CHAPTER ONE

LITERATURE REVIEW

1.1 INTRODUCTION

The history of vaccination go back as far as the middle ages when the Chinese developed the practice of inhaling a powder made of smallpox scabs as protection against future infection. On 14th May 1796, Edward Jenner used cowpox, material taken from the hand of Sarah Nelmes, to "vaccinate" 8 year old James Phipps, who he later challenged with smallpox and showed that he was protected. This led to the principle of vaccination and founded the science of immunology.

Smallpox, caused by the variola virus, was finally eradicated in 1977 and certified by the World Health Organisation (WHO) (Fenner *et al.*, 1988). This was nearly 200 years after the introduction of prophylactic inoculations with cowpox and vaccinia virus, the benign relative of variola. Vaccination contributed to present concepts of infectious disease and immunity. Research on poxviruses did not languish following the eradication of smallpox. The development of vaccinia virus as a live recombinant expression vector provided a tool for immunologists and biochemists as well as an alternative approach to the development of vaccines against a variety of infectious agents, although in theory, any poxvirus has the potential to be adapted and manipulated to serve as a live virus vector.

Recombinant live viral vectors have the advantage that they represent a viral infection, therefore it has the ability to induce both antibody- and cytotoxic T-cell responses (Bennik et al., 1984; Wiktor et al., 1984). A single dose is usually sufficient to produce long term immunity. This eliminates the need for booster injections (Levy et al., 1994). Vaccinia virus has an extremely large DNA genome of 191.6 kilobase pairs (Baxby, 1993). Large non-essential regions of its genome are available for the insertion of foreign DNA into multiple cloning sites that enable the construction of polyvalent vaccines that express multiple antigens simultaneously (Perkus et al., 1985).

Except for all the advantages associated with vaccinia as a live vaccine vector, a number of disadvantages also exist. Vaccinia has a very wide host range and is capable of infecting

animals of many different species, in addition to the target species. The use of vaccinia-based recombinant vaccines may lead to a wide dissemination of vaccinia with the possibility of recombination occurring with other orthopoxviruses present in the non-target animals. The ability of vaccinia to infect humans can, in some cases, cause serious effects. It may lead to death in especially immuno-compromised individuals (Romero *et al.*, 1993).

For many important diseases, a vaccine or a better one is still needed and it is expected that these will only be developed by application of newer biotechnology. Among all the approaches that this may imply, only live vectors will not require the addition of carriers, activators or adjuvants. The characteristics of an ideal live vector can be summarised under the five headings of safety, efficacy, the nature of the induced immune responses, the approach to molecular design and utility in the field.

Poxviruses other than vaccinia are being used as vectors with promising results and have advantages over vaccinia. Many animal poxviruses have restricted host ranges and their use as vaccine vectors may have less ecological impact than vaccinia. In particular viruses not pathogenic for humans can be used as veterinary vectors, thus bypassing one of the main objections to the use of vaccinia virus. According to Taylor *et al.*, (1988a), a great advantage of host-restricted poxviruses is that although infection outside their host-range lead to incomplete replication, the level of expression of foreign genes are sufficient to induce a specific immune response. Capripoxviruses do not infect man and have a very limited host - range which include cattle, sheep, goats and possibly buffalo, therefore making them more suitable candidates for live vaccine vectors expressing immunogenic antigens of veterinary importance (Romero *et al.*, 1994).

1.2 A GENERAL INTRODUCTION TO POXVIRUSES

1.2.1 Introduction

Poxviruses are large DNA-containing animal viruses that infect both vertebrate and invertebrate hosts (reviewed by Moss, 1996). The general properties of *Poxviridae* include (a) a large complex virion containing enzymes that synthesise mRNA, (b) a genome composed of a single linear double-stranded DNA molecule of 130-300 kilobase pairs (Kb) with a hairpin loop at each end, and (c) a cytoplasmic site of replication. The family *Poxviridae* is divided into two subfamilies: *Chordopoxvirinae* (poxviruses of vertebrates) and *Entomopoxvirinae* (poxviruses of insects). These in turn consist of a number of genera (Table 1.1). The *Orthopoxvirus*, vaccinia virus (VV), has been most intensively studied and is the prototypical member of this family. The major antigens of the orthopoxviruses, containing the most well-known poxviruses namely variola (causative agent of smallpox) and its benign relative vaccinia, are unrelated to those from the other poxvirus genera. Within one genus however, there is little antigenic diversity (Kitching *et al.*, 1986).

