# CHAPTER 1

# LITERATURE REVIEW

## 1.1 INTRODUCTION TO POXVIRUSES

Poxviruses are large DNA-containing viruses which infect both vertebrate and invertebrate hosts and were the first viruses to be visualised by light microscopy (as reviewed by Moss, 2001). An important characteristic of poxviruses is their ability to replicate in the cytoplasm of infected cells. The family *Poxviridae* is divided into two subfamilies, namely the *Chordopoxvirinae* (vertebrate poxviruses) and the *Entemopoxvirinae* (insect poxviruses). These in turn consist of a number of genera (Table 1.1). The *Orthopoxvirus* genus contains the two most well-known poxviruses; variola, which was the causative agent of smallpox and its benign relative, vaccinia virus, which was used successfully as a vaccine against variola and played an important role in the global eradication of smallpox. However, laboratory stocks of variola virus still exist and the potential threat of its use in bio-terrorism has resulted in renewed preparations for mass-vaccination programmes, especially in the USA. Avipoxviruses have been isolated from many avian species and the type member, fowlpox virus, has caused considerable economic losses to the poultry industry (as reviewed by Ghildyal *et al.*, 1989). Capripoxviruses, infecting the livestock species of cattle, sheep and goats, are also economically important (as reviewed by Hunter and Wallace, 2001; Gershon and Black, 1988; Rao and Bandyopadhyay, 2000).

In southern Africa a number of members of the *Poxviridae* family occur. Lumpy skin disease virus (LSDV) of cattle is the only member of the *Capripoxvirus* genus known to occur in South Africa (Weiss, 1968), although all three members of the genus (including sheeppox and goatpox virus) are found throughout the rest of Africa. Avipoxviruses have been isolated from a number of species of southern African birds (Kow, 1992).

Besides vaccinia virus, many poxviruses have been attenuated by serial passage in either cell cultures or on the chorioallantoic membranes of embryonated chicken eggs to serve as live attenuated vaccines (Van Rooyen *et al.*, 1969; Kirmse, 1969; as cited by Taylor and Paoletti, 1988; Winterfield and Reed; 1985).

The development of a recombinant vaccinia virus provided a new strategy for vaccine development (Sam and Dumbell, 1981). Foreign genes could be inserted into non-essential regions of the poxvirus genome using naturally occurring homologous recombination. Expression of the genes, under control of poxvirus promoters, was obtained and it was shown that protective immune responses could be elicited against the expressed proteins. Other

TABLE 1.1 : CLASSIFICATION OF POXVIRUS GENERA*

| SUBFAMILIES | GENERA | SELECTED MEMBER VIRUSES |
|---|---|---|
| *CHORDOPOXVIRINAE* (vertebrate poxviruses) | ORTHOPOXVIRUS | vaccinia, variola |
| | AVIPOXVIRUS | fowlpox, canarypox |
| | CAPRIPOXVIRUS | lumpy skin disease, goatpox, sheeppox |
| | PARAPOXVIRUS | orf |
| | LEPORIPOXVIRUS | shope fibroma |
| | SUIPOXVIRUS | swinepox |
| | YATAPOXVIRUS | tanapox |
| | MOLLUSCIPOXVIRUS | Molluscum contagiosum |
| *ENTEMOPOXVIRINAE* (insect poxviruses) | A | *Melontha melontha* |
| | B | *Amsacta moori* |
| | C | *Chironimus luridus* |

(* - adapted from Moss, 2001)

poxviruses were also then developed as vectors for recombinant vaccines, especially due to concerns arising from complications experienced with vaccinia virus in immunocompromised persons and its broad host-range (as cited by Cadoz *et al.*, 1992). Avipoxviruses and capripoxviruses have been investigated as alternatives (Taylor *et al.*, 1988; Cadoz *et al.*, 1992; Romero *et al.*, 1993), as well as highly attenuated strains of vaccinia virus which are missing a number of genes, especially those associated with virulence and host-range (Scheiflinger *et al.*, 1998). Avipoxviruses have found tremendous favour as they are even

able to express foreign antigens in mammalian cells though unable to complete a productive infection cycle (Taylor and Paoletti, 1988; Stannard *et al.*, 1998).

Capripoxviruses, and in particular strains of LSDV, are being investigated as vaccine vectors for use in the veterinary field for a number of reasons: they are highly host-range restricted (Weiss, 1968), stable (Wallace, 1994), and a number of effective attenuated vaccines have been developed from virulent field isolates (Van Rooyen *et al.*, 1969).

Where they are to be used as vectors for recombinant vaccines in animals which they naturally infect, they also have potential as dual vaccines: a northern African vaccine strain of LSDV (Kenya Sheep-1) expressing either the fusion protein or haemagglutinin protein of rinderpest virus protected cattle against both virulent rinderpest and LSDV challenge (Ngichabe *et al.*, 1997).

Although recent sequence data comparisons have shown that a virulent northern African isolate of LSDV and a virulent southern African isolate share a high degree of sequence homology (Kara *et al.*, 2003), it has been reported that northern African isolates are able to infect more than one ruminant host species (Kitching *et al.*, 1987; Kitching *et al.*, 1989; Gershon and Black, 1989a), whereas there have been no reports of natural capripoxvirus infections in any animals in southern Africa besides cattle (Weiss, 1968). Although the northern African vaccine strains of LSDV have proven safe and effective thus far, there are no guarantees that under the right conditions they might not be able to revert to virulence and infect other ruminant species. There is evidence that at least one isolate of capripoxvirus was formed by a recombination event during its evolutionary history (Gershon *et al.*, 1989b). For these reasons it is inadvisable to use northern African isolates of LSDV as a vector for recombinant vaccines in southern Africa.

Restriction endonuclease studies on the DNA of a number of southern African isolates of LSDV collected over a 40 year period from different geographical locations have shown that the isolates are stable and highly conserved (Wallace, 1994). Thus, the high degree of host-range specificity of the southern African isolates of LSDV makes them prime candidates for development as vectors for recombinant vaccines for use in southern Africa, as well as in other countries where use of a highly host-range restricted vector is a necessity.

The southern African vaccine strain of LSDV was developed from a virulent field isolate and has been in use for over 40 years (Van Rooyen *et al.*, 1969). This highly attenuated strain has proven safe and effective and provides long-term immunity, although the original assertion that protection is life-long has been challenged recently and more frequent vaccinations are now recommended (as reviewed by Weiss, 1968; Hunter and Wallace, 2001).

## 1.2 AIMS OF THIS INVESTIGATION

In lieu of the need for alternative vectors for poxvirus-vectored recombinant vaccines, the high degree of host-range restrictiveness, the potential for use as a dual vaccine and long history of safe and effective use in the field, the South African vaccine strain of LSDV is the obvious choice for development as a vector for recombinant vaccines for use in the veterinary field in southern Africa. In the late 1980's work began in this regard. A number of the earlier studies concentrated on mapping the virus (Perlman, 1993), the use of selectable marker genes for recombinant virus selection (Brand, 1993), identification of unique restriction enzyme sites as potential insertion sites (Wallace, 1994) and identification of potential non-essential genes or intergenic regions as insertion sites (Cohen and Cox, personal communication). More recently Fick (1998) studied transcriptional control and cloned and characterised a bi-directional LSDV promoter (Fick and Viljoen, 1994; Fick and Viljoen, 1999).

The results of these studies provided essential information for developing the virus as a vector. Two independent studies were then initiated to investigate the best route for generating and selecting recombinants. The first study (undertaken at the University of Cape Town's Medical School) concentrated on the use of the ribonucleotide reductase gene as the insertion site and the *Escherichia coli* guanine phosphoribosyltransferase (gpt) gene for dominant selection (Aspden *et al.*, 2002).

The second study, which forms the basis for this doctoral thesis, undertook to investigate the feasibility of using the viral thymidine kinase (TK) gene as the insertion site and on the use of the resulting thymidine kinase-negative phenotype of the virus as the means for recombinant virus selection. The aim of this study was thus as follows:

To evaluate LSDV (type SA vaccine) as a vector for recombinant vaccines against economically important livestock diseases using the viral TK gene as the insertion site.

However, in order to achieve this aim a number of areas had to be addressed, as follows:

(a) Develop a LSDV transfer vector suitable for the insertion of foreign genes into the viral thymidine kinase gene.

(b) Obtain, or develop, a LSDV-permissive cell line suitable for use in thymidine kinase selection of recombinant viruses.

(c) Develop and evaluate a suitable method for generating and selecting recombinants.

(d) Select the recombinants to homogeneity and evaluate stability.

(e) Generate recombinants expressing genes of veterinary importance.

(f) Evaluate the recombinants in animals for their ability to induce a protective immune response.

By way of introduction, the following literature survey provides a general overview of poxvirus biology, with special reference to all aspects of LSDV and the development of poxviruses as vectors for recombinant vaccines.

## 1.3 BIOLOGY OF POXVIRUSES

Vaccinia virus has been extensively characterised and thus serves as the best model to describe general poxvirus characteristics.

### 1.3.1 Virion structure

Vaccinia virions appear by electron microscopy to be oval or brick-shaped bodies approximately 300-400 x 170-260 nanometers (nm) (Westwood *et al.*, 1964). Early studies of electron microphotographs of vaccinia virus revealed the presence of two types of particles; the one form, thought to be the complete form, consisted of a nucleoid with surrounding envelope material and the other, incomplete form, consisted of envelope material with or without nucleoid components (Nagington and Horne, 1962). Thin-sections of virions reveal a lipoprotein bilayer, called the outer membrane, surrounding a central core that encases the viral DNA. The core is surrounded by a palisade layer of rod-shaped molecules (Dales and Pogo, 1981) (Figure 1.1). In vertebrate poxviruses, the core appears biconcave with two structures, called lateral bodies, nestled in the concavities. The lateral bodies are attached to the outer membrane and are ellipsoidal in shape (Medzon and Bauer, 1970). The outer surface of the outer membrane is studded with randomly arranged surface tubule elements (MW 58,000) which give the virion its textured surface (Dales, 1963; Stern and Dales, 1976). Vaccinia virus particles released naturally from cells are called extracellular enveloped virions as they contain an additional lipid-bilayer, called the envelope (Roos *et al.*, 1996). The outer membrane and envelope contain a number of virus-encoded proteins, some of which are important in eliciting an immune response in infected hosts (Payne and Kristensson, 1985).

### 1.3.2 Genomic structure and organisation

Poxvirus genomes consist of a linear, double-stranded DNA molecule which is covalently cross-linked at the terminals (Berns and Silverman, 1970; Gershelin and Berns, 1974; Black *et al.*, 1986). These terminal sequences have been shown to contain repeat sequences which are inverted with respect to one another (Wittek *et al.*, 1978). The inverted terminal repeats of the vaccinia virus genome (strain WR) are each 10 kilobase pairs (kbp) in length (Baroudy *et al.*, 1982; Winters *et al.*, 1985). The termini are incompletely based-paired (bp) giving rise to 104 bp inverted loops which are A-T rich. The termini consist of two sets of 70 bp tandem direct repeats. The first set, consisting of 13 direct repeats, is disrupted by a 325 bp region of unique sequence, and is then followed by the second set of 18 direct repeats.

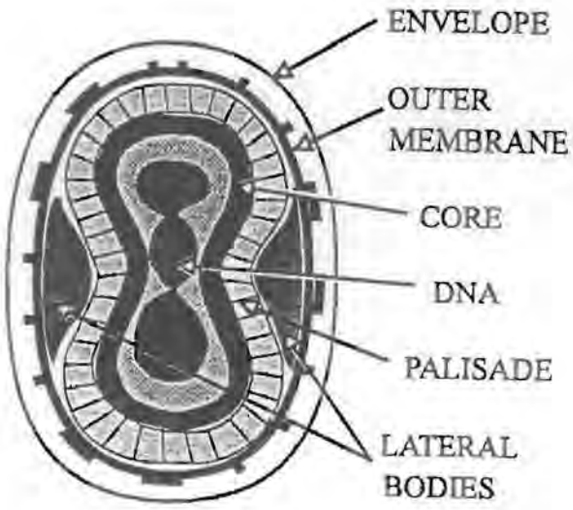



**Figure 1.1** Schematic representation of a poxvirus virion. (Adapted from Dales and Pogo, 1981)

The genomes of poxviruses are large. The genome length of vaccinia virus (Copenhagen) is 191.6 kilobases (kb), potentially coding for 263 proteins greater than 65 amino acids in length (Goebel *et al.*, 1990). At least 30 of these are structural proteins, and the majority are viral enzymes concerned with nucleic acid synthesis and processing (e.g. a multisubunit DNA-

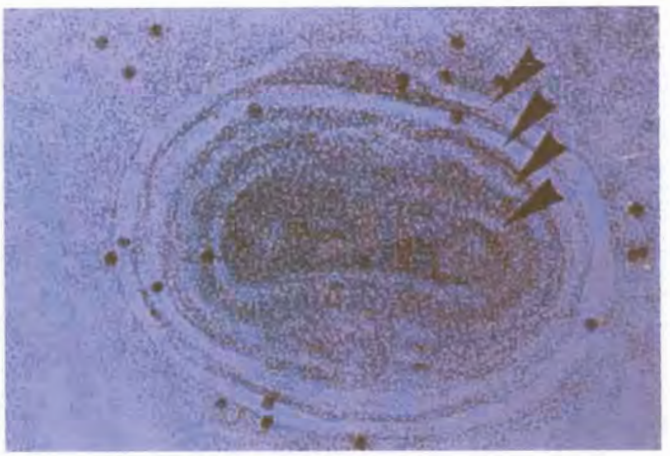


**Figure 1.2.** Electronphotomicrograh of a cryosection through a vaccinia virus EMV (x 80 000) (from Schmelz *et al.*, 1994). Arrowheads indicate membranous layers.

dependant RNA polymerase and a DNA polymerase) (Broyles and Moss, 1986; Challberg and Englund, 1979).

To date 26 complete genome sequences of various poxviruses have been elucidated (Gubser *et al.*, 2004). Chordopoxvirus genomes were found to be considerably more divergent toward their termini than in their central regions. Host-range, virulence and genes associated with interactions with the host immune systems have been mapped towards the termini of poxvirus genomes whereas genes coding for structural proteins and enzymes are placed more centrally (Gillard *et al.*, 1985; Moss, 2001).

### 1.3.3 Virus cell entry

Electron micrographs revealed attachment of enveloped and non-enveloped vaccinia virus to the plasma membrane at the surface of the cell (Dales and Kajioka, 1964). A number of mechanisms have been proposed for virus cell entry. The results of morphological studies suggest that vaccinia may obtain entry by endocytic mechanisms and by fusion of the viral envelope with the host plasma membrane. Experiments using methylamine and chloroquine [lysomotropic agents (weak bases which have the ability to raise the pH of endosomes, thus blocking proteolysis)] showed that these agents increased the *infectivity of vaccinia virus* (Janeczko *et al.*, 1987). Subcellular fractionation studies also revealed the absence of viral polypeptides from endosomes, whereas an abundance of these proteins co-migrated with the plasma membrane fraction. Since plaque formation is not inhibited by lysomotropic agents, pH-independent fusion with the plasma membrane may be the most important entry

mechanism. The exact mechanism of entry now appears to rely upon the type of virion particle: IMVs entering via surface membrane fusion and EEVs by fusion and entry in endosomes with the loss of the outer membrane (Vanderplasschen *et al.*, 1998).

Once inside the cell, the virus undergoes at least two discrete stages of disassembly (Pedley and Cooper, 1987): The first stage is associated with the loss of virion coat proteins and lipids, although the genome is still protected within the core (Holowczak, 1972; Sarov and Joklik, 1972). During the second stage the genome becomes accessible to DNase. A putative, viral-encoded uncoating protein of 23 kDa with trypsin-like activity is thought to be involved in uncoating (Pedley and Cooper, 1987).

### 1.3.4 Gene expression

Almost immediately after entering the cell, and after uncoating, transcription takes place within the core particles producing functional and capped polyadenylated messenger RNA (mRNA) (Brakel and Kates, 1974). ATP is required for the extrusion of transcripts into the cytoplasm where they bind ribosomes for translation. RNA-DNA hybridisation studies revealed that about one-half of the genome is transcribed prior to DNA replication (review by Moss, 2001). Transcription factors have been identified which bind to, and initiate transcription from viral promoters (Li and Broyles, 1993). Although the large genomes are able to code for most of the enzymes required for gene expression, the host cell nucleus is required for the production of infectious virions (Yuwen *et al.*, 1993). Vaccinia virus encoded proteins have been located in the nucleus and one of these (E3L gene product) is able to bind double-stranded RNA. Viral induction of the synthesis of host factors has been reported (Moss and Filler, 1970). These factors are thought to be required for the translation of viral mRNA. During the infection cycle, however, the virus eventually causes the complete inhibtion of host protien synthesis.

Vaccinia virus gene expression is temporal, with classes of early, intermediate and late genes being identifiable (Moss, 2001). Early genes are expressed from 20-100 minutes post infection, intermediate genes from 100-120 minutes post infection and after the onset of DNA replication, whereas late genes are expressed only after replication (140 minutes post infection) (Baldick and Moss, 1993). A number of regulatory mechanims, controlling the switch from early to late gene expression, have been proposed (Moss, 2001). One of these suggests the involvement of regulatory factors which might suppress early gene transcription by binding to transcription factors or early gene promoters (McAuslan, 1963). In addition, early and intermediate gene transcripts have short half lives (30 minutes) providing the basis

for rapid changes in gene expression (Baldick and Moss, 1993). Early protein synthesis is terminated once late gene products are being synthesised, unless the early genes contain late promoter sequences in addition to their early promoters (Cochran *et al.*, 1985). Late gene products consist mainly of structural proteins and enzymes to be packaged within the progeny virions.

The functional elements of promoter sequences are conserved between poxvirus genera; evidence of this is provided by studies demonstrating the ability of the TK promoter of fowlpox virus to function in a recombinant vaccinia virus and of vaccinia virus promoters to function in a recombinant fowlpox virus (Coupar *et al.*, 1990; Prideaux *et al.*, 1990).

A class of early promoters has been identified (Davison and Moss, 1989). They are A-T rich and contain a critical region for activity which corresponds to the downstream site of transcription initiation (AAAAATGAAAAAAAA). Probably the most important enzyme required for transcription is the vaccinia virus RNA polymerase and subunits (Broyles and Pennington, 1990). Another important gene is the DNA topoisomerase (Fogelsong and Bauer, 1984). Inhibition of the activity of this enzyme results in inhibition of transcription of viral genes from intact core particles. Vaccinia virus also codes for an early transcription factor (VETF) which consists of two subunits (70 and 83 kDa) and has ATPase activity required for early gene transcription (Broyles *et al.*, 1988; Broyles and Moss, 1988; Li and Broyles, 1993). An 85 kDa protein (called RAP94) has been identified which is tightly associated with the RNA polymerase, and along with the VETF is important for transcription of early genes.

The 5' ends of early mRNA transcripts are capped by a viral-encoded capping enzyme (Moss *et al.*, 1976) and the 3' ends are polyadenylated by a poly(A) polymerase (Nevirs and Joklik, 1977). A transcription termination factor (VTF) was found associated with the capping enzyme (Shuman *et al.*, 1987). Termination signals (TTTTTNT) were discovered 20 to 50 bp upstream of the transcription termination sites (Yuen and Moss, 1987).

