equal volume of chloroform:isoamylalcohol (24:1) was added to the recovered solution. It was then vortexed and centrifuged at 13000rpm (Gene Company, Eppendorf Centrifuge 5417C) for 2 minutes at room temperature. Again two layers were seen, the bottom layer consisting of the carried over phenol solution, trapped by chloroform:isoamylalcohol, and the top layer consisting of the DNA. DNA was transferred to a sterile 1.5ml microfuge tube. This procedure was repeated 1~2 times to ensure purity of the DNA. One tenth volume of ice cold 3M sodium acetate was added to the solution, followed by 2.5 volume of 100% ethanol. This was to precipitate the DNA. The solution was first mixed by inversion and then incubated at -80^{o}C for 1 hour or at -20^{o}C over night. Products were centrifuged at 13000rpm (Gene Company, Eppendorf Centrifuge 5415R) for 15~30 minutes at 4^{o}C in order to pellet the DNA. Supernatants were discarded and DNA pellets were washed with ice cold 70% ethanol and finally resuspended in 50µl HPLC H_2O.

The DNA concentration of the purified DNA products was determined by nanodrop (NanoDrop® Spectrophotometer ND-1000).

4.2.4 Cloning

The next steps in the construction of the transfer vector involved several cloning procedures, which required preparation of competent cells, several ligation reactions and transformations and selection of positive recombinant plasmids, as described below.

4.2.4.1 Preparation of Competent cells for Transformation

Competent cells were prepared according to the method of Chung (1988) immediately before transformation; this was to ensure maximum competency of the cells.

DH5α cells were inoculated into 5ml of 2x Yeast-Tryptone (YT) broth (1.6% Tryptone, 1.0% yeast extract, 0.5% NaCl, pH 7.0), in a sterile standard container, and incubated at 37^{o}C overnight with shaking.

The overnight culture was diluted 100x in 50ml prewarmed 2YT broth in a 500ml flask. The diluted culture was then placed on a shaker and incubated at 37^{o}C for 2~2.5 hours. This was to allow the growth of the culture to reach early log phase. Early log phase can be measured by reading the absorbance of the culture at 600nm wavelength, using a spectrophotometer. A culture in early log phase will show an absorbance of 0.2 - 0.4 at OD_{600}.

The bacterial cells in early log phase were pelleted by centrifugation, at 5000rpm for 5 minutes at 4^{o}C. Centrifuge tubes were precooled at 4^{o}C prior to centrifugation. The supernatant fluid was discarded and the bacterial pellets were resuspended in 1/10

volume of cold TSB solution [1.6% Peptone, 1.0% yeast extract, 0.5% NaCl, 10% PEG (MW:3350-4000), 5% DMSO, 10mM $MgCl_2$, 10mM $MgSO_4$] and 15% (v/v) glycerol.

4.2.4.2 Transformation and Competency Testing

The competency of the cells was determined in every transformation experiment. This was to ensure the cells were competent enough for transformation. Plasmid DNA with known concentration was added to 100µl of competent cells and left on ice for 30-60 minutes. Cells were heat shocked at 42^{o}C for 2 minutes; this was to enhance the transformation efficiency. 0.9ml of 2YT broth was added to the competent cells and they were incubated at 37^{o}C for 30 - 60 minutes. This was to provide an antibiotic-free environment for the bacteria to express the antibiotic resistance gene. 100µl of the contents were plated on Luria agar containing 10mg/ml ampicillin and incubated at 37^{o}C overnight. The colonies were counted and the competency of the cells determined as the number of colonies / µg of vector.

4.2.4.3 Cloning of PCR products into pGEM T Easy vector

Standard DNA cloning entails the ligation of DNA products that have been digested with restricted enzymes which result in either an overhang of specific base sequences or blunt-ends, which can ligate to a vector that has been digested to have complementary ends. However, *Taq* DNA polymerase, used to generate PCR products, lacks 3' to 5' exnuclease activity which is associated with most DNA polymerases. This means synthesized PCR product often have a single template

independent nucleotide at the 3'end of the DNA strand (Newton *et al.*, 1998), thus preventing one from cloning the PCR products into standard circular plasmid vectors such as pUC21 and pLW51. Amplified fragments were first cloned into pGEM T Easy (supplied by Promega, United States), a vector designed for the cloning of PCR amplified products which have poly-A tails. Following cloning into pGEM T Easy the desired fragment can easily be purified after appropriate restriction enzyme digestion.

Reagents were added as described in the pGEM-T Easy vector user manual. Vector and control DNA, supplied by Promega, United States, were first centrifuged to collect the DNA. The following equation was used to determine the amount of vector and insert DNA to use in ligation reactions:

ng of insert = {(ng of vector x kb size of insert) / (kb size of vector)} x ratio of insert to vector

Ligation reactions were carried out according to the manufacturer's instructions (Promega, Madison, Wisconsin, United States) and then used to transform competent E. Coli DH5α cells (sections 4.2.4.1 and 4.2.4.2).

During the cloning procedure, competent cells were transformed with the original plasmid DNA in order to calculate the competency of the cells. In addition, cells were transformed with ligated plasmid to select for recombinant plasmid. The vectors pGEM T Easy and pUC21 have a lacZ gene thereby allowing for blue-white selection. These vectors were originally designed in such a way that the multiple cloning sites were situated within the β-galactosidase gene. Cloning of foreign DNA into the multiple cloning sites would lead to disruption of the β-galactosidase gene; hence when one plated out on selective media containing IPTG and X-gal, no β-

galactosidase would be available to utilize X-gal. Therefore E. Coli transformed with the vector containing an insert would produce white colonies (Sambrook *et al.,* 1989).

Transformed competent cells were plated onto selective medium containing ampicillin (10mg/ml), IPTG ($5x10^{-5}$ M) and X-gal (5mg/100ml). Media with ampicillin allowed for selective growth of cells containing a plasmid coding for ampicillin resistance. DH5α cells alone were unable to grow in the presence of ampicillin, whereas the vectors used in the cloning procedures all contained an ampicillin resistance gene. Therefore during the transformation process, if DH5α is transformed by a plasmid, such a cell would confer resistance to ampicillin. IPTG is an inducer for β-galactosidase, it induces transcription of the β-galactosidase gene from the vectors. Expressed β-galactosidase utilizes X-gal present in the selective media and turns the bacterial colony blue as mentioned above. Hence to select for the recombinant plasmid, one would select the white colonies.

4.2.4.4 Plasmid Isolation (small-scale preparation)

The alkaline lysis method for minipreparations of plasmid DNA was followed, as described in Sambrook (1989), in order to identify colonies with the desired recombinant plasmid.

Single colonies were plated onto a gridded plate and added to sterile tubes with 5ml sterile 2YT medium. The plates and liquid cultures were incubated at 37^{o}C overnight; the liquid cultures being placed under constant agitation.

The gridded plate was kept as a reference plate so that each possible recombinant

colony could be assigned a defined number, and once the desired recombinant plasmid was identified by analyzing the extracted DNA, one could then trace back to the original colonies and mass produce the identified colony for further experimentation.

Two millilitres of the cell suspension was microcentrifuged for 2 minutes at 13200rpm, (Gene company, Eppendorf Centrifuge 5417C) at room temperature to pellet the cells. The supernatants were discarded. Another 2ml of the overnight culture was added to the pellet, microcentrifuged for 2 minutes at 13200rpm (Gene Company, Eppendorf Centrifuge 5417C) and the supernatant fluids were discarded. This was to collect as many cells as possible. The pellets were resuspended in 200µl of ice cold resuspension solution [50mM glucose, 25mM Tris-HCl (pH 8.0), 10mM EDTA (pH 8.0)] and left at room temperature for 10 minutes. 200µl of lysis solution (1% SDS, 0.2M NaOH), which acts as a detergent, was added to each tube in order to lyse the cells. The solutions were mixed by inversion 7~8 times and placed on ice for 10 minutes. 300µl of neutralization solution (5M potassium acetate, pH 4.8) was added to each tube, mixed via inversion 7-8 times, and left on ice for another 10 minutes. The tubes were centrifuged at 13200rpm (Gene Company, Eppendorf Centrifuge 5417C) for 5 minutes at room temperature. This was to pellet all unwanted debris.

The supernatant fluids were transferred to sterile 1.5ml microfuge tubes and 600µl isopropanol was added. The contents were mixed via inversion and placed at room temperature for 2 minutes. Plasmid DNA was pelleted by centrifuging the tube at 13200rpm (Gene Company, Eppendorf Centrifuge 5417C) for 10 minutes at room temperature. The supernatants were carefully removed, the pellets were washed with

200µl ice cold 70% ethanol, all ethanol was removed, the pellets were dried and resuspended in 50µl HPLC H_2O or TE buffer. Plasmid DNA was stored at 4^oC or -20^oC.

4.2.4.5 Plasmid Isolation (large-scale preparation)

Once the desired recombinant plasmids were identified, large-scale (maxi) preparations were made of the recombinants. The kit used for Plasmid Isolation (Maxi) was supplied by QIAgen, Germany, and the procedure was followed according to the manufacturer's instruction provided by the supplier.

A colony was grown in 100ml Luria Broth in a 1 litre flask. The culture was incubated overnight, on a shaker at 37^oC. Cultures were centrifuged at 4000rpm for 5 minutes at 4^oC. Supernatants were discarded and 2ml of suspension buffer with RNase was added to resuspend the pellet. 4ml of lysis buffer was added, the suspension was well mixed by inverting the tube 6-8 times and the tube was placed on the bench for 2-3 minutes to allow the cells to be lysed. 4ml of pre-cooled neutralization buffer was added to the suspension and well mixed by inversion (6-8 times). At this point a homogenous suspension appeared, which was incubated on ice for 5 minutes.

A piece of filter paper was placed in a funnel; the funnel was inserted into a sterile container. The filter paper was first equilibrated with equilibration buffer before the addition of lysate to the filter. Flow through (containing the plasmid DNA) was collected in the sterile container while the lysed cells were left on the filter. The filtering procedure was repeated in order to ensure the purity of the flow through.

A sealing ring provided by the kit was mounted to the column and the column was mounted onto a sterile 50ml tube. The column was then equilibrated with 2.5ml of equilibration buffer. Flow through was discarded and the cleared lysate, purified by filtration, was added to the equilibrated column. While the lysate flowed through the column, DNA was trapped within the column. This procedure was repeated to increase the amount of DNA collected on the column.

DNA on the column was washed with 5ml of washing buffer. Buffer was allowed to filter through the column. The process was repeated to ensure the purity of the DNA. DNA was eluted from the column by adding pre-warmed (50^{o}C) elution buffer to the column. The eluted DNA was aliquotted (900ul per tube) into sterile 1.5ml microfuge tubes and 600µl of Isopropanol was added to each tube to precipitate the DNA. The DNA was centrifuged at 13200rpm for 30 minutes at 4^{o}C. The supernatant was discarded and the DNA pellet was washed with 600µl ice cold 70% ethanol. The pellet in each tube was resuspended in 50µl of HPLC water. All resuspended DNA was then transferred into one sterile 1.5ml tube.

The DNA concentration was determined by NanoDrop®, Spectrophotometer ND-1000. DNA was then subjected to standard agarose gel electrophoresis to confirm its integrity. The plasmid identity was confirmed by restriction enzyme digestion and agarose gel electrophoresis.

Endotoxin free plasmid was prepared as described above with the addition of endotoxin removal reagent, for transfection experiments (described in Chapter 5).

4.2.4.6 Restriction Enzyme Digestion

Restriction enzyme digestion was used during the cloning procedure to cleave the desired fragments from recombinant plasmids, to linearize vectors and to confirm the identity of recombinant plasmids.

Restriction endonuclease enzymes were supplied by Fermentas, United States. Instructions were followed as described in the manual provided by the suppliers. All digestions were incubated at the temperature indicated in the manual, for 2-2.5 hours, to ensure complete DNA digestion. Digested DNA was examined by standard agarose gel electrophoresis, as described below.

4.2.4.7 Agarose Gel Electrophoresis

The method for agarose gel electrophoresis was followed as described by Sambrook (1989). The purpose of agarose gel electrophoresis was to separate nucleic acids according to the sizes of the fragments. The apparatus used for electrophoresis consists of two buffer tanks containing positive and negative electrodes respectively, which are connected to a voltage supply. The gel lies between the two tanks. Nucleic acid fragments migrate through the agarose gel matrix when exposed to an electrical current. Because the net charge of DNA is negative, DNA fragments will migrate towards the positive electrode. Smaller fragment will move faster through an agarose matrix than larger ones; hence the separation of fragments according to size.

All gels were made up in 1xTris-Borate-EDTA electrophoresis buffer (90mM Tris,

90mM Boric acid and 2mM EDTA). After the agarose gel had been placed in the electrophoresis apparatus, 1x TBE was added to cover the agarose gel. This buffer served to conduct the voltage between the positive and negative electrodes during electrophoresis. The percentage agarose was adjusted according to the size of the DNA fragments to be separated. A high percentage of agarose results in a smaller matrix within the gel, and is therefore more suitable for the separation of small DNA fragments.

Ethidium bromide (EtBr) was used to detect DNA fragments on agarose gels. It is a DNA interchalating agent which fluoresces under UV light. EtBr was either added to the agarose gel before the gel polymerized, at a final concentration of 0.1mg EtBr/100ml of agarose, or, after gel electrophoresis in which case it was added to a staining buffer at a final concentration of 0.1mg/100ml. DNA fragments were visualized on a transilluminator UVP Box.

The actual sizes of the DNA fragments could be determined by comparing the fragment positions to those of molecular weight markers with known sizes (Appendix II).

4.2.4.8 Purification of DNA Fragments by Gel Extraction

During transfer vector construction, one of the procedures was to clone a section of one plasmid into another plasmid. To prevent vector religation or cloning of the wrong fragment, and hence a low yield of desired recombinant plasmid, it was essential to gel extract the fragment of interest.

Restriction endonuclease digestion was carried out as described in section 4.2.4.6. This was to cleave the desired DNA fragment from a plasmid. Digested DNA was subjected to standard agarose gel electrophoresis to separate the desired fragment from the vector. Once electrophoresis had taken place the gel was removed from the apparatus and the band consisting of the desired DNA fragment was cut out from the gel and placed in a sterile 2ml microfuge tube.

A gel extraction kit was supplied by QIAgen, and the following procedure was followed from the user manual, supplied by QIAgen.

