An Investigation into the Specific Function of the Vaccinia Virus 13.8 kDa Protein Encoded by the N1L Gene

By

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SUBMITTED TO THE UNIVERSITY OF CAPE TOWN

In fulfilment of the requirements for the degree

MSc (Med)

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Acknowledgements

I would like to thank my supervisor, Professor Girish Kotwal, for his continued support, encouragement and optimism. Thank you to my fellow lab members of the Medical Virology Biotechnology Laboratory for their assistance, advice and moral support throughout my project.

I am enormously grateful to my family for their loving support and for encouraging me to pursue my studies.

I am grateful to the following people for their assistance during the course of my project: Mr. Rodney Lucas of the Biovac Institute for assistance with my animal work, Dr. Sharon Prince and Dr. Fadiel Essop for the use of their luminometers and Dr. Wolfie Katz of the Biovac Institute for providing the SA vaccine Lister vaccinia virus.

Thank you to the Poliomyelitis Research Foundation, Medical Research Council and University of Cape Town for funding over the course of my study.

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Abbreviations

A = adenine

ATPase = adenosine triphosphatase

C = cytosine

Ca = calcium

$CaCl_2$ = calcium chloride

Cl = chlorine

CO_2 = carbon dioxide

ddH_2O = double distilled water

DNA = deoxyribonucleic acid

dsDNA = double-stranded deoxyribonucleic acid

dNTP = deoxyribonucleoside triphosphate

EDTA = ethylenediaminetetracetate

EtOH = ethanol

G = guanine

g = gram(s)

h = hour(s)

HCl = hydrochloric acid

HIV = human irnmunodeficiency virus

kbp = kilo base pairs

kDa = kilodalton(s)

KOH = potassium hydroxide

M = molar

MDa = megadalton(s)

ml = millilitre(s)

Mg = magnesium

mg = milligram(s)

$MgCl_2$ = magnesium chloride

min = minute(s)

mm = millimetre(s)

mRNA = messenger ribonucleic acid

mU = milliunits

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MW = molecular weight

NaCl = sodium chloride

NaOH = sodium hydroxide

ng = nanogram(s)

pmole = picomole(s)

RNAse = ribonuclease

rpm = revolutions per minute

T = tyrosine

U = unit(s)

UV = ultraviolet

v/v = volume/volume

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Abstract

Vaccinia virus is the most extensively studied, prototype vertebrate poxvirus, which was used as a vaccine in the eradication of smallpox. The genome of this virus has characteristic variable termini encoding open reading frames that are not essential for virus replication in cell culture. One such open reading frame, N1L situated at the left terminal region of the neurovirulent Western Reserve (WR) vaccinia virus strain, encodes a protein 13.8 kDa in size. *In vivo* studies in mouse brains revealed that a recombinant virus, vGK5, lacking the expression of the 13.8 kDa protein was rendered replication deficient in the brain. An essential requirement of poxviruses for their replication is the energy molecule adenosine triphosphate (ATP). The supply of this molecule in the brain to support replication of a virus is limited due to the high-energy requirements and small energy reserves of this organ. The specific function of the vaccinia virus 13.8 kDa protein in relation to viral replication in the brain was investigated.

The South African (SA) Lister vaccinia virus strain was confirmed to encode an identical N1L gene to that of the WR vaccinia virus by amplification, cloning and sequencing of the Lister N1L open reading frame. The Lister vaccinia virus and a 13.8 kDa deletion strain (vGK5) were cultivated and used to intracranially infect mice. Using a luciferin/luciferase bioluminescence assay system the ATP levels in Lister and vGK5 vaccinia virus-infected mouse brains were measured and found to differ significantly after a 5-day infection period. The SA vaccine Lister vaccinia virus strain was found to be a slow growing virus in the brain. Subsequently, a possible role for the vaccinia virus 13.8 kDa protein in influencing ATP levels in the brain was postulated, yet a neurovirulent wild type strain is needed for further studies to consolidate this result. The 13.8 kDa protein was successfully expressed in the *P.pastoris* yeast expression system and positively identified by immunodetection studies.

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The Vaccinia Virus

Poxviruses are a diverse group of vertebrate and invertebrate pathogens belonging to the Family *Poxviridae* (Moss, 2001). The best known poxvirus is likely the human pathogen variola virus, the causative agent of smallpox, however the most extensively studied or prototype poxvirus is the smallpox vaccine, vaccinia virus (Fenger, 1984). Poxviruses which infect vertebrates make up the *Chordopoxvirinae* subfamily and include the genus orthopoxvirus to which the vaccinia virus belongs (Baxby, 1984).

Numerous strains of vaccinia virus, having various levels of safety and efficacy were used in the eradication of the smallpox virus (Henderson, 1988). This included the Lister strain of vaccinia virus developed in the United Kingdom (Fenner *et al*, 1988a). Adverse effects of vaccinations, which were observed particularly in immunocompromised individuals, included post-vaccinial encephalitis, vaccinia necrosum and eczema vaccinatum amongst others (Goldstein *et al*, 1975). Worldwide routine childhood vaccinations were stopped in 1982, following which concerns regarding a safer smallpox vaccine were related to the re-emergence of the virus through bioterrorism and have been exacerbated by the rapidly increasing population of HIV/AIDS and other immunocompromised individuals (Cassimatis et *al*, 2004).

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While its origin and natural host remain unknown, its characteristic size and morphology led to this virus becoming the first animal virus to be seen under a microscope, grown in tissue culture or physically purified (Moss, 2001). Vaccinia virus particles have a rounded rectangular or brick shaped morphology (Moss, 2001) and consist of a biconcave core containing a large DNA genome, flanked by two lateral bodies and surrounded by a viral membrane coated in surface tubules (Baxby, 1984) (see **Figure 1**).

Poxviruses are able to replicate exclusively in the host cell cytoplasm (Fenger, 1984) in cytoplasmic inclusion bodies (Baxby, 1984). Once mature,

virus particles exist as intracellular mature virions (IMVs) or extra cellular enveloped virions (EEVs) (see **Figure 1**) which are responsible for spread of the virus (Payne, 1980).

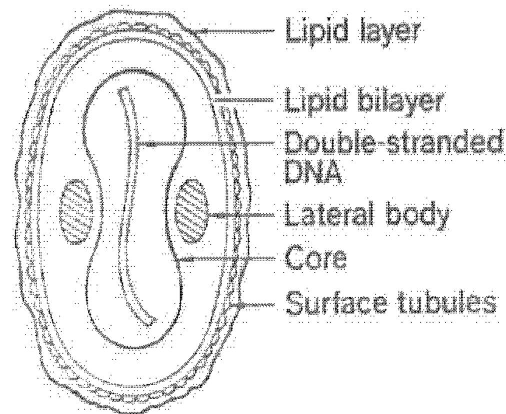

Figure 1 Model of an extra cellular vaccinia virus particle having an extra lipid layer acquired from the host cell membrane (Fenger, 1984).

The vaccinia virus double-stranded DNA genome (**Figure 2**) is 185 kbp in length and is covalently linked at both ends by an A-T rich incompletely base-paired hairpin loop (Baroudy *et al*, 1982; Geshelin *et al*, 1974). Upon denaturation, the genome forms a single-stranded circular structure (Geshelin *et al*, 1974). A 10 000 base pair inverted terminal repeat (Wittek *et al*, 1978; Garon *et al*, 1978) containing tandem repeats of various lengths (Baroudy & Moss, 1982; Wittek & Moss, 1980) is situated at both ends of the virus genome. According to a nomenclature system introduced by Defilippe (1982) each vaccinia virus open reading frame (ORF) is named according to the DNA fragment on which it lies following digestion of the genome with the *Hin* dIII restriction enzyme, which cuts the genome into 15 segments. The largest fragment is assigned the letter A and the smallest the letter O (Defilippe, 1982). Each gene is also assigned a number according to its ORF within the *Hin* dIII fragment and the letter L (left) or R (right) according to the orientation of the ORF on the genome (Moss, 2001).

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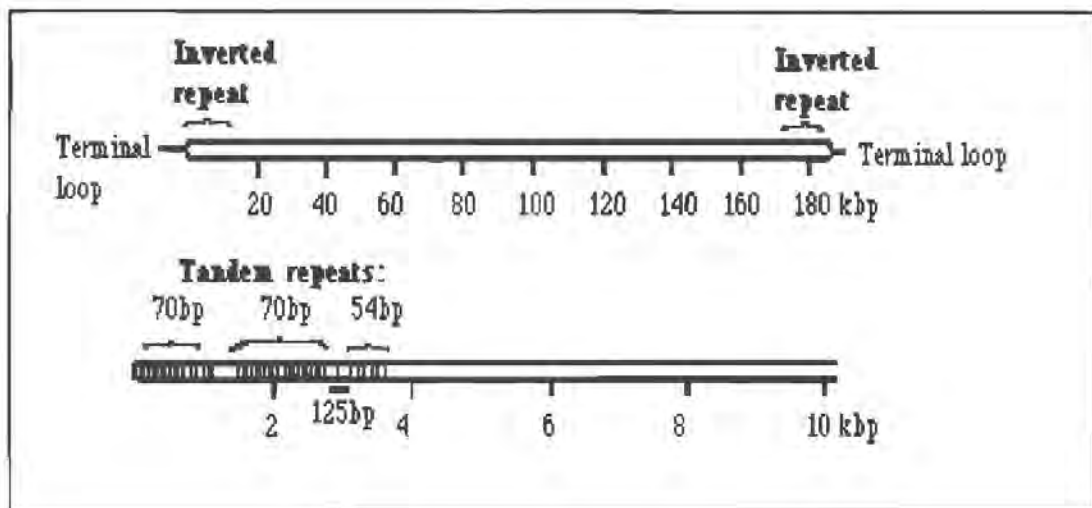

Figure 2 The vaccinia virus genome (www.micro.msb.le.ac.uk).

Variability in poxvirus genomes is generally seen in the genome termini which encode non-essential genes that are not required for growth in tissue culture (Perkus *et al*, 1991), whereas conserved genes that are essential for replication and other functions common to orthopoxviruses are centrally situated (Mackett & Archard, 1979). Non-essential genes also include those which encode proteins known as virokines (Kotwal & Moss, 1988a). Virokines, the first of which was discovered during the mapping of the vaccinia virus termini (Kotwal & Moss, 1988a) enhance viral replication and virulence by facilitating evasion of the host immune system (Smith & Kotwal, 2002).

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Virokines

These viral proteins bear structural as well as functional similarities to host proteins e.g. cytokines and cytokine receptors, chemokines and chemokine inhibitors and complement regulatory proteins (Smith & Kotwal, 2002). They are speculated to have been host-derived and structurally modified to be accommodated by the virus genome and to display increased potency (Smith & Kotwal, 2001).

Cytokine and cytokine receptor homologs produced by the virus disrupt cytokine signalling and keep the infection 'hidden' from the host's immune

system (Smith & Kotwal, 2001). The virally encoded chemokine homologs and inhibitors facilitate viral evasion and replication by attracting susceptible cells to the site of infection (Smith & Kotwal, 2001). Virokines involved in interruption of the complement system include the extensively studied vaccinia virus complement control protein (VCP), a major secretary protein able to block both the classic and alternative complement activation pathways by binding to complement proteins and subsequently reducing the host's inflammatory response (McKenzie *et al*, 1992).

Vaccinia virus strains that have been mutated by disruption or deletion of certain non-essential genes have been reported to display reduced virulence (Dallo & Esteban, 1987). One such vaccinia virus spontaneous mutant, strain 6/2, was found to be lacking a large portion (17 open reading frames) of the left end of the virus genome due to a transposition event (Kotwal & Moss, 1988b). This resulted in considerable attenuation (Buller *et al*, 1985). One of the missing ORF's, the N1L gene, encodes a protein of 13.8 kDa. This protein was later identified as the most potent virulence factor in the WR strain of vaccinia virus (Kotwal *et al*, 1989), a neurotropic laboratory strain produced by intracerebral passaging in mice (Lee *et al*, 1992).

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The 13.8 kDa Protein

Kotwal and Moss (1988a) observed two secreted proteins in the media of vaccinia virus infected cells, one of which was 35 kDa in size and was identified as the complement regulatory protein VCP, and the other a 12 kDa protein. This unidentified protein was absent from the media of cells infected with the vaccinia virus mutant strain 6/2 and could thus be concluded to be encoded by a gene situated at the left end of the vaccinia virus genome (Kotwal & Moss, 1988b). The exact location of the gene encoding this protein was mapped to the N1L ORF of the WR strain of vaccinia virus (Kotwal *et al*, 1989).

The role of this protein was determined by insertional inactivation of the N1L ORF with a *lacZ* expression cassette to produce a 13.8 kDa knockout strain of vaccinia virus called vGK5 (Kotwal *et al*, 1989). Intracranial inoculation experiments in mice revealed that the recombinant vGK5 strain was less virulent than the wild type WR strain by a factor of 1.6 x 10^4 (Kotwal *et al*, 1989). Later studies making use of intranasal and intradermal infection models in mice illustrated the significant enhancement of vaccinia virus virulence in the presence of the N1L gene product when comparing wild type, knockout and revertant mutant strains (Bartlett *et al*, 2002).

Billings *et al* (2004) consolidated the finding of a marked reduction in neurovirulence demonstrated by the vGK5 strain. These authors observed that, 96 hours post-infection, mice infected with the WR strain of vaccinia virus displayed viral titres 10^4 plaque forming units (PFU) higher than that of vGK5 strain infected mice. With this virus, the infection also remained largely localised to the site of infection. In this way, they were able to conclude that the reduced neurovirulence of the vGK5 strain was directly associated with a reduced ability of the virus to replicate (Billings *et al*, 2004).

Despite this extensive attenuation, the virus' immunogenicity was largely maintained with respect to specific antibody response to infection, whilst eliciting a reduced cell mediated or cytotoxic response (Kotwal *et al*, 1989). In addition, the vGK5 strain was able to stimulate an adequate immune response in mice to protect against challenge with a lethal dose of the wild type WR vaccinia virus strain (Billings *et al*, 2004). Similar observations were made with attenuated vaccinia virus mutants produced by Dallo and Esteban (1987). The investigation of one of these mutants, which had an 8-Mda deletion within the left termini of the virus genome, allowed these authors to relate left-end deletions in the vaccinia virus genome to reduced virulence. (Dallo & Esteban, 1987). This mutant virus was, however, still able to carry out normal viral DNA replication and protein production, stimulate a humoral and cell-mediated immune response and protect against challenge within a host organism despite reduced virulence. Possibly, the immature virus

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progeny produced by such deletion mutants after initial infection were unable to infect other cells resulting in loss of virulence (Dallo & Esteban, 1987).

Further studies aimed at characterising the 13.8 kDa protein conducted by Bartlett *et al* (2002) expanded on the structure of the protein. The N1L gene product was described as a non-glycosylated, largely alpha helical, non-covalent homodimer that was predominantly present within infected cells and was completely dispensable for growth in cell culture (Bartlett *et al*, 2002). Due to the lack of a conventional N-terminal signal peptide, the protein was postulated as being released only at a late stage following infection as a result of infected cell leakage or via an unknown pathway and was not likely to be a true secreted protein as previously described (Bartlett *et al*, 2002).

Kotwal *et al* (1989) found a limited degree of structural similarity between the 13.8 kDa protein and a tumour necrosis factor and adenovirus protein. Later studies making use of computer software and protein database analyses found structural similarities of the N1L gene product to a range of different proteins leading to the development of various theories as to the specific function of this protein and how it enhances virus replication.

While focussing on the 13.8 kDa protein intracellular properties which they discovered, Bartlett *et al* (2002) proposed that this virokine is likely to be involved in cell signalling pathways or programmed cell death as opposed to mediation of cytokines, chemokines or complement activity. This group found structural similarity between the 13.8 kDa protein and numerous intracellular alpha-helical proteins (Bartlett et al, 2002).

In agreement with this, Billings *et al* (2004) postulated that on the basis of the similarity in immunogenicities of the vGK5 and WR vaccinia virus strains (Kotwal *et al*, 1989), the 13.8 kDa protein is not an immunomodulatory virokine. Their protein database search revealed 3-dimensional structural similarities of the N1L gene product to the ααα and αβα protein families which include the adenylate kinase enzyme (Billings *et al*, 2004). Adenylate kinase catalyses the interconversion of the energy molecule adenosine triphosphate

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(ATP) and its hydrolysed forms adenosine diphosphate (ADP) and adenosine monophosphate (AMP) (Stryer, 1995).

Diperna *et al* (2004) conducted a database search revealing a significant similarity between the 13.8 kDa protein and a vaccinia virus A52R protein. This protein functions in the inhibition of intracellular Toll/Interleukin-1 receptor (TIR) domain activity responsible for signal transmission from Toll-like receptors which recognise pathogen and induction of pro-inflammatory cytokine production via activation of the NF-κB and interferon regulatory factor 3 (IRF3) during the innate immune response (Diperna *et al*, 2004). Based on their findings that the N1L gene product inhibits NF-κB activation and IRF3 signalling, a contrasting theory relating to the role of the 13.8 kDa protein was presented in which it was stipulated to have an immunomodulatory function, specifically related to the innate immune response (Diperna *et al*, 2004).