1.2.2 Virion structure

The virions of poxviruses are larger than those of other animal viruses and are just discernible by light microscopy. Vaccinia virus appear as smooth, rounded rectangles of approximately 350 by 270 nm by cryoelectron microscopy (Dubochet *et al.*, 1994). Thin sections of virions reveal a lipoprotein bilayer and the outer membrane that surrounds a homogenous central core containing viral DNA. The core appears dumbbell-shaped with lateral bodies in the concavities of the core. The lateral bodies are attached to the outer membrane and are ellipsoidal in shape (Medson and Bauer, 1970). An additional lipid-bilayer, called the envelope surrounds mature virus particles (Moss, 1996).

Table 1.1 Family *Poxviridae*: Genera and members

Subfamilies	Genera	Member viruses
CHORDOPOXVIRINAE (Vertebrate poxviruses)	*Orthopoxvirus*	Vaccinia, variola
	Parapoxvirus	Orf
	Avipoxvirus	Fowlpox, canarypox, pigeonpox
	Capripoxvirus	Goatpox, sheeppox, lumpy skin disease
	Leporipoxvirus	Rabbit (Shope) fibroma
	Suipoxvirus	Swinepox
	Molluscipoxvirus	Molluscum contagiosum
	Yatapoxvirus	Tanapox
ENTOMOPOXVIRINAE (Insect vertebrate)	A	*Melontha melontha*
	B	*Amsacta moori*
	C	*Chironimus luridus*

(Adapted from Moss, 1990)

1.2.3 The poxvirus genome

Poxviruses have linear double-stranded (ds) DNA genomes that vary from about 130 Kb in parapoxviruses to about 300 Kb in avipoxviruses. Inverted terminal repetitions (ITR's), which are identical but oppositely orientated sequences at the two ends of the genome (fig. 1.1) are present in all poxviruses examined. The inverted terminal repeats of the vaccinia virus genome are each 10 kilobases in length (Baroudy *et al.*, 1982). The two strands of vaccinia virus are covalently closed forming incompletely base-paired hairpin loops that are A+T-rich at each end of the genome (Baroudy *et al.*, 1982). The variability of the ITR's of poxvirus genomes is consistent with the suggestion that the region contains most of the non-essential genes.

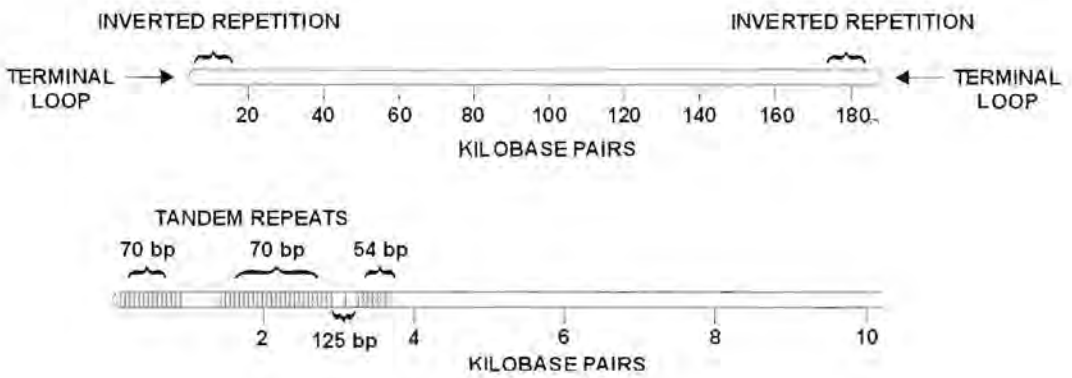

Fig. 1.1 Structural features of vaccinia viral DNA. A representation of the entire double-stranded DNA genome and an expansion of the 10 000 bp inverted terminal repetition (Moss, 1996).

1.2.4 Replication of poxviruses

An unique characteristic of poxviruses is that their DNA replication takes place in the cytoplasm of the host cell due to the fact that the infectious viral cores contain a complete system for the transcription of early class genes (Kates and McAuslen, 1967). Gene expression of vaccinia virus is temporal with classes of early, intermediate and late genes (Baxby, 1993) depending on the stage of infection in which they are activated. Regulation

occurs primarily at the transcriptional level and the genes from each class have distinct cis-acting elements and trans-regulatory factors.