Expression of these genes is dependant upon DNA replication and requires transcription factors coded for by early genes (Wright and Moss, 1989). There appear to be two classes of intermediate genes, viz. early intermediate (consisting of at least two genes) (Vos and Stunnenberg, 1988) and true intermediate (consisting of three genes coding for late gene transcription factors; A1L, A2L and G8R) (Baldick and Moss, 1993). The promoter sequences of the true intermediate genes have been found to contain two critical regions for gene expression (AAATAA and TAAA) (Baldick *et al.*, 1992).

Late gene expression is dependant on replication of the viral genome (Belle *et al.*, 1981) and translation of the three late gene transcription factors. Most late genes occur in the centre region of the genome and code for structural proteins and many of the enzymes destined for packaging within progeny virions. There are two classes of late genes; one which is expressed immediately after DNA replication and the other which is delayed. The promoter sequences of late genes consist of a critical region with the consensus sequence: TAAATG (Weir and Moss, 1984).

A major difference between early and late mRNA transcripts is that late transcripts do not have defined 3' ends and continue downstream through early genes, not recognising early gene termination sequences (Mahr and Roberts, 1984). The 5' capped ends of the late mRNAs also differ from those of early transcripts (Boone and Moss, 1977) and include poly(A) tracts which are thought to be important for ribosomal binding (Ahn and Moss, 1989).

Vaccinia late transcription factor (VLTF-1), VETF and RNA polymerase have been identified in cell extracts purified late in the infection cycle.

Proteolytic maturation of some vaccinia virus proteins (e.g. late gene core proteins 4a, 4b and 25K) occurs via cleavage of precursor polypeptides (Van Slyke *et al.*, 1991). A conserved "cleavage signal" sequence (Alanine-Glycine-Alanine) has been identified.

For LSDV not much work has been performed on gene expression. Sequence comparisons have revealed that the overall genomic organisation is very similar to that of other chordopoxviruses such as suipoxvirus, yatapoxvirus and leporipoxvirus (Tulman *et al.*, 2001). What has been established is that the synthesis of early LSDV mRNA is initiated immediately after infection and continues for nine hours. The transition to late gene transcription occurs approximately 10 hours post-infection and requires DNA replication (Fick and Viljoen, 1994). The identification and characterisation of a LSDV bi-directional promoter showed substantial structural similarities with other poxvirus promoters (Fick and Viljoen, 1999). In transient expression assays the temporal nature of the promoter was verified.

### 1.3.5 DNA replication

Poxviruses replicate in the cytoplasm of infected cells (Dales and Pogo, 1981; Harford *et al.*, 1966). Most, or all of the genes required for DNA replication are encoded by the virus as it is still able to replicate in enucleated cells, although the nucleus is required for the production of

infectious particles (Pennington and Follet, 1974; Yuwen *et al.*, 1993). Replication begins within the first three hours after infection (Harford *et al.*, 1966). Discrete "virus factories" have been identified which are electron dense regions in the cytoplasm where the virus replicates (Moss, 2001). No specific origins of replication have been found in the genome and it is speculated that replication occurs via nicking of the DNA at random points, followed by self-priming and replication giving rise to large concatemeric-branched structures. These might be resolved into unit genomes at a later stage. Concatemeric forms have been isolated when inhibitors of late protein synthesis are added to infected cells. Resolution occurs upon reversal, however, indicating that the concatemers are replicative intermediates. A number of viral enzymes are required to facilitate the high levels of DNA synthesis. Two of these include the vaccinia virus single unit DNA polymerase (MW 110,000) (Challberg and Englund, 1979; Traktman *et al.*, 1984) and a DNA ligase (Moss, 2001). Another enzyme involved in replication is a thymidine kinase (TK) enzyme. It is 20 kDa in size and appears to exist as a tetramer. The TK is important for nucleotide metabolism and is thus needed early in infection, especially in non-replicating infected cells with a low level of thymidylic acid. Other viral enzymes important for replication include a ribonucleotide reductase, which converts ribonucleotides into DNA precursors and a serine/threonine kinase (Banham and Smith, 1992).

Recombination of poxvirus DNA has been linked to replication and could explain the considerable amount of variation in the terminal regions of poxvirus genomes (as reviewed by Buller and Palumbo, 1991). Genetic recombination between orthopoxvirus genomes has been well documented (Ball, 1987; Sam and Dumbell, 1981) and it is thought that a member of the *Capripoxvirus* genus arose by genetic recombination between two other members (Gershon *et al.*, 1989b). The ability of poxvirus genomes to recombine, and to replicate after regions of their genomes have been deleted and replaced with foreign DNA, has resulted in the development of recombinant vector vaccines (Taylor *et al.*, 1988; Cadoz *et al.*, 1992; Taylor *et al.*, 1992).

### 1.3.6 Virus assembly and release

Many of the assembly proteins undergo proteolytic processing prior to assembly. Assembly is initiated in the cytoplasm and the first step is the appearance of crescent-shaped shells with a border of "spicules" on the convex surface and granular material adjacent to the concave surface (Dales and Pogo, 1981). These crescent-shaped shells were thought to consist of a double lipid bilayer membrane derived from the intermediate compartment

between the cellular endoplasmic reticulum and the golgi stacks of the Golgi apparatus (Sodeik *et al.*, 1993; Griffiths *et al.*, 2001), however high resolution electron microscopy points to a single lipid bilayer membrane independent of intermediate compartments (Hollinshead *et al.*, 1999). The spicules (MW 65,000) are thought to provide a scaffolding for the viral assembly (Essani *et al.*, 1982). The immature envelope circularises and the nucleoproteins and DNA genome enter just before it is completely sealed (as reviewed by Buller and Palumbo, 1991). The immature virion then undergoes internal differentiation producing the core and lateral bodies. The surface spicules are replaced by surface tubular elements and the virion attains its brick-like morphological appearance. Mature virions are transported to the cell periphery either in vacuoles or bound in a double membrane (or envelope). There is much controversy over the origin of this second envelope as evidence exists to support an early endosomal origin (Tooze *et al.*, 1993) and an equal degree of evidence exists to support a trans Golgi network origin (Schmelz, 1994). A viral-encoded 42 kDa glycoprotein has been shown to be nesessary for this second enveloping event (Wolffe *et al.*, 1993). Virions may also become associated with a proteinaceous A-type inclusion body which is thought to protect the virus from the external environment (Ichihashi *et al.*, 1971). The virions are released from the cell either by fusion of the surrounding membrane with the plasma membrane or via budding from the cell surface, especially at sites on the microvilli.

### 1.3.7 Host interactions

In evolutionary terms poxviruses are ancient and due to their complexity it is not surprising that they have evolved various mechanisms to interact with their hosts including evading detection by the host's immune system (Gubser *et al.*, 2004).

Shortly after infection viral factors are responsible for shutting down host protein synthesis (Pedley and Cooper, 1984) and DNA replication (Jungwirth and Launer, 1968). Viral homologues of epidermal growth factor actually have a stimulatory effect on cell growth (Buller *et al.*, 1988). However, it is the effects of a broad class of poxviral proteins which help the virus to evade or suppress the host's immune system which have been receiving the most attention in recent years. Poxviruses code for homologues of cellular chemokines, chemokine receptors and intracellular proteins that are involved in signalling pathways and possibly anti-viral activities (Smith *et al.*, 2000; Cao *et al.*, 2002; Harte *et al.*, 2003; as reviewed by Johnston and McFadden, 2003). The proteins have been divided roughly into three classes: virostealth, virotransduction and viromimicry (Nash *et al.*, 1999). Virostealth is characterised by masking of the visible signals associated with virus infection, for example, by reducing the capacity of effector leukocytes to recognise and eliminate infected cells. Virotransducers are intracellular viral proteins that inhibit innate antiviral pathways, such as apoptosis,

proinflammatory cascades, or the induction of the antiviral state. Virotransducers can also target host signal transduction pathways that influence host range.

Viromimicry proteins consist of the virokines and viroreceptors, which are virus-encoded proteins which mimic host cytokines or their receptors respectively. These proteins block extracellular communication signals and promote a protected microenvironment for the virus within normally immuno-exposed tissues.

As the functions of these viral proteins have been elucidated, especially with the use of gene knockout technology (Johnston and McFadden, 2004), their potential for use in immunosuppressive medicine and other therapies has been realised (Dabbagh *et al.*, 2000; Anderson *et al.*, 2003; Essajee and Kaufman, 2004).

## 1.4 – LUMPY SKIN DISEASE VIRUS (LSDV)

### 1.4.1 History and epidemiology

In Zambia in 1929 a new disease of cattle was first noted which manifested itself as large skin nodules (Fig. 1.3) (Morris, 1931). The disease then spread rapidly north and southwards until by the late fifties it was well characterised throughout sub-Saharan Africa (including Madagascar) (von Backstrom, 1945; Thomas and Mare, 1945; Haig, 1957; Burdin and Prydie, 1959; MacOwan, 1959; Ramisse *et al.*, 1969; Nawathe *et al.*, 1978; as reviewed by Woods, 1988). By the late eighties lumpy skin disease (LSD) had spread via Egypt into the Middle East and was noted in an Arabian oryx in Saudi Arabia in 1989 (Ali *et al.*, 1990; House *et al.*, 1990; Abraham and Zissman, 1991; Davies, 1991; Khalafalla *et al.*, 1993; Greth *et al.*, 1992a; Yeruham *et al.*, 1995). The disease is of major economic importance and during one of the first large-scale outbreaks in South Africa, over 8 million cattle were affected with a mortality rate of over 75% (Diesel, 1949). Although more recent outbreaks generally show lower mortality rates, long-term debilitating effects are frequent (Green, 1959; Kitching el al., 1989).

Many attempts were made to isolate the causative agent of LSD. A number of different viruses were isolated from skin lesions on cattle displaying LSD-like symptoms (Alexander *et al.*, 1957; Haig, 1957) and eventually a virus belonging to the poxvirus family was identified as causing true LSD in cattle (Alexander *et al.*, 1957; Munz and Owen, 1966). One of the first purified isolates was a South African isolate named the Neethling isolate, and, thus the virus became known throughout Africa as lumpy skin disease virus (LSDV) (type-Neethling) (Alexander *et al.*, 1957; Weiss, 1963).

LSDV belongs to the *Capripoxvirus* genus (along with goatpox and sheeppox viruses) (Matthews, 1982). Although LSDV is a very recent disease of cattle, sheep and goatpox viruses have been known for centuries and their distribution was widespread, ranging throughout Europe, the Middle East, central Asia and northern Africa. Strict eradication programmes have since removed them from Britain and parts of Europe, and a number of live attenuated vaccine strains have helped control their spread in other regions, although they continue to cause significant economic losses (Kitching, 1983; Kitching, 1986a; Kitching, 1986b; Carn, 1993; Bhanuprakash *et al.*, 2004). Due to the close relatedness of capripoxviruses it has been suggested that they be referred to as one viral species with regional adaptations to specific host species. However, restriction endonuclease analysis, cross-hybridisation studies and, more recently, genomic sequence analysis reveals that the viruses can be distinguished from one another (Davies and Otema, 1981; Kitching and Taylor, 1985; Black *et al.*, 1986; Gershon and Black, 1988; Tulman *et al.*, 2002; Kara *et al.*, 2003).

The three viruses share a common major precipitating antigen (Kitching *et al.*, 1986) making them difficult to distinguish on the basis of serology alone (Davies and Otema, 1981), but, at the same time allowing for the use of heterologous virus for protection (Kitching and Taylor, 1985). For example, in Kenya an attenuated sheeppox virus isolate is used for controlling LSD in cattle (Coakley and Capstick, 1961; Carn, 1993). This phenomenon about LSDV raises some interesting questions as to its origins and natural host reservoirs. LSD is a relatively recent disease of cattle (Morris, 1931). In southern Africa LSDV has only been isolated from cattle and the sub-continent is free from goat and sheeppox viruses (Weiss, 1968). However, in parts of Africa all three capripoxviruses occur simultaneously and in Kenya isolates of LSDV have been recovered from both cattle and sheep (Davies, 1982 – with reference to Davies, 1976; Gershon and Black, 1987; Gershon and Black, 1989a). Serological surveys of game animals in Kenya and Tanzania detected antibodies to LSDV in buffalo (*Syncerus caffer*), greater kudu (*Tragelaphus strepsiceros*), waterbuck (*Kobus ellipsiprymnus* and *K. defassa*), reedbuck (*Redunca arundinum*), impala (*Aepyceros marsupialis*) and giraffe (*Giraffa camelopardalis*) (Davies, 1982; Hedger and Hamblin, 1983; Hamblin *et al.*, 1990; as cited by Greth *et al.*, 1992a). However, it took until 1989 for the first isolation of LSDV from a naturally infected game animal, namely the Arabian oryx (*Oryx leucoryx*) (although antibodies had been detected in captive animals during an earlier survey) (Greth *et al.*, 1992a; Greth *et al.*, 1992b). It is not surprising that this took so long as diseased animals would prove easy prey for large predators such as lions or leopards. Experimental infection of game animals by Young *et al.* (1970) showed a high susceptibility of giraffe and impala to LSDV, whereas buffalo and wildebeest (*Connochaetes gnou*) showed no adverse effects, suggesting their possible role as long-term maintenance hosts.

### 1.4.2 Diagnosis

Diagnosis of LSD is not always easy as the symptoms often resemble those caused by bovine herpes mammalitis virus (originally called "Allerton virus") (Haig, 1957; Burdin, 1959; Capstick, 1959; Prydie and Coakley, 1959; Davies *et al.*, 1971; Woods *et al.*, 1996; as reviewed by Hunter and Wallace, 2001). A pyrexia of 40.0-41.5 °C often occurs quickly after infection, lasting anywhere from one to 10 days (Davies, 1991). This is accompanied by lachrymation, increased nasal and pharyngeal secretions, anorexia, dysgalactia, depression and a disinclination for moving. Within a couple of days large nodules (5-50 mm in diameter) appear on the skin. These may cover the entire body, but this is not always the case (Carn and Kitching, 1995a). Regional lymph nodes become enlarged and may be oedematous. Lesions may also develop in the larynx and trachea, throughout the alimentary tract and especially in the abomasums, where they eventually become necrotic and ulcerate. Mucopurulent nasal discharges, persistent dribbling of infected saliva, coughing and distressed respiration, inflammatory and oedematous swellings of the limbs and genitalia often result and conjunctivitis and keratitis commonly occurs. The skin lesions also become necrotic, and, while some remain *in situ*, where they are recognizable for at least a year, others slough away to leave holes in the skin. This is where the risk of secondary bacterial infection becomes high. Lesions on the udder and teats may cause a mastitis and oedema with secondary infections and can result in sloughing of mammary tissue. Pneumonia is a common and often fatal complication of LSD (de Boom, 1947). Abortion frequently follows the acute infection in cows, and infertility has been a problem in the months succeeding an outbreak. Bulls may have painful lesions of their genitalia, which can prevent them from mounting females and they may remain infertile for four to six months after onset of disease symptoms.

Virus can be isolated from the skin lesions, semen, nasal, lachrymal and pharyngeal secretions, milk and blood (Thomas and Mare, 1945; Henning, 1949; Weiss, 1968; Irons *et al*; 2005). Electron microscopy of skin biopsy samples (Figure 1.4), growth in cell culture and fluorescent antibody staining are just some of the techniques routinely used to detect the presence of virus and antibodies in infected animals (Plowright and Witcomb, 1959; De Lange, 1959; Davies *et al.*, 1971; Prozesky and Barnard, 1982; Binepal *et al.*, 2001). More recently molecular-based tests, including Western-blot analysis, indirect enzyme-linked immunosorbant assays (I-ELISAs) and a diagnostic PCR test have been developed for detecting capripoxviruses allowing for more sensitive and specific detection of viral antibodies and DNA respectively (Chand *et al.*, 1994; Carn *et al.*, 1994a; Carn, 1995; Ireland and Binepal, 1998; Heine *et al.*, 1999; Markoulatos *et al.*, 2000; Hosamani *et al.*, 2004).

**Figure 1.3** Young calf showing undisrupted lumps due to lumpy skin disease virus infection (Photograph by D. Wallace).

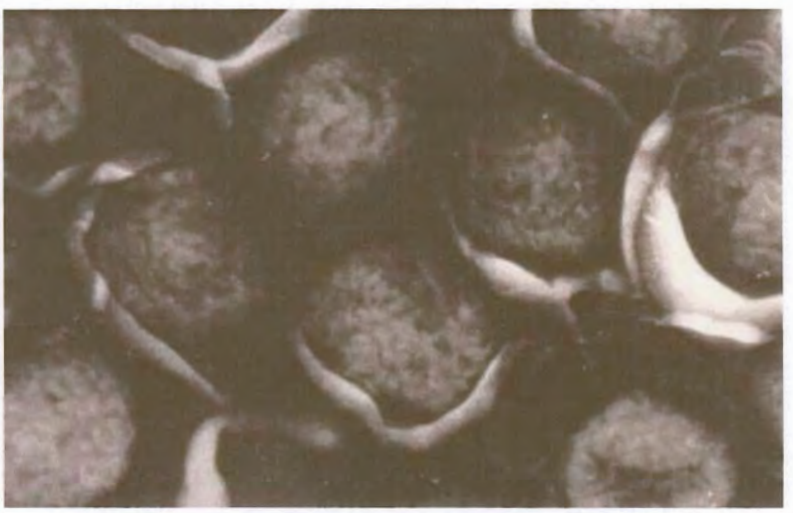


**Figure 1.4** Electronphotomicrograph of clustered lumpy skin disease virus particles (x 75 000). (Photograph kindly supplied by L.M. Stannard, Medical School, University of Cape Town)

### 1.4.3 Transmission

During the first outbreaks of LSD in South Africa in 1944 there was speculation that the transport of cattle was responsible for the spread of the disease, however, once watercourses were reached it spread rapidly along low-lying areas (Thomas and Mare, 1945), suggesting that insects might also be playing an important role. However, many new outbreaks of LSD in sub-Saharan Africa have been related to cattle movements both within a country and from one country to another (Davies, 1991). The introduction of LSD to Egypt followed the importation of cattle from Somalia, where there had been recent outbreaks of LSD. Infected animals might not have shown clear symptoms of the disease, thus avoiding detection by inspectors.

Weiss (1968) described the isolation of virus from the biting fly, *Stomoxys calcitrans* and from *Biomyia fasciata*. Natural capripox infections occur throughout the endemic areas as a result of contact between diseased and susceptible animals (Davies, 1976), however, transmission studies suggest that the most prevalent method for the spread of LSD is mechanical transmission by insects such as *S. calcitrans* (Kitching and Mellor, 1986; Carn and Kitching, 1995b; as reviewed by Carn, 1996), and *Aedes aegypti* mosquitoes (Chihota *et al.*, 2001). Mosquitoes that had fed upon lesions of LSDV-infected cattle were able to transmit virus to susceptible cattle over a period of two to six days post-infective feeding. Virus was isolated from the recipient animals in five out of seven cases. The clinical disease recorded in the animals exposed to the infected mosquitoes was generally of a mild nature, with only one case being moderate.

The outbreak of LSD in Israel in 1989 is thought to have been caused by the aerial spread of LSDV-infected *S. calcitrans* from foci of the disease at El Arish in northern Sinai, or at Ismailiya and the Nile delta in Egypt, a minimum distance of over 85 kms (Yeruham *et al.*, 1995). Epizootics of LSD are associated with high temperatures and high humidity. Cold weather reduces its rate of spread, whereas in warm, moist conditions the disease spreads rapidly (Kitching and Mellor, 1986).