Buffer QG was added to the 2ml microfuge tube at a ratio of 1mg gel: 3µl buffer QG. This was then incubated at 50^{o}C until the gel had dissolved. Isopropanol was added to the mix at the ratio of 1mg gel: 1µl isopropanol. The solution was first mixed via inversion for 6~8 times and then transferred to a spin column. The column was centrifuged at 13200rpm for 1 minute; this was to allow the DNA to bind to the column and the debris to pass through the column. 750µl of buffer PE with ethanol was added to the column, and the column was again subjected to centrifugation at 13000rpm for 1 minute. Buffer PQ served to wash the DNA that was bound to the column. Flow through was discarded and to ensure all residue in the column was removed, the column was centrifuged again at 13000rpm for 1 minute. 50µl of HPLC water was added to the column to elute the DNA, and the column was centrifuged at 13000rpm for 1 minute.

4.2.4.9 Vector Preparation

Vectors were first linearized with the desired restriction endonuclease(s). Digested

vectors were subjected to DNA purification as described in section 4.2.3. This was to remove the enzymes and buffers in the solution. After purification, to prevent vector religation, the linearized vector was treated with calf intestinal phosphatase (CIP) prior to ligation with the desired DNA fragment.

4.2.4.9.1 Treatment of Digested Vector with Calf Intestinal Phosphatase (CIP)

CIP enzyme and buffer were supplied by Sigma Aldrich, United State. Experiments were carried out as stipulated in the user manual.

1U of CIP enzyme was used to treat 1 pmol of DNA. To calculate the amount of DNA to use, the following equation was used:

DNA (pmol) = ($2X10^6$ x µg of plasmid) / (bp of plasmid x 660) (Sambrook *et al.*, 1989)

Reagents were added as stipulated in the user manual. The solution was incubated at 37^oC for 1 hour. DNA subjected to CIP was purified as described in section 4.2.3 prior to the ligation step, so as to remove the CIP enzyme and buffer.

4.2.4.10 Ligation and Transformation

After the vector had been treated with CIP and purified, it was ready to be ligated with the desired DNA fragment. T4 DNA ligase and its buffer were supplied by Roche, Germany; and ligation was carried out as instructed in the user manual. Ratios of insert:vector used were 5:1 and 10:1 to increase the chances of insert ligating to vector.

The following equation was used when determining the amounts of insert and vector to use.

ng of insert =

(ng of vector x kb size of insert) / (kb size of vector) x ratio of insert to vector

Experiments were performed as tabulated below:

	Insert (µl)	Plasmid (CIP treated) (µl)	Plasmid (not CIP treated) (µl)	Undigested Plasmid (µl)	Ligase Buffer (10x) (µl)	Ligase (µl)	H_20 (µl)
Experiment 1 Ratio 5:1	ng amount of insert and vector		0	0	2	1	Add up to 20
Experiment 2 Ration 10:1	ng amount of insert and vector		0	0	2	1	Add up to 20
Contamination control	0	0	0	0	2	1	Add up to 20
Competency test	0	0	0	100ng	2	1	Add up to 20
	0	0	0	10ng	2	1	Add up to 20
	0	0	0	1ng	2	1	Add up to 20
Cip Control	0	Xng*	0	0	2	1	Add up to 20
	0	0	Xng*	0	2	1	Add up to 20
Ligase control	0	0	Xng*	0	2	0	Add up to 20

* For CIP control and Ligase control, the amount of vector DNA was equal to the amount of vector DNA used in Experiment 1 and 2.

Ligation was carried out at room temperature for 4 hours. DH5α cells were then transformed with 10ul ligation mix as described in section 4.2.4.2 Plasmid DNA was prepared from single colonies as described in section 4.2.4.4.

4.2.4.11 Preparation of Glycerol Stocks

Stocks of E. Coli cells with the desired recombinant plasmid were prepared as described below and stored at -80^{o}C.

A single bacterial colony with the desired plasmid was incubated in 3ml of 2YT Broth (with 10mg ampicillin) at 37^{o}C overnight. Overnight cultures were aliquotted into 1.5ml cryotubes and sterile glycerol was added to the aliquot such that the final concentration of glycerol solution was 15%. Aliquots were then clearly labeled, snap-frozen and stored at -80^{o}C.

4.3 Results

4.3.1 Extraction of DNA from PEPV and VACV

PEPV and VACV DNA was extracted for PCR amplification of the 2 flanking sequences of the PEPV TK gene and the VACV K1L gene respectively as described in section 4.2.1. The extracted VACV and PEPV DNA was examined by standard agarose gel electrophoresis as shown in Figure 4.6, lanes 2 and 3 respectively. Poxvirus DNA is in the order of 200kb. From the result shown the largest molecular weight marker is 23.13kb and the poxvirus DNA looks to be the same size. This is because standard agarose gel electrophoresis limited its ability to resolve fragments of sizes larger than 20kb. The DNA concentration was measured by NanoDrop®, Spectrophotometer ND-1000. The viral DNA was diluted to 1-5ng/µl for use in PCR reactions (section 4.3.2).

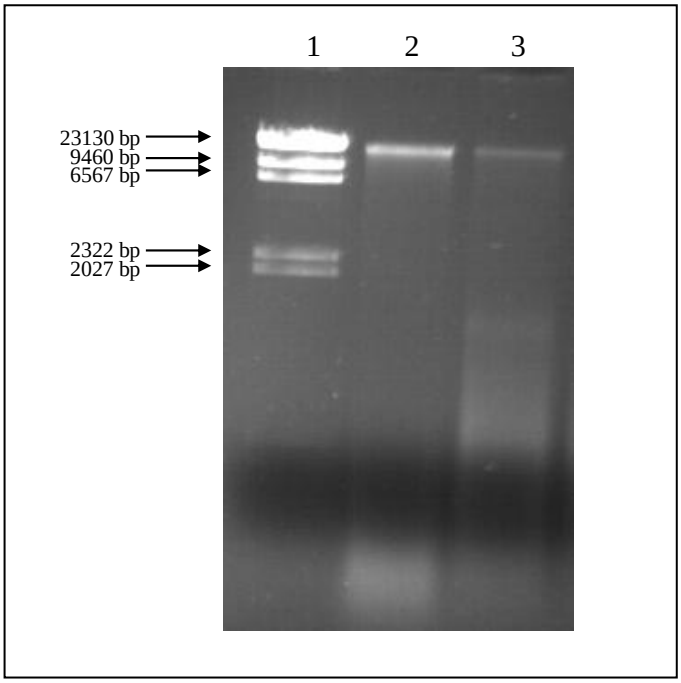

Figure 4.6. Agarose gel electrophoresis of the DNA extraction products from VACV and PEPV. Lane 1 - lambda *Hin*d III DNA molecular weight marker (see Appendix II); Lane 2 – VACV DNA; Lane 3 – PEPV DNA

4.3.2 PCR Amplification of VACV K1L and PEPV TK Flanking Sequences

The first step in the transfer vector construction was to amplify VACV K1L and PEPV TK fragments by PCR (Figure 4.3A) before the cloning procedure.

PCR amplification was carried out as described in section 4.2.2. A fragment of approximately 290bp (Figure 4.7, lanes 3 and 4) was amplified from PEPV genomic DNA using the TK left flank primer pair (see Table 4.1); whereas a fragment of approximately 200bp (Figure 4.7, lane 6 and 7) was amplified from PEPV genomic DNA using the TK right flank primer pair (see Table 4.1). This was as expected, as deduced from the PEPV TK sequence (Figure 4.4), with no non-specific amplification.

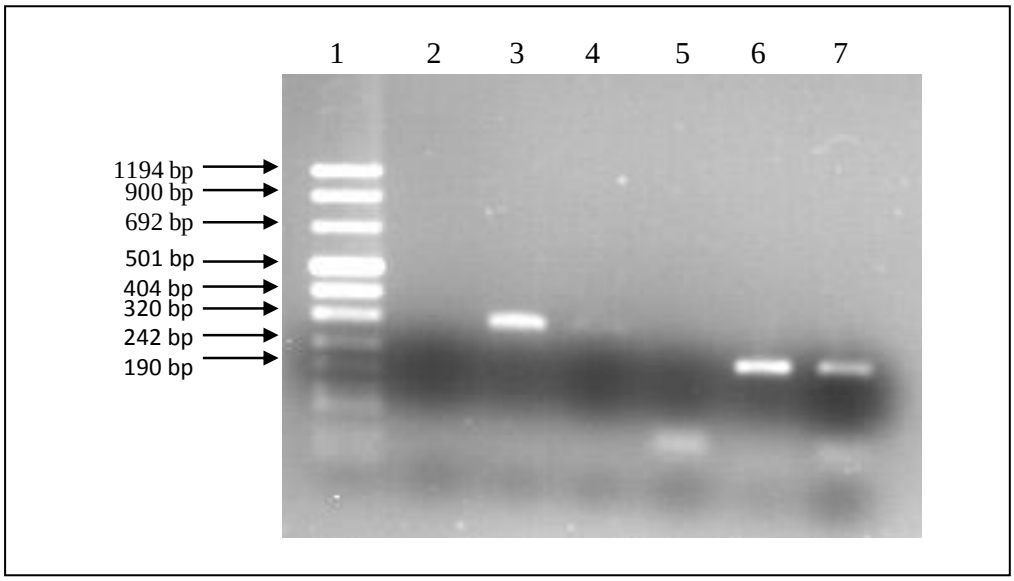

Figure 4.7. Agarose gel electrophoresis of the PCR products amplified from PEPV genomic DNA. Lane 1 – 1kb DNA molecular weight marker (see Appendix II); Lanes 2 and 5 - negative controls with no DNA used in the PCR procedure; Lanes 3 and 4 are duplicate PCR reactions (using 5ng and 1ng of DNA respectively) using TK left primers; Lanes 6 and 7 are duplicate PCR reactions (using 5ng and 1ng of DNA respectively) using TK right primers.

The PCR product, amplified from VACV genomic DNA using the VACV K1L primer pair (see Table 4.1) was approximately 1kb when examined by standard agarose gel electrophoresis (Figure 4.8, lane 3). This size matches the expected size of the K1L gene as deduced from sequence analysis (1088bp, see Figure 4.5, confirmed by sequencing).

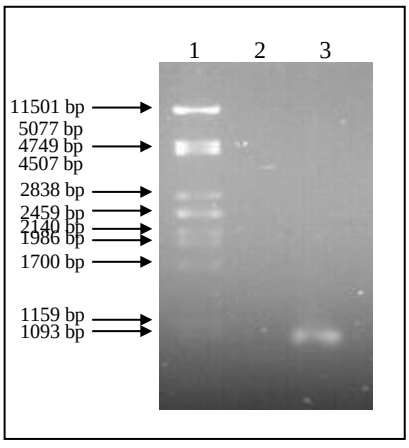

Figure 4.8. Amplification of VACV K1L from VACV genomic DNA, using K1L forward and reverse primers. PCR products were subjected to 1% agarose gel electrophoresis. Lane 1 – lambda *Pst* I DNA molecular weight marker (see Appendix II); Lane 2 – negative control, no DNA; Lane 3 – amplified VACV K1L DNA

All 3 PCR products (VACV K1L, TK left and TK right) were cloned into pGEM T Easy vector (see Figure 4.3A), as described in the next section.

4.3.3 Cloning of PCR-Amplified Fragments into pGEM T Easy and gel extraction of Required Fragments, TK Left, TK Right and VACV K1L

All three amplified PCR products described in the previous section (section 4.3.2) were cloned into the pGEM T Easy vector as described in section 4.2.4.3. For the positive control, provided by the PCR cloning kit, there were many white colonies (no blue colonies), showing that there had been no vector religation and successful ligation of the control DNA insert to the vector. No colonies were seen on the negative control plates; this indicated that there was no contamination in the competent cells nor in any other stocks such as ligase or vector. A large number of colonies were seen on the test plates. However, among these colonies, half of them were blue colonies (no insert) and half of them were white colonies (with insert). Several white colonies were picked and plasmid DNA isolation (mini) was carried out on these colonies as described in section 4.2.4.4.

For cloning of TK left, the plasmids isolated from white colonies were digested with *Hin*d III and *Pst* I. A fragment of 290bp was present in all 14 plasmid preparation (Figure 4.9A). Large-scale DNA isolation (maxi) was applied to one of these clones with the TK left flank as described in section 4.2.4.5. From the large-scale DNA isolation, 5μg of the amplified plasmid was digested with the restriction enzymes, *Hin*d III and *Pst* I to cleave the PEPV TK left flank from the pGEM T Easy vector. The TK left fragments was extracted by gel extraction (Figure 4.9B) as described in section 4.2.4.8.

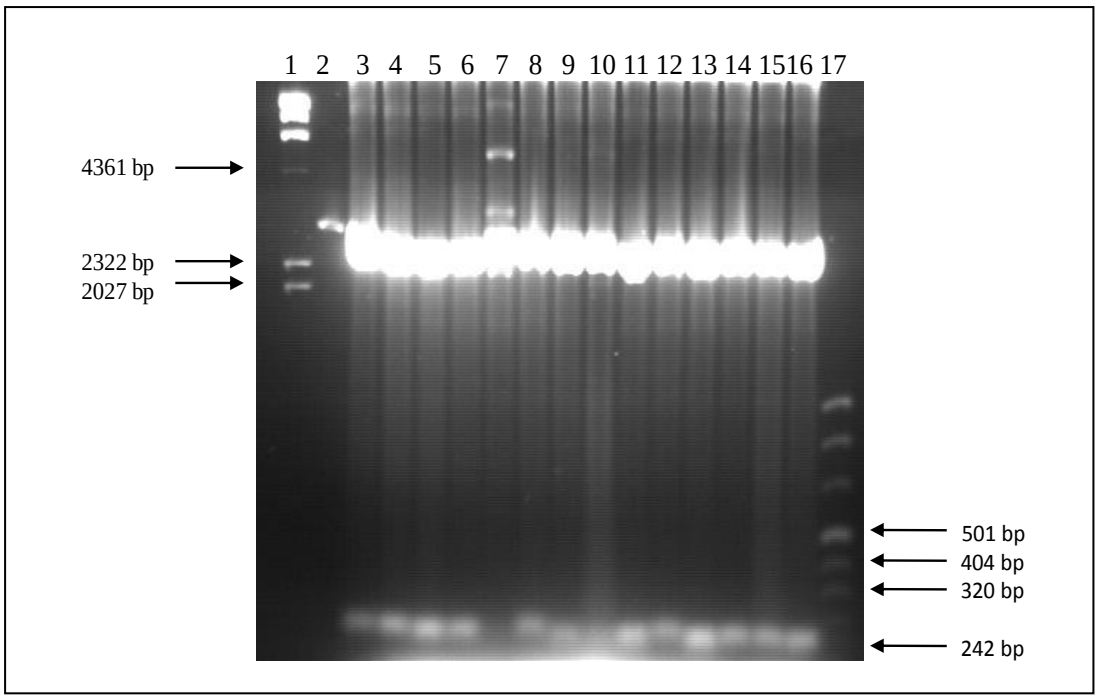

Figure 4.9A. Standard agarose gel electrophoresis of plasmid DNA digested with *Hin*d III and *Pst* I. Lane 1 - λ *Hin*d III DNA molecular weight marker; Lanes 3 ~ 16 - selected clones double digested with *Hin*d III and *Pst* I. Lane 17 - 1kb DNA molecular weight marker. Lane 2 - original pGEM T Easy vector double digested with *Hin*d III and *Pst* I.