The function of the 13.8 kDa protein in relation to enhancement of viral replication in the brain specifically has however not been addressed. To do so the life cycle of the vaccinia virus within a host cell in general and the relationship between vaccinia virus and the brain in particular during active viral infection need to be taken into account.

The Life Cycle of the Vaccinia Virus

In order to accomplish transcription, translation and replication of their genomes, animal viruses make use of host cell machinery and enzymes, yet the extent to which they are dependant on their host cells varies according to the capacity of their genomes (Fenger, 1984). With the largest genomes of all known viruses, poxviruses are able to replicate largely independently of their host (Fenger, 1984; Broyles, 2003), having sufficient viral DNA to encode the enzymes required for the various stages of their replication (Baxby, 1984). This can be seen by a brief description of the vaccinia virus life cycle.

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Due to the different forms of infectious virus particles, the entry of poxviruses into cells is a process which remains to be completely characterised (Moss, 2001). The vaccinia virus appears to infect a broad range of cells yet a specific receptor to which it binds as well as the specific means of cell entry are still to be identified (Moss, 2001).

Upon entering the host cell, the virus particle undergoes an initial uncoating stage releasing the virion core, which contains the genetic material as well as enzymes functional in gene transcription, into the cell cytoplasm (Baxby, 1984). Enzymes present in the core include a DNA-dependant RNA polymerase (Munyon *et al*, 1967), a vaccinia virus early transcription factor (VETF) and all other enzymes functional in mRNA synthesis and modification of products such as an mRNA capping enzyme, poly(A)polymerase and protein kinase (Broyles, 2003; Moss, 2001; Paoletti & Moss, 1972).

Vaccinia virus gene transcription is a temporally regulated process divided into three stages: early, intermediate and late gene transcription (Broyles, 2003). The products of each stage are essential for the transcription of genes in the stage to follow (Broyles, 2003). The virus-encoded RNA polymerase is responsible for transcription of DNA in all three afore-mentioned stages (Broyles, 2003). Transcription of early genes occurs within the virion core and produces proteins which function in synthesis of DNA, expression of intermediate genes (Moss, 2001) and immune system evasion (Kotwal & Moss, 1989). Transcriptional and translational activities of the host cell are inhibited following initiation of viral gene transcription (Fenger, 1984).

A second uncoating phase in which the early gene mRNA products and genomic DNA within the virion core are released into the cell cytoplasm follows early gene transcription (Fenger, 1984). The transcription of intermediate genes which mediate late gene transcription, and late genes, whose products are essentially structural but also include enzymes to be packaged in the virion core, is preceded by replication of the poxvirus genome (Moss, 2001). Numerous proteins encoded by the virus are involved in DNA replication. These include DNA polymerases, DNA ligases and

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glycosylases; however the mode of replication of the poxvirus genome is not completely understood and various replication models have been proposed (Fenger, 1984; Moss, 2001).

In the final stages of the vaccinia virus life cycle, replicated viral DNA and viral proteins combine in immature particles which are then each surrounded by an envelope made up of lipids and viral proteins (Fenger, 1984). The proteins and DNA within the particles rearrange to form lateral bodies and a core structure, and surface tubules are formed on the outer surface of the envelope resulting in the production of intracellular mature virions (Fenger, 1984).

The majority of proteins necessary for expression of vaccinia virus genes are encoded by the virus itself, yet of the three stages of vaccinia virus transcription, early gene transcription is the only stage to occur completely independently of host cell machinery (Broyles, 2003). This is probably because this process is restricted to the interior of the virion core (Broyles, 2003). Interestingly, however, approximately half of the vaccinia virus genome is transcribed prior to viral DNA replication indicating that an extensive portion of the genome is made up of early transcribed genes (Paoletti & Grady, 1977).

Despite the independent nature of poxvirus replication, viruses as a whole are entirely reliant on host cells for energy (Joklik, 1985). With this in mind, the provision of energy for virus activities is likely a primary role of the host cell in vaccinia virus replication.

Adenosine Triphosphate (ATP)

ATP is a nucleic acid molecule which functions as the principal energy supply of all cells, powering cellular activities ranging from chemical reactions to movement and transportation of molecules within cells and in their

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surrounding environment (Raven & Johnson, 1996). The energy status of tissues can be measured by their ATP content (Khan, 2003).

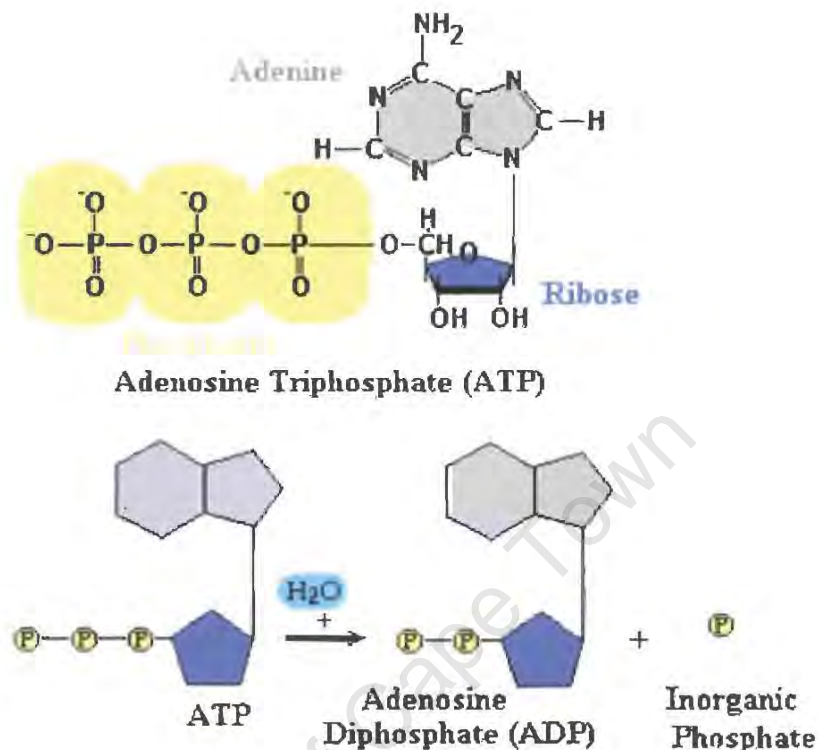

Figure 3 The structure of the energy molecule adenosine triphosphate (ATP) and its hydrolysis (www.people.virginia.edu/~rjh9u/atpstruc.html)

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Energy is provided by the hydrolytic cleavage of the outermost phosphate group of an ATP molecule (see **Figure 3**) and the subsequent conversion of ATP to adenosine diphosphate (ADP) and an inorganic phosphate (Pi) (Raven & Johnson, 1996).The turnover of ATP within cells is very high as ATP is not a long-term storage form of energy, but is rapidly utilised and regenerated from ADP and Pi (Stryer, 1995).

The Vaccinia Virus and ATP

ATP is an essential component in vaccinia virus early gene transcription (Broyles & Moss, 1988; Gershowitz *et al*, 1978). The high ATP level

requirements of the *in vitro* synthesis of RNA have been described (Kates *et al*, 1967; Munyon *et al*, 1967).

Gershowitz *et al* (1978) demonstrated the multiple roles of ATP in the virion core associated synthesis and processing of vaccinia virus mRNA. These roles were summarised as the requirement for ATP in the initiation of RNA synthesis, the termination of RNA chain elongation, the polyadenylylation of synthesised RNA and the extrusion of this RNA from the virion core into the cell cytoplasm, all of which were obstructed by the substitution of ATP with an ATP derivative having a non-hydrolysable γ-phosphate (Gershowitz *et al*, 1978).

The need for phosphorylation of vaccinia virion core proteins prior to the initiation of early gene RNA synthesis in the presence of ATP as a nucleoside triphosphate source has also been described (Paoletti & Moss, 1972). Among the ATP requiring virion core enzymes is the DNA-dependant ATPase nucleoside triphosphate phosphohydrolase I (NPHI) which requires ATP for its role in the elongation of mRNA transcripts and the termination of gene transcription (Deng & Shuman, 1998). A core-associated protein kinase uses ATP as a phosphate source for the phosphorylation of other viral proteins (Paoletti & Moss, 1972) and the vaccinia virus termination factor (VTF) enzyme requires this nucleoside triphosphate for transcriptional termination (Hagler *et al*, 1994).

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Broyles *et al* (1991) illustrated the requirement for ATP in viral early gene transcription specifically related to the vaccinia virus early transcription factor (VETF), which has a DNA-dependant ATPase activity (Broyles & Moss, 1988). This VETF activity was speculated to be the reason for the high-energy demands of early gene transcription (Broyles & Moss, 1988; Broyles, 1991).

Vaccinia virus and the Brain

Post-vaccinial encephalitis is a complication of smallpox vaccination with the vaccinia virus and observed in seemingly normal individuals with no history of predisposing disorders (Goldstein *et al*, 1975). Encephalitis is characterised by inflammation of the brain and is usually related to a viral infection (Geddes & Grosset, 2000).

The intracranial (LD50) injection model, a model used to determine the lethal virus dose for 50% of the infected population, was previously used to evaluate the virulence of poxvirus strains (Smith & Kotwal, 2002). Increased neurovirulence was found to correlate with increased replicative ability (Soekawa *et al*, 1974; Lee *et al*, 1992).

Not all strains of vaccinia virus are able to proliferate to the same extent in the brain as demonstrated by Soekawa *et al* (1974) who discovered the occurrence of strains which were able to replicate extensively and demonstrated greater lethality versus strains which induced similar histological changes in brain cells yet could be cleared in a matter of days.

The cell tropism of different strains of vaccinia virus during the initial stages of infection in brain tissue has also been investigated (Beranek *et al*, 1982). The neurovirulent strain WR was found to predominantly infect oligodendrocytes, whereas the dermotropic Elstree strain typically infected epithelial cells or fibroblasts (Beranek *et al*, 1982).

The pathogenesis of a neurovirulent viral strain can be determined by a single protein as illustrated by Phillips *et al* (1999). The spike protein of a highly neurovirulent strain of the mouse hepatitis virus (MSV), a coronavirus, when combined with a mildly neurovirulent strain was shown to result in a marked increase in neurovirulence causing increased inflammation in the brain (Phillips *et al*, 1999).

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ATP and the Brain

The brain has the highest energy demand of all the organs in the body, requiring 25% of the total body glucose utilisation (Magistretti *et al*, 2000). Energy is required for activities which include vegetative metabolism, sodium and calcium influxing, processing of neurotransmitters, intracellular signalling and axonal and dendritic transport among others (Ames III, 2000).

The brain may experience temporary energy imbalances during which the requirement for energy outweighs the availability thereof due to the generally small energy reserves of this organ (Ames III, 2000). With the limited amount of energy available for normal physiological activities of the brain, it is unlikely that this environment could support the replication of viruses such as the vaccinia virus. This may imply that viruses which are able to propagate in the brain have developed means of sequestering or producing energy to support their replication.

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Hypothesis

The vaccinia virus 13.8 kDa protein enhances viral replication in the brain by altering ATP utilisation to support the high energy requirements of viral propagation in an environment where the energy resources are limited.

Future Potential

A protein, which is able to enhance the production or utilisation of energy in the brain, may have application in treatment of brain injuries or traumas as well as optimising the use of ATP in any energy requiring reaction or process.

The vGK5 strain of vaccinia virus, which lacks the 13.8 kDa protein, is a safer smallpox vaccine candidate due to its impaired replication in the brain and subsequent greatly reduced potential for inducing post-vaccination encephalitic disease.

Aim

To determine the influence of the vaccinia virus 13.8 kDa protein on ATP levels and utilisation in the brain tissue of infected mice.

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Introduction

1. The production of pure poxvirus stocks

In 1962, W.K.Joklik described a 'rapid and widely applicable' method for growing up poxviruses, which was found to be very efficient in producing pure poxvirus preparations (Joklik, 1962). The virus is grown up on a membrane known as the chorio-allantoic membrane or CAM. It is situated below the shell of a fertilised hen's egg and is rich in blood vessels (Kotwal & Abrahams, 2004). This membrane is able to support the replication of almost all poxviruses (Joklik, 1962), without having to be adapted by passage (Fenner *et al*, 1988b). As the virus grows and spreads, virus particles present as pocks, which are greyish-white foci of diameter ranging from 0.4 mm to 4 mm depending on the poxvirus strain, that are produced by a combination of membrane hyperplasia and cell infiltration (Fenner *et al*, 1988b).

Due to one of the contra-indications for immunisation with vaccinia virus, MRA was not vaccinated. Thus, the SA vaccine Lister vaccinia virus was used because it is less virulent and safer than the WR strain used in previous studies relating to the N1L ORF.

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2. The vaccinia virus N1L gene

The amino acid sequence encoded by the N1L gene is highly conserved, not only among different strains of vaccinia virus, but also among the major orthopoxviruses (**Figure 4**) (Bartlett, 2002; Billings *et al*, 2004).

Preliminary work upon which this study is based made use of the Western Reserve strain of vaccinia virus as a wild type strain (Billings, 2001). In order to consolidate or extend this work, it was necessary to ascertain whether the SA vaccine Lister strain of vaccinia virus encoded an identical N1L gene. This could then be assumed to be expressed as an identical protein and to function in an identical manner.

Multiple sequence alignment

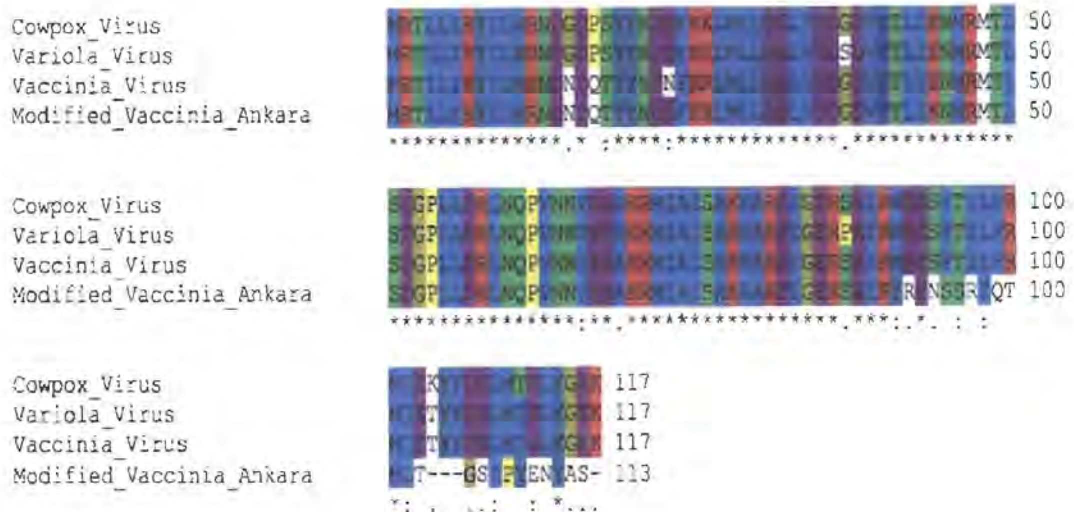

Figure 4 An amino acid multiple sequence alignment of the N1L gene product in 4 different orthopoxviruses. Residues identical in all sequences are indicated by asterisks and conserved and semi-conserved substitutions are indicated by colons and dots respectively. Due to its extensive attenuation, the Modified Vaccinia Ankara strain of vaccinia virus displays lesser amino acid sequence homology in the N1L gene (Billings *et al*, 2004).

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3. Isolation and identification of genes from genomic DNA

In order to characterise a single gene from a virus genome, it is necessary to amplify and isolate that gene and determine its nucleotide base sequence, which can then be compared to known sequences stored in a gene database. The techniques utilised to accomplish these are outlined in the sections to follow.

3.1 The Polymerase Chain Reaction (PCR)

Introduced in 1983 by Kary Mullis, this technique involves the use of two oligonucleotides known as primers, which are complementary to opposite strands of a selected stretch of DNA template, for the amplification of that selected region, wherein the product of one round of amplification becomes

the template for the next round thus creating a chain reaction (Bartlett & Stirling, 2003).

PCR is usually performed as a 3 step process (Hyndman & Mitsuhashi, 2003) in which the template DNA to be amplified is denatured at high temperatures, primers are annealed to their complementary sequences along the template at a reduced temperature and lastly the primers are extended by the addition of nucleotide bases at an elevated temperature (Erlich, 1989). Primer extension at high temperatures was made possible by the discovery of a highly thermostable DNA polymerase, *Taq* polymerase, isolated from the *Thermophilus aquaticus* bacterium (Bartlett & Stirling, 2003).

Specific criteria have to be met in the design of PCR primers, including primer length, specificity (Hyndman and Mitsuhashi, 2003) and nucleotide base composition (Sambrook & Russell, 2001). Good primers are typically 18-28 bases in length, their GC base content ranges between 40 and 60% to increase specificity of binding to the target DNA segment, and their nucleotide base sequence does not contain complementary regions within a single primer or between two primers in a primer set (Grunenwald, 2003). The formation of secondary structures such as hairpin loops (Hyndman & Mitsuhashi, 2003) or primer dimers are in this way avoided (Grunenwald, 2003).