However, not all mechanical insect transmission studies have successfully shown transmission from infected to susceptible cattle suggesting that the other mechanisms might also be involved (Chihota *et al.*, 2003). Attempts to obtain transmission by biting lice (*Hydrotea irritans*) and suckling lice (*Damalina* spp.) were also unsuccessful (Kitching and Mellor, 1986). Carn and Kitching (1995a) attempted to obtain infection by inoculation of LSDV onto the conjunctival sac of susceptible British cattle – however, this also failed. Intradermal inoculation produced local lesions in 80% of animals and generalised infection in the

remaining 20%. By contrast, the intravenous route produced generalised lesions in almost 80% of animals, supporting the role of intravenously feeding arthropods in disease spread.

### 1.4.4 Control

Effectual control in limiting outbreaks of LSD is possible as a number of live, attenuated vaccine strains of LSDV are available. Over 40 years ago a South African field isolate (the "Neethling" isolate) of LSDV was attenuated by serial passage in the chorio-allantoic membranes of embryonated hen's eggs (Weiss, 1963; van Rooyen *et al.*, 1969). Cattle inoculated with this attenuated virus only developed mild local reactions and produced antibodies which persisted upwards of 3 years and were protected from virulent challenge (Weiss, 1968). Originally it was accepted that protection was life-long, however, more recently there have been reports of vaccine failures and annual immunisation is now recommended (Hunter and Wallace, 2001). Not all cattle produce circulating antibodies after vaccination, but they are still resistant to challenge demonstrating the protective role of cell-mediated immunity (Andrew *et al.*, 1989). Those animals that don't sero-convert are unable to confer collostral antibody protection to suckling calves and there are reports of vaccinated dams giving birth to calves with full-blown LSD (Hunter and Wallace, 2001).

Capstick and Coackley (1961) describe the tissue culture production of the Kedong Valley strain of sheeppox virus for use as a vaccine for protecting cattle against LSDV. Conversely, Davies (1976) (as cited by Davies, 1982 and Kitching *et al.*, 1987) describes the isolation of the 0240 capripox virus isolate from a sheep. Attenuation of this isolate gave rise to a vaccine to control sheep and goatpox (called Kenya sheep and goatpox virus, later re-named Kenya Sheep-1 [KS-1]). Sequence analysis of KS-1 revealed that it was almost identical to Kenya Cattle-1 (KC-1), a cattle isolate of LSDV (Gershon and Black, 1989b; Gershon *et al.*, 1989a).

Carn *et al.* (1994b) investigated the possibility of using purified capripoxvirus P32 antigen as a subunit vaccine for priming the immune response of exotic animals from capripoxvirus-free countries before introduction into capripoxvirus-endemic regions. Their results showed favourable responses, although they concluded that complete immunity would best be obtainable with conventional live vaccines.

Except in Israel due to the rapid response of the authorities in the diagnosis and slaughtering of all diseased and in-contact cattle, LSD has not been eradicated from any country in which it has appeared (Davies, 1991). LSD is classified as a group A infectious disease by the OIE and thus countries at risk are encouraged to follow certain guidelines to control the spread of the disease. Annual vaccination is recommended with newly born calves receiving vaccine within 10 days of birth. As the natural host reservoirs of LSDV are unknown, and, as farmers don't always vaccinate their animals, especially during years in which there is disease

quiescence, sporadic outbreaks continue to occur. Until stricter control measures are enforced and more is learnt about the disease, LSD will continue to be of major economic importance.

### 1.4.5 Genomic characterisation

Over the past 20 years molecular tools have been used to compare the genomes of capripoxviruses using restriction enzyme analysis and sequence data (Black *et al.*, 1986; Gershon and Black, 1987; Gershon and Black, 1988). *Hind*III, *Pst*I, *Ava*I and *Sal*I restriction enzyme maps of the genomes of cattle, sheep and goat capripoxvirus isolates have been compared with each other and with those of isolates from related animals from different geographical regions. The results of these comparisons have allowed the division of the capripoxviruses into 5 groups: group 1 isolates are restricted to sheep (e.g. India sheep-1), group 2 isolates to goats (e.g. Iraq goat-1), group 3 isolates infect sheep, goats and cattle, but, are restricted to Africa (e.g. KS-1), group 4 isolates infect either sheep or goats in Africa or the Middle East and group 5 isolates are restricted to cattle in southern Africa (e.g. LSDV-Neethling) (Gershon *et al.*, 1989a).

A comparison of the whole genome organisation of the sheep LSDV isolate, KS-1, with the Western Reserve (WR) strain of vaccinia virus (VV) using cross-hybridisation studies revealed a similar pattern of gene arrangement as observed within the *Orthopoxvirus* genus (Esposito and Knight, 1985; Gershon *et al.*, 1989a) with housekeeping genes constituting the majority of genes found within the highly conserved central regions and host-range and virulence genes situated towards the more divergent termini. KS-1 lacks approximately 45 kb of DNA found in the terminal regions of VV making it one of the smallest poxvirus genomes along with the parapoxviruses (Robinson *et al.*, 1987). Early estimates of the genome lengths of northern African isolates of capripoxviruses, including KC-1, using pulse-field gel electrophoresis technology arrived at an average genome length of 148 kbp (Gershon and Black, 1987; Gershon and Black, 1988). Using similar methodology, the genome length of the South African vaccine strain of LSDV was estimated to be 152,6 kbp (Perlman, 1993). Recently, however, the genomes of the 2490 Kenyan field isolate of LSDV (Tulman *et al.*, 2001), the South African vaccine strain, and a field isolate from an outbreak of LSD in 2001 in the Northern Province in South Africa (Warmbaths) (Kara *et al.*, 2003) have been sequenced to completion (except for the terminal hairpin loops). The assembled sequences yielded genome lengths of 150,8 kbp, 150,5 kbp and 150,8 kbp respectively consisting of 156 putative genes. The sequences of the geographically distinct virulent field isolates were highly conserved with only 18% of the genes containing between one and a maximum of three amino acid changes. On the other hand, the highly cell-passaged and attenuated SA vaccine

strain contains amino acid changes in 76% of its genes compared to the Warmbaths isolate. A thorough investigation of these changes may help to elucidate some of the mechanisms of attenuation which might help in developing an even more effective LSDV vaccine.

From the genome sequences, LSDV has been shown to most closely resemble leporipoxviruses in gene content and organisation, although it also contains homologues of interleukin-10 (IL-10), IL-1 binding proteins, G protein-coupled CC chemokine receptors, and epidermal growth factor-like proteins which are found in other poxviruses (Gershon and Black, 1989a; Cao *et al.*, 1995, Tulman *et al.*, 2001).

## 1.5 – POXVIRUSES AS VECTORS FOR RECOMBINANT VACCINES

### 1.5.1 Introduction

A number of poxviruses from different genera are being developed as potential vectors for recombinant vaccines for use in the medical and veterinary fields. Poxviruses are able to present foreign antigens on the surface of infected cells, which in turn induce cellular and humoral immune responses depending upon the specific poxvirus promoter controlling their expression (Coupar *et al.*, 1986; Andrew *et al.*, 1989).

VV was the first poxvirus to be developed as a eukaryotic cloning and expression vector, and thereafter as a vector for recombinant vaccines (Mackett *et al.*, 1982; Smith *et al.*, 1983a; Smith *et al.*, 1983b). One of its most successful early applications was as a vector for a recombinant rabies vaccine developed for oral vaccination of foxes against rabies in central Europe (Kieny *et al.*, 1984; Blancou *et al.*, 1986; Brochier *et al.*, 1991). Yet there is concern over the safety of VV, especially in immunocompromised persons - in September 2000 a pregnant woman in Ohio (USA) was bitten by her dog while attempting to remove a VV-rabies recombinant vaccine bait from its mouth (Rupprecht *et al.*, 2001). The woman subsequently developed fever, swelling of the bitten arm (with blistering) accompanied by inflammation. Electron microscopy, polymerase chain reaction (PCR) and serology confirmed the presence of the recombinant vaccine from various tissue samples. She fortuitously recovered fully and there were no apparent ill effects to her unborn child. Thus in the veterinary field other poxviruses, including avipoxviruses and capripoxviruses (refer to next section), are being developed as alternatives to VV as vectors as they are more host-range restrictive and have already been used as effective and safe live attenuated vaccines in animals for many years (van Rooyen *et al.*, 1969; Cadoz *et al.*, 1992; Romero *et al.*, 1993; Romero *et al.*, 1994a; Romero *et al.*, 1994b; Wade-Evans *et al.*, 1996; Fries *et al.*, 1996; Karaca *et al.*, 1998;

Amano *et al.*, 1999). A fowlpox virus (FPV) recombinant co-expressing chicken type I interferon and Newcastle disease virus (NDV) hemagglutinin-neuraminidase (HN) and fusion (F) genes protected chickens against challenge with virulent FPV and NDV (Karaca *et al.*, 1998). In a similar study by Viljoen *et al.* (2003) it was shown that expression of just the F gene offered the highest levels and most consistent protection when administered via the wing-web route.

For safer human vaccines a number of poxviruses are being investigated, including avipoxviruses as their ability to undergo early stage gene expression in mammalian cells allows for the induction of an immune response although they are unable to complete a productive infection cycle (Stannard *et al.*, 1998; Vazquez-Blomquist *et al.*, 2002). A canary poxvirus-rabies recombinant vaccine injected into human volunteers induced presumptive protective levels of rabies-specific antibodies (Fries *et al.*, 1996).

Highly attenuated strains of VV lacking virulence and host-range genes, such as modified vaccinia Ankara (MVA) and NYVAC, are also being considered for human use (Blanchard *et al.*, 1998; Hel *et al.*, 2002).

For the development of poxviruses as vectors for recombinant vaccines a number of important factors were taken into consideration including the large size of their DNA genomes (ranging from 140-300 kbp), the lack of infectivity of isolated viral DNA, the packaging of viral enzymes necessary for transcription within the infectious virions, poxvirus-specific promoters, and the cytoplasmic site of virus transcription and replication (Mackett *et al.*, 1982). The largest technological problems which had to be overcome involved insertion of DNA into the large genome, efficient expression of heterologous genes and selection of recombinant virus.

### 1.5.2 Strategies for generating recombinants

Homologous recombination was the first (and still most widely used) approach for inserting the foreign DNA into specific sites in the poxviral genome using transfection of either viral DNA (Sam and Dumbell, 1981) or plasmids containing viral DNA sequences (Weir *et al.*, 1982) into virus-infected cells. Falkner and Moss (1990) provide a useful schematic to describe this process (Fig. 1.5 A).

The coding sequence for the foreign protein to be expressed is inserted downstream of a poxvirus promoter flanked by sequences homologous to a nonessential region of the poxvirus genome (Fig. 1.5 B). The original transfection methods involved the use of calcium-phosphate to precipitate the viral and plasmid DNA (Weir *et al.*, 1982), however, as technology has

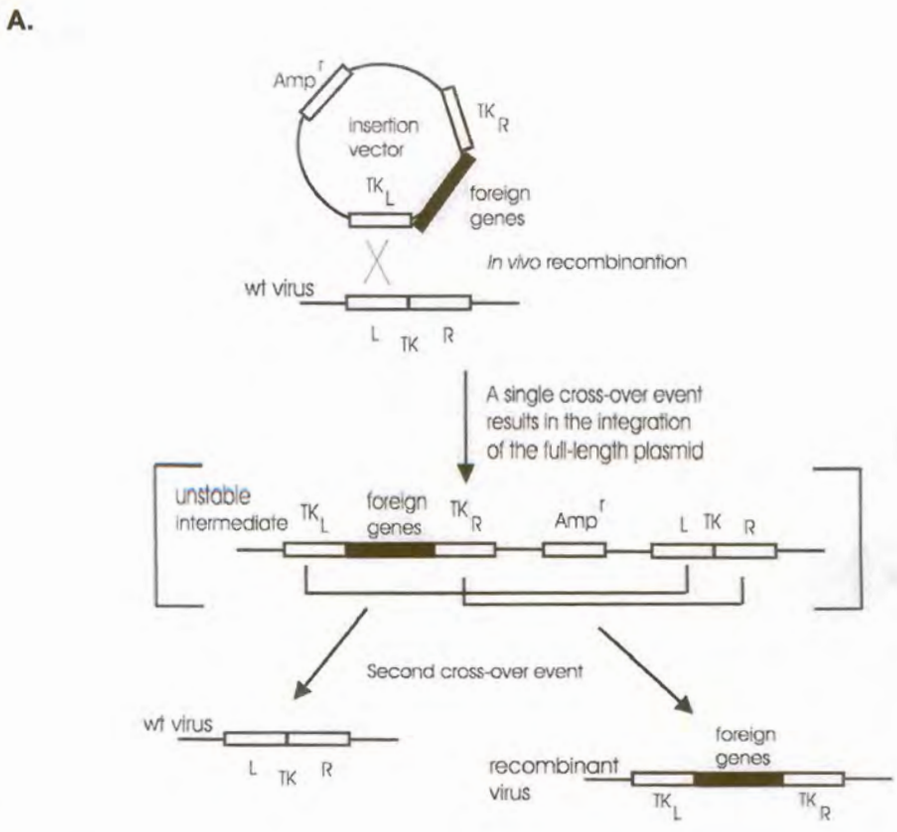



**Figure 1.5** Schematic representation of homologous recombination between viral and plasmid DNA. **A** - The insertion vector/plasmid contains sequences homologous to a non-essential region of the viral genome (e.g. the TK gene) interrupted by the foreign genes (selectable marker genes, reporter genes and immunogenic genes under control of poxvirus promoters – **B**) for insertion into the viral genome. After transfection of virus-infected cells with the insertion vector, homologous recombination occurs between the homologous regions of the viral and plasmid DNA during viral DNA replication. This results in integration of the insertion vector into the viral genome. A second round of recombination then occurs due to the presence of direct repeat sequences, resulting in either wild type (wt) virus, or recombinant virus containing the desired foreign genes (Adapted from Falkner and Moss, 1990).

advanced, better and more convenient methods have been developed e.g. the use of liposomal-based delivery systems (Romero *et al.*, 1993).

*In vitro* direct ligation using a helper virus for recovery of recombinants is another method used for the insertion of foreign genes into the poxviral genome (Merchlinsky and Moss, 1992). Modifications of this method have involved the insertion of unique restriction enzyme sites into the poxviral genome preceded by strong poxvirus promoters to drive efficient expression of the inserted genes (Pfleiderer *et al.*, 1995; Merchlinsky *et al.*, 1997). Although these techniques allow for the insertion of large pieces of DNA, do not require drug selection nor intermediate bacterial plasmid vectors, they have not found general favour due to the difficulties involved in manipulating the large DNA and the need for helper viruses.

A method for overcoming this problem has been to combine *in vitro* ligation with homologous recombination and to use psoralen and long-wave UV irradiation to "create" helper viruses (Timiryasova *et al.*, 2001). This method generates an extremely high percentage (> 90 %) of homologous recombinants without the need for drug selection.

For the expression of the inserted/foreign genes naturally occurring and synthetic poxvirus promoters are inserted upstream to drive transcription (Mackett *et al.*, 1982; Davison and Moss, 1990). It has also been found that due to a high degree of conservation of the structural domains of poxvirus promoters, even between different genera, it is possible to drive expression of foreign genes using promoters from heterologous poxvirus hosts (Prideaux *et al.*, 1990).
As for the selection of sites for the insertion of foreign genes into the poxviral genome, Dubbs and Kit (1964) described the natural occurrence of a number of VV mutants deficient in thymidine kinase (TK) activity, and the selection of these TK-negative mutants in mouse cells using 5-bromo-2'-deoxy-uridine (BUdR). Once the location of the TK gene in the viral genome had been mapped (Weir *et al.*, 1982) it became possible to insert foreign genes into the viral TK gene and to select for recombinants on the basis of their TK-negative phenotype (Mackett *et al.*, 1985). However, TK gene activity does not appear to be non-essential for all poxviruses (Nazerian and Dhawale, 1991; Letellier, 1993; Scheiflinger *et al.*, 1997), and, thus, alternative insertion sites have been utilized (Lorenzo and Blasco, 1998).

### 1.5.3 Selection of recombinants

Besides the insertional inactivation of the viral TK gene as a means for selecting recombinants, a number of other methods have been developed – the common aim of all of them being the eventual selection of a homogeneous pool of recombinants, free from contaminating wild-type (or, parental) virus. The introduction of dominant selectable marker genes, such as the *Escherichia coli* guanine phosphoribosyl transferase (gpt) gene, under control of poxvirus promoters into the DNA insertion vectors, is one such method. Mycophenolic acid (MPA), an inhibitor of purine metabolism, has been shown to block the replication of VV in normal cell lines. However, recombinant viruses expressing gpt are able to replicate in selection medium containing MPA, xanthine and hypoxanthine (Falkner and Moss, 1988). Transient dominant selection allows for the serial construction of recombinants containing several foreign genes; the gpt gene is cloned outside of the viral insertion segments resulting in its loss after selection pressure is removed (Falkner and Moss, 1990). An improvement upon this procedure is called transient marker stabilization in which the marker genes are inserted within the viral flanking regions, however they are flanked by tandem repeated DNA sequences. Thus, when selection pressure is lifted the marker gene is rapidly lost by recombination (Scheiflinger *et al.*, 1998). This method is highly efficient and requires only a few plaque purification steps to select marker-free recombinant virus. The loss of the marker genes from the recombinants is a highly desirable situation, especially for recombinants targeted for vaccine development due to concerns over the release of genetically modified organisms containing antibiotic resistance genes.

Visual markers are also used for selection. The *E. coli* β–galactosidase gene has been widely used due to its property of producing blue plaques in the presence of the chromophore, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) when expressed from recombinant viruses (Chakrabarti *et al.*, 1985; Panicali *et al.*, 1986). Insertion of foreign genes into the β–galactosidase gene provides a further means of selection as plaques then lose their ability to stain blue. A more recent approach utilises the green fluorescent protein gene from the jellyfish, *Aequorea victoria*, as a visual marker by fusing the gene with an antibiotic resistance gene to produce a bifunctional enzyme (BiZyme) (Hansen *et al.*, 2002).

Host-range genes have also been exploited. Their presence confers the ability of the virus to multiply and/or spread in certain additional cell types although their exact functions may be quite diverse (Spehner *et al.*, 1988; Perkus *et al.*, 1989; Smith *et al.*, 1993; Lanar *et al.*, 1996). The VV K1L host-range gene is necessary for growth of the virus in a rabbit kidney cell line (RK-13), but not for growth in monkey kidney cells (Smith *et al.*, 1993). A VV mutant was constructed with parts of this gene deleted. An insertion plasmid containing the hepatitis B surface antigen gene and the complete K1L gene was then used to generate and select recombinant viruses on RK-13 cells.

### 1.5.4 Future prospects

Poxviruses as vehicles for gene expression and recombinant vaccines have come a long way in almost 30 years. This period has seen vast improvements in selection strategies, vaccination regimes and applications (Kwak *et al.*, 2004). Now that the genomes of many poxviruses have been sequenced it has become possible to assign functionality to most poxvirus genes. This has opened up new avenues for the design of improved vectors and for some of these genes to be utilised in areas of immunotherapy and cancer therapy (Jackson *et al.*, 2001; Boyle *et al.*, 2004). Concerns over the use of smallpox virus as a weapon for bioterrorism have accelerated efforts to design better antiviral drugs (Painter and Hostetler, 2004) and recombinant poxviruses are being developed for the testing of these drugs in primates (Jahrling *et al.*, 2004). Poxviruses are even being used as delivery vehicles for gene and anti-tumor therapy (Zhi *et al.*, 2002; Flanagan *et al.*, 2004).