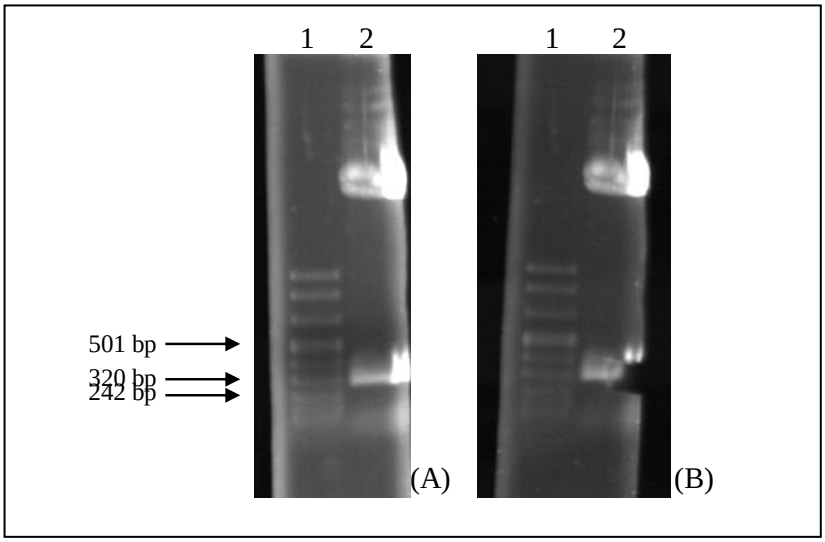

Figure 4.9B. Standard agarose gel electrophoresis showing how TK left fragment was gel extracted. pGEM TK left was digested with *Hin*d III+*Pst* I (lane 2) and the 290bp fragment was eluted from a 1% agarose gel. Lane 1 - 1kb DNA molecular weight marker; DNA was extracted from lanes next to lane 2, which were not stained with ethidium bromide. (A) and (B) show the same gel before and after DNA extraction respectively.

Preparation of VACV K1L gene and PEPV TK right flank were done in the same way as that of TK left. The 200bp PEPV TK right flank was excised from pGEM TKright with *Sma* I and *Eco* RI. The 1kb VACV K1L fragment was excised from pGEM K1L with *Bss* HII.

Once the VACV K1L fragment, TK left and right fragments were gel purified, it was vital to examine whether the extracted DNAs were intact and not contaminated with pGEM T Easy vector, as contamination may cause religation of the vector and inhibit cloning of these fragments into the transfer vector. Figure 4.9C clearly shows that extracted DNA remained intact and was of the correct size and ready for the next procedure, cloning into the vector backbone.

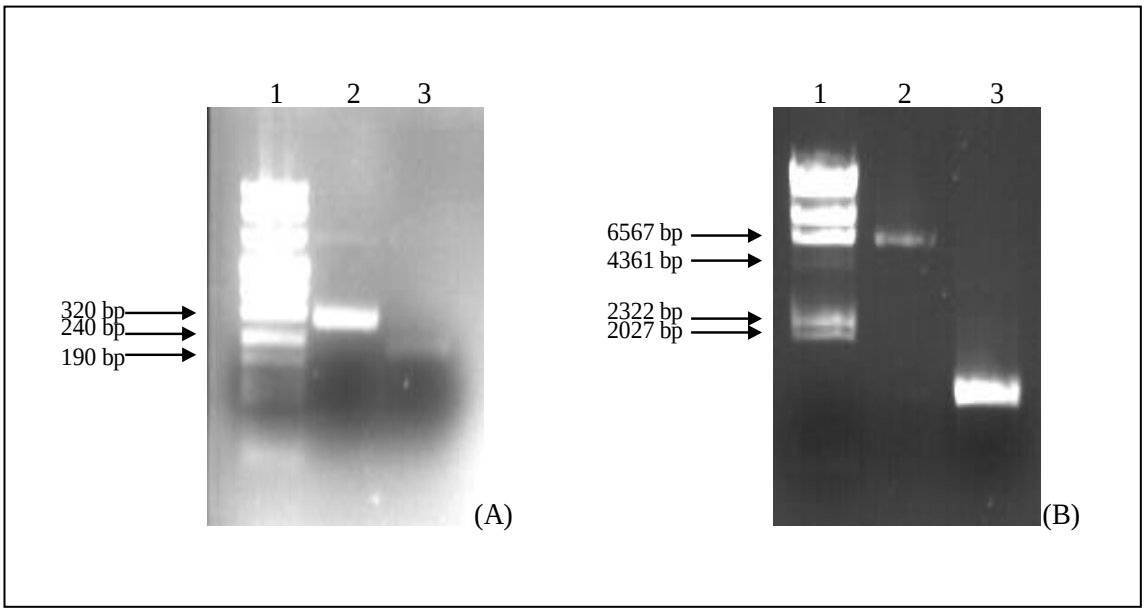

Figure 4.9C. Products from gel extraction of the VACV K1L fragment and the PEPV TK left and right flanks, subjected to standard agarose gel electrophoresis. (A) Lane 1 - 1kb DNA molecular weight marker; Lane 2 - TK left flank (290bp); Lane 3 - TK right flank (200bp). (B) Lane 1 - λ *Hin*d III DNA molecular weight marker; Lane 2 - linear pLW51 vector (6.5kb); Lane 3 - gel extracted VACV K1L fragment (1kb).

4.3.4 Cloning of VACV K1L into pLW51

Figure 4.3B shows a schematic diagram of the next cloning procedure in the construction of transfer vector, pNCH-3, where gel-extracted VACV K1L gene was cloned into pLW51 plasmid.

The extracted and purified VACV K1L gene was ready to be cloned into the vector backbone, but prior to cloning, the vector itself was required to be linearized using *Bss* HII, treated with CIP and purified. These procedures were described in section 4.2.4.9. Figure 4.9C (B) shows the purified K1L fragment (lane 3) and CIP treated pLW51 vector (lane 2). Ligation and transformation were carried out as described in section 4.2.4.10. Unlike the PCR cloning experiment, the selective media for this experiment only contained ampicillin, as blue-white selection could not be utilized in this experiment (pLW51 does not contain a *Lac* Z gene).

After transformation, colonies were picked and plasmid DNA isolation (small-scale) and restriction enzyme digestion were carried out to identify the positive clone containing the K1L gene. Due to the use of a single restriction enzyme (*Bss* HII), one could expect several cloning results: A single insert with either forward or reverse orientation or double inserts with either forward or reverse orientation. To differentiate these different possibilities, restriction enzyme digestion was carried out. Table 4.2 shows the size fragments expected after single *Hin*d III and double *Hin*d III + *Eco* RI restriction enzyme digestion for the different possible recombinants. Calculations were made based on restriction enzyme analysis of the DNA sequences of the insert and pLW51 vector.

Table 4.2. Sizes of fragments expected after single and double restriction enzyme digests to differentiate between different orientations of the insert.

Orientation	Single (forward)		Single (reverse)		Double (forward)		Double (reverse)	
Enzyme	*Hind* III	*Hind* III+ *Eco* RI	*Hind* III	*Hind* III+ *Eco* RI	*Hind* III	*Hind* III+ *Eco* RI	*Hind* III	*Hind* III+ *Eco* RI
Molecular weight	4.5kb	3.1kb	3.9kb	3.7kb	4.5kb	3.1kb	3.9kb	3.7kb
	3.1kb	2.7kb	3.7kb	2.7kb	3.1kb	2.7kb	3.7kb	2.7kb
		1.8kb		1.2kb	1.1kb	1.8kb	1.1kb	1.2kb
						1.1kb		1.1kb

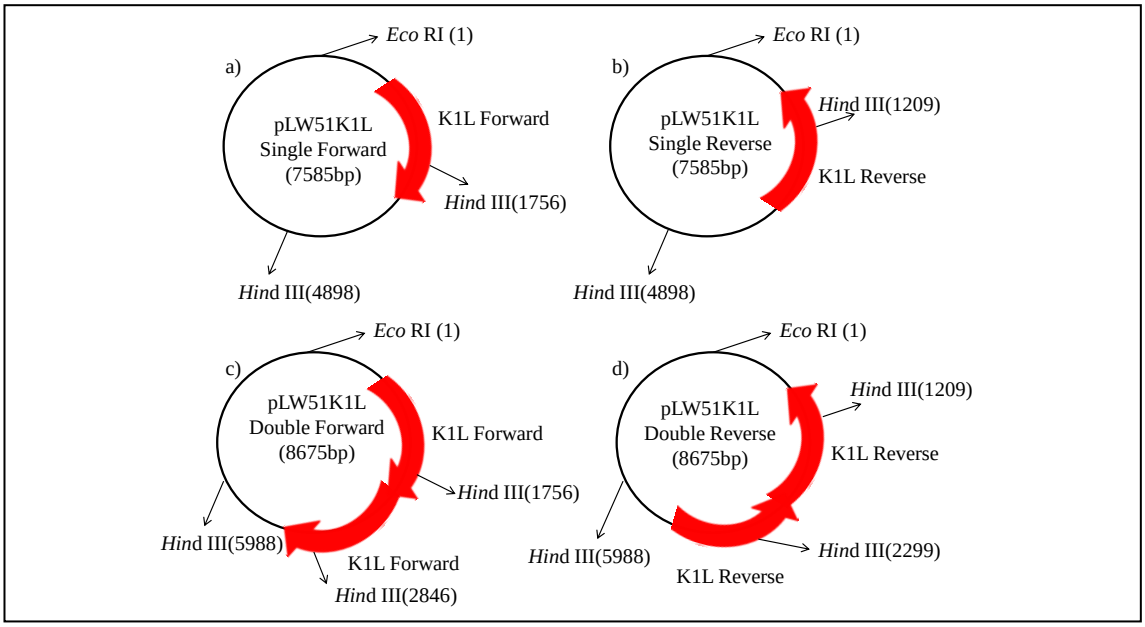

Figure 4.10A. Plasmid maps of K1L cloned into pLW51 in different orientation and as single and double inserts. a) single insert, forward orientation; b) single insert, reverse orientation; c) double insert, both in forward orientation and d) double insert, both in reverse orientation.

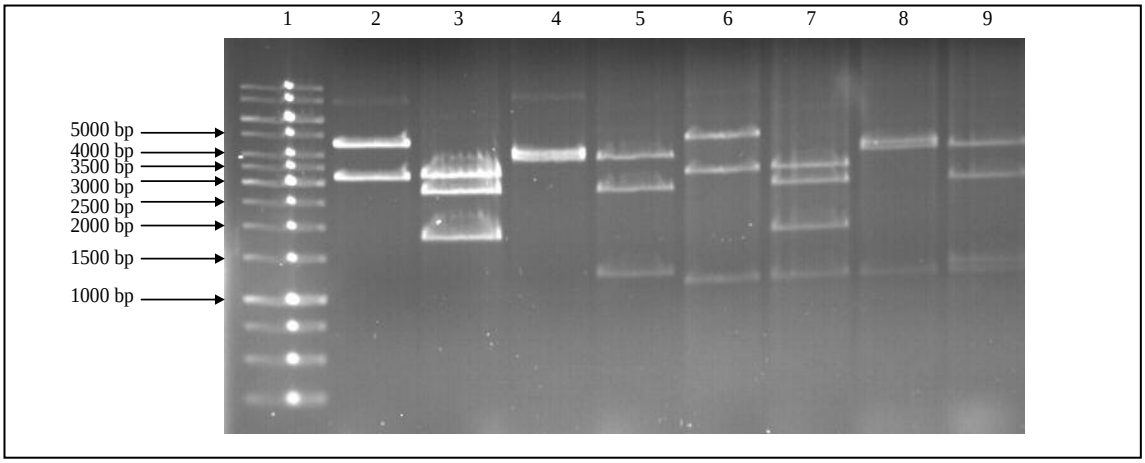

Figure 4.10B. Standard agarose gel electrophoresis showing clones containing K1L in different orientations and with multiple inserts. Lane 1 – GeneRuler^{TM} 1kb DNA molecular weight marker; Lanes 2, 4, 6, and 8 - *Hind* III restriction enzyme digestion of clones 1-4 respectively; Lanes 3, 5, 7, 9 - *Hind* III+*Eco* RI double digests of clones 1-4 respectively.

Analysis based on Table 4.2 and Fig 4.10, confirmed clone 1 contained a single K1L insert, in the forward orientation; *Hin*d III gave fragments of 4.5 and 3.1kb (lane 2) and double digestion with *Hin*d III and *Eco* RI gave fragments of 3.1, 2.7 and 1.8kb (lane 3). Clone 2 contained a single K1L fragment, in the reverse orientation; *Hin*d III produced fragments of 3.9 and 3.7kb (lane 4) and double digestion with *Hin*d III and *Eco* RI produced fragments of 3.7, 2.7 and 1.2kb (lane 5). Clone 3 contained a double K1L insert, both in the forward orientation; *Hin*d III produced fragments of 4.5, 3.1 and 1.1kb (lane 6) and double digestion with *Hin*d III and *Eco* RI produced fragments of 3.1, 2.7, 1.8 and 1.1kb (lane 7). Clone 4 contained a double K1L fragment, both in the reverse orientation; *Hin*d III produced fragments of 3.9, 3.7 and 1.1kb (lane 8) and double digestion with *Hin*d III and *Eco* RI produced fragments of 3.7, 2.7, 1.2 and 1.1kb (lane 9).

A single K1L insert in either forward or reverse orientation was required for the final step in the construction of transfer vector pNCH-3 (Fig 4.3D). Clone 1 was used for future cloning. It contains: the host range gene (K1L) which serves as a selective marker for recombinant PEPV, a *Sma* I site for insertion of a future gene of interest, a reporter gene, GUS, to indicate the presence of a recombinant virus and a direct repeat sequence flanking the K1L and GUS genes.