To determine whether a PCR is successful, one of the most extensively used methods for nucleic acid analysis is employed, gel electrophoresis (Grunenwald, 2003). This technique involves the separation of DNA fragments according to size through a gel matrix usually made up of agarose, a highly purified polysaccharide derived from agar (Perbal, 1988).

DNA fragments, which are negatively charged, migrate through a gel matrix from a negatively charged electrode (cathode) toward a positively charged electrode (anode) (Sealey & Southern, 1990) when a current is applied across the gel in the presence of an electrophoresis buffer (Grierson, 1990). Smaller DNA fragments migrate freely through gel pores whilst the

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movement of larger DNA fragments is restricted (Perbal, 1988). Fragment size can be determined by comparison to a range of DNA bands of predetermined size known as a molecular weight marker (Sealey & Southern, 1990).

A fluorescent dye known as Ethidium Bromide (EtBr) is commonly used to stain agarose gels (Perbal, 1988). It intercalates between DNA bases (Sambrook & Russell, 2001) and glows orange when exposed to UV light (302nm) enabling the visualisation of DNA fragments as bands which can then be photographed and recorded (Perbal, 1988).

For a successful PCR a clear, single band of size corresponding to that of the target DNA should be seen (Grunenwald, 2003). Establishing that the amplified DNA is of the correct size may however not be sufficient for its positive identification. To achieve this, DNA sequencing is required.

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3.2 Cloning of PCR amplified DNA into plasmid vectors

One of the general functions of cloning a fragment of DNA is the sequencing of important regions of various genomes, for which several prokaryotic DNA vectors e.g. plasmids have been developed (Perbal, 1988). A plasmid is an autonomously replicating, circular, extra chromosomal double-stranded DNA molecule derived from a bacterium, which usually codes for an enzyme that confers antibiotic resistance to its bacterial host (Sambrook & Russell, 2001). A DNA ligase enzyme is used to covalently join a DNA plasmid vector and a DNA fragment in a process known as ligation (Perbal, 1988).

Initially, the insertion of PCR products into plasmid vectors was not easily accomplished (Marchuk, 1990). The discovery of an additional activity of *Taq* polymerase, the addition of adenine overhangs to the 3' ends of amplified DNA fragments (Clark, 1988; Mole, 1989), led to the development of a highly efficient method of cloning PCR products (Marchuk, 1990). Restriction enzyme digested plasmid vectors with thymidine overhangs at their exposed 3' ends that could conveniently ligate with the complementary ends of PCR products were created and termed T-vectors (Marchuk, 1990).

Since plasmid vectors are derived from naturally occurring plasmids that are able to replicate to multiple copies within a cell (Perbal, 1988), a recombinant plasmid vector can be inserted into a compatible bacterial cell line to produce high copy numbers by a process known as transformation (Sambrook & Russell, 2001). Transformed DNA can then be extracted and positive clones identified by agarose gel electrophoresis and DNA sequencing.

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Methods and materials

1. Growth of vGK5 and Lister in Embryonated Hen's Eggs

The vGK5 strain of vaccinia virus was obtained from Professor GJ Kotwal (project supervisor). The Lister SA vaccine vaccinia virus strain was obtained from Dr. Wolfie Katz. This smallpox vaccine is a freeze-dried preparation partially purified from sheep skin (Biovac Institute Pinelands, Cape Town) and was supplied at a concentration of 10^6 PFU/ml. Embryonated hens' eggs were obtained from the UCT Animal Unit (Mr. Noel Markgraaff).

1.1 Viral inoculation

Each egg was cleaned with 70% ethanol to avoid bacterial contamination. While examining each with an egg-candler, a pencil marking was made at the air sac and dorsal regions of each egg as illustrated in **Figure 5.**

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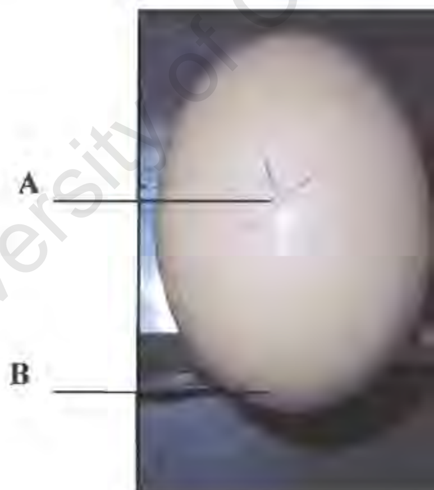

Figure 5 An embryonated hen's egg with markings for dorsal (A) and air sac (B) regions indicated (picture as used by Kotwal & Abrahams, 2004).

Eggs were placed on an egg rack and an 'egg-pricker' was used to create a small opening at each marked region. A small drop of melted candle wax was placed above each dorsal opening. This would later serve to cover this opening to prevent bacterial contamination. A drop of 0.85% physiological

saline was pipetted onto each dorsal opening and while viewing eggs under the egg-candler, a pipette bulb was used to create a steady suction force at the air sac opening of each egg in order to drop the region of the chorio-allantoic membrane immediately below and surrounding the dorsal opening. This region then formed a surface on which the virus could be cultured. The dropping and enlarging of this membrane area was clearly visible in the light of the egg-candler. If the membrane did not appear to drop, the air-sac opening was enlarged or an additional drop of 0.85% physiological saline was pipetted onto the dorsal opening. Eggs were incubated at 37°C for 1.5 h to allow dropped membrane areas to stabilise.

Using a 1 ml tuberculin syringe with 29 gauge needle (Cliniscience), each dorsal opening was inoculated with 100 microlitres of poxvirus suspension containing between 100 and 1000 plaque forming units and covered with the drop of candle wax for reasons previously indicated. Inoculation with a viral titre which is too low will result in poor pock confluence and subsequently a low virus yield, whereas a viral titre which is too strong may kill the membrane and chick embryo (Kotwal & Abrahams, 2004).

Eggs were incubated at 37ºC for 72 – 96 h to ascertain whether higher virus titres could be achieved with longer incubation of attenuated vaccinia virus strains. The Western Reserve strain would ordinarily be incubated for a period of 72 h.

1.2 Viral Harvesting

A pair of sharp scissors was inserted at the air sac opening of each egg and used to cut along either side of the dropped CAM region creating a 'lid' which was lifted to view the infected tissue. Any membranous structures attaching the CAM to the chick embryo were carefully cut and the infected CAM region was removed with forceps.

Membranes were rinsed in 0.85% physiological saline to wash away any blood. This was repeated in fresh saline.

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Membranes were transferred to chilled, thick-bottomed universal tubes containing 1cm of glass beads. The thick bases were required to prevent tube breakage during mechanical lysing of CAMs with the glass beads. All remaining steps were carried out on ice.

A volume of McIlvaine's buffer (pH 7) (0.1 M citric acid, 0.2 M $Na_2HPO_4.12H_2O$) and Arklone X (1, 1, 2 trichloro-1, 2, 2 trifluoroethane), was added in a 3:1 ratio according to the number of membranes per tube. For 6 membranes, 3 ml of McIlvaine's buffer and 1 ml of Arklone X were added. The Arklone X is a strong organic solvent (Stannard, 1998) which dissolves the fatty acid layer around the membrane-bound poxvirus particles allowing them to dissociate, whilst the McIlvaine's buffer is a hypotonic solution in which the viral particles could resuspend (Kow, 1992).

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The universal tubes were wrapped in paper-towel to prevent leakage of infectious material and shaken for 2 min. This served to mechanically break the CAM tissue and release viral particles. The tubes were balanced and centrifuged at 800 rpm for 10 min at room temperature in a Sigma 301K centrifuge. Resulting supernatants were transferred to clean, chilled universal tubes and incubated on ice.

The shaking and centrifugation steps were repeated. These steps can be carried out a further 2 to 3 times depending on the number of membranes harvested and the confluence of the membranes. The pooled supernatants were incubated on ice for 1.5 h to allow for sedimentation of membrane debris followed by centrifugation at 2 000 rpm for 10 minutes at room temperature.

The supernatants were transferred to sterile 50 ml polyallomer centrifuge tubes. A volume of 5 ml of 36% sucrose in Tris-EDTA (TE) buffer pH 9 (10 mM Tris, 1mM EDTA) was slowly pipetted at the base of each centrifuge tube so as to create a sucrose cushion for purification of virus particles. As a dense sugar, the sucrose serves to trap any remaining membrane tissue debris, whilst the small poxvirus particles pass through this cushion and are

pelleted. Tubes were centrifuged at 11 000 rpm for 1 hour at 4°C in a Beckman JA20 rotor.

The resulting supernatants were discarded and the pellets resuspended in 0.5 ml TE buffer pH 9 overnight at 4°C. The alkaline pH of the buffer reduces the aggregation of viral particles (perscomm Dianne Marais). The resuspended virus was stored at -20°C or -80°C.

1.3 Viral Titration

The concentration of poxvirus particles in solution can be determined by transfection of a susceptible cell line with a dilution of the virus and by counting the circular 'gaps' known as lytic plaques produced in the cell layer (Fenner et al, 1988b). One plaque corresponds to one virus particle and by taking into account the dilution of the inoculum used for transfection, the viral concentration or titre can be calculated. The vaccinia virus has a broad host range and is able to produce plaques in a variety of cell lines (Fenner et al, 1988b). All titrations were carried out in a BSC-1 (African Green Monkey Kidney Epithelial Cells, ATCC# CCL26) cell line in a laminar flow hood.

1.3.1 Maintenance of BSC-1 cells

BSC-1 cells were maintained at 37°C in a CO_2 incubator (5% CO_2) in Minimal Essential Medium (MEM) Earle's Base with L-glutamine, non-essential amino acids and $NaHCO_3$ (Highveld Biological). Foetal Calf Serum (FCS) (Highveld Biological) was added to media to a 1% final concentration and a Penicillin, Streptomycin and Fungizone (PSF) (Highveld Biological) mix was added to a 1 X final concentration to prevent bacterial or fungal contamination. Cells at 100% confluency were passaged in a laminar flow hood by treatment with trypsin to loosen cells from flask surfaces and dilution in fresh nutrient media.

For long-term storage of BSC-1 cells, cultures were frozen according to the method described at www.research.umbc.edu/~jwolf/method5.htm. One millilitre alliquots of BSC-1 cells suspended in a 10% DMSO (dimethyl sulfoxide), 90% FCS solution were incubated in 200 ml of isopropanol and frozen at -80°C overnight. Isopropanol does not freeze and thus facilitates

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gradual freezing of cells as the temperature decreases at a rate of 1°C per minute thereby eliminating potential cell damage incurred by ice crystals or dehydration (www.research.umbc.edu/~jwolf/method5.htm). Alliquots were removed from the isopropanol and stored at -80°C until further use.

To cultivate cells from frozen cultures, aliquots were thawed rapidly by hand, mixed with 5 ml of MEM in a 15 ml falcon tube and centrifuged at 4°C for 5 minutes at 100 x g in a Heraeus Multifuge benchtop centrifuge. Cells were sedimented at a low speed to prevent damage by compaction. The resulting supernatant containing the DMSO, which is toxic to growing cells, was discarded. Cells were gently resuspended in 5 ml of MEM (10% FCS, 1% PSF) in a 25 cm^2 culture flask and incubated at 37°C (5% CO_2) overnight. Media was drawn off and replaced with 5ml of fresh MEM (10% FCS, 1% PSF). Cells were grown to 100% confluency and maintained by passaging.

1.3.2 Titration of vaccinia virus stocks

BSC-1 cells were grown to 100% confluence in 2 ml of MEM (10% FCS, 1% PSF) per well of a 6-well plate. A volume of 1.5 ml of media was removed from each well and transferred to a sterile 15 ml falcon tube. The poxvirus stock was mixed with a vortexer to disrupt aggregated viral particles or sonicated for 20 seconds using a VirSonic Ultrasonic Cell Disruptor 100 at low voltage. A 1:1 000 dilution of poxvirus preparation was made in fresh MEM and vortexed to mix. One microlitre of dilution was transferred to a well labelled with the corresponding dilution value i.e. 10^{-6}, and mixed thoroughly by pipetting up and down. One hundred microlitres of this dilution was transferred to the adjacent well labelled with the corresponding dilution value i.e. 10^{-7} and mixed as described. Subsequent dilution steps were carried out in 3 of the remaining 4 wells to make 10^{-8}, 10^{-9} and 10^{-10} virus dilutions respectively. The final well was left virus free as a negative control.

Cells were incubated at 37°C (5% CO_2) for 2 hours to allow virus particles to infiltrate the cell monolayer. A volume of 1.5 ml of the previously removed media was added to each well and cells were incubated at 37°C (5% CO_2) for 48hrs or until plaques were visible under the microscope. Media was

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removed and wells were stained with 200-500 microlitres of a 10% crystal violet dye solution (Merck) (10 ml crystal violet solution, 10 ml formaldehyde, ddH_2O to 100 ml) for 1.5 h at room temperature. The dye was removed and wells were allowed to dry. Plaques were visible as clear circular areas disrupting the stained cell layer.

2. DNA extraction from poxvirus preparations

In a 1.5 ml microcentrifuge tube, an equal volume of lysis buffer (50 mM Tris, 700 mM NaCl, 10 mM EDTA, 1% SDS) was added to 100 microlitres of poxvirus suspension in TE buffer (pH 9). The tube was inverted gently to mix and incubated at room temperature for 10 minutes to allow for lysis of the viral particles. Sodium dodecyl sulphate (SDS) is an anionic detergent which denatures proteins (Roskams & Rodgers, 2002) including nucleases (Grierson, 1990). It also inhibits nuclease activity (Sambrook & Russell, 2001) and would thus protect the DNA. The SDS and anionic salt (Cl) present in the lysis buffer removes proteins bound to duplex DNA (Sambrook & Russell, 2001) and the EDTA chelates divalent cations (Sealey & Southern, 1990).

A volume of 2 microlitres of a 50 mg/ml Proteinase K solution was added. Proteinase K is a highly active serine protease which hydrolyses peptide bonds and digests a range of native proteins released following whilst maintaining its activity in the presence of detergents such as SDS (Sambrook & Russell, 2001). The tube was gently inverted and incubated at 56°C for 1.5-2 h.

An equal volume of phenol: chloroform: isoamyl (25:24:1) was then added to the solution. The mixture separated into 2 distinct phases, of which the upper aqueous phase contains the nucleic acid and the bottom organic phase is made up of the phenol: chloroform mix (Sambrook & Russell, 2001). Phenol (carbolic acid, hydroxyl benzene) originally used as a detergent (Perbal, 1988), denatures proteins which aggregate at the interface of the two phases

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(Sambrook & Russell, 2001). In addition to denaturing of proteins the chloroform facilitates phase separation due to its density and the isoamyl alcohol prevents foaming (Sambrook & Russell, 2001). The tube was inverted to mix and centrifuged at top speed (14 000 rpm) in a benchtop Eppendorf centrifuge for 10 minutes. The top phase (aqueous layer) containing the extracted nucleic acid was carefully pipetted off and transferred to a clean 1.5 ml eppendorf tube.

The phenol: chloroform: isoamyl extraction step was repeated on this aqueous solution and the upper phase was again transferred to a clean 1.5 ml eppendorf tube.

Two and a half volumes of ice-cold 95% ethanol and 0.1 volumes of ice-cold 3 M sodium acetate (NaAc) was added to the nucleic acid solution and following mixing by tube inversion, the solution was incubated at -20°C overnight or -80 °C for 1 h. Ethanol precipitates DNA by disrupting its hydration shell and exposing charged phosphate groups which are then neutralised by the sodium (Na^+) ions in the sodium acetate causing it to come out of solution (Sambrook & Russell, 2001).

The DNA was pelleted by centrifugation at top speed for 20 minutes. The supernatant was discarded and the pellet washed by a 10 minute centrifugation step at top speed in 200 microlitres of ice-cold 70% ethanol. This facilitated the removal of salts bound to the DNA pellet (Sealey & Southern, 1990), which make the pellet appear white

The supernatant was discarded and the pellet was dried at room temperature to evaporate residual ethanol, which may lower the DNA density resulting in problems with loading of DNA into wells for agarose gel electrophoresis (Sealey & Southern, 1990). The pellet was resuspended in 50 microlitres of TE buffer (pH 8), a standard buffer used for resuspension and storage of DNA (Roskams & Rodgers, 2002). DNA can be stored for long periods without degradation in TE buffer due to the protease inhibiting properties (Sambrook & Russell, 2001) of EDTA (ethylenediaminetetracetate).

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The viral genomic DNA was diluted in ddH_2O and quantified using a Thermo Spectronic HeΛios light spectrophotometer. DNA absorbance is read at a wavelength of 260 nm at which 1 OD (optical density) unit corresponds to 50 micrograms/ml of double-stranded DNA (Sambrook & Russell, 2001). The DNA was stored at -20°C for long-term storage.

3. Amplification of the N1L gene by the Polymerase Chain Reaction (PCR)

3.1 Primer Design

The Western Reserve (WR) N1L gene sequence was obtained from http://www.ncbi.nlm.nih.gov/entrez (Entrez Nucleotide reference AF451287).