### 1.5.5 Development of capripoxviruses as vectors for recombinant vaccines

Attenuated vaccine strains of capripoxviruses have been in use for many years. These have proven safe, reliable and provide long-term protection, although there have been reports of severe adverse reactions to vaccination of dairy cattle with the Kenyan KS-1 (0240) vaccine strain of LSDV (Yeruham *et al.*, 1994). Capripoxviruses are highly host-range restricted, although certain strains appear able to cross-infect the three host livestock species. These characteristics have made the vaccine strains ideal candidates as vectors for recombinant vaccines against other livestock diseases, with the added advantage of the potential for dual protection. A number of recombinant vaccines using the KS-1 isolate of LSDV as vector have been developed and tested in field trials in Kenya (Romero *et al.*, 1994a; Romero *et al.*, 1994b). Recombinants expressing either the fusion or haemagglutinin genes of rinderpest virus were used successfully in field trials in Kenya to protect cattle against both rinderpest and LSD (Ngichabe *et al.*, 1997), whereas KS-1 recombinants expressing the major core structural protein (VP7) of bluetongue virus (BTV) only provided partial protection to sheep against a virulent heterotypic BTV challenge (Wade-Evans *et al.*, 1996).

Although both LSDV (Neethling) and KS-1 fall within the *Capripoxvirus* genus, they exhibit a number of significant differences in their epidemiological characteristics, geographical distribution and genomic structure (Weiss, 1968; Black *et al.*, 1986). The Neethling isolate of LSDV was discovered in the North Western Province of South Africa in 1944 (Thomas and Mare, 1945). Since then, epidemics have occurred every few years in South Africa, as well as in neighbouring countries. An early field isolate was attenuated by passage on the

chorioallantoic membrane of chicken embryos to produce an effective vaccine (van Rooyen *et. al.*, 1969). Genomic studies on the vaccine and field isolates from southern Africa have revealed minor differences in their terminal regions, whereas comparative studies with isolates from Kenya showed more major differences (Gershon and Black, 1988; Perlman, 1993, Wallace, 1994). These differences might also be reflected in the host-range restrictiveness of LSDV (Neethling), and closely related strains, to natural infection in only cattle, whereas the Kenyan strains are able to infect cattle, sheep and goats (Weiss, 1968; Gershon and Black, 1988).

Sequencing of a virulent field isolate of LSDV from Kenya and comparison with a field isolate from South Africa and the South African vaccine strain has revealed that only minor differences occur between the field isolates, whereas the vaccine strain exhibits many deletions by comparison (Tulman *et al.*, 2001; Kara *et al.*, 2003). It will be interesting to determine which of the genetic differences between the northern and southern field isolates are responsible for the differences in the degree of host-range restrictiveness.

Due to these differences independent studies were initiated to develop both the northern and southern vaccine strains of LSDV as vectors for recombinant vaccines.

For the development of the first capripoxvirus-vectored recombinant vaccines the TK gene of KS-1 was chosen as the insertion site as this gene was shown to be non-essential for VV (Romero *et al.*, 1993). The fusion (F) gene of rinderpest virus (RPV) was inserted under control of the VV late P11K promoter and the *E. coli* gpt gene was used for positive selection of recombinant viruses. Resulting recombinants were confirmed for the presence of the F and gpt genes by Southern transfer and radioactive probe hybridisation. Expression of the F gene was confirmed using immunofluorescence with an anti-F monoclonal antibody. Virus was then grown to high titres and cattle were immunised. Three weeks later they were challenged with virulent RPV and three weeks after that with virulent LSDV. All the vaccinated animals survived both challenges proving the effectiveness of the KS-1 strain of LSDV as a recombinant vaccine vector able to offer dual protection (Romero *et al.*, 1994a).

A similar recombinant vaccine was then developed expressing the haemagglutinin (H) gene of RPV also under control of the VV P11K promoter. In this trial it was shown that although most cattle were protected from virulent RPV challenge, the presence of neutralising antibodies was not always enough for protection, thus suggesting that cell-mediated immunity plays an important role (Romero *et al.*, 1994b). A mixture of both recombinants provided long-term protection against both RPV and LSDV challenge in cattle (Ngichabe *et al.*, 1997).

These constructs were also shown to protect goats against challenge with virulent peste des petits ruminants virus (PPRV) (Romero *et al.*, 1995), proving more effective than a mixture of live, attenuated vaccines against PPRV and capripox (Martrenchar *et al.*, 1997).

For an even more efficient KS-1 vectored recombinant vaccine against PPRV, the F gene of PPRV was inserted under control of the VV P7.5K early/late promoter (Berhe *et al.*, 2003). Proteins under control of this promoter are known to stimulate both a humoral and cell-mediated immune response (Coupar *et al.*, 1986; Andrew *et al.*, 1989). Using the sonication and PCR testing method (as developed and described in this thesis, Chapter 3) they were able to obtain pure recombinants which conferred protection to goats against virulent PPRV challenge at a low dose of 0.1 plaque forming units (pfu).

Work on developing the southern African vaccine strain of LSDV as a recombinant vaccine vector has been ongoing since 1988. As mentioned earlier ("Aims of this investigation"), initial efforts were aimed at mapping the viral genome, identifying suitable insertion sites, selection markers and promoters. While the direction taken by ourselves at the Onderstepoort Veterinary Institute was that of the viral TK gene as an insertion site (as presented in this thesis), our colleagues at the University of Cape Town's Medical School (under the leadership of Professor Keith Dumbell and Dr Anna-Lise Williamson) investigated alternative insertion sites (Cohen and Cox, personal communication). They identified the ribonucleotide reductase (RR) gene and an intergenic region as potential insertion sites. The RR gene was inserted into a transfer plasmid interrupted by the *E. coli* gpt positive selectable marker gene, the lacZ visual marker gene and the rabies virus glycoprotein gene under control of the fowlpox virus early/late pAF promoter (Aspden *et al.*, 2002). Recombinants were generated and selected and inoculated into cattle once expression of the glycoprotein gene had been confirmed. They were able to show that both humoral and cell-mediated immune responses were elicited against the glycoprotein. They were also able to show the potential of the recombinant to be used as a vaccine in non-ruminant hosts (Aspden *et al.*, 2003). One drawback of their work, however, was that their recombinant was not purified to homogeneity, and thus upscale growth of the recombinant virus should be performed under selection pressure. The development of a repeatable method for selecting recombinants to homogeneity forms an important part of this doctoral thesis.

In the following chapters methods for generating recombinants using the TK gene of LSDV (Neethling) as the insertion site are described as well as a method for selecting the resulting recombinants to homogeneity. Recombinants expressing immunogenic genes of viruses of veterinary importance were then generated and their ability to protect animals against virulent challenge evaluated.

# CHAPTER 2

# GENERATION AND SELECTION OF TK-DISRUPTED LSDV RECOMBINANTS

## 2.1 INTRODUCTION

A number of methods have been developed over the years for the generation and selection of poxvirus recombinants (Mackett *et al.*, 1982; Mackett *et al.*, 1985; Chakrabarti *et al.*, 1985; Evans *et al.*, 1988; Guo *et al.*, 1989; Falkner and Moss, 1990; Merchlinsky and Moss, 1992; Cadoz *et al.*, 1992; Scheiflinger *et al.*, 1998; Timiryasova *et al.*, 2001; Boyle *et al.*, 2004).

In order to generate LSDV recombinants using the SA vaccine strain of LSDV our laboratory decided to follow the approach most commonly used to generate VV recombinants (Mackett *et al.*, 1985; Boyle and Coupar, 1986), viz. foreign gene insertion into the viral thymidine kinase (TK) gene making use of naturally occurring homologous recombination with selection on the basis of the resulting TK-negative viral phenotype (generally referred to as the TK-negative selection method). This method was possible as the TK gene of VV was found to be non-essential for normal growth of the virus (Dubbs and Kit, 1964). The same situation has been found for most, but not all poxviruses (Nazerian and Dhawale, 1991). For example with the avipoxvirus, pigeonpox virus, insertion into the TK gene resulted in the generation of unstable recombinants (Letellier, 1993). A similar situation was found for certain highly attenuated strains of fowlpox virus (Scheiflinger *et al.*, 1997). For both poxviruses other non-essential regions had to be utilised for generating stable recombinants.

Recombinants have been successfully generated from the northern African KS-1 strain of LSDV using the viral TK gene for insertion (Romero *et al.*, 1993; Wade-Evans *et al.*, 1996) and thus this site should prove suitable for use with the South African vaccine strain of LSDV.

A distinct advantage of using the TK-negative selection approach is that selection is on the basis of the resulting TK-negative phenotype, and thus does not rely upon the inclusion of a selectable antibiotic marker gene (such as the E. *coli* gpt gene), which hold certain environmental concerns. However it does rely upon the availability of a TK-negative cell line for the selection of recombinants. It is possible to use certain mutagens in cell culture medium to induce and select for mutants which are deficient in TK activity, but fortuitously for this study it was possible to acquire a Madin-Darby bovine kidney (MDBK) cell line (from Dr L.J. Bello, University of Pennsylvania, USA) which is resistant to high levels (up to 100 μg/ml) of the TK selection drug, BUdR. These cells, renamed BU100 cells, were shown to contain less than 5% of the TK activity found in the normal parental MDBK cells (Bello *et al.*, 1987). BUdR is a thymidine analogue which results in strand breakage when phosphorylated and incorporated into newly synthesized

DNA by active TK. Thus only viruses which have had their TK genes inactivated, and for which TK activity is non-essential, are able to survive in the presence of BUdR (Dubbs and Kit, 1964).

As poxviruses code for most of their own genes, including regulatory genes, expression of foreign genes needs to be under control of poxvirus promoters. The expression of poxvirus genes is temporal and is dependant on the "class" of promoter which regulate their expression (Coupar *et al.*, 1986; Kumar and Boyle, 1990). Promoters can be divided into early, intermediate, late and early/late depending on when they are activated during the infection/replication cycle. A number of synthetic promoters have been developed containing optimised sequences for the crucial regulatory regions (Chakrabarti *et al.*, 1997). It has also been found that the different "classes" stimulate different types of immune responses: early promoters stimulate a cell-mediated immune (CMI) response and late promoters a humoral response (Coupar *et al.*, 1986).

In developing a vaccine system it would be an advantage if both arms of the immune response were stimulated, and thus a promoter active early and late in the infection cycle would be ideal. The P7.5K promoter of VV is an early/ate promoter (Cochran *et al.*, 1985) and has been used successfully in a number of potential vaccinia virus-vectored recombinant vaccines in which good levels of immunity were induced (Smith *et al.*, 1983a; Collett *et al.*, 1987), including the highly protective VV-rabies recombinant vaccine used to control rabies in foxes in Europe and in raccoons in the USA (Kieny *et al.*, 1984; as reviewed by Paoletti, 1996). As the functional elements of poxvirus promoters have been shown to be active in heterologous poxviruses (Coupar *et al.*, 1990; Prideaux *et al.*, 1990), this promoter was chosen to drive the expression of foreign genes in this study.

The calcium phosphate coprecipitation method (Drillien and Spehner, 1983) was decided upon for transfection as this method has proven successful for the generation of many other poxvirus recombinants (Weir *et al.*, 1982; Smith *et al.*, 1983a; Kieny *et al.*, 1984) and has been used successfully in our laboratory for generating VV recombinants. As the rates of the infection-cycles of LSDV and vaccinia virus differ markedly (Fick and Viljoen, 1999), minor adaptations were implemented to help maximize the chances for successful homologous recombination (Fig. 2.1).

To assist with the selection process for potential recombinants the *E. coli* lacZ reporter gene was inserted into the LSDV transfer vector. Expression of this gene results in the production of a blue colouration by utilising the chromogenic substrate, X-gal (Panicali *et al.*, 1986).

After many attempts at generating LSDV recombinants using this approach, as well as introducing modifications such as transfecting the cells 12 hours after infection which would synchronise the time of the DNA (viral and plasmid) entering the cells with the early stages of viral replication (thus enhancing the chances for homologous recombination), no evidence for recombinants could be found. In a parallel study, 4 VV recombinants (each containing either the VP2, 3, 5 or 7 genes of African horse-sickness virus type 3) were successfully generated and

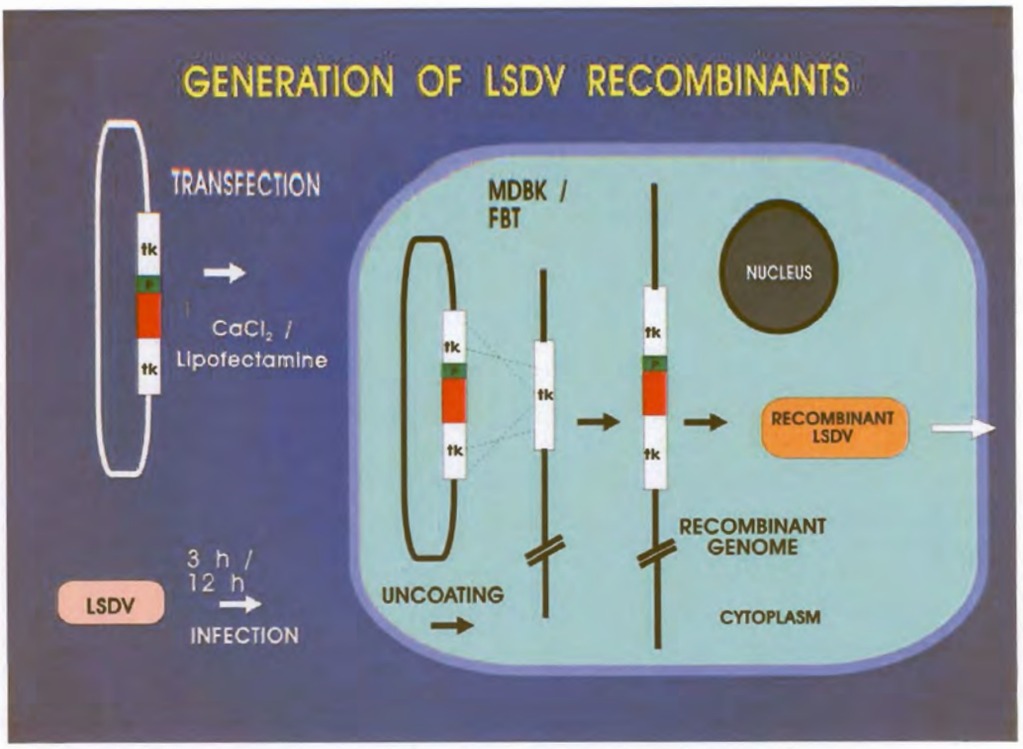



**Figure 2.1** Schematic representation of the processes involved in generating LSDV recombinants using homologous recombination and the viral TK gene as the insertion site. Cells are infected with wtLSDV. A number of hours later, coinciding with the early stages of viral DNA replication in the cytoplasm, they are transfected with plasmid DNA containing the necessary viral insertion sequences (tk) flanking a foreign gene under control of a poxvirus promoter (P). Homologous recombination occurs between the viral and plasmid DNA resulting in incorporation of the foreign gene into the newly synthesised viral DNA. This DNA is then packaged and released from cells as recombinant virus. Schematic prepared by Marco Romito.

selected using the same basic protocol, thus demonstrating that the reagents and general methodology were correct.

An alternative approach was thus attempted, incorporating the *E. coli* gpt gene as a dominant selectable marker and following the methodology of Romero *et al.* (1993) with minor adaptations. This time success was achieved in generating and selecting homogeneous LSDV recombinants which were shown to be stable.

## 2.2. MATERIALS AND METHODS

### 2.2.1 Viruses and cells:

Freeze-dried virus of the South African vaccine strain of LSDV (also referred to as "Neethling-type virus" or "parental control LSDV" or "wild-type LSDV") was reconstituted in sterile distilled water and passaged 5 times in Madin-Darby bovine kidney (MDBK) cells (obtained from the American Type Tissue Culture Collection, USA). Stocks were prepared from clarified cell lysates, and were stored at -20 °C. Aliquots were removed for titration on MDBK cells as described (Wallace, 1994).

Primary cultures of foetal bovine testis (FBT) cells were prepared using standard procedures (Freshney, 1987) and were used for the generation and selection of recombinant viruses.

BU100 cells, a derivative of MDBK cells, which are tolerant of BUdR to a concentration of 100 μg/ml, were kindly supplied by L.J. Bello (University of Pennsylvania, USA).

All cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 nutrient supplement (Highveld Biological Products, South Africa), 10% foetal calf serum (FCS) (Highveld Biological Products, South Africa) and antibiotics (100 μg/ml penicillin, 100 μg/ml streptomycin and 250 μg/ml amphotericin) (Highveld Biological Products, South Africa).

### 2.2.2 Plasmid DNA analysis, cloning and purification:

Restriction enzyme digestions

Restriction enzyme (R.E.) digestions were carried out at optimal temperatures and in appropriate buffers as specified by the manufacturer (Roche, Germany). Routine digestions were performed for one to three hours, however, when complete digestion was required for cloning purposes they were performed overnight. Double-digests were performed in the same buffer where

possible, or, the first digest in the buffer requiring the lowest salt concentration and then with the addition of salt to increase the final concentration to that required for the second enzyme. Where buffers were totally incompatible the DNA was digested with the first enzyme, run on an agarose gel, the appropriate fragment excised, purified and digested with the second enzyme. Partial DNA digestions were optimized according to each individual application by varying combinations of enzyme concentration, incubation temperature and duration of digestion.

Analysis and/or separation of DNA

Digested DNA was analysed or separated using horizontal agarose (0.8 %) gel electrophoresis using a mini-sub DNA cell (7x10 cm) (Pyramid, SA) and a EC 105 power-pack (E-C Apparatus Corporation, USA). Gels were prepared in a 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) (Sambrook *et al.*, 1989) containing 0.5 µg/ml ethidium bromide and were run at a constant rate of 7 V/cm in order to avoid heat build-up and distortion of the DNA bands. DNA molecular weight markers were prepared from digestion of phage lambda DNA (Promega, USA) with either *Pst*I or *Hind*III enzymes. DNA fragments were viewed under 254 nm wavelength UV light on a transilluminator (UVP, USA). Where necessary, images were captured using a Lumi-Imager (Roche, Germany) gel documentation system and printed on thermal paper (UPP-110HG, Sony, Japan) from a Sony UP-D895 Digital Graphic Printer (Sony, Japan).

Excision and purification of DNA fragments

Gels containing DNA fragments for excision and purification were viewed under 366 nm wavelength UV light to avoid strand damage using a hand-held transilluminator (Camag, UK). The relevant fragments were excised from gels using sterile scalpel blades and the DNA extracted and purified using a Qiaex II Gel Extraction Kit (Qiagen, Germany). DNA concentrations were determined by running aliquots on agarose gels alongside controls of known concentration (pBR322, Promega, USA).

Ligation of DNA fragments

For the ligation of DNA fragments with incompatible R.E. digested termini, the termini were first blunted using the Klenow fragment of *E. coli* DNA polymerase I (USB, USA). Termini of vector DNA were prevented from re-ligating upon themselves by dephosphorylation using calf intestinal phosphatase (Roche, Germany).

Sticky-end ligations were routinely carried out at 4-8 °C for 18 hours using a 1:2 vector to insert molar ratio using T4 DNA ligase (Roche, Germany) and blunt-end ligations were routinely carried out at 23 °C for 18 hours (or longer) using a 1:4 vector to insert molar ratio. However, when problematic ligations were encountered a range of incubation temperatures, incubation times and vector to insert ratios were applied.