4.3.5 Cloning PEPV TK Flanks into pUC21

Purified and extracted TK left and right flanks from section 4.3.3 were cloned into pUC21 prior to the final transfer vector construction. PEPV TK left flank was first cloned into the pUC21 vector to produce pUC21TKleft. The PEPV TK right flank was then cloned into the recombinant pUC21/TK left flank plasmid. The cloning procedure

was followed as described in section 4.2.4.10. Figure 4.3C shows the cloning strategy followed in the construction of pUC21TKl+r.

To confirm the cloning of TK left flank into pUC21, isolated plasmid DNA was subjected to double restriction enzyme digestion with *Hin*d III + *Pst* I, which cleaved the TK left flank of 290bp from the recombinant plasmid, thus proving its presence (Figure 4.11A). The recombinant plasmid, pUC21 TKleft, was prepared in larger quantities as described in section 4.2.4.5.

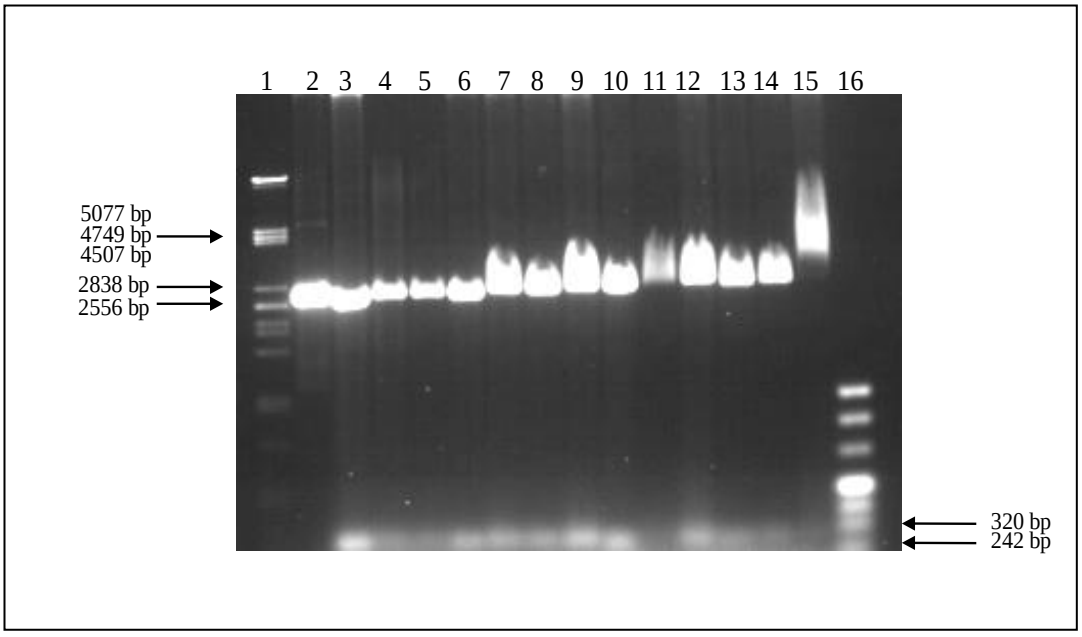

Figure 4.11A. Standard agarose gel electrophoresis showing the presence of PEPV TK left flank cloned into pUC21. Lane 1 – λ *Pst* I DNA molecular weight marker; Lane 2 - pUC21, double digested with *Hin*d III and *Pst* I; Lanes 3 ~15 - Extracted plasmid DNA from 12 different colonies, double digested with *Hin*d III and *P*st I; Lane 16 - 1kb DNA molecular weight marker.

TK right flank was isolated from pGEM TKright on a *Sma* I+*Eco* RI fragment and cloned into pUC21TKleft. Confirmation of the recombinant plasmid, pUC21TKl+r was carried out by restriction enzyme digestion (Figure 4.11B). Restriction enzyme analysis was done based on the sequences of the TK gene and pUC21. A *Hin*d III site is present

in the TK right flank (shown in Figure 4.11B), thus single digestion with *Hin*d III will digest pUC21TKleft vector once, producing a linearized plasmid of approximately 3kb (Figure 4.11C, lane 4) but digestion of pUC21TKl+r with *Hin*d III will produce fragments of 2.7kb and 500bp (Figure 4.11C lane 5).

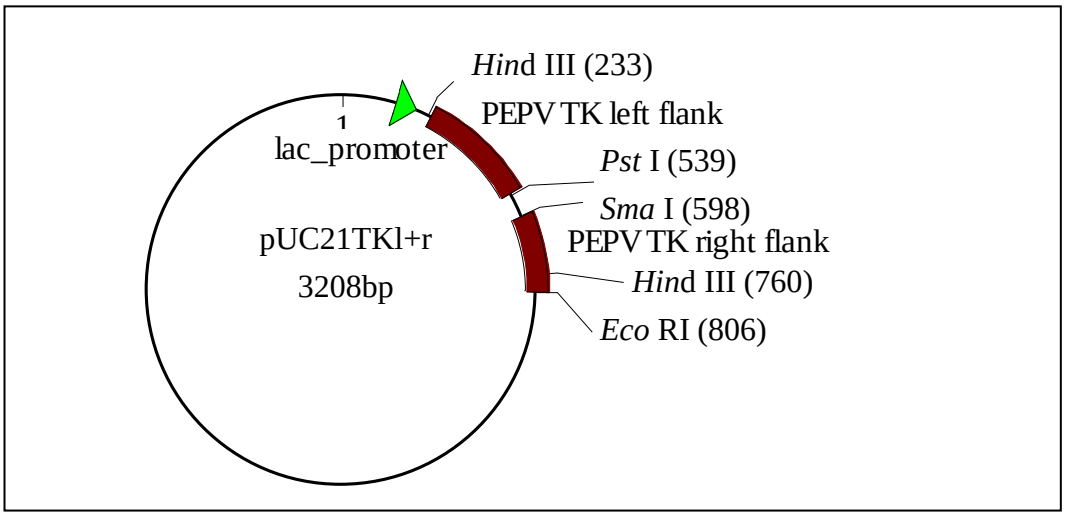

Figure 4.11B. Plasmid diagram representing the pUC21TKl+r plasmid, showing relevant restriction enzyme siteS for the confirmation of PEPV TK flanks.

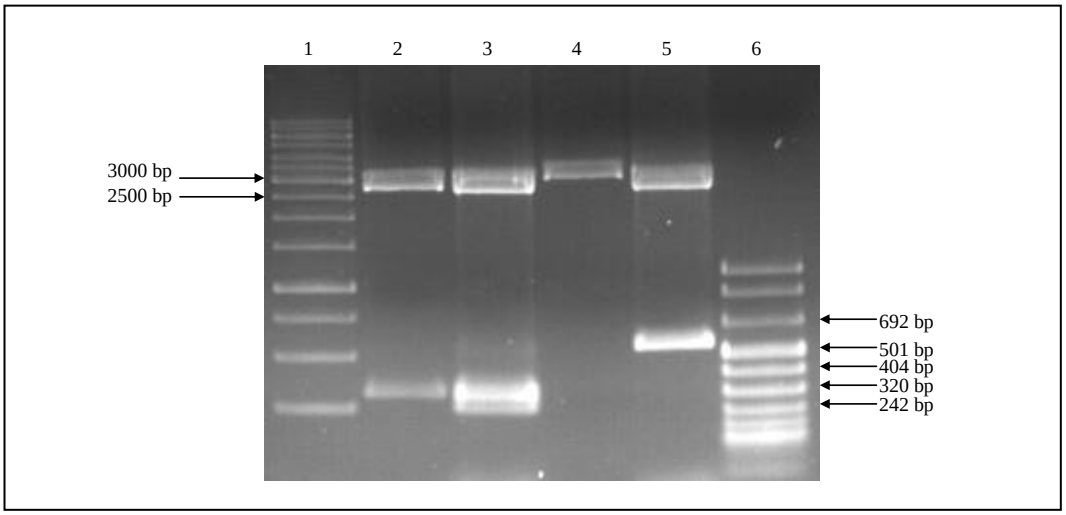

Figure 4.11C. Standard agarose gel electrophoresis showing the presence of both PEPV TK left and right flankS in recombinant pUC21 plasmid. Lane 1 – GeneRuler^{TM} 1kb DNA molecular weight marker; Lane 2 - recombinant pUC21TKleft plasmid, double digested with *Hin*d III+*Pst* I, (producing approximately 300bp fragment); Lane 3 - clone 1, double digested with *Hin*d III+*Pst* I, (producing 2 fragments of approximately 300bp fragment); Lane 4 - recombinant pUC21TKleft plasmid, single digested with *Hin*d III; Lane 5 - clone 1, digested with *Hin*d III, (producing appoximately 500bp fragment); Lane 6 - 1kb DNA molecular weight marker.

Figure 4.11C confirmed the presence of both TKleft and TKright flanks in pUC21TKl+r plasmid, by comparing it with pUC21TKleft (lane 4). After the confirmation of the presence of both TK flanks, one could proceed to the final step of the construction of the transfer vector.

4.3.6 Construction of pNCH-3 Transfer Vector

The ultimate goal of this project was to construct the transfer vector pNCH-3. All the necessary elements for the transfer vector were divided among 2 vector backbones, pUC21TKl+r and pLW51K1L. Thus the final step, shown in Figure 4.3D, was to insert the fragment from pLW51K1L, containing K1L, GUS, the direct repeats and the *Sma* I site downstream of the pmH5 promoter, between the two TK flanks in pUC21TKl+r.

4.3.6.1 Extraction of K1L-GUS Fragment from Recombinant Plasmid pLW51K1L

When pLW51K1L is digested with C*la* I+B*am* HI, two fragments of 3.9kb and 3.7kb are generated (Figure 4.12). The 3.9kb fragment (required fragment) consists of a pair of direct repeat sequences, GUS reporter gene, K1L gene and a SmaI site for insertion of foreign gene(s) (Figure 4.12). The 3.7kb fragment consists of the remainder of pLW51K1L. These two fragments (3.9 and 3.7kb) do not separate easily on an agarose gel; hence, for the isolation of the required 3.9kb fragment, after double digestion with C*la* I + B*am* HI, the enzymes were heat inactivated at 65^{o}C for 15 minutes and the DNA was digested with a third enzyme, *Eco* RI. This was to digest the vector fragment and produce 3 fragments in total, a 3.9kb fragment (the original desired insert), a 3.2kb fragment (partial backbone) and a 0.5kb fragment (partial backbone) (Figure 4.12). Gel extraction was done as described in section 4.2.4.8.

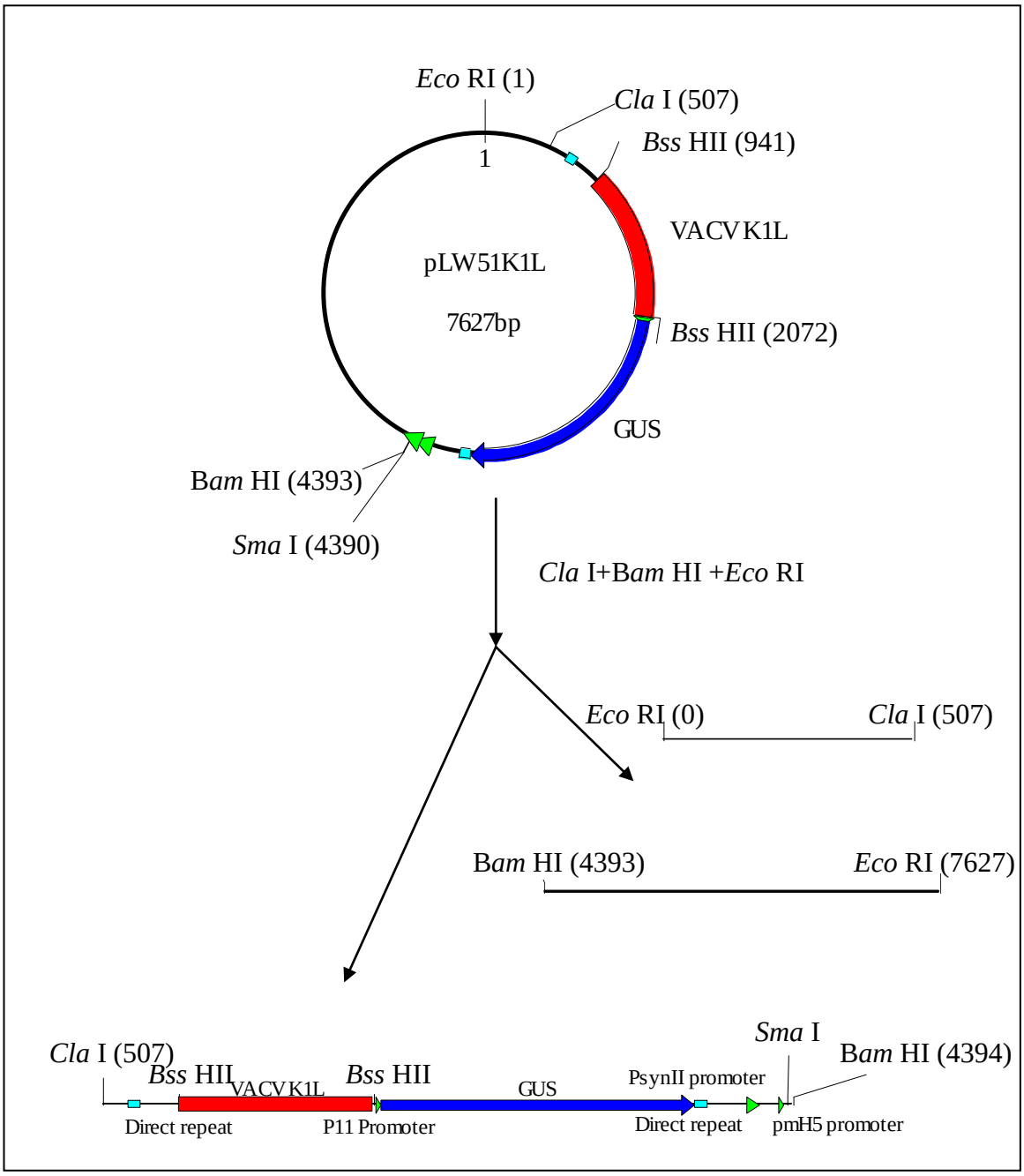

Figure 4.12. Diagram of pLW51K1L showing fragments generated by digestion with C*la* I + B*am* HI + *Eco* RI. The numbers at the ends of the fragments indicate their sizes in bp.

Once the C*la* I–B*am* HI fragment was extracted, it was important to examine if the gel extracted product was intact and pure, i.e. no contamination with the original pLW51 vector. The identity of the fragment was confirmed by *Bss* HII digestion, which cleaves the 3.9kb fragment twice into fragments of 2.4kb, 1kb (K1L) and 500bp (Figure 4.13).