Vaccinia virus strain WR N1L gene:

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  1 atgaggactc tacttattag atatattctt tggagaaatg acaacgatca aacctattat
 61 aatgatgatt ttaaaaagct tatgttgttg gatgaattgg tagatgacgg cgatgtatgt
121 acattgatta agaacatgag aatgacgctg tccgacggtc cattgctaga tagattgaat
181 caaccagtta ataatataga agacgctaag cgaatgatcg ctattagtgc caaagtggct
241 agagacattg gtgaacgttc agaaattaga tgggaagagt cattcaccat actctttagg
301 atgattgaaa catattttga tgatctaatg attgatctat atggtgaaaa ataa
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(Bartlett, N., Symons, J.A., Tscharke, D.C. and Smith, G.L.)

The purpose of a PCR experiment can dictate the location of the primers to be used (Hyndman & Mitsuhashi, 2003), thus as the N1L gene needed to be cloned and sequenced, primers were designed to bind to the terminal regions of the sequence so as to amplify the entire gene.

Forward primer (26mer):

5' GAA TTC ATG AGG ACT CTA CTT ATT AG 3'

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The primer was designed to incorporate the first 20 bases at the 5' end of the WR N1L gene sequence. An *Eco* RI restriction enzyme recognition site (highlighted bases) was added to the 5' end of the primer.

Reverse primer (26mer):
5' GCG GCC GCT TAT TTT TCA CCA TAT AG 3'

The primer was designed to be 100% complementary to the last 20 bases at the 3' end of the WR N1L gene. A *Not* I restriction enzyme recognition site (highlighted bases) was added to the 5' end of the primer.

The 5' ends of primers can be modified to include restriction enzyme recognition sequences, which would then be incorporated into the amplified DNA product during PCR (Scharf, 1986). *Eco* RI and *Not* I restriction enzyme recognition sites were incorporated into the forward and reverse primer sequences respectively to allow for cloning of the amplified gene into a yeast cloning vectors cloning vector with identical restriction enzyme recognition sites (pPIC9), thereby providing the option of expressing the gene in yeast for future applications.

Both primers were analysed by the Gene Runner computer software programme (www.generunner.com) for potential secondary structures, primer dimerisation events and Tm (melting temperature) values or temperatures at which 50% of the template DNA is hybridised (Hyndman & Mitsuhashi, 2003) according to the nucleotide base composition. Both were found to fit the criteria for a successful PCR.

Primers were synthesized by the Oligo 100M DNA Synthesizer, Beckman Instruments Inc.

3.2 PCR

PCR was carried out on the purified Lister vaccinia virus genomic DNA with the use of designed primers (see section 3.1). Using the Roche PCR Core Kit

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(Version 3) according to manufacturer instructions, PCR reaction mixes for control and experimental reactions were set up and are outlined in Table 2. All reactions were set up on ice.

Table 1 Control and experimental reaction mixes for PCR of the SA vaccine Lister vaccinia virus N1L gene

Reagents:	**Experimental** (microlitre)	**Positive control** (microlitre)#	**Negative control** (microlitre)
Viral DNA (240 ng/microlitre) *	1	1	0
10x PCR Buffer (with 1.5 mM $MgCl_2$)	5	5	5
dNTP mix	1 (appr.0.2 mM)	1	1
Forward primer (20 pmol/microlitre)	2	1	2
Reverse primer (20 pmol/microlitre)	2	1	2
Taq polymerase	0.5 (1-5 U/100 microlitres)	0.5 (1-5 U/100 microlitres)	0.5 (1-5 U/100 microlitres)
ddH_2O (microlitre)	38.5	40.5	39.5

*The concentration of template for the amplification of genomic DNA should be between 100 and 500ng (Grunenwald, 2003) # Primers for positive control obtained from Dr.Tayo Odunuga at concentration of 50pmol/ulfor amplification of VCP gene.

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The $MgCl_2$ in the 10 x PCR buffer is an essential component to PCR, affecting both specificity and yield (Saiki, 1989), as it stimulates *Taq* polymerase activity (Gelfand, 1989). Mg^{2+} ions are quantitatively bound by dNTPs, thus the dNTP concentration is selected so as to leave sufficiently high levels of free $MgCl_2$ (Saiki, 1989).

The negative control allows one to determine the occurrence of contamination, a commonly encountered problem in PCR (Stirling, 2003). In

the absence of template DNA, the occurrence of bands on an agarose gel would indicate contamination of one or more of the reagents.

A positive internal control makes use of primers specific to another region of the same template DNA that are known to produce a successful PCR reaction (Stirling, 2003) and would thus allow one to confirm that the reagents e.g. *Taq* polymerase are functional. In the event that only the positive control PCR is successful, the possibility of non-functional reagents can be ruled out and thus trouble-shooting for unsuccessful experimental PCR reactions will be narrowed down to optimisation of PCR parameters or $MgCl_2$ concentration.

<u>Table 2</u> Parameters for PCR amplification of the SA vaccine Lister vaccinia virus N1L gene

STAGE 1 (Denaturing)	94°C for 2 minutes (1 cycle)
STAGE 2 (Denaturing, Annealing and Extension)	94°C for 1 minute 55°C for 1 minute } 30 cycles 72°C for 1 minute
STAGE 3 (Final elongation step)	72°C for 10 minutes (1cycle)

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Very high temperatures are required for denaturation, or strand separation, of duplex DNA (Saiki, 1989). A temperature of 94°C is sufficient for denaturation (Saiki, 1989) and can be tolerated by Taq polymerase for 30 or more PCR cycles without being undergoing excess damage (Sambrook & Russell, 2001).

Primer annealing temperatures can be calculated at 5 degrees lower than the Tm (melting temperature) of the primers, yet temperatures ranging from 55°C to 70°C have been described as providing the best results in general (Grunenwald, 2003).

For primer extension the temperature is increased to that which is optimal for *Taq* polymerase activity, 72°C (Bartlett & Stirling, 2003). At this temperature the rate of Taq polymerase activity is approximately 2000 nucleotides/minute and 1 minute is usually assigned for every 1000 bases of template DNA (Sambrook & Russell, 2001). The final extension step (see stage 3 Table 2) ensures that all the amplified DNA fragments are fully extended (Grunenwald, 2003).

The PCR parameters were programmed into the Thermo Hybaid PCRSprint thermo cycler and the reaction mixes were stored at 4°C after programme completion.

PCR products were run on a 1% agarose gel in 1 X TBE (Tris Borate EDTA) electrophoresis buffer (10.8 g Tris, 5.5 g boric acid, 0.6 g EDTA, ddH_2O to 1 litre) and visualised by Ethidium Bromide (EtBr) staining. A 6 x bromophenol blue (0.025 g bromophenol blue powder, 3 ml glycerol, ddH_2O to 10 ml) loading dye solution was added to DNA samples prior to gel loading. Loading dyes usually contain sucrose or glycerol (Roskams & Rodgers, 2002), which increases sample density and facilitates settling of DNA into gel wells, and at the same time providing colour (Roskams & Rodgers, 2002) and enabling visual monitoring of DNA migration along the gel (Bartlett, 2003).

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3.3 Purification of PCR products

This procedure 'cleans up' the PCR reaction mix by removing reagents such as dNTP's and *Taq* polymerase, which interfere with future reactions using the amplified viral DNA such as restriction enzyme digestion (Crowe, 1991). The 'GFX PCR DNA and Gel Band Purification Kit' supplied by Amersham was used according to manufacturers instructions. The kit contained GFX columns containing glass fibre matrices which trap DNA, 2 ml microcentrifuge collection tubes, a capture buffer which contained acetate and chaotrope and a wash buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 80% EtOH).

A GFX column was placed into a 2 ml collection tube. A volume of 500 microlitres of capture buffer followed by 40 microlitres of PCR reaction mix

was pipetted into the GFX column. The column and collection tube were centrifuged at maximum speed for 30 seconds in an Eppendorf Centrifuge 5415D. Proteins in the PCR mix were denatured by the reagents in the capture buffer and the PCR DNA was trapped in the fibre matrix during centrifugation. The resulting flow-through was discarded and 500 microlitres of wash buffer was pipetted into the filter tube which was centrifuged once again at top speed for 30 seconds. The flow-through containing salts and other contaminants washed away by the ethanol component of the buffer, was discarded and the centrifugation step repeated to dry the column.

The column was transferred to a sterile 1.5 ml eppendorf tube. A volume of 50 microlitres of TE Buffer (pH8) was pipetted directly onto the centre of the GFX column to access a maximum amount of trapped DNA. The set-up was incubated at room temperature for 1-2 minutes to elute the DNA, followed by centrifugation at top speed for 1 minute. Flow-through of resuspended purified PCR DNA preparation was stored at -20ºC.

4. Cloning of amplified Lister N1L gene

4.1 Preparation of competent cells

Competent cells were prepared according to the calcium chloride incubation procedure described by Dagert and Ehrlich (1979) with modifications from (Seidman *et al*, 1987; BSc (Med) (Honours) techniques manual, 2004).

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Five millilitres of LB (Luria Bertani) Broth (10 g tryptone, 5 g yeast extract, 5 g NaCl, to 1 litre with ddH_2O) was inoculated with a single *E.coli* XL-Blue colony and incubated at 37ºC, with shaking, overnight. A 1:100 dilution of the overnight culture was made in a final volume of 50 ml LB broth in a 500 ml conical flask. A large flask was required for proper aeration of the culture (Seidman *et al*, 1987). The diluted culture was incubated at 37ºC with shaking and OD_{600} readings were taken initially at 1 hour intervals and later at 30 minute intervals on a Thermo Spectronic Heλios light spectrophotometer until an OD_{600} reading of between 0.2 and 0.6 was obtained. At this optical density reading, the culture should be in the log

phase of growth, beyond this point the cells transformation efficiency decreases (Seidman *et al*, 1987).

The culture was centrifuged in a Heraeus Multifuge 3 L-R benchtop centrifuge at 4000 rpm for 5 minutes at 4ºC to gently pellet the cells. The supernatants were discarded and all remaining steps were carried out on ice. It was necessary to handle the cells very gently from this stage so as not to disrupt their integrity and render them unable to take up DNA.

The cell pellets were resuspended in 0.5 volumes of ice-cold 0.1 M $CaCl_2$ and incubated on ice for 3.5 h. Cell competency increases with time of incubation on ice (Mandel & Higa, 1970). Cells were once again centrifuged at 4 000 rpm for 5 minutes at 4ºC, supernatants were discarded and the resulting pellets were resuspended in 0.1 volumes of ice-cold 0.1 M $CaCl_2$.

Ice-cold sterile glycerol was added to a 10% (v/v) final concentration and the cells were incubated on ice for 30 minutes. Two-hundred microlitre aliquots of cells were pipetted out into sterile 1.5 ml eppendorf tubes and stored at -80ºC. Glycerol does not freeze and thus reduces loss of cell competency by mechanical lysing when aliquots are thawed.

4.2 Ligation of Lister N1L gene and pGEM-T Easy cloning vector

The pGEM-T Easy Vector System was supplied by Promega and included a 2 x Rapid Ligation Buffer, T4 DNA ligase, pGEM-T Easy vector and Control insert DNA. This T-vector system, specifically designed for the cloning of PCR products, was developed by cleavage of the Promega pGEM-T Easy Vector with the *Eco* RV restriction enzyme followed by the addition of a 3' terminal thymidine nucleotide base to each cleaved end (Promega). The system was used according to manufacturer instructions.

Reagents were combined in 10 microlitres final volume reaction mixes as outlined in Table 3 and incubated at 4°C for 72 h. A longer incubation period increases the probability of efficient ligation reactions (Promega).

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Table 3 Reaction mixes for ligation of SA vaccine Lister vaccinia virus N1L PCR DNA fragments and the pGEM-T Easy vector

Reagent	Experimental Mix (microlitre)	*Positive control (microlitre)	Background control (microlitre)
Insert DNA	3	2	0
pGEM-T Easy Vector (50 ng/microlitre)	1	1	1
T4 DNA ligase (3 U/microlitre)	1	1	1
2 x Rapid Ligation Buffer	5	5	5
ddH_2O	0	1	3

* Control insert DNA supplied at a concentration of 4 ng/microlitre.

The amount of insert DNA should be equivalent to or more than that of vector DNA for successful ligation reactions (Perbal, 1988). Details of the pGEM-T Easy Vector are described in appendix. The T4 DNA ligase enzyme facilitates the formation of phosphodiester bond between 3' hydroxyl group and 5' phosphate of two dsDNA molecules and requires ATP (Perbal, 1988), which is provided by the 2 x Rapid Ligation Buffer (Promega).

The positive control incorporates an insert which is known to produce successful ligation reactions and provides a means of confirming that reagents and ligation reactions are efficient, whereas the background control provides an indication of the prevalence of undigested or non-T tailed pGEM-T Easy vector plasmids which are in circular form and can thus be taken up by competent cells (Promega). To determine the outcome of control and experimental ligation reactions, a volume of each ligation reaction mix must be cloned into competent cells.

4.3 Transformation

The transformation procedure was carried out as according to the University of Cape Town BSc (Med) (Hon) techniques manual (2004) with modifications

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obtained from the pGEM-T Easy Vector System Technical Manual No. 042 (Promega).

E.coli XL Blue competent cell aliquots were thawed on ice and 100 microlitres of cells was added to 1 microlitre of each ligation reaction mix (see section 4.2) in a sterile 1.5 ml eppindorf tube. The mixture was incubated on ice for 30 minutes. This allows the DNA to adhere to the cells (Seidman *et al*, 1987). A heat shock was applied to each tube at 42°C for 1 minute. This step facilitates entry of DNA into the cells (Seidman *et al*, 1987).

Cells were incubated on ice for 2 minutes. A volume of 900 microlitres of LB broth was added to each cell mix and tubes were incubated at 37°C for 1 hour with shaking. This incubation period allows expression of the ampicillin resistance gene (Seidman *et al*, 1987) encoded by the plasmid (see appendix 1).

Using asterile techniques, LB agar plates (10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g Agar, ddH_2O to 1 litre) containing ampicillin at a final concentration of 0.1 mg/ml were spread-plated with 100 microlitres and 200 microlitres of experimental transformation mix respectively and 100 microlitres of each control transformation mix (see section 4.2) was spread-plated onto individual plates. Ampicillin is a penicillin derivative which is bactericidal to growing cells due to its ability to prevent the cross-linkage of cell wall peptidoglycans and interrupt bacterial cell wall synthesis (Roskams & Rodgers, 2002). The plasmid vector ampicillin resistance marker thus facilitates the selective growth of transformed cells (Seidman *et al*, 1987). Plates were incubated at 37°C overnight and examined for colony growth.

For the isolation of transformed recombinant plasmid DNA by small-scale plasmid preparation, single colonies which grew on selective media were picked and grown up in 4ml of ampicillin containing LB Broth with shaking at 37°C for 18 hours.

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4.4 Small-scale plasmid preparation

The QIAprep Spin Miniprep Kit from Qiagen was used according to manufacturer instructions to purify the recombinant pGEM-T Easy vector DNA from cell culture. Reagent and protocol information was also obtained from http://mbclserver.rutgers.edu/Courses/IMBBR04/31504_lab/31504_Lab3.pdf.

Cells were pelleted by centrifugation Eppendorf Centrifuge 5415D in 1.5ml eppindorf tubes at 5 000 rpm for 5 minutes at room temperature. A volume of 250 microlitres of Buffer P1 (50 mM glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA pH 8 and 100 micrograms/ml RNase A) was used to thoroughly resuspended each cell pellet. An equal volume (250 microlitres) of lysis buffer, Buffer P2 (0.2 N NaOH, 1% SDS), was added and tubes were inverted 4 to 6 times to mix and incubated at room temperature for a maximum of 5 minutes.

A volume of 350 microlitres of Buffer N3 (3 M Guanidine-HCl pH 4.8) was added to neutralise each solution and tubes were once again inverted 4 to 6 times to mix. Solutions became flocculent as cell debris and SDS were precipitated out. Tubes were centrifuged at 14 000 rpm for 10 minutes at room temperature to pellet precipitants.

Supernatants were transferred to spin columns and centrifuged at 14 000 rpm for 1 minute to settle DNA in the column. Resulting flow-through was discarded and each column was washed with 750 microlitres of Buffer PE (10 mM Tris-HCl pH 7, 50% ethanol) by centrifugation at 14 000 rpm for 1 minute. The flow-through was discarded and the centrifugation step repeated to dry the column and remove any remaining ethanol which may interfere with future applications with the plasmid preparation. A volume of 50 microlitres of elution Buffer EB (10 mM Tris-HCl pH 8.5) was directly dispensed onto the centre of each filter column. Columns were incubated at room temperature for 5 minutes for efficient DNA elution. Each column was transferred to a fresh 1.5 ml eppindorf tube and following a final

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centrifugation step at 14 000 rpm for 1 minute, the flow-through containing the eluted DNA was stored at -20ºC.

4.5 Restriction enzyme digestion of recombinant plasmid

Each plasmid DNA preparation was digested with 10-15 U (units) of *Eco* RI, *Pst* I and *Hin* dIII restriction enzymes respectively in a 20ul final volume. These Class II restriction enzymes recognise and cut specific DNA sequences (Perbal, 1988) that usually have a twofold symmetry (Sambrook & Russell, 2001) and were selected for the frequency of their recognition sites in the pGEM-T Easy vector (appendix 1) and N1L gene insert.