Transformation of competent *E. coli* cells with ligated DNA

Stocks of competent DH5α *E. coli* cells were prepared according to the technique of Inoue *et al.* (1990) and were stored in 100 µl aliquots at – 80 °C (Ultra-low temperature freezer, Specht Scientific, South Africa). When required, an aliquot was thawed slowly on ice and approximately 20 ng of ligated DNA was added. The cell-DNA mixture was incubated on ice for one hour followed by a 30 second heat-shock at 42 °C. The mixture was then returned to ice for 2 minutes and then 800 µl of room temperature SOB medium was added and the cell mixture shaken vigorously for one hour at 37 °C. The mixture was then divided up equally and spread onto four LB-agar plates (containing 100 µg/ml ampicillin [Sigma, USA]) for overnight incubation at 37 °C. Single colonies were picked using sterile tooth-picks and inoculated into 2 ml LB medium for overnight incubation and plasmid DNA extraction. Using this technique it was possible to routinely obtain transformation efficiencies of 2 x $10^8$ transformants/ µg of plasmid pBR322 DNA.

Plasmid purification

Plasmid DNA was extracted from transformed bacterial cells using the modified alkaline lysis method of Ish-Horowicz and Burke (1981) (original method by Birnboim and Doly [1979]). Air-dried DNA was resuspended in sterile distilled water and stored at – 20 °C. For larger scale plasmid preparations and where plasmid DNA of high purity was required (such as for use in transfections) 100 ml bacterial cultures were grown and the DNA purified through a gravity-flow column from a Qiagen Plasmid Midi Kit (Qiagen, Germany). DNA concentrations were determined by measuring the absorbance at 260 nm (1 $OD_{260}$ = 50 µg/ml DNA) and the purity by determining the $OD_{260}/OD_{280}$ absorbance ratio.

### 2.2.3 Construction of LSDV transfer vector, pLSTK7.5:

The DNA plasmid, pLSDTK3c (Figure 2.2), was obtained from Dr Anna-Lise Williamson (University of Cape Town). This plasmid contains the 2.5 kbp *Hind*III fragment of LSDV in which is found the entire viral TK gene, and was used as the base plasmid for the construction of the transfer vector. The VV P7.5K early/late promoter and a restriction enzyme multiple cloning site (MCS) were excised as a single fragment from the VV transfer vector, pGVRW1, using *Nde*I and *Eco*RI (Figure 2.3) and blunt-end inserted into the *Kpn*I site of the LSDV TK gene in pLSDTK3c.

### 2.2.4 Insertion of lacZ reporter gene into pLSTK7.5:

To assist with monitoring the generation and selection of the first TK-disrupted LSDV recombinants the lacZ chromogenic reporter gene was utilized. The lacZ gene was inserted into the pLSTK7.5 transfer vector as follows:

The full-length lacZ gene (3.7 kbp) was removed from pSV-β-gal (Invitrogen, USA) using *Hind*III and *Eco*RI, blunted using Klenow, and inserted into the blunted (and dephosphorylated) *Sal*I site in the MCS of pLSTK7.5 to produce the insertion plasmid, pLSTK7.5-lacZ (Figure 2.7).

### 2.2.5 Transient expression of lacZ and X-gal staining:

MDBK cells were seeded on 6-well cell culture dishes (Nunclon, Denmark) and when 90% confluent they were infected with wtLSDV at an MOI = 1.0. One hour later the cells were washed with PBS and co-transfected with 5 µg of ultra-pure plasmid DNA using the DOTAP (Roche, Germany) liposomal-based transfection reagent. Twenty four hours later the cells were fixed in 4% paraformaldehyde (Merck, Germany) for 5 minutes, washed in PBS and stained with an X-gal stain (1 mg/ml X-gal [Biosolve Ltd, Netherlands], 5 µg/ml potassium ferrocyanate [Sigma, USA], 5 µg/ml potassium ferricyanate [Sigma, USA], and 5 µg/ml magnesium chloride [Sigma, USA]). The fixed cell monolayer was incubated at 37 °C (5% $CO_2$, Forma Scientific, model 3164, USA) until the blue colouration of cells expressing lacZ became visible.

### 2.2.6 Insertion of the positive selectable marker gene, gpt, into pLSTK7.5-lacZ:

When the TK-negative selection method failed to deliver selectable LSDV recombinants, the use of the *E. coli* gpt dominant positive selectable marker gene was investigated as an alternative method for selecting recombinants. This gene was inserted into the pLSTK7.5-lacZ insertion vector as follows (Figure 2.9):

The 1.3 kbp E. *coli* gpt gene was excised from the plasmid pSelp(HS)$G_1G_2$ (supplied by Dr Anna-Lise Williamson, University of Cape Town) using *Eco*RI, blunted and was inserted into the dephosphorylated *Sma*I site of pLSTK7.5-lacZ to produce the insertion plasmid, pLS(g)lac.

### 2.2.7 Generation and selection of LSDV recombinants:

TK-negative selection method

The method was adapted from that described by Drillien and Spehner (1983). In brief, MDBK cells (90% confluency) were infected with LSDV (SA-Neethling vaccine) at a MOI = 1 focus

forming units per cell (ffu/cell). At 12 hours post-infection (to coincide with the early onset of LSDV DNA replication) (Fick and Viljoen, 1999) calcium phosphate-precipitated LSDV DNA (2.5 μg) and 0.5 μg pLSTK7.5-lacZ were added. The cells were then glycerol-shock treated and incubated until 80 % displayed cytopathic effect (cpe) (2-3 days post-infection). These cells were then harvested, freeze-thawed three times, clarified by low-speed centrifugation and the supernatant fraction titrated serially on BU100 cells in the presence of 100 μg/ml BUdR (Sigma, USA).

The infected cells were incubated for 4 days, and areas of the cell monolayer which resembled viral foci were collected in a 50 μl volume using a 200 μl filter pipette tip. Samples were divided into two equal aliquots: one set was freeze-thawed at –20 °C three times and transferred to individual wells of a 24-well cell culture dish (Nunclon, Denmark) containing BU100 cells under TK selection. An X-gal agarose overlay (1% agarose containing 200 μg/ml X-gal) was placed over the cells for the evaluation of lacZ expression (Panicali *et al.*, 1986). The other set of aliquots were prepped for DNA extraction and polymerase chain reaction (PCR) analysis (2.2.5).

MPA-selection method (adapted from Romero *et al.*, 1993):
A 6-well microtitre plate (Nunclon), was seeded with FBT cells. The cells were incubated at 37 °C in a 5% $CO_2$ incubator until they reached 80% confluency. They were then infected with LSDV (SA vaccine) at a MOI = 0.1 ffu per cell in diluent (0.1 mM Hepes, DMEM) for 60 minutes at 37 °C in a 5% $CO_2$ incubator. During this period, the transfection mixes were prepared as follows: 5 μl of the liposomal-based transfection reagent LipofectAMINE (Gibco-BRL, Scotland) was mixed with 95 μl of OPTIMEM (Gibco-BRL) in a 10 ml polystyrene test tube. 5 μg of ultra-pure pLS(g)lac DNA was added to OPTIMEM to a final volume of 100 μl in a separate tube. The solutions were then mixed and left at room temperature (24 °C) for 20 minutes. The volume was made up to 1 ml with DMEM and gently pipetted over the cells once the inoculum had been removed. The cells were returned to the incubator for 7 hours after which time 1 ml of DMEM (10% FCS, no antibiotics) was added to each well, and incubation was continued for 17 hours. The transfection mixture was then replaced with DMEM (10% FCS, including antibiotics) and the cells were incubated for a further 4 days, or until almost 100% of them showed cpe. Cells were freeze-thawed alternately at -20 °C and room temperature three times. A 10-fold serial dilution was made of the cell lysate and an aliquot of each dilution was placed onto fresh FBT cells pre-incubated for 24 hours in the presence of gpt selection medium (20 μg/ml xanthine [Sigma, USA], 20 μg/ml hypoxanthine [Sigma, USA], 30 μg/ml mycophenolic acid [Sigma, USA], DMEM, 2.5% FCS). Cells were incubated under standard conditions (37 °C, 5% $CO_2$), with the medium being replaced every 48 hours until cpe became visible (usually 4-5 days post-inoculation). The cells were washed in PBS and individual foci were picked. These were then freeze-thawed as

described and were inoculated onto fresh cells under selection in 6-well or 12-well culture dishes (Nunclon). Cells were incubated until foci appeared. Foci were again picked, except, this time, only half a focus was removed. The position of each focus in relation to the well was carefully noted. The cells were then fixed and stained with the X-gal stain as described (2.2.5). It was thus possible to determine which foci contained viruses expressing the lacZ enzyme. A number of these foci were then selected for DNA extraction and PCR analysis.

### 2.2.8 Extraction of viral DNA for PCR analysis:

Foci were diluted 1:1 in lysis buffer consisting of 1% SDS [Sigma, USA], 20 mM β–mercaptoethanol [Merck, Germany], and 20 mM EDTA. These were then digested with Proteinase K (Roche, Germany)(100 µg/ml) at 56 °C for 2 hours in order to release viral DNA (approximately 1 ng per sample). Calf liver tRNA (0.5 µl of a 10 mg/ml stock) (Roche, Germany) and 1/10$^{th}$ volume 3 M sodium acetate (pH 5.3) were added and the DNA was extracted with phenol/chloroform [Sigma, USA], and then ethanol precipitated. After air-drying, the DNA was resuspended in 50 µl sterile, ultra-pure water and stored at –20 °C for PCR analysis.

### 2.2.9 PCR analysis:

Two primers were designed, P1 and P2 (Gibco-Brl, Scotland), which bind to the wtLSDV TK gene flanking the *Kpn*I restriction enzyme site used for the insertion of the foreign genes (as shown in Fig. 2.13 A).

P1 - CACCAGAGCCGATAAC
P2 - GTGCTATCTAGTGCAGCTAT
P3 - GAAGTGTCCCAGCCTG
P4 – CTCGCAAGCCGACTGATGCC
P5 - GATCCCCCACCCGCTTTTTATAGTAAGTTTTTCACA

These primers were then used to help distinguish wt from recombinant virus using a GeneAmp 2400 thermal cycler (Perkin-Elmer, USA) and Taq DNA polymerase. Primers P3 and P4 (Gibco-Brl, Scotland), binding to the gpt and lacZ marker genes, were used as internal controls. Primer P5 binds to the VV P7.5K promoter and was used to confirm insertion of this promoter (along with the MCS) into the LSDV transfer vector, pLSTK7.5.

A 50 µl reaction volume was used consisting of 5 µl 10X PCR buffer (containing 20 mM $MgCl_2$) (Takara Biomedicals, Japan), 4 µl 2.5mM dNTPs (Takara Biomedicals, Japan), 0.5 U Taq DNA

polymerase (TaKaRa Ex Taq™, Takara Biomedicals, Japan), 1µl of each primer [20 pmoles each], 1 µl template DNA (~ 0.1 ng) and 38 µl sterile distilled water. Template DNA was denatured for 45 seconds at 93 °C, primer annealing was carried out at 53 °C for 45 seconds, and strand extension was at 72 °C for 5 minutes (repeated through 35 amplification cycles).

2.2.10 Purification of LSDV:

Preparation of routine virus stocks

The infected FBT cells were harvested, either by shaking the flask vigorously to dislodge the cells (only possible when the cells were heavily infected) or by scraping the cells loose using a "rubber policeman" or by removing them gently using 0.1% trypsin (Difco Laboratories, USA). The medium containing the loose cells was dispensed into 20 ml glass bottles. These bottles were balanced and centrifuged at low speed in a benchtop centrifuge at 1500 rpm (250g) for 10 minutes at 4 °C. The cell pellets were lysed in 2 ml McIlvain's hypotonic buffer (Lennette and Schmidt, 1969) and left on ice for 10 minutes. The lysed cells were centrifuged at 2000 rpm (450g) for 10 minutes and the supernatant fluid was collected. The cell debris was resuspended in McIlvain's buffer and was again centrifuged at 2000 rpm (450g) for 10 minutes. The supernatant fluid was collected and pooled with the first virus-containing supernatant fluid and an aliquot was removed for virus titre determination (Wallace, 1994). The rest of the virus suspension was diluted in glycerol to produce a final concentration of 70 % glycerol and was dispensed into glass ampoules and frozen at -70 °C.

Preparation of pure LSDV for DNA extraction (Esposito, 1981)

The culture medium was removed from flasks containing infected cells exhibiting 90 % cpe. The infected cells were harvested by using trypsin to release them from the flasks. The cells were pooled with the medium, dispensed into centrifuge tubes and 0.5 ml of 36 % sucrose (in PBS) was layered at the bottom of each tube. The virus/cell suspension was centrifuged at 11 000 rpm (19000g), 60 minutes, 4 °C in a Beckman J2-21 (Beckman Instruments, USA) high speed centrifuge in a JS13-1 (Beckman Instruments, USA) rotor. The pellet was resuspended in 9 ml of McIlvain's buffer (4 mM) and left on ice for 10 minutes. ß-Mercaptoethanol (26 µl) and 1 ml of 10 % Triton X-100 (Merck, Germany) (in McIlvain's buffer) were then added and the suspension was further incubated on ice for 10 minutes to disrupt the cells. The cell debris was removed by centrifugation at 2000 rpm (450g) for 5 minutes, 4 °C in a benchtop centrifuge and the supernatant fluid was collected. The cell debris was resuspended in McIlvain's buffer and the centrifugation was repeated (2000 rpm (450g) for 5 minutes, 4 °C in a benchtop centrifuge). The supernatant fluid was collected and pooled with the first virus-containing supernatant fluid. This fluid was transferred to a centrifuge tube and 0.5 ml of 36 % sucrose [prepared in TE (10 mM

Tris, 1 mM EDTA, pH 9.0)] was layered beneath. Virus was then pelleted [centrifuged at 11 000 rpm (19000g), 60 minutes, 4 °C in a Beckman J2-21 high speed centrifuge in a JS13-1 rotor]. The pellet was resuspended in 0.5 ml of TE buffer. Lysis buffer (450 µl 4% sodium lauroylsarcosinate, 54% sucrose, 100 mM Tris (pH 7.8) mixed with 50 µl 200 mM β-Mercaptoethanol, 100 mM Tris (pH 7.8)) and Proteinase K (Roche, Germany) (final concentration = 100 µg/ml) were then added and the virus mixture was incubated for 3 hours at 56 °C (Massung and Moyer, 1991).

### 2.2.11 Purification of viral DNA (adapted from Sambrook *et al.* (1989):

The viral DNA was subjected to one round of phenol extraction, followed by one round of phenol and chloroform extraction (24:1 chloroform:isoamylalcohol) (Sigma, USA), and finally, one round of chloroform extraction. DNA was precipitated by addition of $1/10^{th}$ volume sodium acetate (3 M, pH 5.2) and 2.5 volumes 96% ethanol, followed by a wash in 70% ethanol. Resuspension of the DNA was in sterile distilled water or TE buffer and storage was at -20 °C.

### 2.2.12 Southern transfer and radioactive $^{32}$P-labelled probe hybridisation:

Purified DNA from wtLSDV and LSDV-lacZ recombinant (from purified virions and from the cellular debris fraction after low speed centrifugation) were cut with *Hind*III and run on a 0.8% agarose gel. The separated DNA fragments were then Southern transferred to a Hybond$^{TM}$N$^{+}$ nylon membrane (Amersham, USA) using alkaline buffer for transfer (Sambrook *et al.*, 1989).
A $^{32}$P-dATP labelled DNA probe was prepared from the *Hind*III fragment of LSDV containing the TK gene using nick translation (Nick Translation Kit, Promega, USA). The probe was then hybridised to the membrane, washed under stringent conditions (Sambrook *et al.*, 1989) and the washed membrane was then exposed to Kodak Biomax$^{TM}$ MS-1 Scientific Imaging Film (Sigma, USA) at –70 °C. The film was then developed and the resulting autoradiograph analysed.

## 2.3 RESULTS

### 2.3.1 Construction of the LSDV DNA transfer vector, pLSTK7.5:

In order to insert foreign genes into the TK gene of LSDV it was necessary to construct a transfer vector containing the viral TK sequence interrupted by a restriction enzyme MCS, into which the foreign genes could be inserted. A poxvirus promoter is required to drive expression of the foreign genes. For this purpose the VV early/late P7.5K promoter was chosen. A distinct

advantage of using a dual early/late promoter to drive the expression of foreign genes is that it has been shown that proteins expressed early and late in the course of poxviral infections are able to stimulate both humoral and cell-mediated immune responses in animals (Andrew *et al.*, 1989). Recombinant LSDVs resulting from double cross-over homologous recombination between the disrupted plasmid TK gene and the intact parental viral genomic TK gene would thus contain an inactive TK gene. The TK-negative phenotype of resulting recombinants formed the basis for selection in the TK-selection method.

A plasmid containing the full-length LSDV TK gene (pLSDTK3c) was utilized as the base plasmid for the construction of the transfer vector (with kind permission of AL Williamson, UCT). A convenient *Kpn*I site is located close to the middle of the TK gene (Figure 2.2) and this site was used for the insertion of a MCS downstream of the VV P7.5K promoter.

The MCS and promoter were excised as a single 250 bp fragment from the VV transfer vector (pGVRW1) using *Nde*I and *Eco*RI and blunt-end ligated into the blunted *Kpn*I site in the TK gene in pLSDTK3c (Figure 2.3). Competent DH5α cells were transformed with the ligation mixture and plasmid DNA was extracted from 24 transformed bacterial colonies growing on the ampicillin-agar plates. DNA was then cut with *Hind*III and the resulting fragments were separated using agarose gel electrophoresis (Figure 2.4 A). Although difficult to see on the photograph, a number of DNA patterns looked correct by direct visualization on a UV transilluminator through a protective Perspex screen. One of these preps (#13) was then compared with a *Hind*III digest of pLSDTK3c (Figure 2.4 B). *Hind*III cuts pLSDTK3c to produce two fragments of 3.4 and 2.5 kbp (Figure 2.2). However, with the insertion of the 250 bp DNA fragment containing the P7.5K promoter and MCS from pGVRW1, the additional *Hind*III site in the MCS will result in three fragments of 3.4, 1.9 and 0.85 kbp. These fragment sizes are obtained for prep #13 cut with *Hind*III (Figure 2.4B and Figure 2.5A). Prep #13 was then cut with *Pst*I for further confirmation and the expected fragment sizes of approximately 2.85, 1.90 and 1.35 kbp were obtained (Figure 2.5A).

To be certain that the P7.5K promoter was included in the insert in prep #13, a PCR was performed (including a number of other preps showing the correct *Hind*III pattern) using a primer (P5) binding specifically to the P7.5K promoter and a primer (P1) which binds towards the right end of the LSDV TK gene (refer to figure 2.13 A). PCR conditions were as described (2.2.9) except that extension was 60 seconds.

An amplification product of 450 bp would be produced if the promoter was in the correct orientation. In Figure 2.5 (B) it can be seen that for a number of the preps (#13, 16, 11, 20 and 3) this amplification product is produced, although not for prep #5 (lane 7), nor for pLSDTK3c (lane 8) which lacks the P7.5K promoter. Thus it is probable that for prep #5 the insert is in the opposite orientation to the other preps.

It can be concluded from the results of these experiments that the P7.5K promoter and the MCS were successfully inserted into the LSDV TK gene in pLSDTK3c to produce a LSDV transfer vector (pLSTK7.5, Figure 2.6) with a number of unique R.E. sites for the insertion of foreign genes.