The poxvirus virion, containing a ds DNA genome, RNA polymerase and transcription factors, attach to the host cell and fuse with the cell membrane. Once inside in the cell, the virion uncoats releasing cores into the cytoplasm. In the cores early mRNAs are synthesised that are translated into a variety of proteins, including growth factors, immune defence molecules, enzymes, and factors involved in DNA replication and intermediate transcription. Followed uncoating of cores the viral DNA is replicated and the intermediate genes are transcribed. Their translation products include late transcription factors. The late genes are transcribed and the mRNAs are translated to form virion structural proteins involved in viral assembly, enzymes and early transcription factors. These are packaged into a newly formed progeny particle and is transported to the periphery of the cell. Fusion of the virion with the plasma membrane results in release of an extracellular enveloped virus that can start a new reproductive cycle (Moss, 1996).

1.2.5 Capripoxviruses

Capripoxviruses have been classified according their ability to cause sheeppox in sheep, goatpox in goats or lumpy skin disease (Neethling) in cattle. Work done by Kitching and Taylor (1985), confirmed earlier observations from Capstick and Coakley (1961) that strains of capripoxvirus can infect more than one species of ruminant although they show a preference for growth in either sheep, goats or cattle. The pathology of the lesions produced by capripoxvirus infection in sheep and cattle is very similar and strains obtained from cattle, sheep and goats cannot be distinguished by direct or indirect immunofluorescence or serum neutralisation tests (Davies and Oedema, 1981). A common major antigen of 67 K is also shared by the three capripoxviruses (Kitching *et al.*, 1986).

The close antigenic relationship between strains of capripox was shown by passively immunising sheep with serum against capripoxviruses isolated from a sheep and from a goat. Sheep immunised with immune serum to Oman sheeppox or Yemen goatpox resisted challenge with Yemen goat pox or Nigeria sheeppox respectively. These results showed that immune serum from Oman and Yemen isolates of sheeppox and goatpox were equally

effective in neutralising homologous and heterologous strains of capripoxvirus (Kitching *et al.*, 1986).

Another example to confirm the close antigenic relationship was demonstrated by cross-protection between isolates of capripoxvirus provided by passive immunisation. Lambs born to sheep hyperimmunised with isolates of capripox from Sudan, India and Nigeria were also protected against challenge with Yemen goatpox (Kitching, 1986).

1.2.6 Genomic relationship between capripoxviruses

Capripoxvirus DNA of 12 field and vaccine isolates from sheep, goats and cattle were analysed by digestion with *Hind* III followed by agarose gel electrophoresis. The patterns of fragments obtained with the restriction enzyme studies are sufficiently similar showing that the capripoxviruses are closely related although patterns for 10 different isolates could be correlated with the animal of origin. The close relatedness between the different capripoxviruses was also demonstrated by the high level of sequence homology (Black *et al.*, 1986).

Nucleotide sequence divergence values, calculated from the numbers of conserved and non-conserved *Hind* III, *Pst* I, *Ava* I and *Sal* I sites on the genomes of typical sheep, goat, and cattle isolates, indicated that the genomes of the three groups were between 95.8 % and 97.0 % homologous at nucleotide sequence level. The compared values were an indication that the typical cattle and sheep isolates are more closely related to one another than either is to the typical goat isolate (Gershon and Black, 1988).

Site maps obtained with *Hind* III, *Pst* I, *Ava* I and *Sal* I of the capripoxvirus from sheep, goats and cattle demonstrated that relatively small deletions were observed on the genomes when compared to one another. These deletions were not defined to the terminal or near-terminal regions of the genome where host-range genes would be expected as described for orthopoxvirus (vaccinia), therefore the suggestion that a more restricted set of genes are responsible for the host-range of capripoxviruses (Gershon and Black, 1988).

1.3 LUMPY SKIN DISEASE VIRUS (LSDV)

1.3.1 History

As early as 1929, a skin disease of cattle "pseudu-urticaria" was noticed in the territory then known as Northern Rhodesia (today Zambia). The disease was presumably lumpy skin disease, but during that time the lesions were thought to be caused by the bites of insects (Mac-Donald, 1931). Fourteen years later (1943), it appeared in Bachuanaland (Botswana) and spread throughout the country. Towards the end of 1944 the disease made its first appearance in Transvaal, South Africa (Thomas and Mare, 1945; as cited by Weiss, 1968) and developed into an explosive epidemic despite enforced control measures. By 1947 the disease had become established and enzootic in South Africa (Weiss, 1968) and has been reported from neighbourhood countries. The first outbreak of the disease in Kenya occurred towards the end of 1957 (MacOwen, 1959; as cited by Weiss, 1968). In South Africa, sporadic outbreaks of lumpy skin disease have occurred within the last fifty years. A major outbreak was reported in 1990 suggesting that although there is an effective vaccine, (discussed below), improper or inadequate use was made of the vaccine.