The *Eco* RI enzyme has two recognition sites within the pGEM-T Easy vector multiple cloning region; each one situated on either side of the 3' thymidine overhangs (see appendix 1). It was thus used to excise the DNA insert. An enzyme which cleaves the vector only, *Pst* I, was used to linearise the plasmid DNA and *Hin* dIII, which cuts only the N1L gene insert, was used to confirm positive clones.

Eco RI and *Pst* I digests were carried out in a 10 x high salt buffer and the *Hin* dIII digests in a 10 x medium salt buffer. Digests were incubated at 37°C overnight to ensure efficient cleavage of plasmid DNA. One unit of enzyme is sufficient to completely digest 1ug of bacteriophage λ DNA in 1 hour at 37ºC (Perbal, 1988). Digested plasmid preparations were run on a 1% agarose gel in 1 X TBE electrophoresis buffer and visualised by Ethidium Bromide (EtBr) staining.

4.6 Sequencing of the Lister N1L gene insert

Plasmid recombinants that were positively identified by restriction enzyme digestion as having an insert of size corresponding to that of the N1L gene were sequenced using the ABI Prism 3100 Genetic Analyzer, University of Stellenbosch.

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Results

1. Viral stocks grown up in embryonated hens eggs

Poxvirus stocks were grown up on the chorio-allantoic membrane (CAM) of 9-day-old embryonated hens' eggs and produced titres ranging from 10^7 to 10^9 plaque forming units per millilitre (PFU/ml) of poxvirus suspension.

Viral titres obtained from different incubation periods are indicated in Table 4. Higher titres were achieved for the knockout strain (vGK5) when eggs were incubated for an additional 24 h.

Table 4 Recombinant vGK5 vaccinia virus titres obtained at 72 and 96 hour egg incubation periods

Incubation period (hrs)	vGK5 (PFU/ml)
72	$7x10^7$
96	$3.8x10^9$

2. Amplification and cloning of Lister N1L gene

The Lister vaccinia virus N1L gene was amplified by PCR using 26-mer oligonucleotide primers complementary to the respective termini of the WR vaccinia virus N1L gene. The PCR products were analysed by agarose gel electrophoresis.

A clear, single DNA band of expected size (354 bp) was observed for the N1L gene PCR product (see **Figure 6**). Similarly the internal positive control produced a single band of expected size (840 bp) with no DNA bands observed for the negative control PCR confirming that no contaminating DNA was present.

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Figure 6 1% Agarose gel of the Lister vaccinia virus N1L gene PCR product. Lane (1) 1 kb DNA ladder MW Marker (Promega); (2) N1L gene PCR product using 1 microlitres Lister genomic DNA template; (3) N1L gene PCR product using 3 microlitres Lister genomic DNA template; (4) no sample loaded; (5) VCP gene PCR product, internal positive control; (6) no template DNA, negative control.

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The Lister vaccinia virus N1L gene PCR product was ligated into a cloning T-vector using the pGEM-T Easy Vector system (Promega) and restriction enzyme digestion was used to confirm positive clones. Digested DNA products were visualised on a 1% agarose gel (see **Figure 7**) by Ethidium Bromide (EtBr) staining.

Plasmid digestion with the *Eco* RI restriction enzyme produced a DNA band corresponding to the size of the pGEM-T Easy plasmid vector (3 015 bp) (**A**) and a band less than 500 bp in size (**B**). This was expected as the *Eco* RI enzyme cuts on either side of the plasmid multiple cloning site, excising the DNA insert. The *Pst* I and *Hin* dIII restriction enzymes, which have single cut sites in the vector and insert respectively, produced linearised fragments

which ran above the *Eco*R1 digested plasmid confirming the presence of the an insert (see lanes 4 and 5).

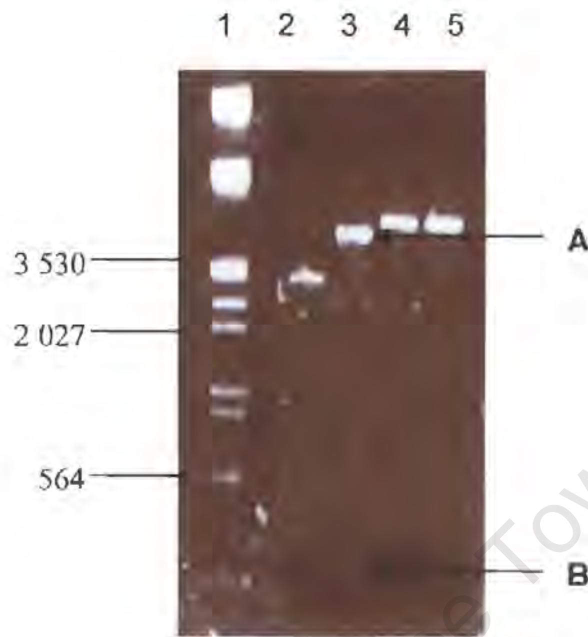

Figure 7 1% Agarose gel of restriction enzyme digested plasmid preparations carrying the vaccinia virus N1L gene from transformed XL Blue *E.coli* colonies. Lane (1) Lambda DNA/*Eco*R1 + *Hin*d III Marker (Promega); (2) uncut plasmid preparation; (3) *Eco*R1 digested plasmid preparation; (4) *Hin* dIII digested plasmid preparation and (5) *Pst*1 digested plasmid preparation.

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3. Sequencing of the Lister N1L gene insert

Electronic sequencing results were analysed using the Chromas software (www.technelysium.com.au/chromas.html) programme and entered into a gene database to compare its homology to genes of preciously sequenced genomes using the nucleotide-nucleotide BLASTn programme (www.ncbi.nlm.nih.gov/BLAST/). The SA vaccine Lister vaccinia virus was found to be 100% homologous to its corresponding gene in the Western Reserve (WR) vaccinia virus genome (**Figure 8**).

```
Lister N1L   CGCGGGAATTCGATTGAATTCATGAGGACTCTACTTATTA   120
WR N1L       .....................ATGAGGACTCTACTTATTA    19

Lister N1L   GATATATTCTTTGGAGAAATGACAACGATCAAACCTATTA   160
WR N1L       GATATATTCTTTGGAGAAATGACAACGATCAAACCTATTA    59

Lister N1L   TAATCATGATTTTAAAAGCTTATGCTTGTTGGATCAATTG   200
WR N1L       TAATCATGATTTTAAAAGCTTATGCTTGTTGGATCAATTG    99

Lister N1L   GTAGATGACGGCCATGTATCTACATTCATTAAGAACATGA   240
WR N1L       GTAGATGACGGCCATGTATCTACATTCATTAAGAACATGA   139

Lister N1L   GAATGACGCTGTCCGACGGTCCATTGCTAGATAGATTGAA   280
WR N1L       GAATGACGCTGTCCGACGGTCCATTGCTAGATAGATTGAA   179

Lister N1L   TCAACCAGTTAATAATATAGAAGACGCTAAGCGAATGATC   320
WR N1L       TCAACCAGTTAATAATATAGAAGACGCTAAGCGAATGATC   219

Lister N1L   GCTATTAGTGCCAAACTGGCTACAGACATTGGTGAACGTT   360
WR N1L       GCTATTAGTGCCAAACTGGCTACAGACATTGGTGAACGTT   259

Lister N1L   CAGAAATTAGATGCCAAGAGTTATTCACTATACTCCTTAG   400
WR N1L       CAGAAATTAGATGCCAAGAGTTATTCACTATACTCCTTAG   299

Lister N1L   GATGATTGAAACATATTTTGATGATCTAATGATTGATCTA   440
WR N1L       GATGATTGAAACATATTTTGATGATCTAATGATTGATCTA   339

Lister N1L   TATGGTGAAAAATAAGCGGCCGCAATCACTAGTGAATTCG   480
WR N1L       TATGGTGAAAAATAA                            354
```

Figure 8 A multiple sequence alignment of the SA vaccine Lister and Western Reserve vaccinia virus N1L genes

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Discussion

Higher viral titres were achieved for the vGK5 strain of vaccinia virus with an additional 24 h incubation period in embryonated hens' eggs.

The Lister vaccinia virus N1L gene was successfully amplified by the polymerase chain reaction using primers complementary to respective ends of the Western Reserve strain N1L gene. Sequence analyses of the amplified gene product revealed 100% identity between the Lister and WR vaccinia virus strains N1L gene. It could thus be concluded that the SA vaccine Lister vaccinia virus strain would produce an identical 13.8 kDa protein.

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Chapter 3: Determining Adenosine Triphosphate (ATP) Levels in Vaccinia Virus Infected Mouse Brain Tissue

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Introduction

1. Detection of ATP Using Firefly Luciferase Bioluminescence

To determine whether the vaccinia virus 13.8 kDa protein allows viral replication in the brain by altering brain ATP levels or utilisation, ATP levels in brain tissue infected with a vaccinia virus wild type and 13.8 kDa negative strain needed to be measured. When working with animal tissues, the use of a biochemical assay for adenosine 5' triphosphate (ATP) detection is generally preferred (Khan, 2003). The firefly luciferase bioluminescence system is one which is specific for ATP (Deluca & McElroy, 1978). Luciferases are enzymes, which catalyse light generating reactions in bioluminescent organisms by the oxidation of a substrate known as luciferin (Sambrook & Russell, 2001).

This bioluminescent detection system is based on the conversion of luciferin substrate to oxyluciferin (Kamidate, 1996) in the presence of ATP, magnesium ions and oxygen, and is accompanied by the emission of light at 560 nm (see **Figure 9**) (DeLuca & McElroy, 1978). The amount of light emitted is linearly related to the concentration of ATP present (Kamidate, 1996).

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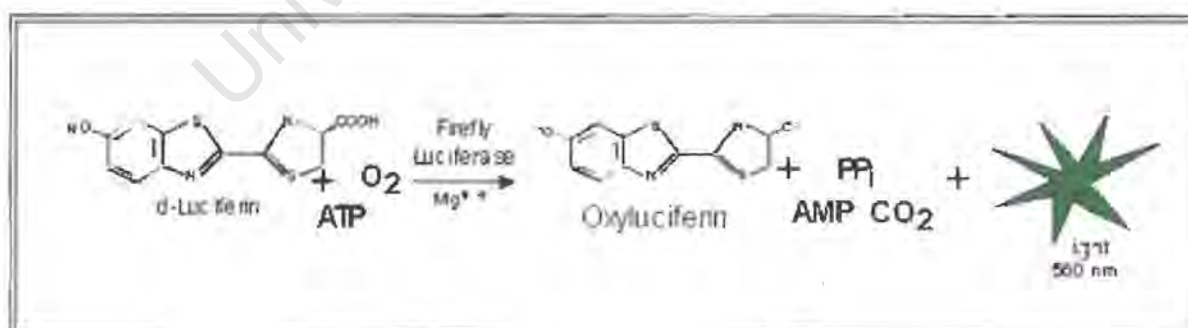

Figure 9 Bioluminescent reactions catalyzed by firefly luciferase (www.biotek.com/products/tech).

In the initial reaction, the luciferase enzyme forms a complex with the luciferin and adenosine monophosphate (AMP) from ATP and two inorganic

phosphates are released (Lemasters & Hackenbrock, 1978). Magnesium ions are essential as magnesium-bound ATP is the actual substrate for activation of the luciferase enzyme (DeLuca & McElroy, 1978). The enzyme complex then reacts with molecular oxygen resulting in the production of oxyluciferin, AMP, carbon dioxide (CO_2) and light (Lemasters & Hackenbrock, 1978). The reaction takes place rapidly wherein light emittion occurs within milliseconds and a peak is reached after approximately 0.3 seconds (DeLuca & McElroy, 1974). The amount of light is measured by a luminometer in relative light units (RLU).

Extraction of ATP from cells requires consideration of specific factors including effective cell lyses for the release of ATP, an extractant buffer which inactivates ATP converting enzymes (ATPases) and which does not interfere with the activity of the luciferase enzyme (Yang, 2002).

Khan optimised the detection of ATP by firefly luciferase bioluminescence in mouse brain striata (Khan, 2003) by comparing different tissue homogenisers for the disruption of cells as well as different solutions for extracting. Optimal results were obtained when disrupting tissue with an Ultraturax homogeniser in the presence of 10% perchloric acid (Khan, 2003). Perchloric acid ($HClO_4$) is a strong acid, which extracts ATP whilst inactivating ATPases (Yang, 2002).

The luciferase enzyme is subject to inhibition by anions such as $HClO_4$ in perchloric acid (www.pathtech.com), however this interference can be minimised by acid neutralisation with potassium chloride (KOH) and appropriate sample dilution and a highly stable luminescence signal can be achieved (Khan, 2003).

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Methods and Materials

1. Intracranial infection of BALB/c mice

Mr. Rodney Lucas of the Biovac Institute, Pinelands carried out all animal work in the P2 Isolation Laboratory of the University of Cape Town Animal Unit. Animals were housed in individually ventilated cages (IVC's) and were provided a constant supply of food and water.

Three- to four-week-old male and female BALB/c mice (Animal Unit, University of Cape Town) were separated into 2 experimental and 1 control group, anaesthetised by administration of a Ketamine/Xylazine cocktail and infected via an intracranial route with inoculums and doses as outlined in Table 5. Mice are used at this age as their skulls are soft and easily penetrable for the intracranial injection procedure (Smith & Kotwal, 2002). Each inoculum was administered in a 25 microlitres final volume using a 1cc Tuberculin syringe with 29 gauge needle (Cliniscience).

Table 5 Inoculation and dosage of 3 groups of BALB/c mice

Group	**1**	**2**	**3** (control)
Inoculum	SA vaccine Lister vaccinia virus	vGK5 vaccinia virus	Physiological saline 0.85%
Number of mice	6	6	3
Dose of virus particles (PFU)*	100 to 1 000	100	0

*PFU = plaque forming units.

2. Extraction and homogenisation of infected mouse brain tissue

A maximum of 3 mice per group were sacrificed by cervical dislocation at 24 h intervals for 5 days. Brains were extracted and immediately frozen in liquid nitrogen in a 50% glycerol solution and stored at -80°C. Rapid freezing

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prevents the loss of ATP prior to processing the brain tissue (Wan, 1999) and the glycerol solution protects the tissue whilst it is in a frozen state.

Each mouse brain was thawed on ice and homogenised in 1 ml ddH_2O at low speed using an Ultra-turax T8 tissue homogeniser (IKA Labortechnik) with a 5 mm diameter dispenser tool. Homogenisation was carried out on ice to prevent degradation of ATP due to sample heating.

3. Extraction and titration of vaccinia virus from infected mouse brain tissue

A volume of 500 microlitres of each mouse brain tissue homogenate was subjected to 3 freeze/thaw cycles in 1 ml of Minimal Essential Medium (MEM) (Highveld Biological). Repeated freezing at -80°C and thawing at 37°C facilitated mechanical lysing of cells and the release of virus particles. Lysates were then sonicated using a VirSonic Ultrasonic Cell Disruptor 100 for 20 seconds at low voltage and stored at -80°C. For virus titration, lysates were thawed at room temperature, briefly mixed with a vortexer and transfected into BSC-1 cell monolayers in 6-well-plates as described in Chapter 2 (section 1.3.).

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4. ATP bioluminescence assay

The extraction and detection of ATP from mouse brain tissue was carried out as described by Khan (2003) with modifications. A half volume of each whole brain tissue homogenate (volumes ranging from 600 – 900 microlitres; average volume = 750 microlitres) was treated with an equal volume of pre-cooled 20% perchloric acid ($HClO_4$). All remaining steps were carried out on ice.

Homogenates were centrifuged in a Heraeus Multifuge 3 L-R bench top centrifuge (Kendro Laboratory Products) at 4 500 rpm for 10 minutes at 4°C to pellet all cell debris. A volume of 500 microlitres of each supernatant was

transferred to a sterile 15 ml falcon tube and 200 microlitres of a 2.5M KOH (potassium hydroxide) solution was added to each aliquot. This strong base neutralised the perchloric acid solution. The mixture became flocculent and was centrifuged at 4 500 rpm for 5 minutes at 4°C to pellet the precipitant. The resulting supernatants were diluted 1:40 in 0.1 M TE buffer pH 7.75 (100 mM Tris; 2 mM EDTA). This pH is optimal for luciferase activity (www.pathtech.com).

A volume of 100 microlitres of each diluted extract was loaded into wells of a sterile MultiScreen Opaque 96-well-plate (Millipore). Opaque plates prevent light carryover between adjacent wells and allow for rapid light measurement reading over a series of wells (Thorpe *et al*, 1985). A volume of 100 microlitres of each concentration of a dilution series of adenosine 5' triphosphate (ATP) disodium salt (Sigma-Aldrich) was loaded into respective wells and used for an ATP standard curve from which ATP content for experimental samples could be extrapolated. A volume of 100 microlitres of TE Buffer pH 7.75 was loaded in triplicate to obtain an average background ATP level reading.

Using the Luminoskan Ascent (Amersham) or Veritas microplate luminometer (Turner Biosystems), an ATP measurement for each well was obtained in relative light units (RLU) following the addition of 50 microlitres of the ENLITEN rLuciferin/Luciferase reagent (Promega) by luminometer dispensers and a brief shaking step to allow mixing of reagents. The average background ATP level as determined from the RLU readings given for wells containing TE buffer pH 7.75 was subtracted from all other sample readings.