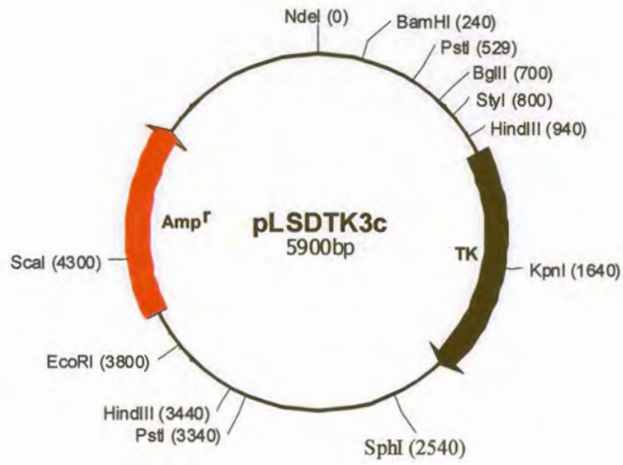



**Figure 2.2** Restriction enzyme map of plasmid pLSDTK3c (supplied by Dr Anna-Lise Williamson, University of Cape Town).

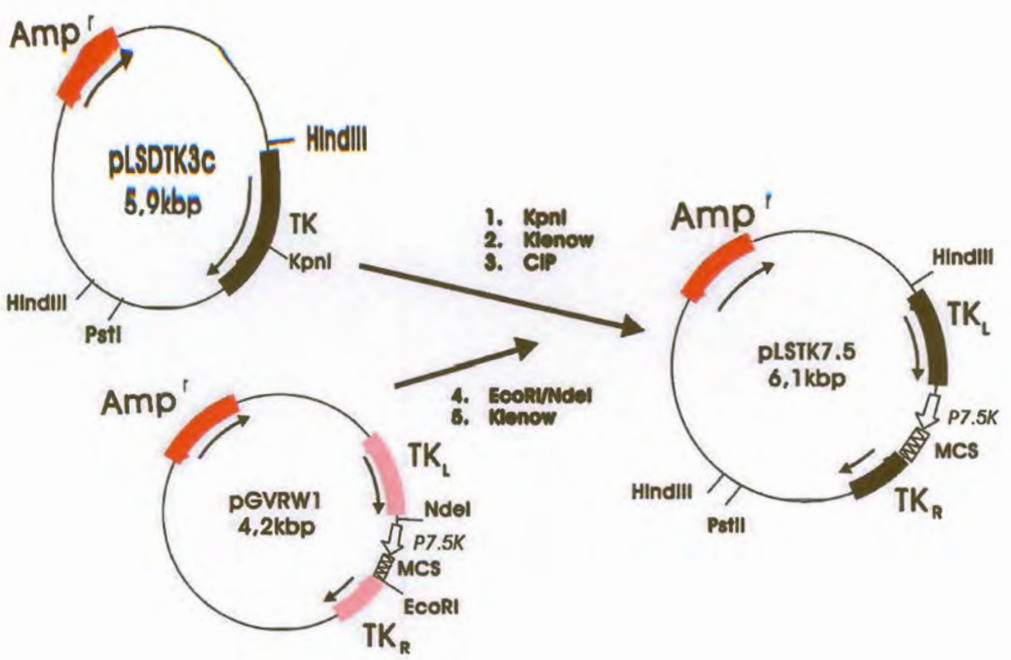
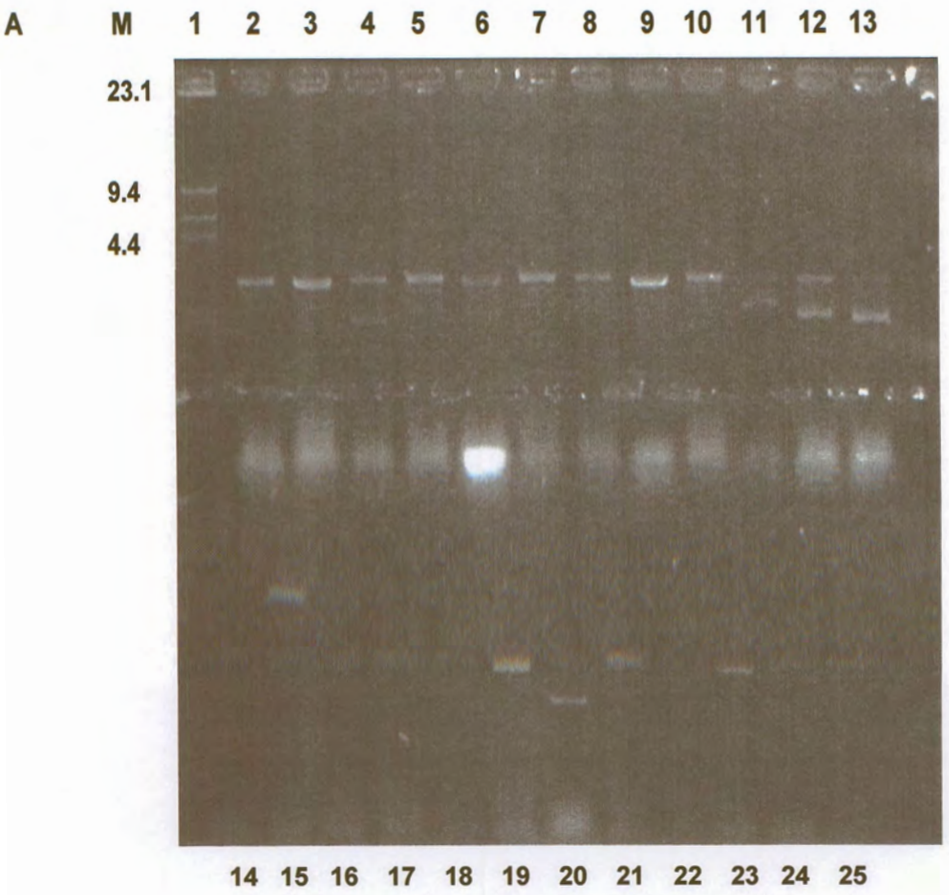



**Figure 2.3** Construction of the LSDV transfer vector, pLSTK7.5. The VV early/late P7.5K promoter and downstream multiple cloning site (MCS) were removed from the VV transfer vector, pGVRW1, and inserted into the KpnI site of the LSDV thymidine kinase (TK) gene in pLSDTK3c. The resulting LSDV transfer plasmid, pLSTK7.5, contains an inactive TK gene with convenient restriction enzyme sites for the insertion of foreign genes under control of the P7.5K promoter.



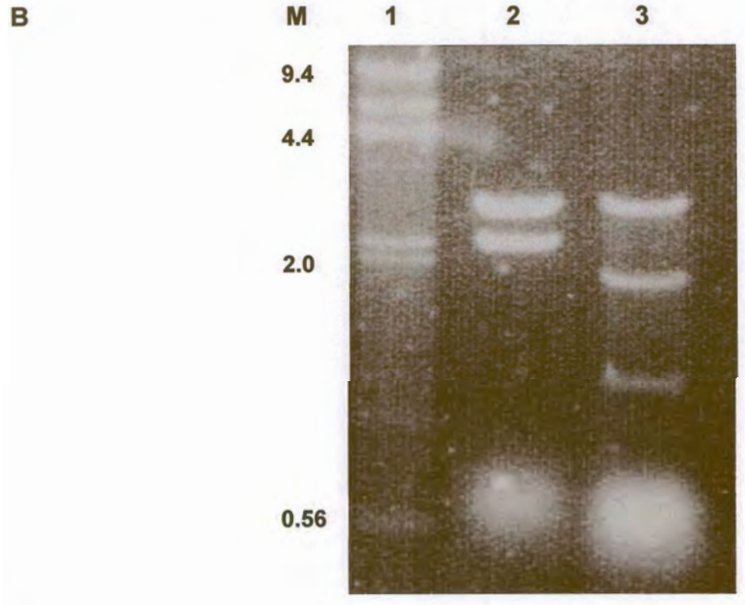




**Figure 2. 4** Agarose gel electrophoretic analysis of miniprep DNA for the construction of the LSDV transfer vector, pLSTK7.5. **A** – miniprep DNA cut with *Hind*III (lanes 2 to 25). Lane 1 = lambda *Hind*III marker. **B** – *Hind*III cut pLSDTK3c (lane 2) and miniprep #13 (lane 3). Lane 1 = lambda *Hind*III marker. M – sizes of DNA fragments in kbp.

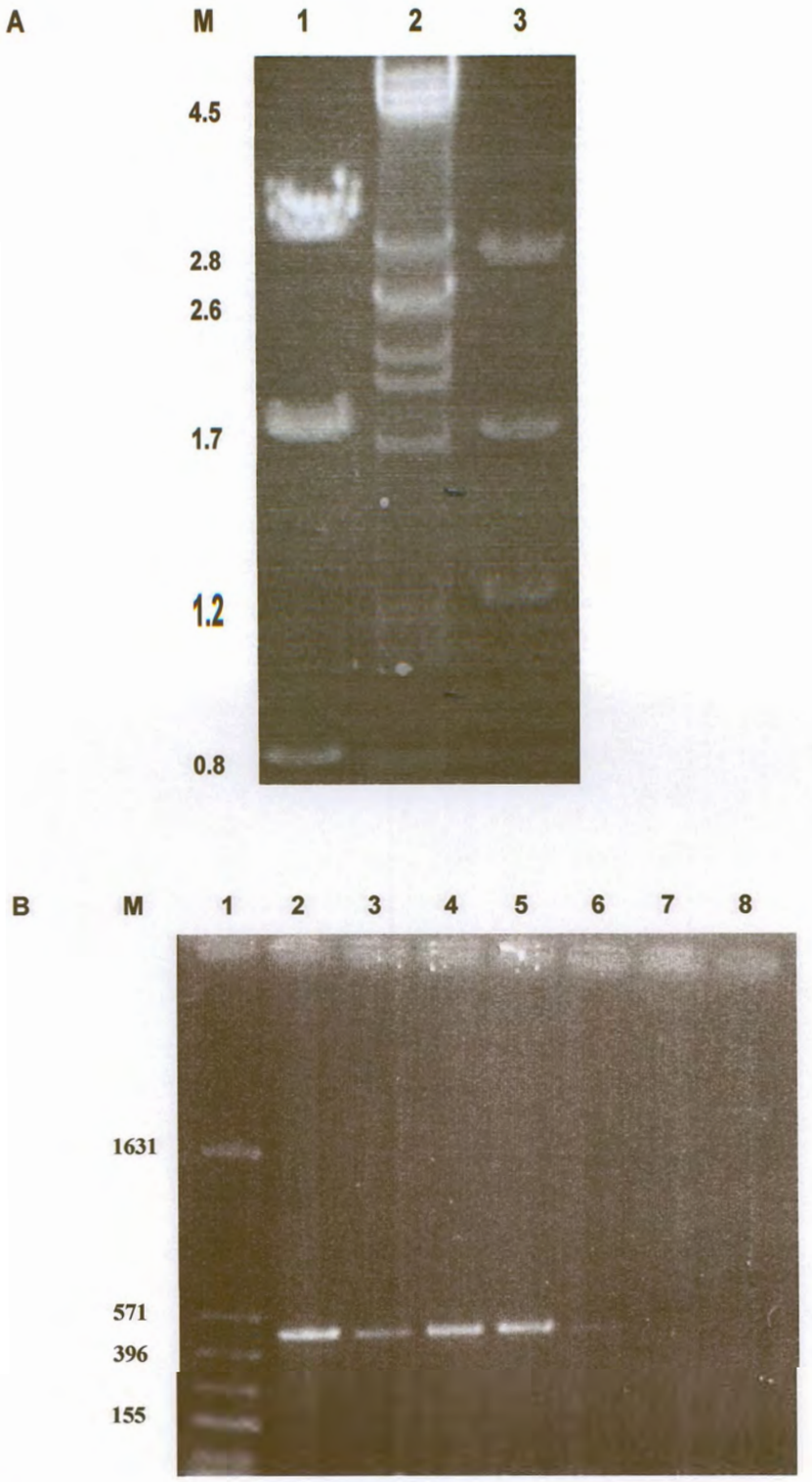



**Figure 2.5** Agarose gel electrophoretic analysis of miniprep DNA for the construction of the LSDV transfer vector, pLSTK7.5. **A** – *Hind*III (lane 1) and *Pst*I (lane 3) cut prep #13 with a lambda *Pst*I DNA marker (lane2). **B** – PCR-amplified DNA from preps #13 (lane 2), #16 (lane 3), #11 (lane 4), #20 (lane5), #3 (lane 6), #5 (lane 7) and pLSDTK3c (lane 8) using primers P1 and P5. Lane 1 = pAT153 *Hin*fI DNA marker. M = DNA fragment sizes in kbp.

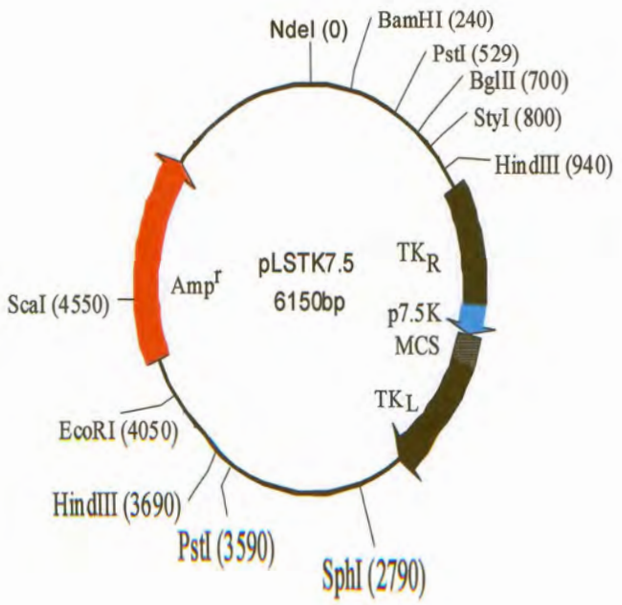



**Figure 2.6** R.E. map of the LSDV transfer vector, pLSTK7.5 showing the restriction enzyme sites in the MCS (R.E. sites starting from P7.5K promoter: - *Bgl*II, *Pst*I, *Sal*I, *Bam*HI, *Hind*III, *Xba*I, *Kpn*I, *Sph*I, *Eco*RV, *Sac*I, *Xma*I, *Ava*I, *Sma*I, *Eco*RI - )

2.3.2 Insertion of lacZ reporter gene into the LSDV transfer vector, pLSTK7.5:

With the construction of a suitable LSDV transfer vector, it was then possible to insert foreign genes into the vector for the generation of TK-disrupted LSDV recombinants expressing the foreign genes. However, as the chosen approach (TK-negative selection) for generating and selecting the recombinants was unproven for LSDV, the first recombinants were to be generated expressing a reporter gene to allow easier evaluation of the selection strategy. For this purpose the *E. coli* lacZ reporter gene was selected as a means for colour screening of recombinants and was to be inserted into the pLSTK7.5 transfer vector under control of the P7.5K promoter.

A copy of the lacZ gene was obtained from the pSV-ß-gal mammalian expression vector (Promega, USA). The gene was removed using *Hind*III and *Bam*HI (a 3.7 kbp fragment) and was inserted blunt-ended into the *Sal*I site (also blunt-ended) in the MCS of pLSTK7.5 (Figure 2.7). As the gene was inserted as a blunt-ended product, it was important to determine its orientation with respect to the P7.5K promoter. Plasmid mini-preps were cut with *Sac*I and the DNA fragments were separated using agarose gel electrophoresis (Figure 2.8). The lacZ gene contains a *Sac*I site 1.6 kbp from its 3' end and the pLSTK7.5 vector contains a *Sac*I site

downstream of the *SalI* site (Figure 2.6). Thus, for the correct orientation, cutting with *SacI* should produce two bands of 8.2 kbp and 1.6 kbp – as is the case for preps in lanes 2, 5 and 6 (Figure 2.8). The rest of the preps contain the gene in the wrong orientation. Prep #2 was then cut with *EcoRI*, *PstI*, *SacI* and *SmaI* and run on an agarose gel to confirm that the construct was correct. The expected fragment patterns were obtained (results not shown) and the plasmid (now named pLSTK7.5-lacZ) was grown to large scale and purified through a Qiagen Plasmid Midi Kit gravity-flow column for storage at – 20 °C.

In order to ensure that the P7.5K promoter and lacZ gene contained no mutations which could interfere with lacZ expression, standard practice would have been to sequence the expression cassette. However, it was quicker to evaluate the construct by co-transfecting it into LSDV-infected MDBK cells and monitoring for transient expression of lacZ by fixing and staining the cells with X-gal. MDBK cells were co-transfected as described (2.2.5) with pLSTK7.5-lacZ (or, pLSTK7.5 as a negative control) and were then fixed and stained with an X-gal stain 24 hours post-infection. Transient expression of lacZ was observed in cells co-transfected with the pLSTK7.5-lacZ construct as evidenced by the production of a strong blue colouration. (Figure 2.12 B). However, no such colouration was observed for cells co-transfected with pLSTK7.5 (Figure 2.12 A) proving that it was expression of the lacZ gene from pLSTK7.5-lacZ that was responsible for the production of the blue colouration.

Thus the pLSTK7.5 transfer vector was modified by the insertion of the lacZ reporter gene under control of the VV P7.5K promoter. This construct could now be used in the generation of TK-disrupted LSDV recombinants and the lacZ gene would prove a useful marker for assisting with the evaluation of the selection process.

### 2.3.3 Generation and selection of LSDV recombinants using the TK-negative selection method:

The construction of an insertion vector (pLSTK7.5-lacZ) containing the lacZ reporter gene under control of a poxvirus promoter and flanked by viral TK sequences made it possible to assess the TK-negative selection method as a means to generate and select TK-disrupted LSDV recombinant viruses. The availability of a LSDV-permissive cell line (BU100 cells) able to tolerate the BUdR selection drug further assisted with this process.

For the generation of recombinants, MDBK cells were infected with wtLSDV at an MOI = 1 and were co-transfected 12 hours later with calcium-phosphate precipitated LSDV genomic DNA and pLSTK7.5-lacZ as described (2.2.7). Once the cells displayed 80% cpe they were harvested, freeze-thawed three times, clarified by low-speed centrifugation and the supernatant fraction titrated serially on BU100 cells in the presence of 100 µg/ml BUdR. Four days later areas of the

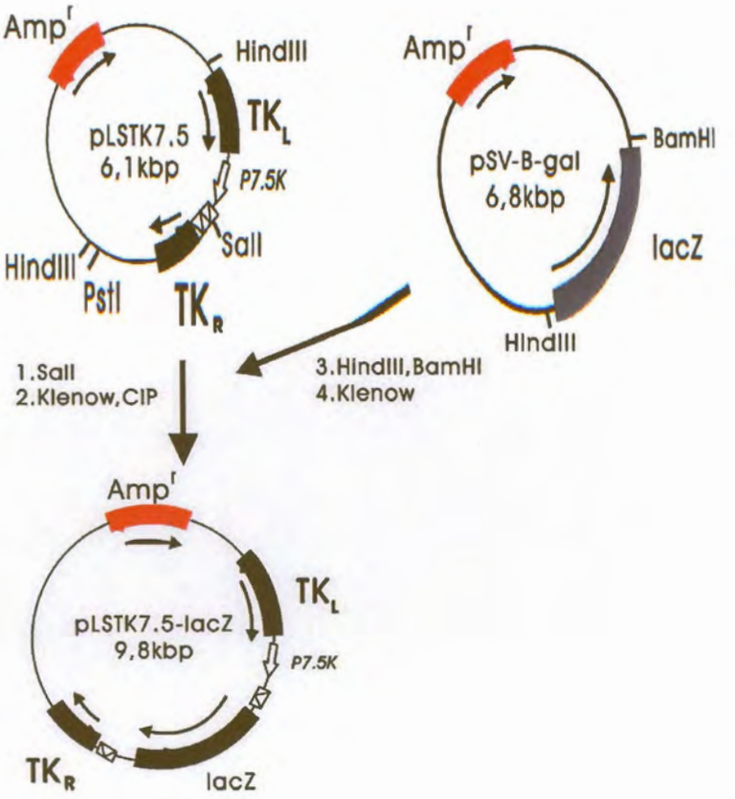



**Figure 2.7** Construction of the LSDV insertion vector, pLSTK7.5-lacZ. The complete β–galactosidase gene (lacZ) was removed from the mammalian expression vector, pSV-β-gal, and blunt inserted into the *Sal*I site in the MCS of pLSTK7.5. The lacZ gene serves as a useful reporter gene to monitor the progression and efficiency of poxviral recombinant generation and selection.