Figure 4.13 (lanes 3 and 4) shows *Bss* HII digested gel extracted products produce 2 visible fragments of 2.4kb and 1.0kb. The fragment of 500bp was too small to be visible on the gel. This indicated the gel extracted product contained the K1L gene as predicted.

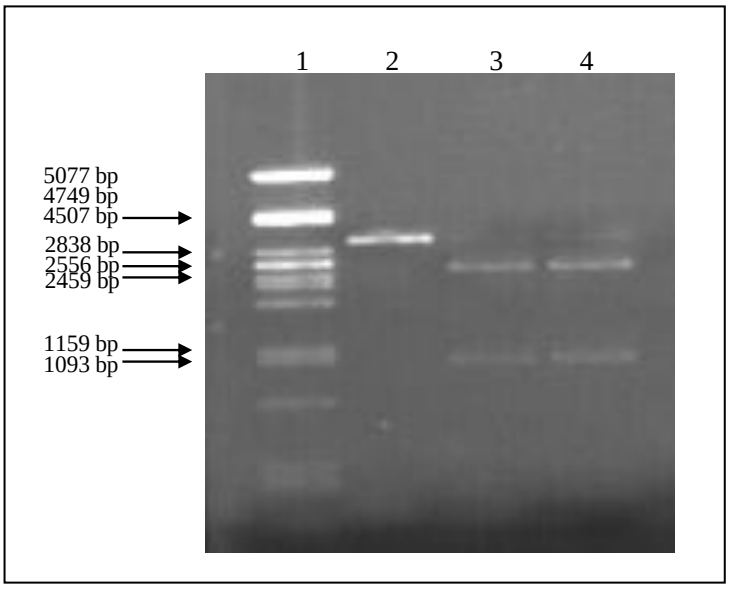

Figure 4.13. Standard agarose gel electrophoresis confirming the presence of *Bss* HII sites in the gel extracted C*la* I – B*am* HI fragment and showing vector pUC21TKl+r was ready for the final cloning procedure. Lane 1 – λ *Pst* I DNA molecular weight marker; Lane 2 – CIP treated pUC21TKl+r. Lanes 3 and 4 - gel extracted product, digested with *Bss* HII.

4.3.6.2 Cloning of the Gel Extracted K1L-GUS Fragment into pUC21TKl+r

To prepare the vector for cloning, several steps were required to be done prior to the final cloning step (Figure 4.3D). The pUC21TKl+r vector was digested with *Sma* I restriction enzyme and immediately treated with CIP. Figure 4.13 (lane 2) shows linearized pUC21TKl+r after digestion with SmaI and CIP-treatment. *Sma* I created blunt-ended sites for insertion of the fragment. The extracted fragment however, consisted of sticky-ends (C*la* I and B*am* HI). Thus the fragment was blunt-ended by using T4 DNA polymerase to fill the ends with appropriate dNTPs. The blunt-ended

fragment was then ligated to the prepared pUC21TKl+r plasmid. Competent cells were transformed with the ligation mix, as described in section 4.2.4.10. Plasmid DNA isolation (small-scale) was done on the transformed colonies in search of the final recombinant plasmid, pNCH-3.

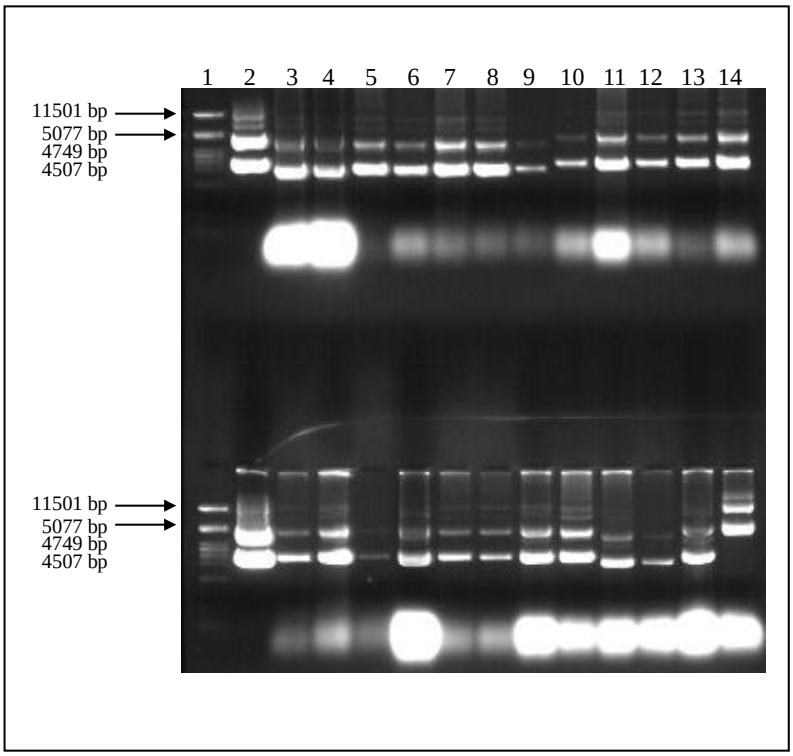

Figure 4.14A. Standard agarose gel electrophoresis of plasmid DNA isolated in search of pNCH-3. Top and bottom Lanes: Lane 1 – λ *Pst* I DNA molecular weight marker; Lane 2 – undigested pUC21TKl+r vector; Lanes 3-14 undigested plasmid DNA isolated from picked colonies.

The pUC21TKl+r vector is 3.2kb. The desired plasmid (pNCH-3) is 7kb. Therefore one could see a major difference in migration between the vector (Figure 4.14A, lane 2) and the recombinant (Figure 4.14A, lane 14, bottom half of gel). This potential recombined (labelled I24), was digested with *Bss* HII to confirm the presence of the insert.

Figure 4.14B (lane 3) shows the potential clone (I24) produced a 1kb fragment after digestion with *Bss* HII. This confirmed the presence of the insert.

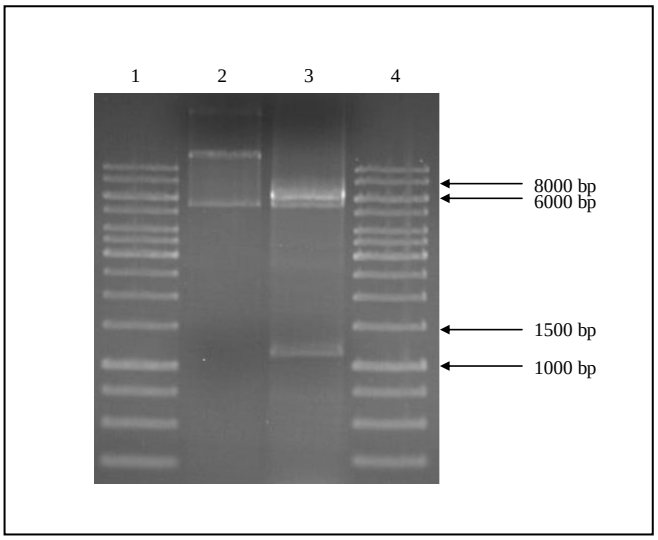

Figure 4.14B. Standard agarose gel electrophoresis of the plasmid I24 subjected to restriction enzyme digestion with *Bss* HII. Lane 1 – GeneRuler^{TM} 1kb DNA molecular weight marker; Lane 2 – I24 undigested; Lane 3 - I24 (from Figure 4.14A, bottom lane 14), digested with *Bss* HII; Lane 4 - GeneRuler^{TM} 1kb DNA molecular weight marker.

Due to the blunt-ended cloning, the cloned insert could be in one of two orientations. Primers were designed for the sequencing of I24, and the plasmid was sent to the University of Stellenbosch Sequencing Unit, together with the primers, to be sequenced. The sequencing data was assembled using BioEdit (version 7.0.5.2). The sequence showed K1L to be closer to the PEPV TK right flank and GUS to be near the TK left flank. Figure 4.15 shows a diagram of the transfer vector pNCH-3.

The pNCH-3 DNA sequence is shown in Figure 4.16, coloured sequences indicate the PEPV TK flanks, VACV promoters, direct repeats, GUS reporter gene and selective marker gene, K1L. Present in this transfer vector is a SmaI site downstream of the pmH5 promoter, which is suitable for the cloning of foreign genes such as those encoding HIV-1 antigens / epitopes. This is the scope of a future project.

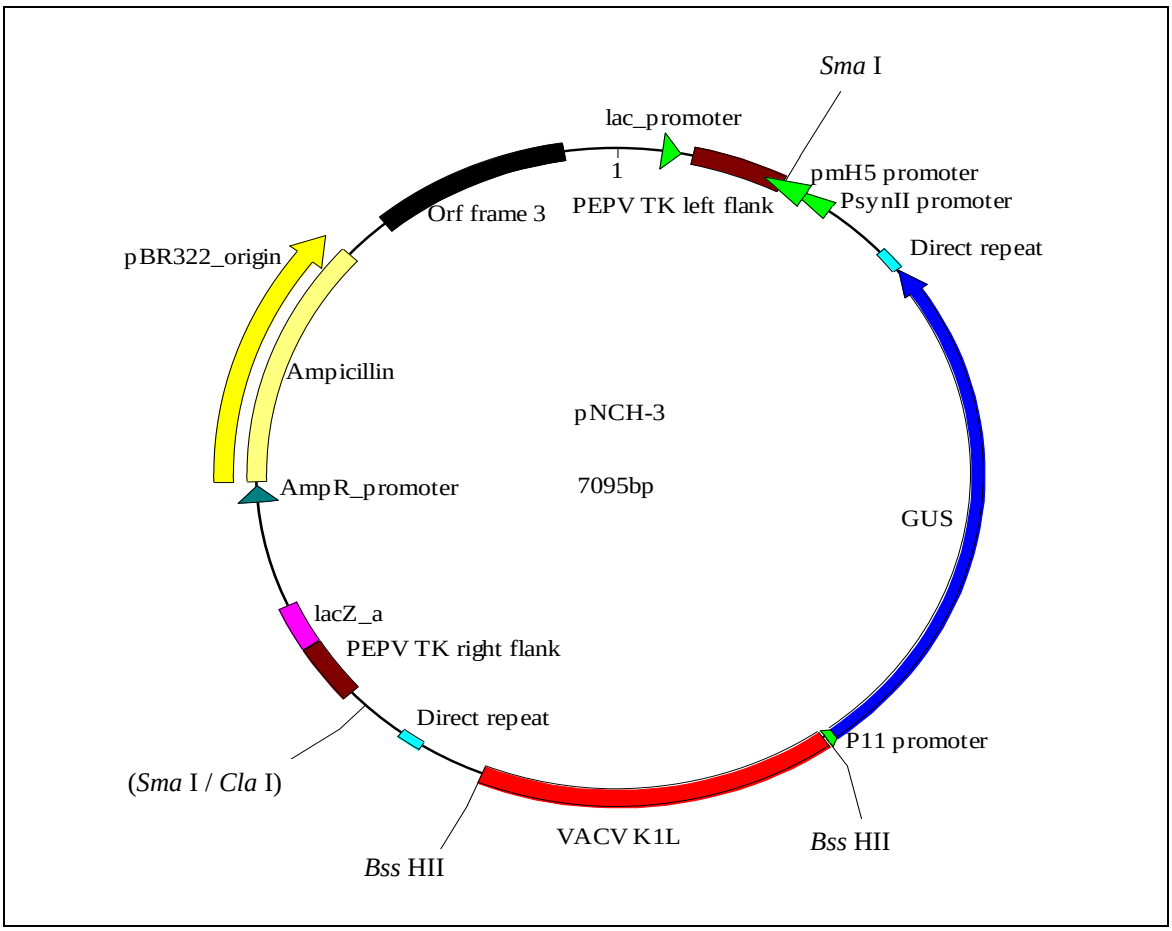

Figure 4.15. Plasmid diagram representing the final transfer vector pNCH-3, showing all elements present within the vector.

pNCH-3 was tested for its ability to rescue the growth of PEPV in RK-13 cells and this is described in the next chapter. At the same time attempts were made to isolate a recombinant virus expressing K1L and GUS. This would show that it is possible to use the transfer vector pNCH-3 for the future construction of recombinant PEPV expressing specific antigens, the genes of which would be cloned into the *Sma* I site of pNCH-3.

GCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCC
GACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTA
CACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATG
ACCATGATTACGCCAAGCTTATGGCTTCCGGAAGTATCCATGTTATTACAGGTCCTATGTTTTCAGGTAAAACAT
CAGAACTAGTAAGAAGAATAAAAAGANNTTTATGCTATCTAACTTTAAATGTATTATTATTAAACATTGTGGAG
ATAATAGATATAACGAAGATGATATAAATAAAGTATGNNTACACACGATAGATTGTTTATGGAAGCTATAGCATC
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CCTTTGTATTTATTTTCAATTTTTAGGTTGGGTACCAGCGGCCGCTATTTATTATTTATAGCATAGAAAAAAAGA
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CACCGAAGTTCATGCCAGTCCAGCGTTTTTGCAGCAGAAAAGCCGCCGACTTCGGTTTGCGGTCGCGAGTGA
AGATCCCTTTCTTGTTACCGCCAACGCGCAATATGCCTTGCGAGGTCGCAAAATCGGCGAAATTCCATACCTG
TTCACCGACGACGGCGCTGACGCGATCAAAGACGCGGTGATACATATCCAGCCATGCACACTGATACTCTTCA
CTCCACATGTCGGTGTACATTGAGTGCAGCCCGGCTAACGTATCCACGCCGTATTCGGTGATGATAATCGGCTG
ATGCAGTTTCTCCTGCCAGGCCAGAAGTTCTTTTTCCAGTACCTTCTCTGCCGTTTCCAAATCGCCGCTTTGGA
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GAACATTACATTGACGCAGGTGATCGGACGCGTCGGGTCGAGTTTACGCGTTGCTTCCGCCAGTGGCGCGAA
ATATTCCCGTGCACCTTGCGGACGGGTATCCGGTTCGTTGGCAATACTCCACATCACCACGCTTGGGTGGTTTT
TGTCACGCGCTATCAGCTCTTTAATCGCCTGTAAGTGCGCTTGCTGAGTTTCCCCGTTGACTGCCTCTTCGCTG
TACAGTTCTTTCGGCTTGTTGCCCGCTTCGAAACCAATGCCTAAAGAGAGGTTAAAGCCGACAGCAGCAGTT
TCATCAATCACCACGATGCCATGTTCATCTGCCCAGTCGAGCATCTCTTCAGCGTAAGGGTAATGCGAGGTAC
GCCCTTCACTGCCACTGACCGGATGCCGACGCGAAGCGGGTAGATATCACACTCTGTCTGGCTTTTGGCTGTG
ACGCACAGTTCATAGAGATAACCTTCACCCGGTTGCCAGAGGTGCGGATTCACCACTTGCAAAGTCCCGCTA
GTGCCTTGTCCAGTTGCAACCACCTGTTGATCCGCATCACGCAGTTCAACGCTGACATCACCATTGGCCACCA
CCTGCCAGTCAACAGACGCGTGGTTACAGTCTTGCGCGACATGCGTCACCACGGTGATATCGTCCACCCAGG
TGTTCGGCGTGGTGTAGAGCATTACGCTGCGATGGATTCCGGCATAGTTAAAGAAATCATGGAAGTAAGACTG
CTTTTTCTTGCCGTTTTCGTCGGTAATCACCATTCCCGGCGGGATAGTCTGCCAGTTCAGTTCGTTGTTCACAC
AAACGGTGATACGTACACTTTTCCCGGCAATAACATACGGCGTGACATCGGCTTCAAATGGCGTATAGCCGCC
CTGATGCTCCATCACTTCCTGATTATTGACCCACACTTTGCCGTAATGAGTGACCGCATCGAAACGCAGCACG
ATACGCTGGCCTGCCCAACCTTTCGGTATAAAGACTTCGCGCTGATACCAGACGTTGCCCGCATAATTACGAAT
ATCTGCATCGGCGAACTGATCGTTAAAACTGCCTGGCACAGCAATTGCCCGGCTTTCTTGTAACGCGCTTTCC
CACCAACGCTGATCAATTCCACAGTTTTCGCGATCCAGACTGAATGCCCACAGGCCGTCGAGTTTTTTGATTT
CACGGGTTGGGGTTTCTACAGGACGTACCATTTATAGCATAGAAAAAAACAAAATGAAAGGCGCGCGTATGG
GTACGGTGTAAGGAATCATTATTTTATTTATATTGATGGGTACGTGAAATCTGAATTTTCTTAATAAATATTATTTT
TATTAAATGTGTATATGTTGTTTTGCGATAGCCATGTATCTACTAATCAGATCTATTAGAGATATTATTAATTCTGG

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GATAGTGGTAACATGCAAACGGTAAAACTGTTTGTTAAGAAAAATTGGAGACTGATGTTCTATGGGAAAACT
GGATGGAAAACTTCATTTTATCATGCCGTCATGCTTAATGATGTAAGTATTGTTTCCTACTTTCTTTCAGAGATA
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GGCCCGTCGACTCTAGAGCTCTGCAGAGAATTTAGTGAATCTATGGCTAATTTAGGTAAAATAGTTATTATAGC
CGCGCTTAATGGTGATTTTAAACGCGAATTATTTGGAAACGTGTGTAAGCTATTACCATTAGCAGAGACAGTTT
CCAGTTTAACAGCTATTTGTGTGAAATGCTATCGCGAAGCTTCGTTTTCAAAACGCATTACAGAGAGTAAAGA
AGTAATGGATGAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAA
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AACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTT
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CGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTG
GGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAA
CGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGC

GGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCC
CGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCT
ACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTA
AGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAA
GGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCG
TCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAA
CAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAA
CTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAA
CTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGT
GTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGT
GCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGC
GCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCA
CGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCG
TCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTT
CCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTAC
CGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAG
CGGAAGA

Figure 4.16. Sequence of pNCH-3 transfer vector. Brown coloured sequences indicate the PEPV TK flanking sequences (PEPV TK left and right); green coloured sequences indicate the poxvirus promoters (pmH5, PsynII and P11); light blue coloured sequences indicate the direct repeats; the dark blue coloured sequence indicates the GUS reporter gene; the red coloured sequences indicate the VACV K1L gene; and the single *Sma* I restriction enzyme site is underlined.

4.4 Discussion

Transfer vectors are used widely in the construction of recombinant poxviruses (Elahi *et al*., 1999; Dvoracek *et al*., 2003; Hahn *et al*., 2000; Perkus *et al*., 1989). This is the first attempt to construct a transfer vector for the generation of a recombinant PEPV. As has been previously used (Kit, 1985; Hahn *et al*., 2000; Perkus *et al*., 1989), the thymidine kinase (TK) gene was chosen as the site of insertion. This gene is not essential for the growth of poxviruses in vitro (see section 4.1); therefore it is the gene of choice for inserting foreign genes into poxviruses, as disruption of this gene through homologous

recombination is not lethal for growth of the virus in vitro. The TK gene is also located in a conserved region of the genome, which would suggest that any recombinant virus containing gene(s) inserted in this locus would be stable.

The PEPV thymidine kinase gene was first identified by Daria Kow (1992), by primer hybridization. However, the first sequencing work of the TK gene was only done during my Honours study (Hu, 2007). During this study, TK sequences of Fowlpox virus and Canarypox virus were aligned and primers were designed from the conserved sequences. The primers were used to amplify 330 bp of the PEPV TK gene. However, this sequence is relatively short to be used as flanking sequences in a transfer vector and the probability of homologous recombination taking place would be small (Yao *et al*., 2001). In 2008, most of the PEPV genome was sequenced by Olivia Carulei, Division of Medical Virology, Department of Clinical Laboratory Sciences, UCT. The complete sequence of PEPV TK (552 bp) was obtained, and used in this study for the construction of a PEPV transfer vector. The combined total of the homologous sequence used in this study is 496bp (290bp TK left flank and 206bp TK right flank). Yao's group has shown the ideal sequence length for homologous recombination to occur is 366bp in VACV. Although Yao's study was based on VACV, one can apply it to PEPV, therefore it is likely for homologous recombination to occur between the transfer vector constructed in this study and the PEPV genome.

Stannard *et al,* used a plasmid containing a reporter gene, *LacZ*, under the control of the VACV P11 promoter. (Stannard *et al*., 1998). In her study no expression was seen from the reporter gene after transient expression of the plasmid in PEPV infected RK-13 cells, indicating no transcriptase was present to modulate the late promoter and hence initiate the transcription of the reporter gene (Stannard *et al*., 1998). In this study, the P11

promoter was used to express the reporter gene, GUS. If GUS is expressed, then the presence of K1L would have rescued late gene expression and it could be possible to rescue recombinant PEPV in RK-13 cells using VACV K1L.

The next chapter describes the rescue of PEPV growth in RK-13 cells using VACV-K1L. If this were to be successful then the next step would be to clone an antigen gene(s) of interest into the *Sma* I site of pNCH-3 (Figure 4.15). Once the entire gene cassette is inserted into the PEPV genome, producing blue plaques in RK-13 cells, one could then passage the virus on chick CAMs in order to remove the K1L and GUS genes by means of spontaneous deletion of the sequence between the 2 direct repeats. The final PEPV recombinant would have only the gene of interest inserted into the TK locus. This desired recombinant could be identified by PCR of individual picked pocks.

Chapter 5: Rescue of PEPV Growth in RK-13 Cells and Attempts to Construct Recombinant PEPV

5.1 Introduction

The first VACV host range genes were discovered when researchers attempted to generate temperature sensitive VACV mutants. During the screening of mutagenized viral stock, VACV host range mutants were discovered. These mutants were found to exhibit altered plaque morphology on permissive cell-lines or showed restricted growth in previously permissive cell-lines (Drillien *et al*., 1981). K1L was the first host range gene to be discovered. K1L knockout VACV was shown to grow in human cells; however, it showed restricted growth in rabbit kidney (RK-13) cells (Perkus et al., 1990). Later, Sutter showed expression of VACV K1L in RK-13 cells complemented the host range defect of VACV mutants (Sutter et al., 1994). This again was shown when the growth of highly attenuated Modified Vaccinia Ankara, (derived from VACV that was passaged >500 times in primary chick embryo fibroblast (CEF) cells, and which had lost its ability to replicate in RK-13 cells), was rescued by VACV K1L expression (Wyatt *et al*., 1998).

It is not known whether the K1L gene product alone can rescue Avipoxvirus growth in RK-13 cells. It is an early gene product which is required for translation to take place following poxvirus infection of cells. VACV K1L has been shown to bind host PKR (see section 3.1.2), and so is expected to block phosphorylation of elF2 by PKR phosphorylation. In this study, the transfer vector, pNCH-3, was designed in such a way that the promoters expressing both the reporter gene (GUS) and the K1L gene were poxvirus promoters. Poxvirus promoters only function in the presence of Poxvirus transcription factors. Hence expression of the reporter gene would show that both infection and transfection have taken place. Replication and formation of plaques would then show that K1L has rescued late gene expression.

5.2 Materials and Methods

5.2.1 Infection and Transfection of RK-13 Cells

Infection and transfection was performed to determine whether PEPV would recognize the VACV promoters, and whether VACV K1L expression could rescue growth of PEPV in RK-13 cells.

RK-13 cells were prepared as described in section 3.2.1. Fresh RK-13 cells were seeded into sterile 6-well plates. Once the wells were 50% - 70% confluent, plates were infected with PEPV as described in section 3.2.2.1 and transfected with transfer vector pNCH-3.

Transfection was carried out as described in the manufacturer's instruction of a tranfection kit, Effectene (supplied by QIAGEN). This kit contained Effectene, a nonliposomal lipid reagent and an enhancer which condenses DNA. The condensed DNA mixed with Effectene forms small transfection complexes which enables the plasmid DNA to be transported into the cells by fusion through the cell membrane.

For transfection of cells in one well of a 6-well tissue culture plate; 0.4-1µg of plasmid DNA was diluted with buffer EC to a final volume of 100µl, followed by addition of 3.2µl Enhancer. Reagents were mixed thoroughly by vortexing prior to incubation at room temperature for 2-5 minutes. Ten microlitre of Effectene reagent was added after the incubation; the contents were mixed gently, but thoroughly by pipetting up and down gently. The transfection mix was then incubated at room temperature for 5-10 minutes prior to the addition of 600µl DMEM, with PS, fungin and 2%FCS. This mix

was added drop wise to the cell surface; this was followed by the addition of 1.6ml DMEM with PS, fungin and 2%FCS to each well. Plates were then incubated at 37^{o}C with 5% CO_2 for 2-5 day. PEPV was harvested from cells as described in section 3.2.2.2.

5.2.2 Detection of GUS Reporter Gene Expression using X-gluc as a Substrate

The reporter gene, β-glucuronidase (GUS), was included in the transfer vector, primarily to aid in the isolation of recombinant virus containing this gene. It also allows one to monitor the efficiency of transfection of infected cells. GUS enzyme is capable of digesting the substrate X-gluc and turning the RK-13 cells blue.

A stock of 20mg/ml X-gluc was prepared, wrapped in foil paper and stored at -20^{o}C. X-gluc was first diluted to 0.2mg/ml in DMEM with P/S, fungin and 2%FCS. All the medium in the wells was removed and the wells were rinsed with 1xPBS before adding 0.5ml of diluted X-gluc solution to each well.

For agarose overlays, melted agarose was added to the medium containing X-gluc to a final concentration of 1% agarose and 0.5ml of melted agarose overlay was added to each well swiftly after the 1xPBS wash. Working quickly was important to prevent agarose from polymerizing before adding it to the well. The plates were first kept at room temperature for the agarose to polymerize before incubating at 37^{o}C, 5% CO_2 for 1-3 days.

5.2.3 Preparation of Cell Extracts for PCR

For detection of recombinant virus, DNA was extracted from the infected and transfected cells and subjected to PCR analysis. Wells were gently washed with 1xPBS prior to cell lysis. First, 100µl of lysis buffer A (100mM KCl, 10mM Tris-HCl of pH 8.3 and 2.5mM $MgCl_2$) was added to each well. The plates were incubated at room temperature for 5 minutes. Secondly, 100µl of lysis buffer B (10mM Tris-HCl of pH 8.3, 2.5mM $MgCl_2$, 1% Tween 20, 1% NP40 and 120µg/ml Proteinase K) was added to each well. The contents of the well were pipetted into 1.5ml microfuge tubes and incubated at 60^{o}C for 1hr to allow Proteinase K to perform its function of protein degradation. Lastly, the tubes were incubated at 95^{o}C for 10 minutes to inactivate the Proteinase K. DNA was stored either at 4^{o}C or -20^{o}C in 1.5ml microfuge tubes and used as template in the subsequent PCR experiments.

5.2.4 PCR Amplification

PCR was performed as described in section 4.2.2 to determine whether recombination had taken place or not. The K1L reverse primer together with a primer which binds to PEPV DNA downstream of the TK gene (DTK right, 5' TGTATCTTACAAACTGAATTTCAG 3') would amplify a 2.5 kb fragment from the desired recombinant.

5.3 Results

5.3.1 Evidence to Show Growth of PEPV was Rescued by VACV K1L Gene Expression

RK-13 cells were initially infected with 3 known different virus concentrations and transfected with 600ng of pNCH-3, as described in sections 5.2.1. The experiment was performed in a 6-well sterile tissue culture plate as shown in Table 5.1 and Figure 5.1. Infected and/or transfected cells were stained with X-gluc (substrate for GUS reporter gene) 48 hours post infection as described in section 5.2.2. Photographs were taken using an inverted microscope 24 hours post staining.

All 3 negative controls showed no CPE characteristic of viral infection (Figure 5.1A-C) nor blue cells. This indicated that pNCH-3 vector alone could not produce GUS protein and PEPV alone could not produce infectious progeny in RK-13 cells. In all 3 experiments where both PEPV and VACV K1L were present noticeable CPE and/or plaques were visible (Figure 5.1D-F). Severe infection was seen at the highest PEPV concentration of an MOI of one infectious virus particle per cell (Figure 5.1 D).

This experiment, in particular the large blue plaque seen in Figure 5.1F suggests that K1L can rescue the growth of PEPV in RK-13 cells.