A protein estimation using the BioRad protein estimation kit with bovine serum albumin (BSA) protein standards was performed on 50 microlitres of each mouse brain homogenate and used to correlate ATP concentration with average protein content per sample group. Protein content per sample was measured on an Anthos 2010 microplate reader (Separation Scientific). ATP concentrations for SA vaccine Lister vaccinia virus, recombinant vGK5 vaccinia virus and mock-infected mouse brains at each 24 h period post-

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infection were graphically compared and analysed for significant differences by student T tests using Windows XP Microsoft Office Excel (2002) software.

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Results

1. Vaccinia virus replication in mouse brain

Mouse brains infected with 100 PFU of the Lister or vGK5 vaccinia virus were homogenised and their viral concentrations determined and compared following titration in BSC-1 cell cultures (**Figure 10**). Viral titres in vGK5 vaccinia virus-infected mouse brains increased by 4 log over the period of infection. Lister vaccinia virus titres did not increase for the first 4 days of infection (200 PFU/ml). At 5 days post-infection these titres increased by a factor of 4 (800 PFU/ml).

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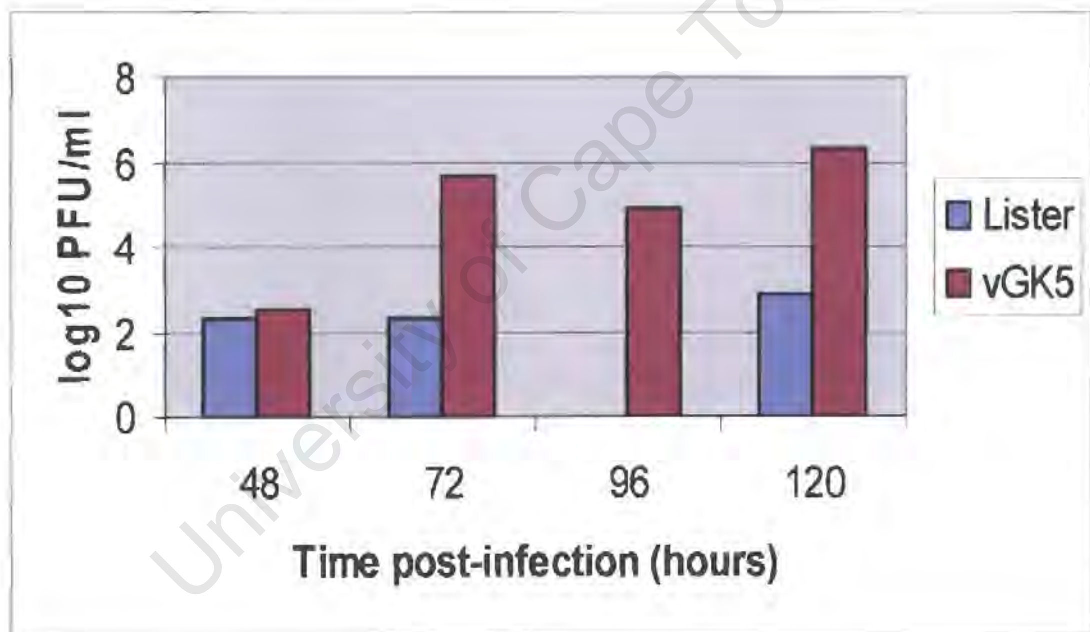

Figure 10 Viral titres of Lister and vGK5 vaccinia virus-infected mouse brains over a 5 day infection period.

2. ATP level analysis

ATP levels in Lister vaccinia virus, recombinant vGK5 virus and mock-infected mouse brain homogenates were determined using a luciferin/luciferase bioluminescence assay. Average ATP concentrations for

each sample group were calculated using the line equation obtained from the linear double log fitting of a standard curve (Santos et al, 2003) of known ATP concentrations (see **Figure 11**) and results were graphically compared (**Figure 12**).

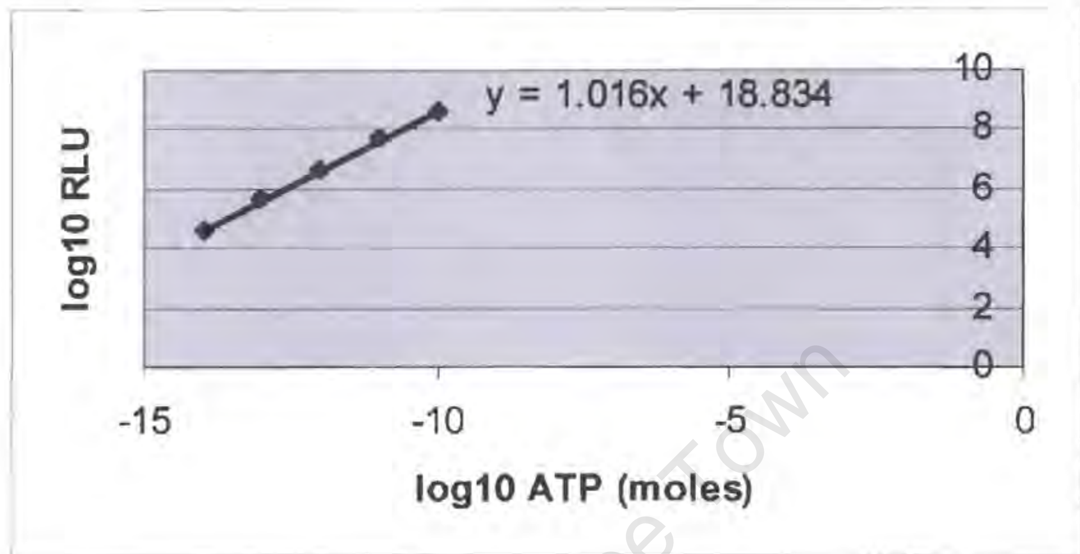

Figure 11 Linear double log fitting ATP standard curve

An initial decrease in ATP was observed within the first 48 h of infection in both Lister and vGK5 vaccinia virus-infected groups, yet in contrast to the vGK5 virus and mock-infected mice, the ATP levels in Lister vaccinia virus-infected brains were unchanged for the remainder of the period of infection (**Figure 12A**). The ATP concentrations in mock and vGK5 virus-infected mouse brains underwent small fluctuations during the intermediate stages of infection and eventually increased to concentrations above those seen at 24 h post-infection (**Figure 12A**). A significant difference (p-value=0.01613) in ATP concentrations between wild type and knockout-infected mouse brains was obtained at 120 h post-infection (**Figure 12B**), with no significant differences at prior stages of infection.

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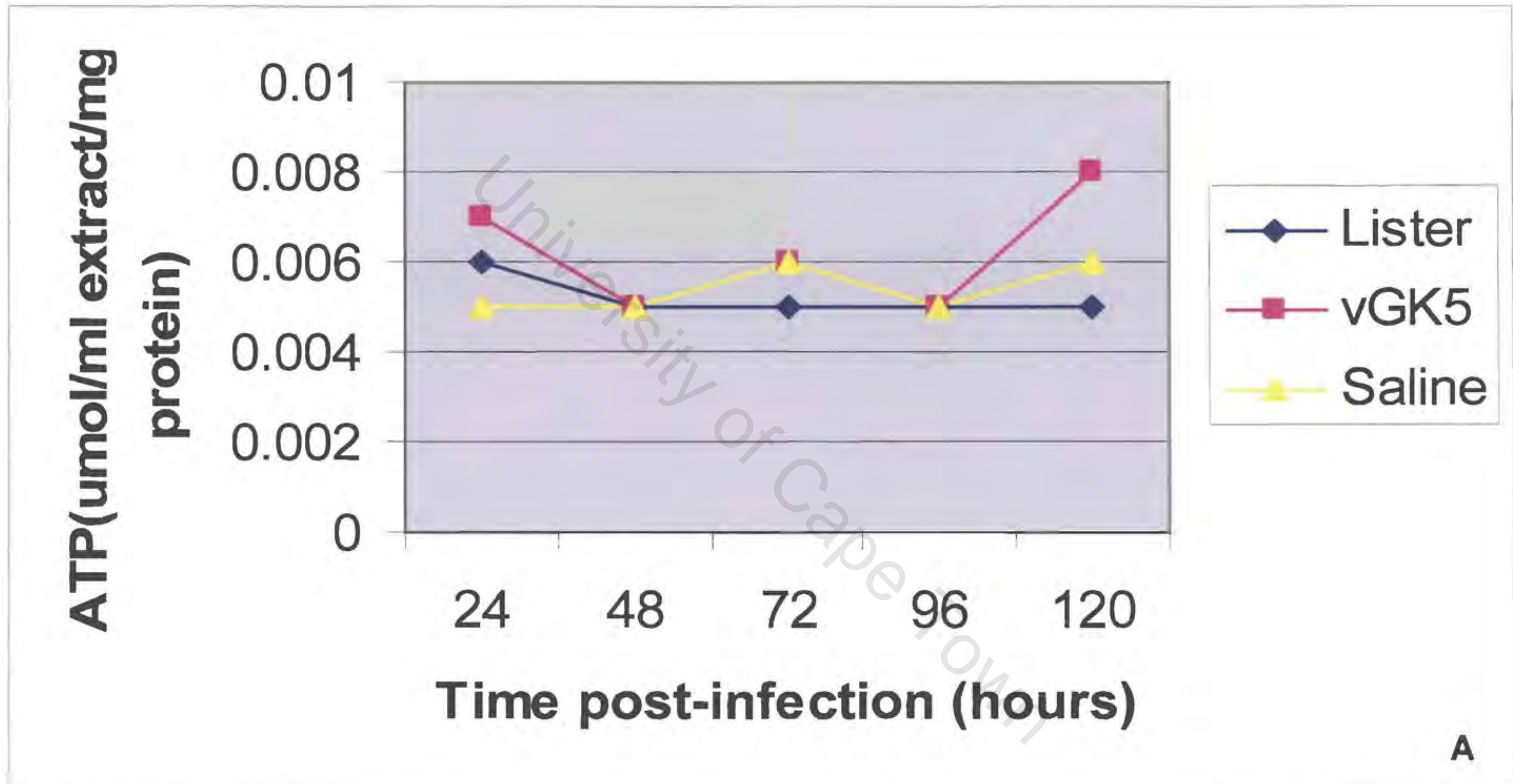

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Figure 12A Line graph of ATP concentrations in Lister vaccinia virus, recombinant vGK5 vaccinia virus and mock-infected mouse brain over a 5 day infection period

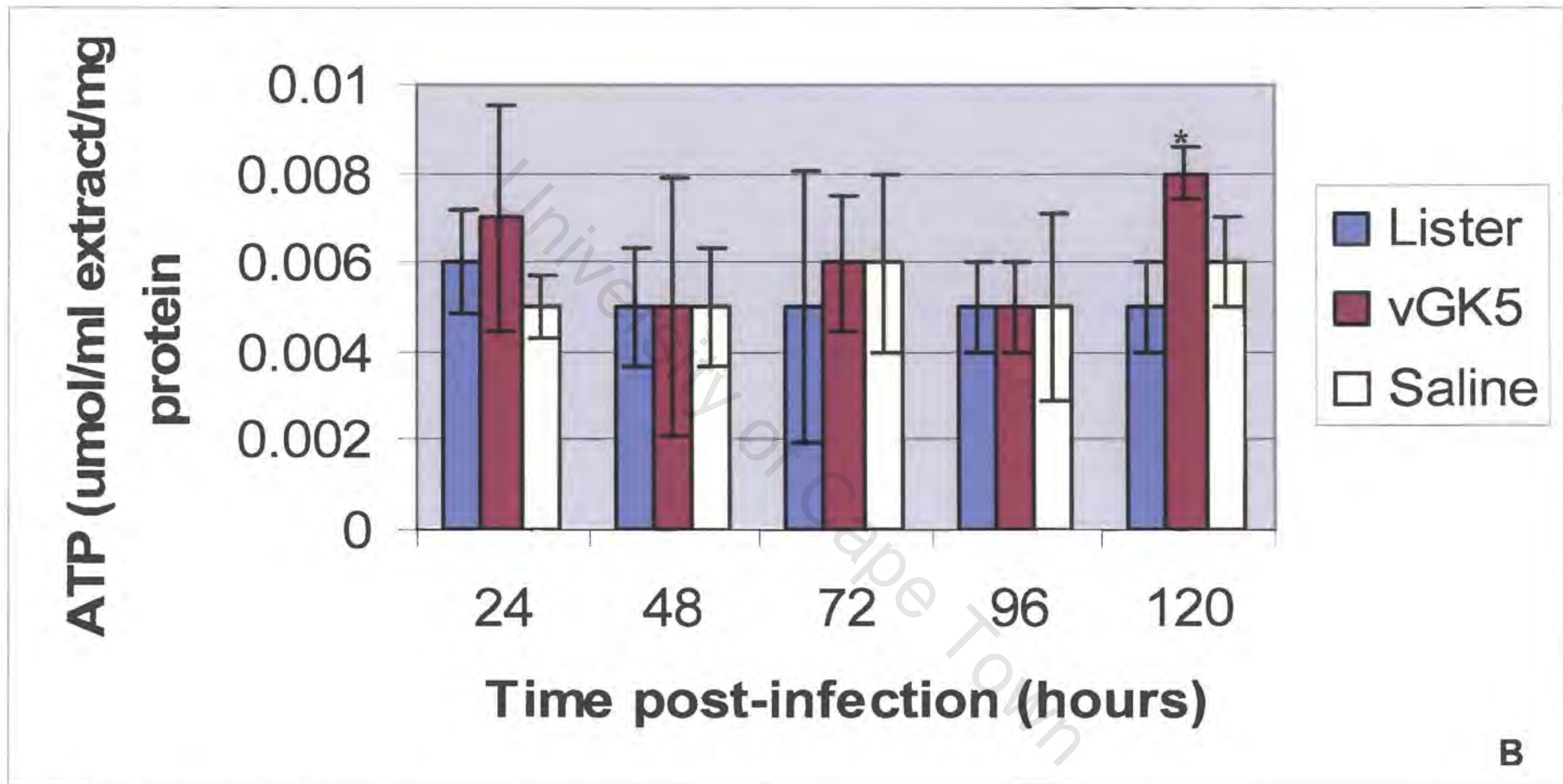

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Figure 12B Bar graph of ATP concentrations in Lister vaccinia virus, recombinant vGK5 vaccinia virus and mock-infected mouse brain over a 5 day infection period with standard deviations indicated by error bars per sample group and point of significant difference (*p-value = 0.01613) in ATP concentration shown.

Discussion

The adenosine 5' triphosphate (ATP) energy molecule plays a vital role in the replication of poxviruses (Gershowitz *et al*, 1978, Broyles, 1991, Broyles & Moss, 1988). A 13.8 kDa protein produced by the vaccinia poxvirus was found to be essential for dissemination of this virus within the brain (Billings *et al*, 2004). By investigating the possible role of this protein in virus-mediated utilisation of ATP in the brain, which has low energy reserves (Ames III, 2000), a significant difference in brain ATP concentration was observed between wild type Lister vaccinia virus-infected and knockout vGK5 vaccinia virus-infected groups of mice at 5 days post-infection.

In vitro studies comparing ATP levels in neuroblastoma cell cultures infected with a wild type Western Reserve vaccinia virus and a corresponding 13.8 kDa-negative strain revealed a rapid depletion of ATP within 48 hours of infection with the wild type virus (Abrahams *et al*, in press). This result may consolidate the virus-mediated consumption of ATP in the brain during viral replication.

In the *in vivo* system studied here, ATP levels in wild type Lister vaccinia virus-infected mouse brains decreased in the first 48 hours of infection and were thereafter unchanged for the remaining period of infection. Likewise, the ATP concentrations in vGK5 vaccinia virus-infected mouse brains dropped during the first 48 hours of infection yet appeared to return to the initial brain ATP concentrations over the final 24 hours of infection. The maintained ATP level in wild type-infected mouse brains could be attributed to a constant ATP turnover; that being a balanced system of ATP production and immediate consumption characterised by no visible periods of ATP level increase or decrease. However, a significant difference in wild type and knockout-infected mouse brain ATP levels was observed only at 120 hours post-infection. A similar *in vivo* study conducted over a longer infection period may be required to establish whether a significant ATP level difference is maintained.

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A contrast in ATP utilisation may however be seen when comparing the pattern of ATP level changes in the different experimental and control groups over the 5-day infection period. A similar pattern of ATP level changes in knockout and mock-infected mouse brains, characterised by slight fluctuations in brain ATP levels over the infection period, was observed. These changes in ATP levels were possibly in response to the injury incurred at the site of intracranial inoculation, yet were not observed in wild type-infected mice.

Thus, from this study, the vaccinia virus 13.8 kDa protein cannot be concluded to have a specific role in directly altering or enhancing ATP utilisation or production in the brain, yet a role in influencing the pattern of ATP utilisation may be implicated.