Animals recovered from apparent or inapparent natural infection developed antibodies in their sera capable of neutralising up to 3 logs of virus and were also resistant to re-infection and developed life-long immunity (Weiss, 1968). Following immunisation of cattle with the modified life virus vaccine, circulating antibodies were detectable, especially in those animals showing a local swelling at the site of inoculation. A rise in antibodies was difficult to demonstrate in cattle that failed to develop local reactions. These cattle as well as those with antibodies were resistant to virus challenge. Calves from immune cows showed a passive immunity derived from the colostrum which persisted for six months (Weiss, 1968).

The LSDV-Neethling strain was recovered on numerous occasions from the skin lesions of infected cattle in South Africa as well as in Kenya. An effective vaccine was prepared from the South Africa Neethling isolate and has been routinely used for many years since the 1960's (Weiss, 1968). Serial passage of the Neethling strain in embryonated eggs were followed by adaption to tissue culture resulted in attenuation of the virus for cattle.

1.3.2 Antigenic relationship between different isolates of LSDV

Lumpy skin disease virus has been isolated from field specimens from widely separated outbreaks of the disease in South Africa, Kenya and Malawi. When analysed, all the poxvirus isolations were antigenically similar and showed complete reciprocal cross-neutralisation with the "Neethling" prototype strain. Cross-immunity between the South African "Neethling" virus and the Londiani strain from Kenya suggests that there is only one immunological virus type responsible for true lumpy skin disease (Prydie and Coackley, 1959).

1.3.3 Epidemiology and transmission of LSDV

Although the different ways of transmission of LSDV have not been established with certainty, evidence suggests that biting insects may play an important role in dissemination of infection. Epidemics of the disease was found to be more prevalent during wet summer months specific in low-lying areas and along water sources where the spread couldn't be controlled by quarantine measures. The virus was recovered from the flies *Stomaxys calcitrans* and *Biomyia fasciata* (Kitching and Mellor, 1986). Successful transmission of the disease was observed when infected and susceptible animals in the same stable were watered at a common drinking trough. This confirm the suspicion that infective saliva might contribute towards the spread of the disease (Weiss, 1968). Further more the development of skin lesions in cattle injected intradermally with the Isiolo strain of sheep pox, suggested that sheep might possibly act as carriers of the lumpy skin disease virus. These observations have not been confirmed in South Africa. In South Africa, capripoxviruses have only been isolated from cattle during outbreaks of LSD, it has never been reported in sheep or goats (Thomas and Mare, 1945; as cited by Kitching et al., 1989).

1.3.4 Clinical signs

Clinical signs of cattle infected with naturally LSDV can usually be observed after an incubation time between 2 and 5 weeks. In animals that develop symptoms, the disease is characterised by a febrile reaction accompanied by a loss of appetite, salivation, lachrymation and a nasal discharge. Within 48 hours of the first rise in temperature, circumscribed, firm, round and raised nodules (0.5 to 5.0 cm in diameter) occurs in the skin of the entire body that may vary from a few in mild cases to several hundreds in severe cases. Similar lesions may

be present in the skeletal muscles and the mucosa of the digestive and respiratory tracts. The nodules usually undergo complete necrosis and start to separate from the surrounding healthy tissue after approximately 7 to 10 days and become hard and dry to form scabs which ultimately fall off 3 to 5 weeks after the first appearance. Apart from the circumscribed skin nodules, a subcutaneous oedema of the limbs and ventral parts of the body and a generalised lymphadenopathy are also characteristic of the disease (Weiss, 1968). A constant feature of the disease is also the marked enlargement of the superficial lymph glands.

The mortality rate in affected animals varies from less than 1-10%. Although the mortality rate is low, the disease is of major economic importance through direct losses resulting from emaciation, temporal or permanent cessation of milk production, infertility in bulls and permanent damage to the hides which adversely affected the quality of the leather (Green, 1959).