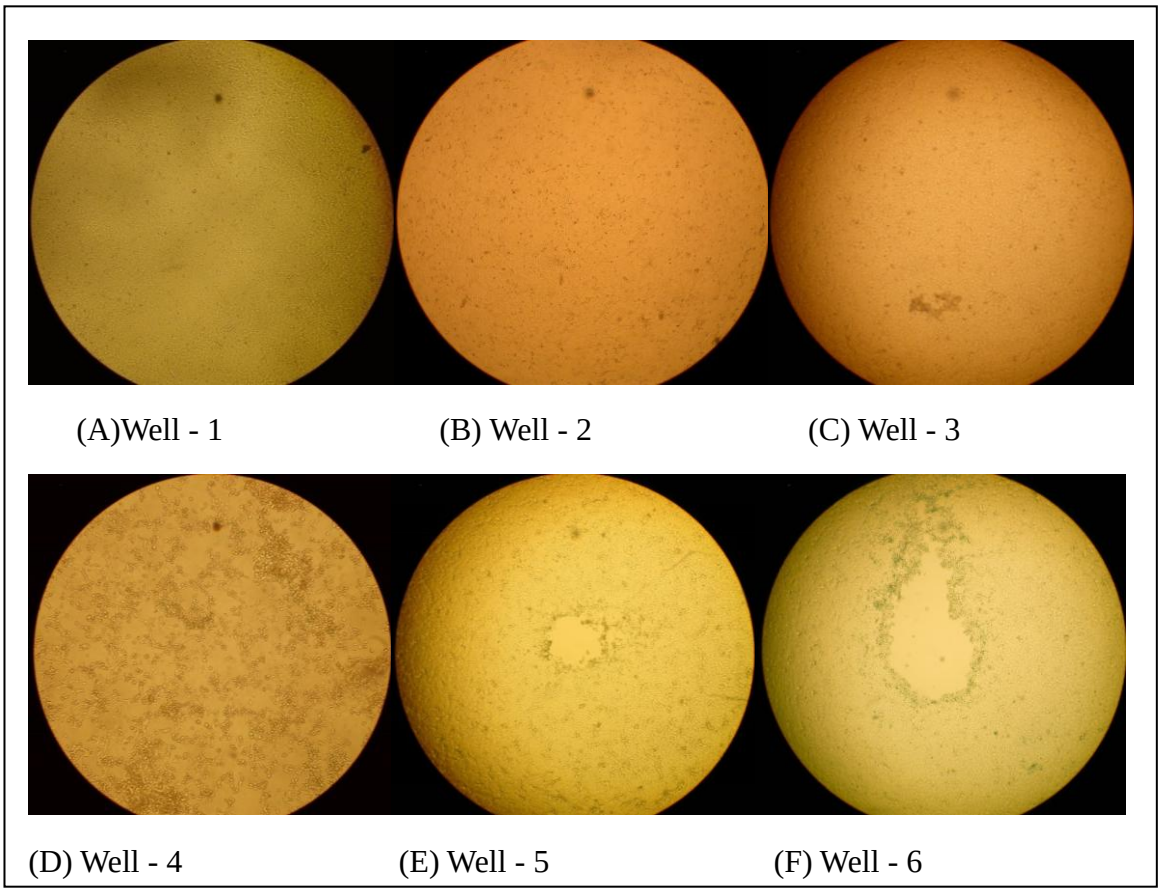
Table 5.1. Contents of individual wells used to examine whether transfection of pNCH-3 can rescue the growth of PEPV in RK-13 cells*

Well 1 - Cells Only (A) - Negative control - No infection - No transfection	Well 2 - pNCH-3 Only** (B) - Negative control - No infection - Transfection with 600ng pNCH-3	Well 3 - PEPV Only*** (C) - Negative control - Infect with MOI of 0.1 - No transfection
Well 4 - PEPV / pNCH-3 (D) - Experiment - Infect with MOI of 1 - Transfection with 600ng pNCH-3	Well 5 - PEPV / pNCH-3 (E) - Experiment - Infect with MOI of 0.1 - Transfection with 600ng pNCH-3	Well 6 - PEPV / pNCH-3 (F) - Experiment - Infect with MOI of 0.01 - Transfection with 600ng pNCH-3

*Images of individual wells are shown below in Figure 5.1.

**Examine if pNCH-3 alone can produce GUS protein.

*** Examine if infection with PEPV alone can produce viral plaques.



(A)Well - 1 (B) Well - 2 (C) Well - 3

(D) Well - 4 (E) Well - 5 (F) Well - 6

Figure 5.1. X-gluc stained RK-13 cells visualized using an inverted microscope at 40X magnification, 3 days post infection. A) Well 1 - Cells only; B) Well 2 - Transfection with 600ng pNCH-3 only; C) Well 3 - Infection with PEPV at an MOI of 0.1; D-F) Infection with PEPV at an MOI of 1, 0.1 and 0.01 respectively followed by transfection with 600ng of pNCH-3.

5.3.2 Attempts to Construct a Recombinant PEPV Which Expresses GUS

The purpose of the construction of the transfer vector pNCH-3 was to engineer a recombinant PEPV. Therefore the next step of this experiment was to search for a recombinant PEPV expressing K1L and GUS.

RK-13 cells were infected and transfected as described in section 5.2.1, harvested as described in section 3.2.2.2 and the cell lysate passaged in RK-13 cells. Plaques were seen after 3 days of the first passage, and used to infect RK-13 cells again. On the second passage, no blue plaues were seen, but CPE was seen after 5-7 days. It was unclear whether this was due to the presence of virus or simply cell death. When stained for GUS expression, no blue coloration was observed. PEPV is unlikely to adapt to growth in RK-13 cells, as shown in Chapter 3. Moreover it was not known whether the plaques seen after the first passage were due to the presence of recombinant virus which did not grow upon further passage; or whether they resulted from cells both infected with PEPV and transfected with plasmid pNCH-3. This was further investigated by PCR analysis of the cell lysates of infected and/or transfected RK-13 cells (see section 5.3.3).

5.3.3 PCR Amplification on the Cell Lysate of Infected RK-13 Cells

PCR was performed in order to determine whether any recombinant PEPV was generated after infection and transfection as described in section 5.2.1. A 6-well plate of RK-13 cells was infected and/or transfected as depicted in the grid below, the same procedure as that described in section 5.3.1 and used to search for a recombinant virus.

Table 5.2. Contents of individual wells used to examine for the presence of recombinant PEPV

Well 1 - Cells Only - Negative control - No infection - No transfection	Well 2 - pNCH-3 Only - Negative control - No infection - Transfection with 600ng pNCH-3	Well 3 - PEPV Only - Negative control - Infect with MOI of 0.1 - No transfection
Well 4 - PEPV / pNCH-3 - Experiment - Infect with MOI of 1 - Transfection with 600ng pNCH-3	Well 5 - PEPV / pNCH-3 - Experiment - Infect with MOI of 0.1 - Transfection with 600ng pNCH-3	Well 6 - PEPV / pNCH-3 - Experiment - Infect with MOI of 0.01 - Transfection with 600ng pNCH-3

After infection and transfection had taken place, the plate was incubated at 37^{o}C, 5% CO_2 for 2-5 days prior to cell lysis. Cells were lysed as described in section 5.2.3. Isolated DNA was subjected to PCR as described in sections 4.2.2 and 5.2.4, using selected primers shown in Table 5.3. Table 5.3 shows all possible outcomes of the PCR amplification of DNA from the infected cell lysates. PCR products were subjected to standard agarose gel electrophoresis, which is shown in Figure 5.2.

Table 5.3. Primer pairs used for PCR amplification of DNA from cell lysates depicted in Table 5.2 and expected sizes of DNA products

	Primer pair 1 (K1L forward + K1L reverse)	Primer pair 2 (UTK left* + DTK right**)	Primer pair 3 (DTK right** + K1L reverse)
Function	Identify K1L fragment (plasmid or recombinant PEPV)	Identify non-disrupted TK gene (wt PEPV)	Identify TK disrupted by K1L (recombinant PEPV)
Forward Primer	K1L forward	UTK left*	DTK right**
Reverse Primer	K1L reverse	DTK right**	K1L reverse
No DNA	No DNA fragment	No DNA fragment	No DNA fragment
Cells only (well1)	No DNA fragment	No DNA fragment	No DNA fragment
pNCH-3 only (well 2)	1 kb fragment	No DNA fragment	No DNA fragment
PEPV only (well 3)	No DNA fragment	1 kb fragment	No DNA fragment
Possible recombinant PEPV (wells 4~6)	1 kb fragment	5kb fragment (too large for amplification)	2.5kb fragment

*Sequence in PEPV that was situated upstream of TK gene
(5' GGAAATAGCTAGAGAAACGCTAA 3')
**Sequence in PEPV that was situated downstream of TK gene
(5' TGTATCTTACAAACTGAATTTCAG 3')

In Table 5.3, primer pair 1 was used to detect K1L (Figure 5.2, lanes 2, 5, 8, 11 and 14), primer pair 2 was used to detect wt PEPV TK (Figure 5.2, lanes 3, 6, 9, 12 and 15) and primer pair 3 was used to detect recombinant virus (Fiure 5.2, lanes 4, 7, 10, 13 and 16). Both the positive controls with appropriate sizes can be seen in Figure 5.2. Lane 8 shows the K1L fragment amplified from pNCH-3 and lane 12 shows the TK fragment amplified from the wtPEPV genome. However no DNA was amplified from primer pair 3, which was specific for recombinant PEPV (Figure 5.2, lane 16) . Lanes 14 and 15 show the presence of pNCH-3 and wtPEPV respectively in the infected and transfected cells.

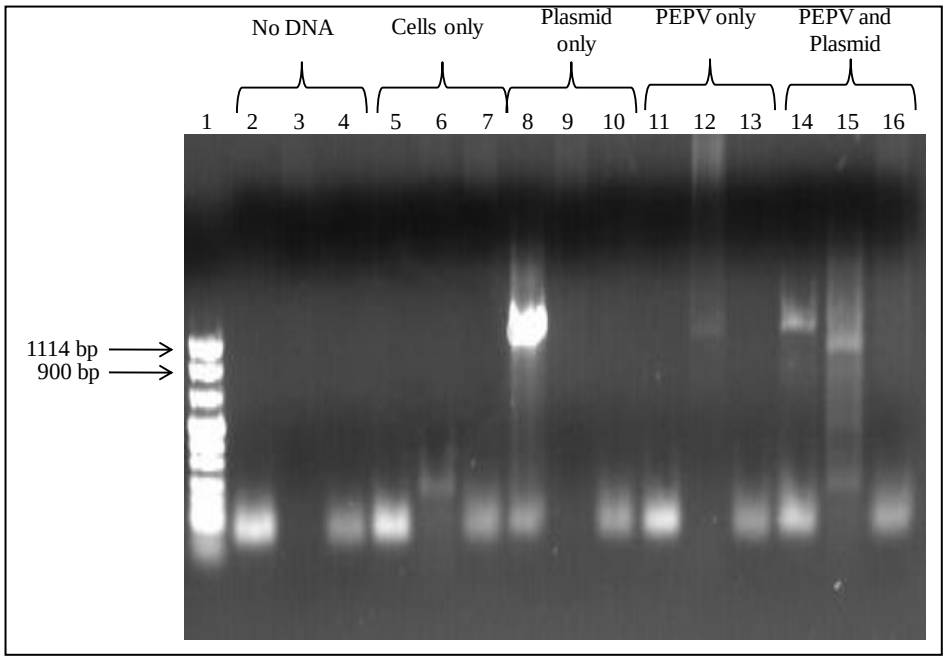


Figure 5.2. Standard agarose gel electrophoresis showing PCR amplified products from cell lysates depicted in Tables 5.2 and 5.3. Lane 1 - 1kb DNA molecular weight marker; Lanes 2-4 - negative control for PCR experiment, using H_2O in place of template DNA; Lanes 5-7- cells only; Lanes 8~10 – pNCH-3 transfected cells; Lanes 11-13 – PEPV infected cells; Lane 14~16 – PEPV-infected and pNCH-3 transfected cells. Primer pair 1 (K1L) was used in lanes 2, 5, 8, 11 and 14; Primer pair 2 (PEPV TK) was used in lanes 3, 6, 9, 12 and 15; Primer pair 3 (PEPV TK+K1L) was used in lanes 4, 7, 10, 13 and 16.

This result suggests that the CPE generated in the cells both infected with PEPV and transfected with pNCH-3 was attributed to transient expression of K1L in cells infected with PEPV and transfected with pNCH-3. However, it is possible that, in addition to transient expression, a small amount of recombinant virus was formed but not detected in the PCR assay due to the relatively large size of the fragment to be amplified (2.5kb) and the somewhat fuzzy bands detected from amplification of viral DNA (Figure 5.2, lanes 12 and 15). The extention time of 4 minutes should have been sufficient to amplify a fragment of 2.5kb. Primers were designed to amplify a fragment of 2.5kb, and not smaller, as it was important that the one primer bind outside of the TK flanking sequence present in the plasmid. The insensitivity of the assay may be due to the presence of PCR inhibitors in the lysates. Sensitivity assays were not performed to test the detection level. This could have been done by spiking the uninfected cell lysate with known amounts of PEPV DNA and determining the number of genomes required to produce a positive PCR result, using primer pair 2 (UTKleft + DTK right).

5.4 Discussion

The VACV K1L gene was used to rescue the growth of PEPV in RK-13 cells, in order to isolate recombinant PEPV in RK-13 cells. During the growth rescue experiment, RK-13 cells were infected with wtPEPV and transfected with the pNCH-3 plasmid. Plaques and CPE were observed in the infected and transfected cells, and after staining with X-Gluc, blue cells were seen surrounding the plaques as shown in section 5.3.1, Figure 5.1. This suggested that expression of K1L was able to rescue the growth of PEPV in RK-13 cells. However, when the “rescued” virus was passaged in fresh RK-13 cells, plaques and CPE were seen in the first passage but not thereafter. PCR analysis was carried out for detection of the recombinant virus and was unable to detect the presence of

recombinant virus (see section 5.3.3). It is possible that recombinant virus is generated at a level too low for detection by PCR following infection and transfection, but needs to be amplified by passage before being detectable.

To explain the inability of recombinant virus to grow upon passage, one has to examine at which stage the blockage in replication of PEPV in RK-13 cells occurs. In the previous work done by Stannard *et al* (1998), no sign of infection or CPE was identified in PEPV infected RK-13 cells. Poxvirus early proteins are responsible for the rounding of host cells, however severe CPE was not observed in the PEPV infected RK13 cells. This may suggest that, after viral entry, viral protein incorporated within the virus package, somehow only transcribes certain early genes, and there is probably no translation (with no K1L equivalent). Hence, severe CPE does not take place and virus alone was unable to progress to the intermediate phase of the replication cycle, thus replication was abolished. During the rescue experiment, CPE was seen after infection with PEPV and transfection with pNCH-3 plasmid. Thus it indicated that K1L was capable of producing the necessary viral early proteins for the virus to progress to a late stage of the replication cycle. The X-gluc staining experiment showed that GUS was expressed. The GUS gene was regulated by a Poxvirus late promoter, P11, indicating that replication of PEPV DNA must have take place, as well as translation of the intermediate proteins, which regulate late gene expression. However, it is not known why rescued PEPV was unable to be passaged.