Despite the fact that the Lister strain of vaccinia virus was confirmed to encode an identical N1L gene, the replicative properties of this strain need to be taken into account. As a vaccine strain, a reduced capacity to replicate is already implicated. This property has proven to be more pronounced in the brain as viral titres were seen to undergo a marginal increase after 120 hours of infection. The poor replication of the Lister (Elstree) vaccinia virus in mouse brain has previously been reported (Soekawa *et al*, 1974; Simon & Werner, 1979). In addition, the wild type Western Reserve vaccinia virus strain from which the recombinant vGK5 strain was derived, is neurotropic and highly neurovirulent as opposed to the dermotropic Lister vaccinia virus (Beranek *et al*, 1982). Thus, the possibility that any differences in ATP consumption between the Lister virus strain and the 13.8 kDa negative strain are due to differences in replication and not due to the protein in question cannot be ruled out.

The 4 log increase in vGK5 vaccinia virus titres in mouse brains over the infection period was unexpected due to the previously observed inability of this virus to replicate in the brain (Billings *et al*, 2004). A possible reason for this occurrence is the presence of revertant mutants within the knockout virus stock. The 13.8 kDa knockout strain was produced by insertional inactivation of the WR vaccinia virus N1L gene with a *lacZ* expression cassette (Kotwal et

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al, 1989). Thus, regular blue/white selection staining of vGK5 vaccinia virus plaques in cell culture for β-galactosidase production would allow for detection of revertant or wild type virus.

A potential role for the recombinant vGK5 strain of vaccinia virus as a safer smallpox vaccine likened to that described for a thymidine kinase knockout vaccinia virus by Buller *et al* (1985) has been proposed. This strain had the ability to replicate comparable to that of its wild type counterparts when administered intradermally, yet displayed a decreased capacity to disseminate to or within the brain, making it less likely to induce vaccination-related complications (Buller *et al*, 1985). The vGK5 vaccinia virus may provide a more promising smallpox vaccine alternative as it has been shown to stimulate an antibody-mediated immune response similar to that of the virulent Western Reserve strain of vaccinia virus (Kotwal *et al*, 1989).

In this study, the presence of the vaccinia virus 13.8 kDa protein was shown to influence ATP levels as well as the pattern of ATP utilisation in mouse brain at late stages of infection. As a potential mediator of ATP acquisition or production in the brain, this virokine could have therapeutic potential for enhanced brain trauma or injury recovery. Additional *in vivo* studies incorporating a neurovirulent wild type strain such as the Western Reserve vaccinia virus strain are expected to effect more pronounced differences in ATP consumption and possibly produce significant ATP level differences earlier in infection.

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Chapter 4: Expression and Immunodetection of the Western Reserve (WR) Vaccinia Virus 13.8 kDa Protein

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Introduction

1. Protein expression in *Pichia pastoris*

The *Pichia pastoris* protein expression system has proved very efficient in the production of high levels of recombinant foreign proteins displaying all necessary post-translational modifications e.g. Hepatitis B surface antigen (Vassileva *et al*, 2001) and green fluorescent protein (Zupan *et al*, 2003). It holds numerous advantages over alternative eukaryotic protein expression systems such as absence of endotoxins, which hamper expression in bacteria or viruses, which contaminate animal cell culture systems (Cino, 1999). This expression system was employed for the production of the WR vaccinia virus 13.8 kDa protein. Mass production and purification of this protein would allow for direct administration of this protein into the brain to determine its specific function.

Pichia pastoris is a methylotrophic yeast able to utilise methanol as its sole carbon source. Catalysed by one of two alcohol oxidase enzymes, methanol substrate is oxidised into formaldehyde and hydrogen peroxide (Cereghino & Cregg, 2000). Alcohol oxidase is encoded by two genes, AOX1 and AOX2, of which the AOX1 gene is predominantly responsible for methanol utilisation, whilst the product of the AOX2 gene displays a reduced methanol metabolising activity (Cregg *et al*, 1989). Alcohol oxidase production is induced at a transcriptional level in the presence of methanol (Ellis *et al*, 1985). *P.pastoris* strains lacking a functional AOX1 gene are termed Mut^s (Methanol utilisation slow) strains as they grow slowly on methanol-containing media (Cregg *et al*, 1989).

Cregg *et al* first described the use of *P.pastoris* as a host system for transformation of foreign DNA carried by plasmid vectors (Cregg *et al*, 1985). Expression of foreign DNA genes requires its integration into the *P.pastoris* genome (Cereghino & Cregg, 2000) by homologous recombination.

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P.pastoris secretes only low levels of endogenous proteins (Cereghino & Cregg, 2000). Thus when recombinant proteins are targeted for secretion, their levels constitute the major portion of total protein expressed, thus simplifying purification of the target protein (Cereghino & Cregg, 2000). Secreted expression of recombinant proteins can be achieved by utilising specific plasmid vectors such as the pPIC9 (**Figure 13**) fusion vector (Invitrogen) which encodes an α-factor secretion signal (S).

This pPIC9 vector is designed for the incorporation of the gene of interest in frame with the initiation codon of the signal sequence. The gene insert is then positioned within the multiple cloning site between a 5' AOX1 gene segment containing AOX1 promoter sequences and a 3' AOX1 fragment containing the AOX1 gene termination sequence (3' AOX1 (TT)), elements common to most *P.pastoris* expression vectors (Cereghino & Cregg, 2000). The 5'AOX1 and 3' AOX1 primer sites of pPIC9 allow for the positive identification of cloned inserts by the polymerase chain reaction (PCR).

The HIS4 gene carried by the vector encodes the histidinol dehydrogenase enzyme and allows for selection of vector recombinants following transformation into *P.pastoris* GS115 strain by complementing the *his*4 genotype of this strain which is unable to produce histidine or grow in its absence (Cregg *et al*, 1985). *P.pastoris* cells carrying the pPIC9 vector thus become His^+ strains. Recombinant pPIC9 vectors can also be grown and selected for in *E.coli* due to their *E.coli* origin of replication, pBR322, and their Ampicillin resistance marker, respectively.

By cleavage of the pPIC9 fusion vector with the *Bgl* II restriction enzyme, His^+ Mut^+ GS115 *P.pastoris* recombinants can be generated by a double recombination event between the 5' and 3' AOX1 homologous regions resulting in the replacement of the GS115 AOX1 gene and subsequent loss of function (Vassileva, 2001).

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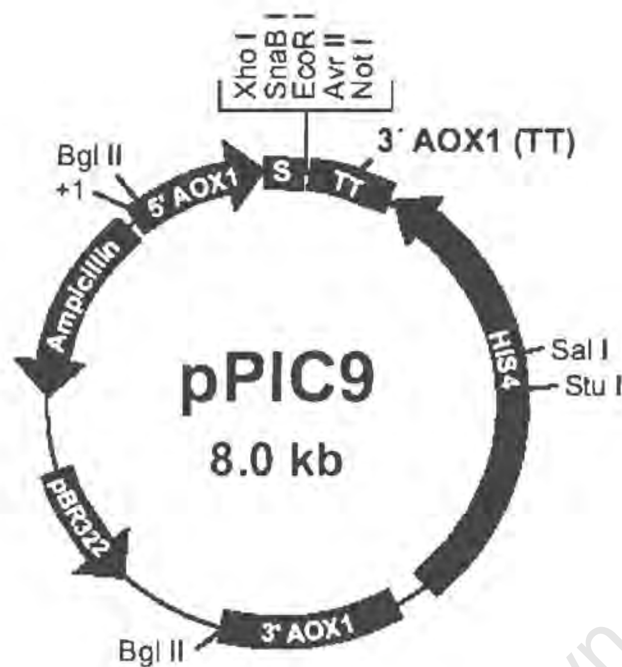

Figure 13 The pPIC9 fusion vector (www.invitrogen.com).

Once positive recombinant *P.pastoris* clones are identified and isolated, mass production of recombinant proteins is made possible by growth in appropriate yeast nutrient media and optimisation of methanol induction. Expressed protein can be concentrated by numerous methods including ammonium sulphate precipitation, filtration or centrifugation.

2. Immunodetection of proteins expressed in *P.pastoris*

Proteins can be identified on the basis of their size and antigenic properties by immunoblotting techniques e.g. western blotting (Rybicki & Purves, 1996; Towbin et al, 1979) or slot-blotting.

Proteins are first separated according to their size (Grierson, 1990) by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Polyacrylamide gels are formed by the polymerisation of acrylamide monomers and their covalent cross-linkage with N, N-methylene-bis-acrylamide (Perbal, 1988) in the presence of a chemical catalyst (Grierson, 1990). These acrylamide cross-links serve as pores through which proteins must pass (Sambrook & Russell, 2001).

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In the presence of an electrophoresis buffer, peptide migration through an acrylamide gel matrix is driven by a current applied between two electrodes of opposite charge and results in peptide migration toward the positively charged electrode, or anode. Due to the amphoteric nature of proteins, which are made up of both acidic and basic residues (Perbal, 1988), an overall negative charge can be inferred on proteins by treatment with the denaturing detergent sodium dodecyl sulphate (SDS). This facilitates protein migration toward the anode during gel electrophoresis. SDS molecules wrap around the polypeptide backbone (Perbal, 1988) and the amount of SDS is proportional to the molecular weight of the protein to which it is bound (Sambrook & Russell, 2001). The size of peptides resolved in this manner can then be established by comparison to molecular weight marker bands of known size (Sambrook & Russell, 2001).

A specific antibody is then used for further identification of the protein of interest. Due to the size restrictive pores of polyacrylamide gels, antibodies are not able to access and bind to proteins whilst still in the gel matrix (Perbal, 1988), thus proteins are first transferred and bound onto a solid support such as a nitrocellulose membrane (Sambrook & Russell, 2001).

An antibody-antigen complex can then be detected by a secondary antibody which recognises common features of the primary antibody and carries a reporter enzyme such as horse-radish peroxidase (Sambrook & Russell, 2001). The peroxidase enzyme catalyses the oxidation of luminol in the presence of hydrogen peroxide creating a reaction intermediate which emits light (Seitz, 1978). This allows detection of antibody-antigen complexes by chemiluminscent techniques. Enhancer molecules such as p-iodophenol, a substituted phenol, may be used to prolong and intensify light emission (Thorpe *et al*, 1985). The light generated by this reaction can be transferred onto an X-ray film and visualised.

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Methods and Materials

1. Methanol induced expression of the 13.8kDa protein in *Pichia pastoris*

P.pastoris GS115 yeast colonies, transformed with pPIC9 cloning vectors containing the Western Reserve (WR) vaccinia virus N1L gene, were obtained from Zhouning Zhang of the University of Louisville. These yeast recombinants all displayed a His^+ Mut^s (Methanol utilisation slow) phenotype.

The growth of *P.pastoris* cultures and induction of protein expression were carried with the use of the *Pichia* Expression Kit (Invitrogen) according to manufacturer instructions with modifications.

Single *P.pastoris* recombinant colonies were streaked onto fresh MMH (Minimal Methanol Histidine) plates (15 g agar in 800 ml ddH$_2$O autoclaved, 1.34% yeast nitrogen base, 4 x 10^{-5}% biotin, 0.5% methanol, 4 x 10^{-3}% histidine, 1 litre final volume) and incubated at 30ºC for 5 days followed by storage at 4ºC. Colonies were sub-cultured in this manner each month to maintain yeast recombinants. For long-term storage of *P.pastoris* clones, 5 ml of YPD broth (10 g yeast extract, 20 g bactopeptone, 20 g dextrose, ddH$_2$O to 1 litre) were inoculated with a single *P.pastoris* colony and incubated at 37ºC on a shaker for 72 h. The culture was separated into 850 microlitres aliquots in sterile 1.5 ml eppindorf tubes. A volume of 167 microlitres of 80% glycerol was added to each aliquot and tubes were shaken gently to mix. Cells in glycerol were stored at -80ºC.

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Single yeast colonies from MMH sub-culture plates were inoculated into respective 5 ml volumes of BMGY (Buffered Glycerol-complex Medium) (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base, 4 x 10^{-5}% biotin, 1% glycerol) in 50 ml conical flasks and incubated at 30ºC in a C25 incubator shaker (New Brunswick Scientists) at 200 rpm for 48 h. The resulting turbid yeast cultures were diluted 1:4 in fresh

BMGY and incubated for a further 48 h with shaking (200 rpm). The glycerol in BMGY is a carbon source which represses heterologous gene expression (Cereghino & Cregg, 2000).

OD_{600} readings were taken at regular intervals. At an OD_{600} reading between 2 and 6, where 1 OD unit corresponds to approximately 5 x 10^7 cells/ml of culture (Invitrogen), cultures were centrifuged at 4 500 rpm in a Heraeus Multifuge 3 L-R benchtop centrifuge for 10 minutes at room temperature to pellet yeast cells. The supernatants were discarded and cell pellets were rinsed briefly in 2 washes of distilled water, resuspended in 0.5 volumes of BMMY (Buffered Methanol-complex Medium) (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base, 4 x 10^{-5}% biotin, 0.5% methanol) and incubated at 30°C with shaking (200 rpm) for 24 h. The methanol in this medium served to initiate the induction of recombinant protein expression.

Both BMGY and BMMY contain phosphate buffer which has a very high buffering capacity and buffers over a range of pH values (Sambrook & Russell, 2001). The yeast extract and peptone in each medium helps to stabilise secreted proteins and limit their proteolysis whilst simultaneously feeding the culture (www.invitrogen.com).

A filter-sterilised 100% methanol solution was added to each BMMY culture to a final concentration of 0.5% every 24 hours for a period of 96 hours. Methanol was added to compensate for loss due to consumption and evaporation (www.invitrogen.com).

Yeast cultures were centrifuged at 4 500 rpm for 10 minutes and the resulting supernatants were further centrifuged at 10 000 rpm for 45 minutes in a RC5B Plus Sorvall centrifuge to sediment remaining cell debris. The supernatants were stored at 4°C for short-term storage or frozen at -20°C in an equal volume of 100% glycerol for long-term storage following filtration through a 0.22 um filter to remove any contaminating bacteria. Filtration was

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carried out at 4°C to prevent protein degradation due to the instability of the 13.8 kDa protein.

A 1 ml aliquot of each yeast culture was taken before each induction step and following the final 24 h incubation step for SDS polyacrylamide gel electrophoresis to establish at which time period and stage of induction optimal recombinant protein expression was achieved. A volume of 7.5 microlitres of loading buffer, made up of 2 parts NuPAGE 4 X LDS (lithium dodecyl sulphate) sample buffer medium (Invitrogen) and 1 part β-mercaptoethanol, was added to 16.5 microlitres of each pre- and post-induction yeast expression supernatant and incubated at 70°C for 10 minutes. The heat combined with the β-mercaptoethanol facilitated the dissociation of proteins before gel loading (Sambrook & Russell, 2001).

The Laemmli (1970) discontinuous SDS-PAGE gel system, which produces optimal protein band resolution (Perbal, 1988) was used. This system is made up of a short stacking gel segment of large pore size in which proteins are placed in a thin stack and thereafter enter a resolving gel in which they are separated according to their size (Sambrook & Russell, 2001). The acrylamide-bisacrylamide concentration of the resolving gel can be varied to resolve different size ranges of protein (Sealey & Southern, 1990) for example; a 15% SDS-PAGE gel separates proteins ranging from 12 to 43 kDa in size (Roskams & Rodgers, 2002).

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A 5% stacking gel (0.67 ml 30% acrylamide-bisacrylamide, 0.5 ml 1 M Tris pH 6.8, 0.04 ml 10% SDS, 0.04 ml 10% ammonium persulphate, 0.004 ml TEMED, 2.7 ml ddH_2O) and 12%-15% resolving gel (5 ml 30% acrylamide-bisacrylamide, 2.5 ml 1.5 M Tris pH 8.8, 0.1 ml 10% SDS, 0.1 ml 10% ammonium persulphate, 0.004 ml TEMED, 2.3 ml ddH_2O) were used for the resolution of *P.pastoris* expression supernatants in a 1XSDS electrophoresis buffer (15.1 g Tris-base, 94 g glycine, 50 ml 10% SDS, ddH_2O to 1 litre). Each acrylamide gel solution was polymerised by the addition of an initiator, ammonium persulphate, and a catalyst, TEMED (N, N, N', N'-tetramethylethylenediamine) (Perbal, 1988).

Gels were stained in Coomassie Brilliant Blue solution (0.25 g coomassie blue dye, 45 ml methanol, 45 ml ddH_2O, 10 ml glacial acetic acid), which binds non-specifically to proteins forming strong, non-covalent bonds and contains methanol and acetic acid which fix proteins prior to staining (Sambrook & Russell, 2001). Following staining in this solution for 30 min at room temperature on an orbital shaker, gels were incubated in destain solution (454 ml methanol, 75 ml glacial acetic acid, ddH_2O to 1 litre) for 40 minutes to 24 h depending on the intensity of the bands of interest. The Coomassie Brilliant Blue stain is able to detect approximately 0.3 micrograms protein per band (Rybicki & Purves, 1996)

2. Concentration of *P.pastoris* expressed proteins

2.1 Concentration by centrifugation

An easier, more convenient method of protein purification involves filtration of *P.pastoris* supernatant containing expressed recombinant proteins through a sterile filter membrane under centrifugal force. A filter of pore-size suitable for the exclusion of proteins larger than that of the protein of interest is utilised and increasing protein concentrations are simply achieved with longer centrifugation periods.

The Millipore YM-10 Centriprep centrifugal filter device was used for concentrating yeast expression supernatants according to manufacturer instructions. This ultrafiltration device contained a 10 kDa cut-off filter membrane.