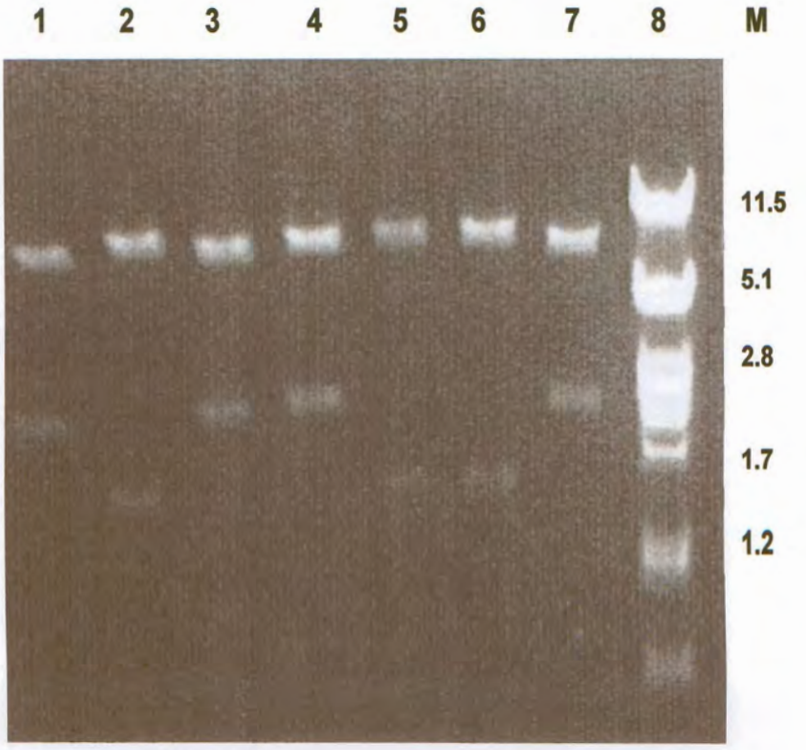



**Figure 2.8** Agarose gel electrophoresis analysis of miniprep DNA for the insertion of the lacZ reporter gene into pLSTK7.5. DNA was cut with *Sac*I. Lanes 1-7 contain *Sac*I cut miniprep DNA and lane 8 a lambda PstI marker. M = DNA fragment sizes in kbp.

cell monolayers which resembled viral foci were picked, divided into two equal aliquots and the one half was treated as described for lacZ expression. Surprisingly none of the aliquots placed under an X-gal agarose overlay produced any clear indication of lacZ expression.

The other half of the aliquots from which DNA had been extracted were then tested for viral DNA (both recombinant and wild type) using PCR analysis (Pasamontes *et al.*, 1991). The primer pair P1 and P2 bind specifically to regions flanking the insertion site in the LSDV TK gene and can be used to distinguish wt from recombinant viral DNA (Fig. 2.13 A). As internal controls, primer P3 which binds to the 3' end of the gpt gene and P4 which binds to the central region of the lacZ gene, were designed. Even though the plasmid controls worked, no PCR products were produced from DNA extracted from the "foci" (data not shown).

A number of modifications were then introduced including increasing the amounts of input viral and plasmid DNA, varying the times between infection and co-transfection of DNA, and leaving out the agarose overlay and fixing and staining the cells directly for lacZ expression (results not shown). However, none of modifications yielded recombinants. Fick (1998) attempted a similar approach to generating LSDV recombinants expressing the VP2 gene of African horsesickness

virus (type 3) and also failed. In parallel experiments, generation of VV recombinants via the TK-negative selection strategy were successful, confirming technical competence (data not shown). This approach therefore totally failed to produce recombinant viruses which were selectable on the basis of their TK-negative phenotype and therefore a change in strategy was required. The MPA selection method had been successfully used by Romero *et al.* (1993) to select TK-disrupted recombinants of the KS-1 strain of LSDV, and therefore this method was now attempted for the South African vaccine strain of LSDV.

### 2.3.4 Insertion of the *E. coli* gpt dominant selectable marker gene into pLSTK7.5-lacZ:

The MPA selection method requires the presence of the *E. coli* gpt dominant selectable marker gene in the recombinant virus during the selection stages. It can either be retained in the final recombinant construct if inserted between the TK flanking sequences in the insertion vector, or, allowed to be deleted once selection pressure has been removed if inserted outside the TK flanking sequences. For the purposes of this study it was decided to insert the gene inside the TK flanking sequences of the insertion vector, pLSTK7.5-lacZ.

The gpt gene, under control of the VV P7.5K promoter, was provided by Dr Anna-Lise Williamson (University of Cape Town) within the plasmid, pSelp(HS)G1G2. The gene was then removed from the plasmid (including the poxvirus promoter) using *Eco*RI and the 1.3 kbp DNA fragment was blunt-end inserted into a unique *Sma*I site downstream of the lacZ gene in pLSTK7.5-lacZ, giving rise to pLS(g)lac (Fig. 2.9). In this construct it was important to ensure that the two P7.5K promoters were in opposite orientations to each other or else there would be the risk of an unwanted recombination event occurring which would drop out the lacZ gene. Thus making use of an internal *Sac*I site in the lacZ gene and a *Sac*I site at the 5' end of the gpt expression cassette (Figure 2.11) miniprep DNA was cut with *Sac*I to enable the orientation of the P7.5K promoter-gpt cassette to be determined. The desired orientation would produce three bands of 8.5, 1.5 and 1.3 kbp. In Figure 2.10 (A) it can be seen that preps in lanes 4, 5, 6, 9 and 11 produced this pattern. Prep #11 was then chosen for further characterization using a number of different restriction enzymes (Figure 2.10 B) – from the R.E. map (Figure 2.11) the expected patterns were produced and large amounts of highly pure DNA were prepared – the insertion plasmid was now called pLS(g)lac.

Further evidence for the integrity of the pLS(g)lac vector is provided in figure 2.13 (B) whereby PCR amplification of certain regions of the vector using different primer pairs (lanes 1 and 4) resulted in amplification products of the expected sizes (5.5 kbp and 1.7 kbp respectively).

Now that the gpt gene had been inserted into the lacZ insertion vector it was possible to attempt the MPA selection strategy for generating and selecting LSDV recombinants.

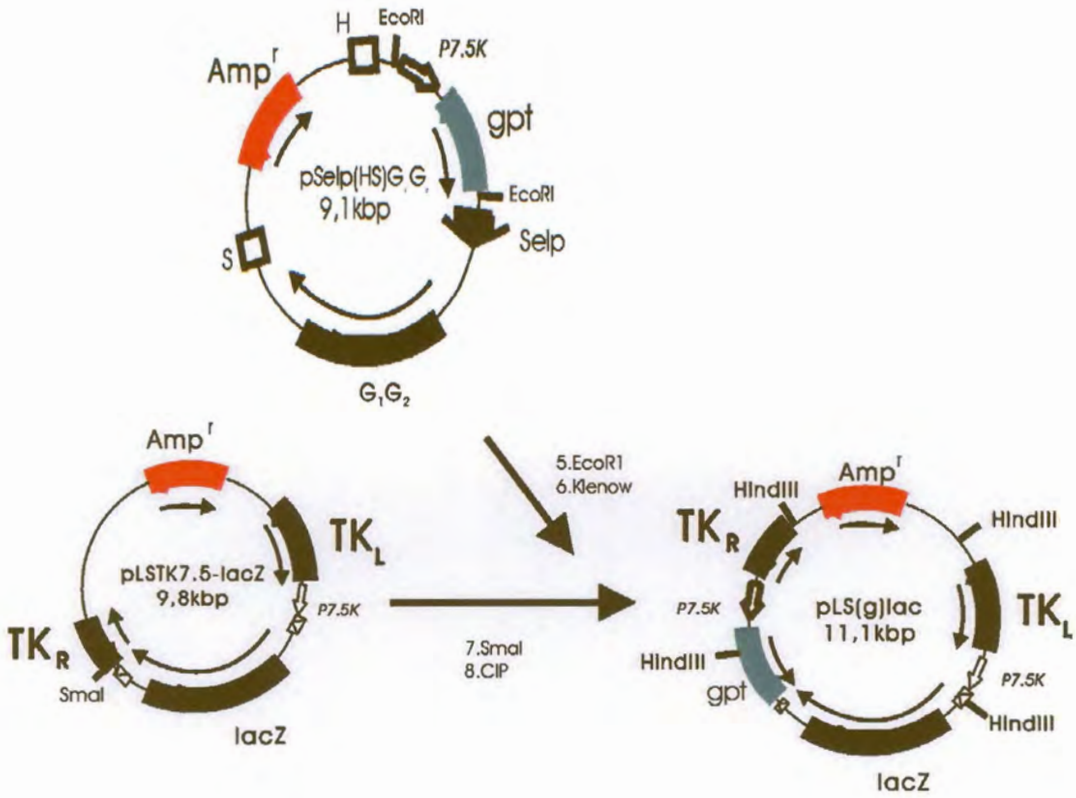



**Figure 2.9** Construction of the insertion vector, pLS(g)lac. The *E. coli* gpt gene (1.3 kbp) was excised from pSelp(HS)$G_1G_2$ using *Eco*RI and blunt-end inserted into the *Sma*I site of the remaining MCS of pLSTK7.5-lacZ. The gpt gene is a positive selectable marker gene and allows for the selection of recombinant virions (expressing the gene) over non-expressing virions when grown in MPA selection medium.

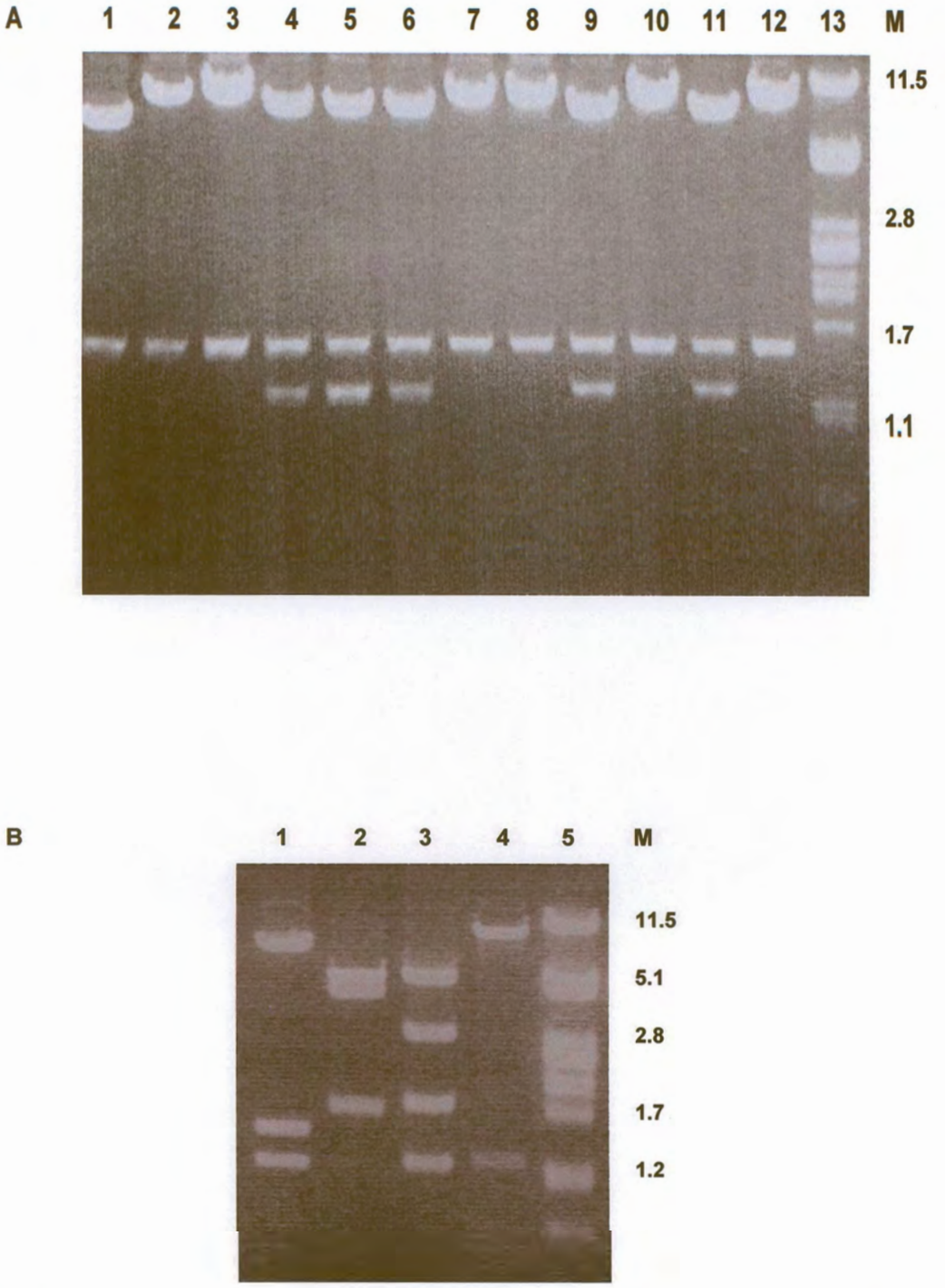



**Figure 2.10** Agarose gel electrophoresis analysis of R.E. digests of pLS(g)lac. **A** – Minipreps digested with *Sac*I (lanes 1-12). Lane 13 = lambda *Pst*I marker. **B** – Prep #11 cut with a number of R.E.s Lane 1 = *Sac*I digest, lane 2 = *Nde*I, lane 3 = *Pst*I, lane 4 = *Sma*I and lane 5 = lambda *Pst*I marker. M = DNA fragment sizes in kbp.

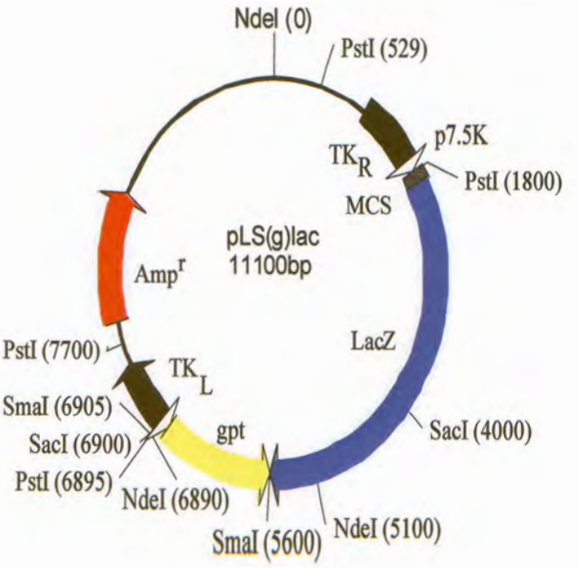



**Figure 2.11** R.E. map of pLS(g)lac.

2.3.5 Generation and selection of LSDV recombinants using the MPA selection method:

This approach was based on the method of Romero *et al.* (1993) and makes use of positive dominant selection in the form of the *E. coli* gpt gene. Before commencing with the generation and selection of recombinants, lacZ expression from pLS(g)lac was first confirmed using a transient expression assay in FBT cells and LipofectAmine as transfecting reagent (2.2.5).

Transient expression of lacZ was observed in LSDV-infected FBT cells co-tranfected with pLS(g)lac and fixed and stained 24 hours post-infection (Figure 2.12 D). No blue colouration was observed in wells in which LSDV-infected FBT cells were transfected with pLSTK7.5 (Figure 2.12 C).

FBT cells were then tested for tolerance to the MPA selection medium. No adverse effects were observed (data not shown).

Cells were also infected with wt LSDV in the presence of selection medium and were observed for the development of viral foci. No viral foci were visible in control wells in which FBT cells were infected with wt LSDV under MPA selective pressure. However, when this approach was used for the generation and selection of LSDV recombinants (2.2.7), progeny virions released

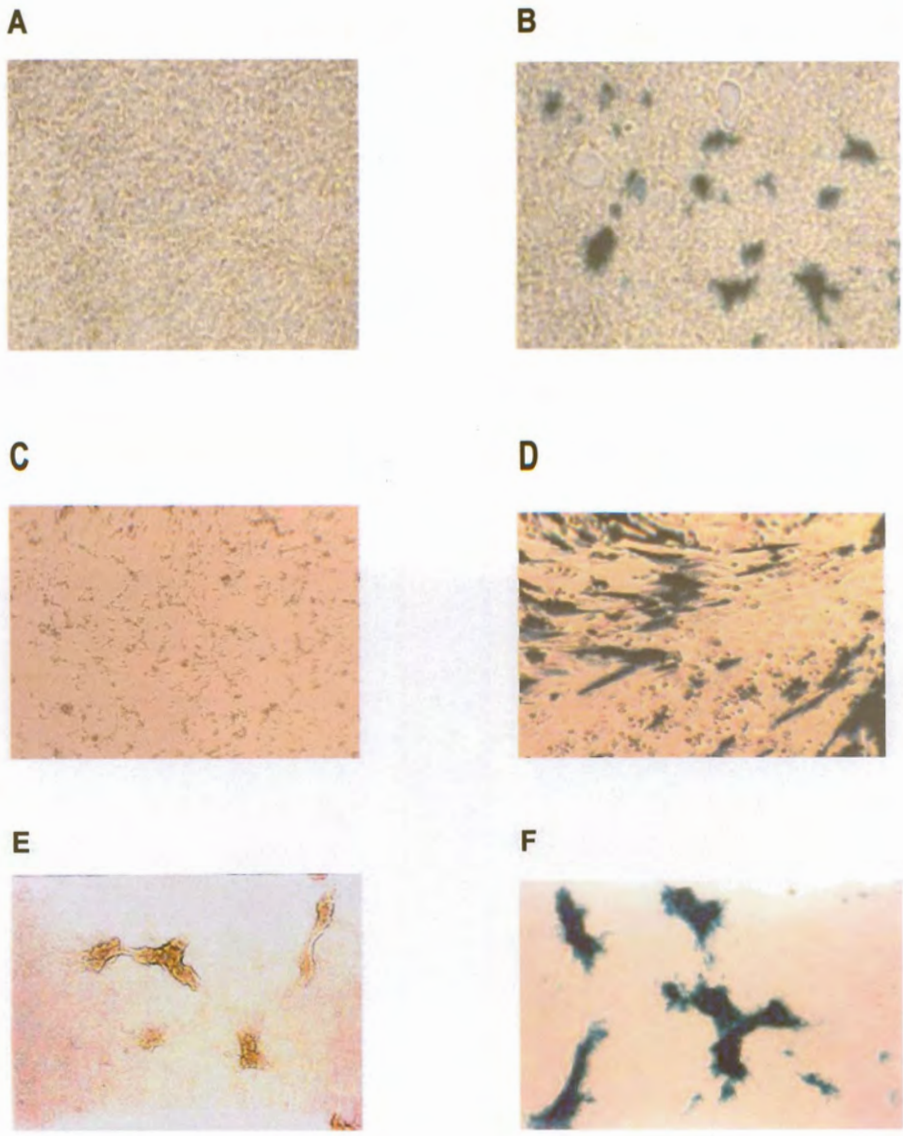
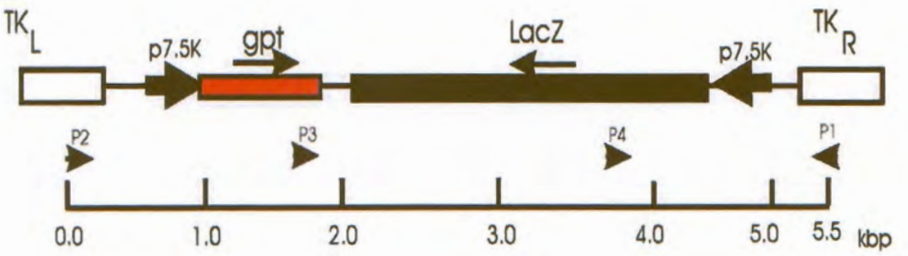



**Figure 2.12** LSDV-lacZ expression: transient and from LSDV-lacZ recombinants. All cells were fixed and stained with an X-gal stain as described (2.2.5). **A** – MDBK cells co-transfected with pLSTK7.5 and wtLSDV (MOI = 1). **B** - MDBK cells co-transfected with pLSTK7.5-lacZ and wtLSDV (MOI = 1). **C** - FBT cells co-transfected with pLSTK7.5 and wtLSDV (MOI = 1). **D** - FBT cells co-transfected with pLS(g)lac and wtLSDV (MOI = 1). **E** – wtLSDV foci on MDBK cells (4 days post-infection). **F** – LSDV-lacZ recombinant virus on MDBK cells (4 days post-infection). Magnification: 250X.