Chapter 6: Conclusion

This thesis describes preliminary groundwork performed in the development of PEPV as a vaccine vector. With no cells known to be permissive for PEPV growth, the virus was grown and titrated on the CAMs of 10-12 day old chick embryos. It was confirmed by growth curves that PEPV cannot multiply in RK-13 cells and does not adapt to growth in these cells upon multiple passage.

In a series of cloning experiments, a transfer vector pNCH-3 was constructed which consists of the selective host range gene, K1L and marker gene, GUS, flanked by direct repeat sequences, outside of which is a unique *Sma* I restriction enzyme site for future cloning experiments, and all of these elements are flanked by PEPV TK sequences. This transfer vector, pNCH-3, containing VACV K1L, was shown to rescue PEPV growth in RK-13 cells. Although infected and transfected cells appeared blue and plaques surrounded by blue cells were visible, no recombinant virus could be recovered after passage of these cell lysate.

This is the first attempt to complement PEPV in a non-permissive cell line with a host range gene to restore virus growth. The results obtained are inconclusive as to whether K1L can be used as gene for selection of recombinant virus, but do provide a foundation for future work on the construction of recombinant PEPV. Even if the K1L host range gene is not employed in the future, the vector pUC21TKl+r containing the PEPV TK flanking sequences could be used to test other positive selection genes such as gpt, if a cell line permissive to PEPV growth is found.

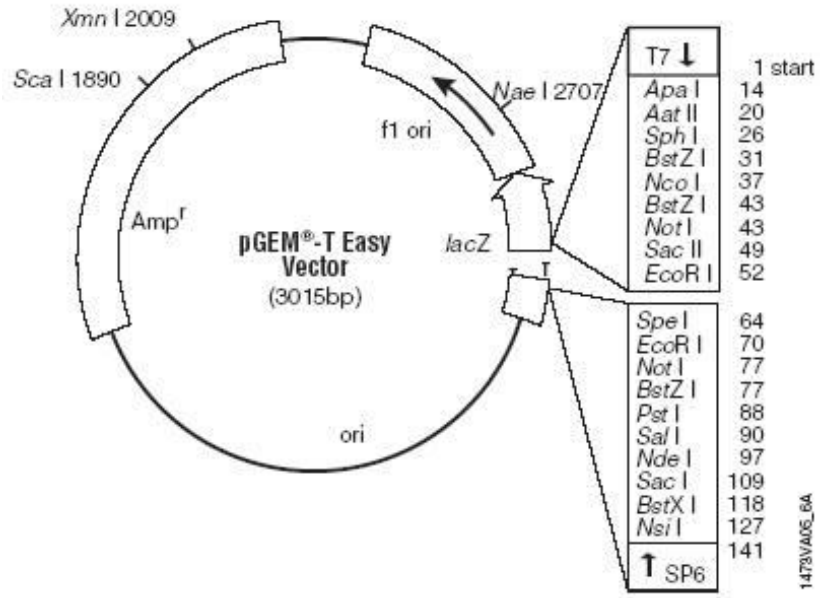
For future work, one could compare the sequence of PEPV with other poxviruses that

are known to grow in RK-13 cells, and search for the possible host range gene that will enable PEPV to complete its replication cycle in RK-13 cells. This study showed K1L was able to rescue early and late gene expression, thus to limit the area of investigation, one could search for late genes that are associated with host range. However, due to time constraints, further investigation into host restriction was not done.

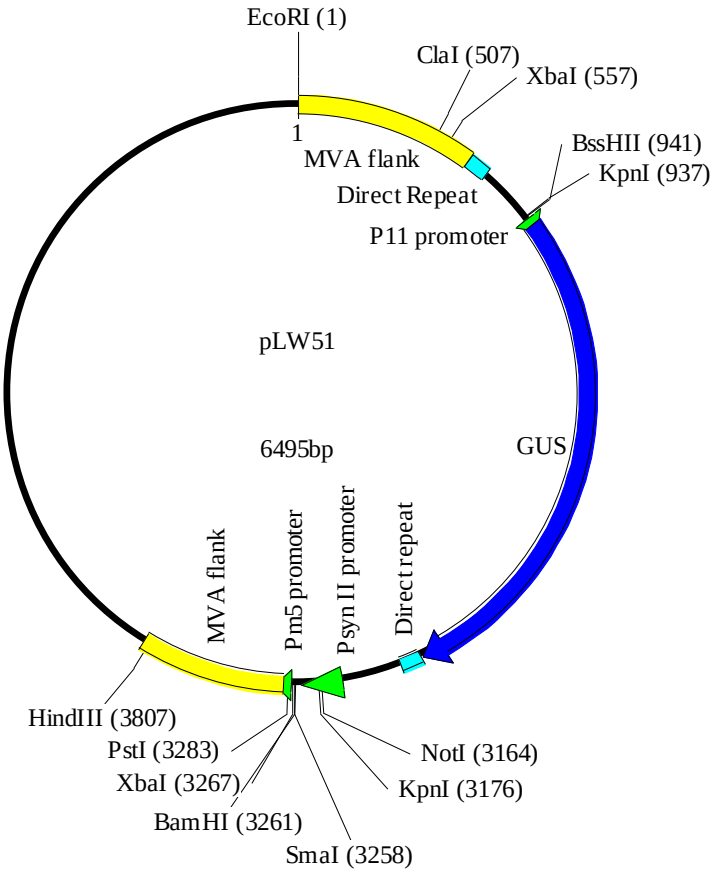
At present PEPV has been unable to complete its replication cycle in any of the tested cell-lines, including CEFs. This unusual nature (most *Avipoxviruses* grow in CEF cells) may indicate that PEPV may be a particularly safe vector to use for a vaccine as PEPV is more restrained compared to other known *Avipoxviruses*. However, to construct a recombinant virus, one is required to grow the PEPV in a permissive cell line; therefore further research still needs to be conducted in the search for a cell-line permissive to the growth of PEPV.

Appendix I: Vector Diagrams

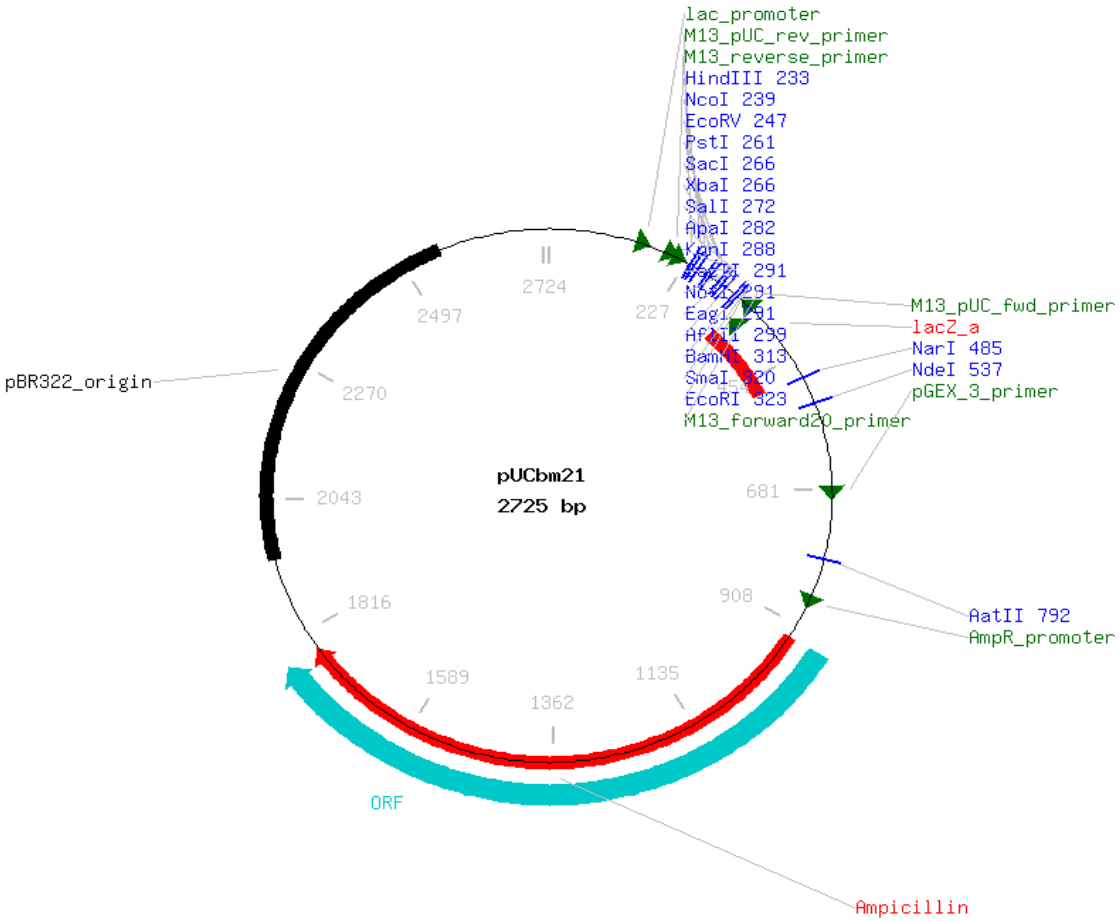
pGEM-T Easy Vector



pLW51 Vector

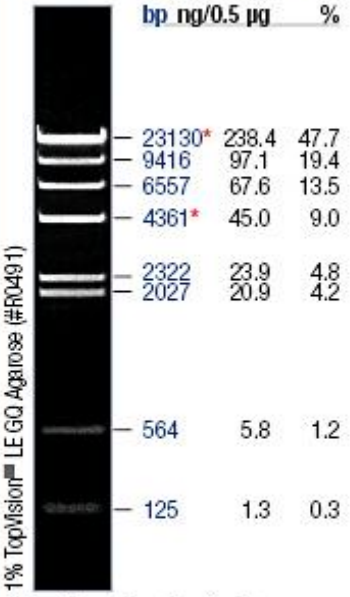


pUC21 Vector



Appendix II: DNA Molecular Weight Markers

Lambda DNA/HindIII Marker, 2



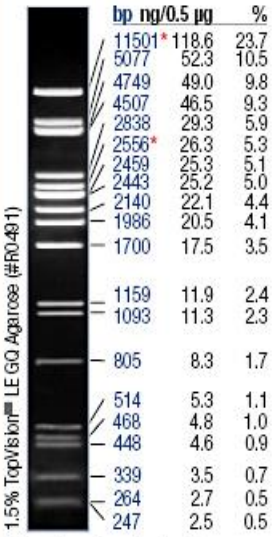
bp	ng/0.5 µg	%
23130*	238.4	47.7
9416	97.1	19.4
6557	67.6	13.5
4361*	45.0	9.0
2322	23.9	4.8
2027	20.9	4.2
564	5.8	1.2
125	1.3	0.3

0.5 µg/lane, 8 cm length gel,
1X TAE, 7 V/cm, 45 min

Range

8 fragments (in bp): 23130*, 9416, 6557, 4361*, 2322, 2027, 564, 125.

Lambda DNA/PstI Marker, 24

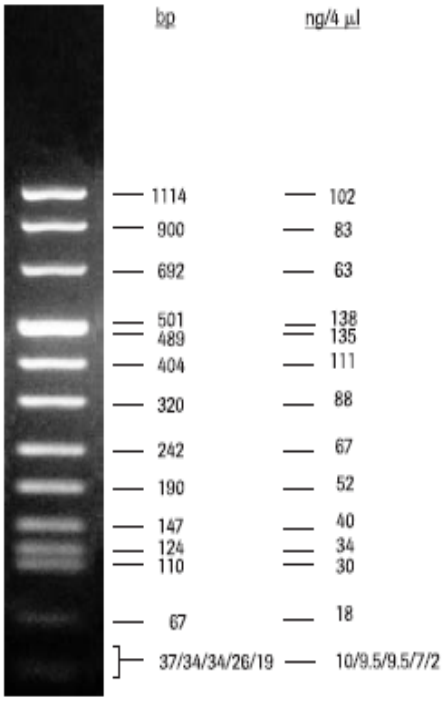


bp	ng/0.5 µg	%
11501*	118.6	23.7
5077	52.3	10.5
4749	49.0	9.8
4507	46.5	9.3
2838	29.3	5.9
2556*	26.3	5.3
2459	25.3	5.1
2443	25.2	5.0
2140	22.1	4.4
1986	20.5	4.1
1700	17.5	3.5
1159	11.9	2.4
1093	11.3	2.3
805	8.3	1.7
514	5.3	1.1
468	4.8	1.0
448	4.6	0.9
339	3.5	0.7
264	2.7	0.5
247	2.5	0.5

0.5 µg/lane, 8 cm length gel,
1X TAE, 7 V/cm, 1 h

Range

29 fragments (in bp): 11501*, 5077, 4749, 4507, 2838, 2556*, 2459, 2443, 2140, 1986, 1700, 1159, 1093, 805, 514, 468, 448, 339, 264, 247, *216, 211, 200, 164, 150, 94, 87, 72, 15 (oblique fragments comprise 2.3%).*

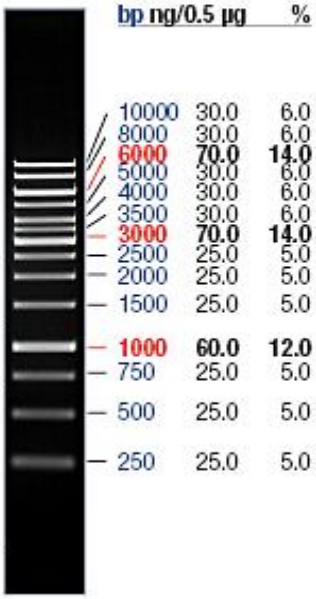


bp	ng/4 µl
1114	102
900	83
692	63
501	138
489	135
404	111
320	88
242	67
190	52
147	40
124	34
110	30
67	18
37/34/34/26/19	10/9.5/9.5/7/2

1.8 %
Agarose
MP Gel

GeneRuler™ 1 kb DNA Ladder

O'GeneRuler™ 1 kb DNA Ladder, ready-to-use



bp	ng/0.5 µg	%
10000	30.0	6.0
8000	30.0	6.0
6000	**70.0**	**14.0**
5000	30.0	6.0
4000	30.0	6.0
3500	30.0	6.0
3000	**70.0**	**14.0**
2500	25.0	5.0
2000	25.0	5.0
1500	25.0	5.0
1000	**60.0**	**12.0**
750	25.0	5.0
500	25.0	5.0
250	25.0	5.0

0.5 µg/lane, 8 cm length gel,
1X TAE, 7 V/cm, 45 min

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