P.pastoris expression supernatants were loaded into the device sample containers and displaced by the insertion of a filtrate collector into the container creating a small amount of hydrostatic pressure. Each device was locked by an air seal cap and during centrifugation the hydrostatic pressure was amplified by the centrifugal force. Filtration occurred in a direction opposite to that of sedimentation.

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A maximum volume of 15 ml of protein supernatant per centriprep sample container was concentrated at 3 000 x g in a Heraeus Multifuge benchtop centrifuge for 40 minutes to reduce the supernatant volume 5-fold. This was carried out at 4ºC to prevent loss of proteins due to the instability of the 13.8 kDa protein. The filtrate was decanted and retentate remaining in the sample container was centrifuged for an additional 10 minutes at 3 000 x g to reduce the supernatant volume a further 3-fold.

The concentrated *P.pastoris* expression supernatants were analysed by SDS-PAGE as previously described and compared to non-concentrated protein samples to determine differences in 13.8 kDa protein concentration and purity.

3. Immunodetection of the 13.8kDa protein

3.1 Slot Blotting

An anti-rabbit polyclonal 13.8kDa antibody was obtained from the Kotwal lab in the USA and stored at -20ºC. The Minifold II Slot-Blot System (Schleicher & Schuell) was used according to manufacturer instructions for the transfer of proteins onto a nitrocellulose membrane.

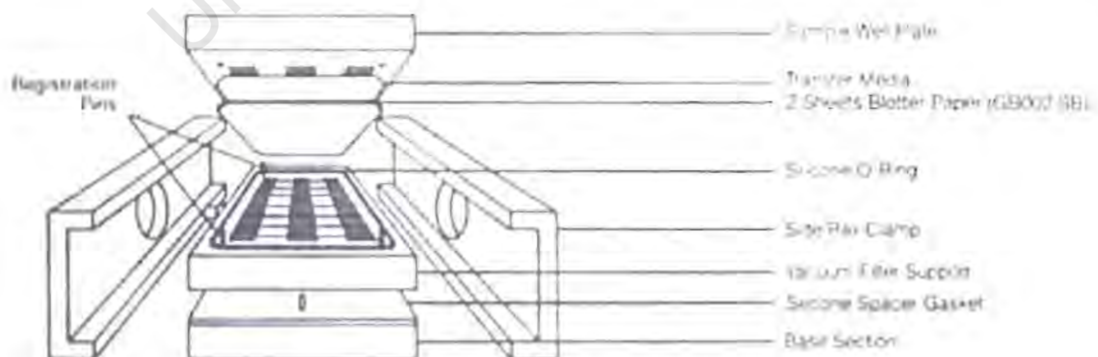

Figure 14 Minifold II Slot-Blot System assemblage

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A sheet of Immobilon-P (Millipore) PVDF (polyvibylidene difluoride) nitrocellulose transfer membrane was pre-wet in 100% methanol for approximately 3 seconds and rinsed in ddH_2O for 3 minutes to remove excess methanol. The nitrocellulose membrane, along with two 3mm Whattman filter paper sheets of like size, was equilibrated in western blot running buffer (48 mM Tris-base, 39 mM glycine, 20% methanol, ddH_2O to 1 litre) for 20 min at room temperature.

The membrane was placed on the underside of the sample well plate (see **Figure 14**) and gently smoothed out to exclude bubbles. The 2 sheets of filter paper were placed on the vacuum filter support (see **Figure 14**). Components were assembled and secured with side rail clamps as indicated in **Figure 14**.

A volume of 20 microlitres of yeast expression supernatant was loaded per well/slot of the sample well plate. A vacuum was applied via a vapour trap connection to the vacuum filter support hose adapter and supernatants were drawn through the nitrocellulose membrane, trapping proteins onto the membrane support and transferring the remaining liquid onto the filter paper sheets. Membrane blocking, antibody probing and antibody complex detection were carried out according to the ECL Western Blotting Detection Reagents Kit (Amersham Biosciences) manufacturer instructions.

The slot-blot apparatus was disassembled and the nitrocellulose membrane was immediately removed and incubated in 5% non-fat milk powder blocking solution in Tris-buffered saline (TBS) pH 7.5 (50 mM Tris-base, 150 mM NaCl, ddH_2O to 1 litre) on an orbital shaker for 1 hour at room temperature or at 4°C overnight. The low fat milk proteins in the blocking solution bind and block regions of the membrane where non-specific antibody binding may occur (Sambrook & Russell, 2001).

The membrane was removed from the blocking solution and briefly rinsed twice in TBS-T (0.1% Tween 20 in TBS). The Tween 20 (polyoxyethylene (20) sorbital monolaurate) in the TBS-T is a non-ionic detergent with weak

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denaturing properties and serves as a good washing solution (Roskams & Rodgers, 2002).

The anti-rabbit 13.8 kDa primary antibody was diluted 1: 10 000 in TBS-T and the membrane was incubated for 1 hour at room temperature with shaking in this dilution. The membrane was rinsed briefly as previously described and thereafter washed in TBS-T for 15 min on an orbital shaker followed by 3 X 5 min successive washes in fresh TBS-T at room temperature.

The ECL Anti-rabbit IgG, peroxidase-linked species-specific whole secondary antibody (from donkey) (Amersham Biosciences) was diluted 1:100 000 in TBS-T and the membrane incubated for 1 hour with shaking at room temperature. The membrane was rinsed and washed as described following primary antibody incubation period.

ECL detection solutions A and B were equilibrated at room temperature and mixed in a ratio of 40:1 (A: B) with a final volume of 0.1 ml/cm^2 of nitrocellulose membrane. In a dark room, the membrane was placed protein side up on a sheet of cling-film and the detection reagent mix pipetted onto the immunoblot so as to cover the entire surface area and incubated at room temperature for 5 min. Excess reagent was then allowed to drain off and the membrane was placed protein side down onto a clean sheet of cling-film and transferred protein side up into a developing cassette. A sheet of X-ray film of like size was placed onto the immunoblot, the cassette closed and the blot exposed for 2 minutes. The X-ray film was remove and replaced with another sheet of film for a longer exposure time period.

Following exposure, the X-ray films were incubated in 1 x developer solution for 2 min, briefly 'swept' through a 2% acetic acid stop solution and finally in 1 x fixing solution for 2 min. The blot was then rinsed briefly in ddH_2O and allowed to dry. Immunoblots were exposed for various time periods to obtain optimal band detection and clarity.

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3.2 Western Blotting

Protein bands resolved on a 15% SDS-PAGE gel were transferred onto a Hybond-ECL nitrocellulose membrane (Amersham Biosciences) using the Bio-Rad Western Blot apparatus.

The gel and nitrocellulose membrane were sandwiched between 2 sheets of Mini-Trans Blot filter paper (Bio-Rad) following equilibration of all sandwich components in pre-cooled western blot transfer buffer. It was essential that the nitrocellulose membrane be of equivalent size to the gel in order to reduce heating effects by minimising the surface area exposed to the electrodes and reducing the amount of current passed through (Rybicki & Purves, 1996).

The sandwich was placed between two electrodes and submerged in transfer buffer. Transfer of proteins onto the membrane was accomplished by applying a voltage (100 volts) perpendicular to the gel (Perbal, 1988) between the two electrode plates for one hour whilst keeping the apparatus cool with a sheet of ice and circulating the buffer on a magnetic stirrer. The methanol in the transfer buffer prevents swelling of the gel (Sambrook & Russell, 2001) and increases the efficiency at which proteins bind to the nitrocellulose membrane, whilst the alkaline pH of the buffer was ensures that all proteins migrate towards the anode (Rybicki & Purves, 1996).

The membrane was removed and stained in Ponceau S solution (0.1 g Ponceau S, 1% glacial acetic acid to 100 ml) for 2 minutes at room temperature on an orbital shaker to confirm transfer. The stain was removed by washing in TBS-T. The membrane was then blocked in 5% non-fat milk powder blocking solution in TBS pH 7.5 and probed with primary antibody as described in section 3.1.

The BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit) (Roche) was used according to manufacturer instructions for further immunoblot processing. Following primary antibody incubation, the blot was washed for two 10 minute sessions with shaking in 30 ml TBS-T each and thereafter in

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two brief washes of 0.5% blocking solution. The IgG-horse-radish peroxidase labelled anti-mouse/rabbit secondary antibody (Roche) was diluted to a concentration of 40 mU/ml in a final volume of 6 ml 0.5% blocking solution and used to probe the immunoblot for 30 minutes at room temperature on an orbital shaker. The membrane was rinsed in TBS-T and washed for four 15 minute sessions in 30 ml TBS-T each.

Detection reagents solution A (luminescence substrate) and B (starting solution) were combined in a 100:1 ratio in a final volume of 125 ul/cm^2 of immunoblot and equilibrated at room temperature. The immunoblot was detected as described in section 3.1 following incubation in the detection reagent mix for 60 seconds. Detected bands were transferred onto X-ray film as previously described.

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Results

1. Expression of the 13.8kDa protein in *Pichia pastoris*

P.pastoris colonies transformed with a pPIC9 fusion vector carrying the Western Reserve N1L gene were grown in nutrient media and recombinant protein expression was induced by the addition of methanol to 0.5% final concentration every 24 hours over a period of 96 hours. Pre- and post-induction expression supernatants were resolved by SDS-PAGE and protein bands were visualised by Coomassie Brilliant Blue staining.

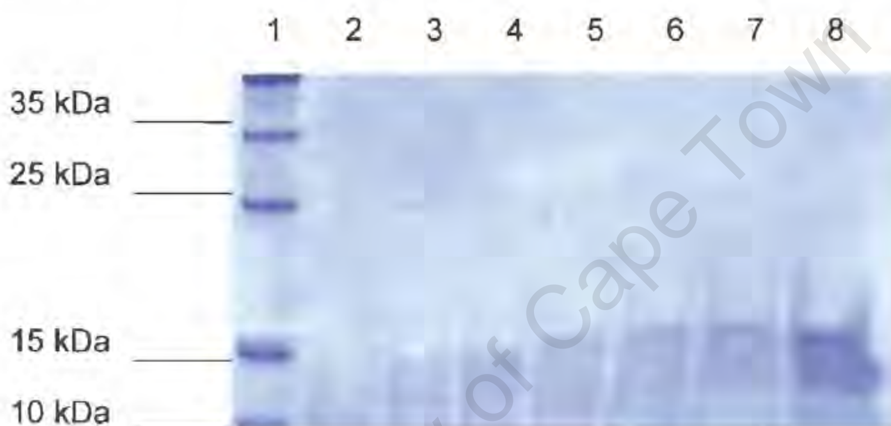

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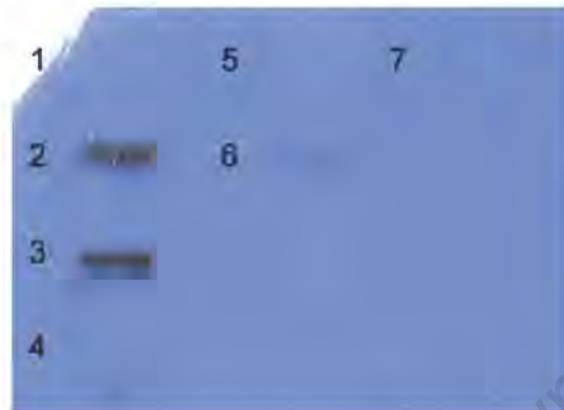
Figure 15 15% SDS-PAGE gel of *P.pastoris* yeast expression supernatants. Lane (1) Promega Broad Range Molecular Weight Marker; protein expression of 13.8 kDa protein (2) pre-induction; (3) 24 h post-induction in BMMY; (4) 24 h; (5) 48 h; (6) 72 h; (7) 96 h post-induction with methanol; (8) YM-10 Centriprep concentrated

2. Immunodetection of the 13.8kDa protein

Proteins contained in *P.pastoris* expression supernatants were immobilised onto nitrocellulose membranes by the slot or western blotting techniques and probed with an anti-rabbit primary antibody. Antibody complexes were detected using chemiluminescent detection techniques.

The 13.8 kDa antibody complex was detected in post-induction expression supernatants of the JL6 and JL4 *P.pastoris* recombinant clones by slot-blot analysis (see **Figure 16**). This complex was not detected in pre-induction samples.



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<u>Figure 16</u> X-ray film of slot-blot of pre- and post-induction *P.pastoris* expressed proteins detected with the 13.8 kDa anti-rabbit primary antibody. Bands correspond to (1) JL6 pre-induction expression supernatant, (2) JL6 expression induced with 1% methanol, (3) retentate and (4) filtrate from concentration of JL6 expression supernatant in YM-10 Centriprep ultrafiltration centrifugal device, (5) JL4 pre-induction expression supernatant, (6) JL6 expression induced with 1% methanol and (7) recombinant VCP (vaccinia virus complement control protein) expressed in *P.pastoris* (negative control).

<u>Figure 17</u> The vaccinia virus 13.8 kDa protein expressed in *P.pastoris* detected by Western blot analysis using polyclonal anti-rabbit 13.8 kDa primary antibody.

Discussion

1. Expression of the 13.8 kDa protein in *Pichia pastoris*

The *Pichia pastoris* yeast expression system proved to be a simple and effective method of protein expression. The Western Reserve vaccinia virus 13.8 kDa protein was successfully expressed and secreted into the extra cellular yeast medium as the major secreted protein with minimal to no *P.pastoris* proteins detected by SDS gel electrophoresis. Rapid protein concentration of small volumes of protein supernatant was achieved by centrifugation in the Millipore Centriprep centrifugal filter device system, which also provided an initial phase of protein purification. A system for the concentration and purification of large volumes of 13.8 kDa expression supernatant remains to be developed.

2. Immunodetection of the 13.8 kDa protein

The Minifold II slot-blot system involves protein sample application over a small surface area and thus produces high signal intensity during detection (Wahl). Due to the presence of numerous *P.pastoris* proteins in expression supernatants, antibody cross-reactivity with proteins other than the recombinant protein of interest cannot be ruled out. In addition, the dense collection of proteins within the slot surface area may obstruct antibody binding to the protein of interest, thus giving no signal output where protein may be present. This may be the case in particular when carrying out a slot-blot on cell extracts.

Western blotting, which incorporates an initial step of protein resolution and separation according to size by SDS-PAGE, is thus a more practical and reliable option for positive identification of recombinant proteins in solutions which have not been purified. Figure 16 displays a single band corresponding to the 13.8 kDa protein. The intensity of this band is less than that of those obtained for the slot blot (**Figure 16**), possibly indicating a loss of protein in

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storage or sample treatment before Western blotting. In conclusion, the vaccinia virus 13.8 kDa was detected and positively identified from *P.pastoris* expression supernatants by two different methods of immunodetection, yet instability in this protein is indicated for long-term storage at 4°C or short-term exposure to temperatures above 4°C.

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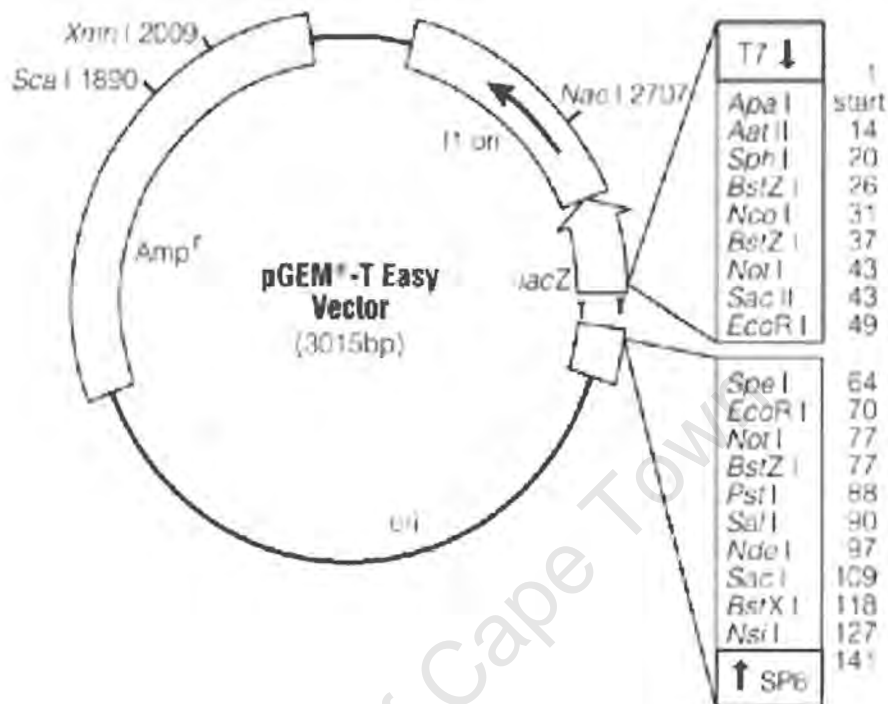
Appendix 1: The pGEM-T Easy Vector



<u>**Figure 15**</u> The pGEM-T Easy cloning vector (www.promega.com). Thymidine (T) residues for the ligation of PCR product adenine overhangs are indicated within the multiple cloning site. Ampicillin resistance (Amp^r) marker as well as SP6 and T7 primer binding sites for sequencing of DNA inserts are indicated.

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