A

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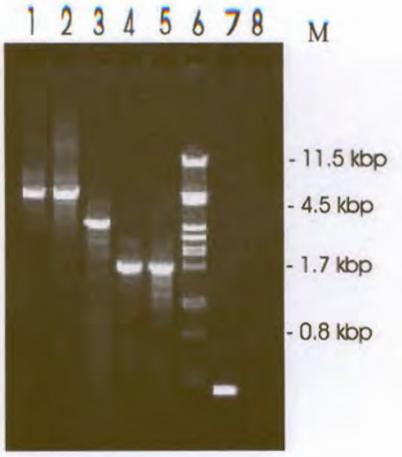



**Figure 2.13** PCR analysis of LSDV-lacZ recombinants. (**A**) Schematic representation of the TK insertion region in the LSDV genome showing the gpt and lacZ genes and their p7.5K promoters. The primers used to analyse the recombinants are shown, including their direction of amplification (arrows). Primer sequences are given in section 2.2.9.

(**B**) Agarose gel of the PCR-amplified products separated by electrophoresis. Lane 1 – pLS(g)lac (primers P1 and P2), lane 2 – recombinant LSDV-lacZ virus (primers P1 and P2), lane 3 – recombinant LSDV-lacZ virus (primers P1 and P3), lane 4 – pLS(g)lac (primers P1 and P4), lane 5 – recombinant LSDV-lacZ virus (primers P1 and P4), lane 6 – lambda *Pst*I DNA marker, lane 7 – wtLSDV ( primers P1 and P2), and lane 8 – $dH_2O$ (primers P1 and P2). M – DNA marker sizes in kbp.

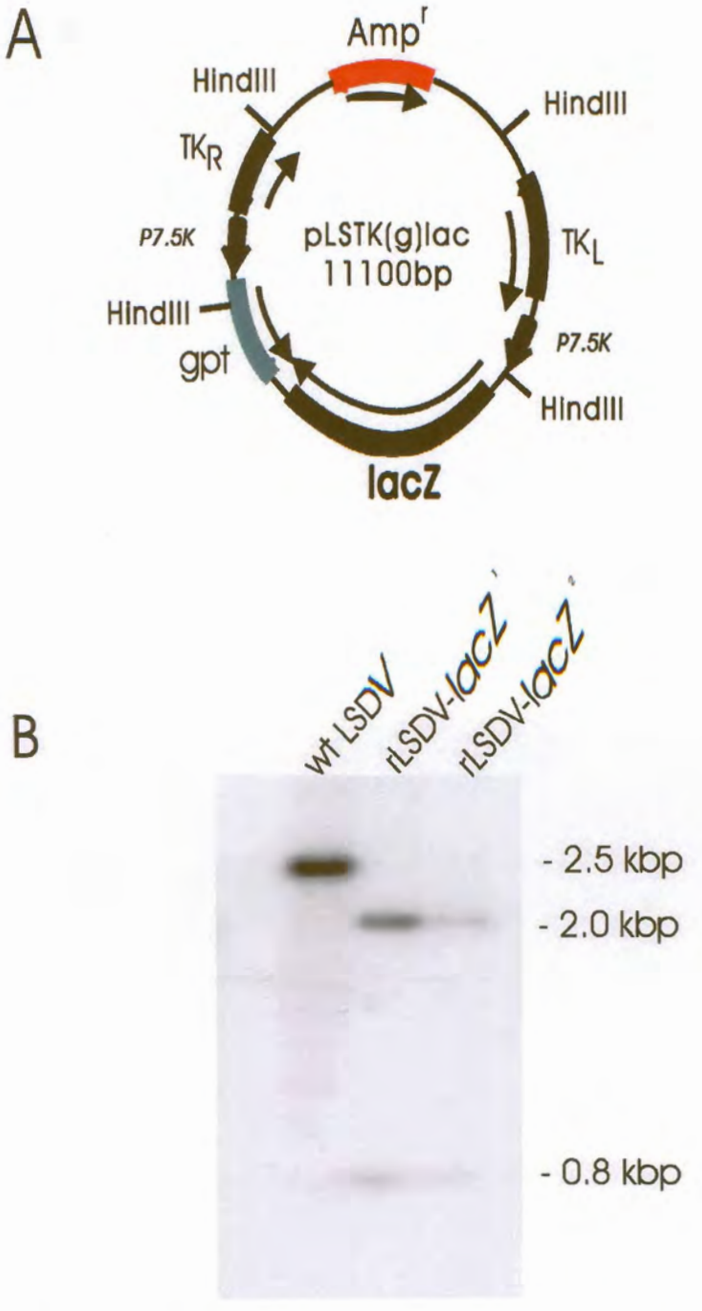



**Figure 2.14** Autoradiograph of wtLSDV and lacZ recombinant *Hind*III digested DNA. **A** - detailed plasmid map of pLS(g)lacZ showing the location of the *Hind*III RE sites. **B** - an autoradiograph of *Hind*III cut wtLSDV DNA and LSDV-lacZ recombinant DNA run on an agarose gel, Southern transferred to a HybondN$^+$ nylon membrane and probed with a $^{32}$P-labelled LSDV TK gene probe. Sizes of the labeled fragments are indicated in kbp on the right.
[1] – DNA from purified virions, [2] – whole DNA extract from infected cells.

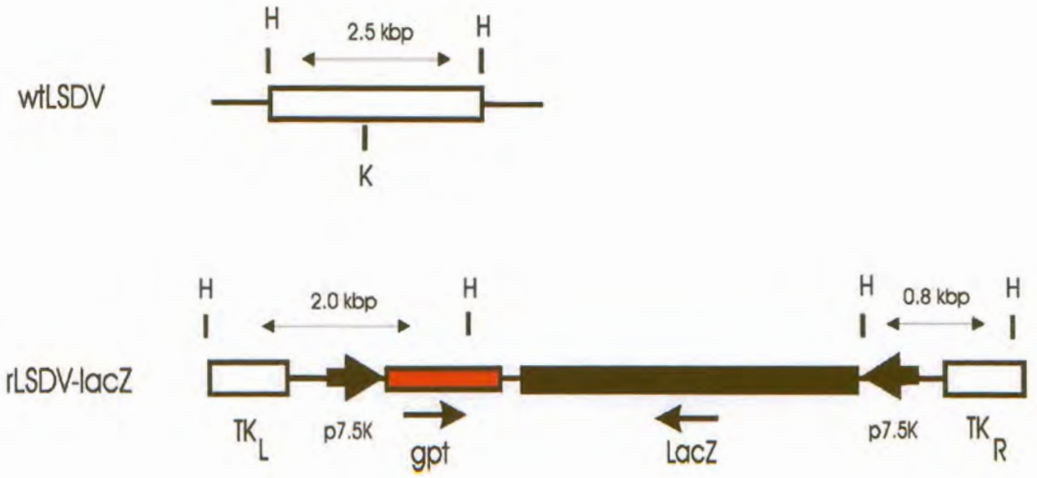
from FBT cells co-transfected with wt LSDV and pLS(g)lac produced distinct viral foci in fresh cells during the second round in selection medium in wells from the lowest dilutions. Media was collected from these wells, treated as described (2.2.7) and titrated further. Cells were then fixed and stained using X-gal (2.2.5). Approximately 90% of the foci stained blue, a strong indication for the presence of recombinants (Figure 2.12 F), whereas no blue colouration was observed for cells infected with wtLSDV (Figure 2.12 E).

An important aspect of the generation and selection of poxvirus recombinants for vaccine purposes is to ensure that they are homogeneous (free from wt parental virus). Mixed populations of wt and recombinant virus would necessitate the constant use of selection media for large-scale virus propagation which would hugely elevate the cost of vaccine production.

*Two molecular-based methods were employed to help evaluate the ability of the modified* Romero method (Romero *et al.*, 1993) to yield homogeneous recombinants, namely PCR and DNA hybridisation.

PCR was utilised as follows: the DNA from a number of viral foci which appeared homogeneous for the expression of lacZ was isolated and subjected to PCR using the TK primers P1 and P2 as described (2.2.9). For at least one of these foci, an amplification product of the full-length insertion region (5.5 kbp) was obtained (Figure 2.13 B, lane 2), providing further support for the recombinant's homogeneity. The presence of an undisrupted TK gene would have produced a band of 450 bp, as can be seen in lane 7 for wtLSDV DNA. Combinations of the internal control primers (P1 and P3, and, P1 and P4) also gave the expected product sizes for DNA amplified from both the pLSTK(g)lac insertion plasmid (lane 4) and this recombinant viral focus (lanes 3 and 5).

Recombinant virus was propagated to high titres from this focus (under selection) and DNA was extracted for additional confirmation of homogeneity using the DNA hybridisation method as follows: DNA from wtLSDV and the recombinant virus was cut with *Hind*III, and the resulting DNA fragments were separated on a 0.8% agarose gel using electrophoresis. The fragments were then Southern transferred to a Hybond$^{TM}N^{+}$ nylon membrane (Amersham, USA) and hybridised with a $^{32}$P-labelled wtLSDV-specific TK gene probe (as described, 2.2.12).
The intact LSDV TK gene is located on a 2.5 kbp *Hind*III DNA fragment. However, as can be seen from figure 2.14 (A) and the schematic below, interruption of the TK gene by insertion of the gpt-lacZ cassette in the *Kpn*I site results in the viral TK gene being split between two *Hind*III fragments (2.0 and 0.8 kbp in size). Thus a radioactive probe produced from the entire viral TK gene would be expected to bind to the 2.5 kbp *Hind*III fragment for wtLSDV DNA and to the 2.0 and 0.8 kbp fragments for rLSDV-lacZ DNA.




As can be seen from the autoradiograph (Figure 2.14 B) this is the case, and, no evidence for the wt TK gene (2.5 kbp fragment) can be seen for the lanes containing the recombinant viral DNA, thus confirming homogeneity.

Another pre-requisite for a pox-vectored recombinant vaccine is that the resulting recombinant is stable. In order to test the stability of the LSDV-lacZ recombinant, selection pressure was removed by growth of the recombinant in normal growth medium (DMEM/Ham's F12, 10% FCS) (no MPA) for 10 passages. DNA was isolated from virus recovered after the $10^{th}$ passage and tested via PCR using the TK primers P1 and P2. Only the recombinant PCR amplification product of 5.5 kbp was obtained (data not shown).
LacZ expression was also tested for progeny virions produced during the $10^{th}$ passage without selection using the X-gal stain. All foci still stained blue.
Collectively, all these results thus confirm that the LSDV-lacZ recombinant was stable and absolutely homogeneous.

## 2.4 DISCUSSION

The aim of this part of the study was to evaluate the viral TK gene of the South African vaccine strain of LSDV as a suitable insertion site for the generation and selection of recombinants. Of the many techniques available for generating and selecting poxvirus recombinants (Mackett *et al.*, 1982; Mackett *et al.*, 1985; Chakrabarti *et al.*, 1985; Guo *et al.*, 1989; Falkner and Moss, 1990; Mason *et al.*, 1991; Cadoz *et al.*, 1992; Merchlinsky and Moss, 1992; Scheiflinger *et al.*, 1992; Ravanello and Hruby, 1994; Stone-Marschat *et al.*, 1996; Scheiflinger *et al.*, 1998;

Timiryasova *et al.*, 2001; Boyle *et al.*, 2004), one of the first and most successfully used methods involves disruption of the viral TK gene with the insertion of foreign genes under control of a poxvirus promoter (Mackett *et al.*, 1985; Boyle and Coupar, 1986). This method has a double advantage as the resulting TK-negative phenotype can provide a means for selection of recombinants and the resulting recombinants are more environmentally acceptable (as no antibiotic resistance markers are present in the final construct). This route was thus the obvious choice for attempting the generation and selection of recombinants of the South African vaccine strain of LSDV earmarked for use as a vector for recombinant vaccines. The success of this method does however reply upon the TK gene being non-essential for growth of the virus. Dubbs and Kit (1964) demonstrated that in mouse fibroblast cells infected with wild type VV there was an induction of TK activity and that the virus encodes a TK gene. They also demonstrated the existence of mutant VV viruses lacking TK activity which were relatively stable. This is also the case for most, but not all poxviruses – TK-disrupted recombinants of pigeonpox virus proved unstable and it was surmised that the gene was essential for certain viral functions (Letellier, 1993). The viral TK gene has been shown to be non-essential for the Kenyan KS-1 vaccine strain of LSDV (Romero *et al.*, 1993), however, this vaccine strain was developed along a different route (and originally for use in sheep and goats) to that of the South African vaccine strain and thus it cannot be assumed that what holds true for one will necessarily hold true for the other (Weiss, 1968; Davies and Otema; 1981; Kitching and Taylor, 1985; Kitching *et al.*, 1987; Gershon and Black; 1989b; Kitching *et al.*, 1989).

Another prerequisite for the TK-negative method of selection is a cell line permissible for growth of LSDV and which is able to tolerate the TK selection drug, BUdR (Boyle and Coupar, 1986). As LSDV is highly host-range restricted (making it ideal for development as a recombinant vaccine vector targeted for use in specific animal species) there are very few cell types which support its growth (Prydie and Coackley, 1959). Surprisingly, attenuation of the South African vaccine strain of LSDV was achieved via repeated passage of a virulent field isolate on the CAMs of embryonated hen's eggs, although subsequent growth in chicken embryo fibroblasts is poor (van Rooyen *et al.*, 1969; Wallace, 1994). An Indian isolate of goatpox virus has been adapted for growth in cells of monkey origin (Vero cells), but, these cells do not support growth of the South African vaccine strain of LSDV (Wallace, 1994; Hosamani *et al.*, 2004). The best growth for LSDV is obtained in cells of bovine and ovine origin (Weiss and Geyer, 1959; Wallace, 1994). It was thus extremely fortuitous to find a bovine kidney cell line (BU100) which had been adapted to tolerate high levels of BUdR (Bello *et al.*, 1987). In preliminary experiments these cells were infected with wild type LSDV (vaccine strain) and observed for cytopathic effects (data not shown) – no cytopathic effects ever developed. However, in the absence of selection pressure, virus grew to levels comparable with growth in normal bovine kidney cells (MDBK cells) (Wallace

and Viljoen, 2002). These results suggest that the viral TK gene was active and propagation of the virus was totally suppressed by the addition of BUdR in the BU100 cells.

A transfer vector which would allow insertion of foreign genes under control of a poxvirus promoter into the LSDV TK gene also had to be developed. pLSTK7.5 was constructed for this purpose with the VV P7.5K early/late promoter upstream of a MCS flanked by the viral TK gene. To assist with monitoring the success rate for the generation and selection of the recombinants the lacZ visual marker gene was included in the insertion cassette. This marker provided a convenient means to rapidly assess whether or not recombinant viruses had been generated, without the need to extract DNA and test via hybridization or PCR. With the construction of the pLSTK7.5-lacZ insertion vector containing the viral TK gene interrupted by the lacZ reporter gene under control of the VV P7.5K promoter it was possible to begin evaluating the TK-negative selection method for generating and selecting recombinants. The calcium phosphate co-precipitation method was used for transfection of the viral and plasmid DNA as this method was being successfully used to generate VV recombinants in our laboratory. However, after many attempts to generate and select recombinants, even after optimising the transfection conditions to match the replication cycle of LSDV, altering the MOI and the amounts of transfecting viral and plasmid DNA, no recombinants could be selected using this approach. At first the reasons for this failure were unknown. Different transfection reagents were even tested (data not shown), but, without success. The only logical explanation was that viral TK activity was important for growth of the virus, but, at this stage it was not possible to test this hypothesis.

Eventually an alternative approach was attempted using positive selection in the form of the *E. coli* gpt gene, however still retaining the viral TK gene as the insertion site – as this approach was found to work for the northern African KS-1 strain of LSDV (Romero *et al.*, 1993). The method does not rely upon a TK-negative cell line for selection. This time success was achieved and it was possible to generate and select LSDV recombinants expressing the lacZ reporter gene, which was evident from the blue colouration of viral foci stained with X-gal.
A single recombinant was selected and was shown to be homogeneous via PCR and DNA hybridisation and indications were that it is highly stable, as removal of the MPA selection drug for 10 passages in FBT cells failed to give any evidence of revertance to wt form (data not shown).

Now that a homogeneous TK-negative LSDV was available, it was possible to evaluate its growth characteristics in a number of different cell types, including the TK-negative BU100 cells. As expected, the recombinant virus was unable to grow in the BU100 TK-negative cell line with or without selection, whereas it was able to grow to high titres in normal TK-producing MDBK, or

FBT cells (Wallace and Viljoen, 2002). These results strongly suggest that some form of TK activity is required for normal growth of LSDV and helps to explain the failure of the TK-negative selection method to yield recombinant viruses. Letellier (1993) found that TK-disrupted recombinants of pigeonpox virus were highly unstable and had a distinct growth disadvantage as compared to TK-positive virus. The importance of TK activity for growth of pigeonpox virus (and fowlpox virus) is highlighted by the inability of others to isolate naturally occurring TK-negative mutants of either virus under BUdR selection (Nazerian and Dhawale, 1991; Letellier, 1993), whereas this is possible for VV (Dubbs and Kit, 1964). Scheiflinger *et al.* (1997) described their inability to isolate TK-negative fowlpox recombinants using a highly attenuated virus strain and they suggest that for such attenuated strains an intact TK gene is required. A similar situation was found for the highly attenuated MVA strain of VV (Scheiflinger *et al.*, 1996), and in this case restoration of viral TK activity by insertion of a functional fowlpox virus TK gene allowed the isolation of recombinants. As the strain of LSDV used in these studies is also a highly attenuated strain, it is not surprising that TK activity is important for viral growth - even if this activity is cellular derived.

Once a strategy had been developed for generating and selecting LSDV recombinants which were shown to be homogeneous and stable, the following step was then to replace the lacZ reporter gene with genes of veterinary importance, as described in the next chapter.

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