1.4 POXVIRUSES AS EXPRESSION VECTORS

1.4.1 Introduction

Despite remarkable success in the use of some vaccines in the control of infectious diseases, vaccines are still not available for some diseases and other vaccines could be improved. Increasingly the techniques of molecular biology are being used to supplement or replace previous methods of attenuation and inactivation that have formed the traditional methods of vaccine development. These techniques have led to the successful introduction of vaccines for infections where the pathogen itself cannot be cultured. A potential problem in the use of live, attenuated, multivalent vaccines is that re-assortment between serotypes can result in the generation of progeny viruses with novel phenotypes in terms of serological and virulence characteristics.

Through the application of molecular biology it is now possible to consider several new approaches for making vaccines, which may combine increased efficacy with greater safety. One of these approaches is to genetically manipulate a virus so that it carries and expresses a foreign gene that codes for a protective antigen for another disease. These have been

based on a variety of virus types - poxviruses, herpesviruses and adenoviruses - and have led to the production of many new potential recombinant live vaccines.

1.4.2 Recombinant vaccines based on mammalian poxvirus vectors

The most thoroughly studied member of the poxvirus family, vaccinia virus, was successfully used as a live vaccine to eradicate smallpox. Medical interest in vaccinia virus was restimulated when live recombinants were shown to be capable of expressing foreign genes and protected immunised animals against infections with influenza virus, herpes simples virus types 1 and 2, hepatitis B, rabies virus and vesicular stomatitis virus (Sekhar *et al.*, 1985).

The potential of using poxviruses as recombinant vaccines include a resemblance to live virus infection, stimulation of both humoral and cellular immunity, economy of production, heat stability and their ease of administration (WHO meeting, 1989). The ability to incorporate large amounts of foreign DNA without a loss of infectivity, the correct processing of expressed proteins and the wide host range of vaccinia virus made this expression system of special value for research purposes (as cited by Sekar *et al.*, 1985).

Vaccinia: Rabies vaccine

Although most pioneering work on recombinant vaccine production was conducted using vaccinia virus, the only vaccinia-based recombinant virus licensed as a veterinary vaccine is the rabies-recombinant vaccine. This vaccine is used for the oral vaccination against rabies of foxes in Europe and raccoons and striped skunks in Northern America; these species being the most important natural reservoirs of rabies on their respective continents. The recombinant rabies vaccine was developed by inserting the glycoprotein G gene of rabies virus into the Copenhagen strain (used as a smallpox vaccine) of vaccinia virus (Yamanouchi *et al.*, 1998). The rabies glycoprotein is a structural component that forms surface projections on the rabies virus particle. The protein is localised on the cytoplasmic membrane of rabies virus infected cells and induces rabies virus neutralising antibodies (Taylor *et al.*, 1988a).

Immunity obtained with oral vaccination of foxes with the rabies-recombinant vaccine conferred a minimum of 12 months in cubs and 18 months in adult animals that corresponds to the duration of the protection required for vaccination of foxes in the field. The safety of

this vaccine for domestic, laboratory and numerous European wild animals were test by carefully controlled and monitored field tests performed in Western Europe. No clinical signs or lesions were observed in any of the vaccinated animals during a minimum of 28 days post vaccination (Pastoret and Brochier, 1996).

Similar developments have been taken place in the United States of America (USA) where raccoons and coyotes were vaccinated. Between 1989 and 1995, approximately 8.5 million doses were dispersed in these areas without any problems, demonstrating the effectiveness of wildlife vaccination using recombinant vaccines on a large scale (Yamanouchi *et al.*, 1998). According to Baxby (1993), the candidate recombinant vaccinia virus was safe in the target species but limited subclinical spread to, and seroconversion of, contact animals have occurred. This has delayed field trials of the vaccine and led to studies on other poxviruses as possible recombinant vaccines, maybe more host-restricted poxviruses as the fowlpox and canarypox viruses (see below).

Vaccinia: Rinderpest vaccine

Rinderpest virus (RPV), a member of the genus Morbillivirus in the family Paramyxoviridae, has a single-stranded RNA genome and remains one of the most devastating and economically important diseases of cattle in Africa, Asia and the Middle East. The haemagglutinin (HA) and fusion (F) surface proteins have been shown to provide protective immunity. A major obstacle to the campaign to eradicate rinderpest is the heat-labile nature of the current rinderpest vaccine, despite its very high efficacy. With the aim of producing a more heat-stable vaccine, three different recombinant vaccines, based on vaccinia virus as a vector, have been developed against rinderpest.

Previous studies concentrated on recombinants that expressed HA or F genes of rinderpest respectively (Yilma *at al.*, 1988). These recombinants produced pocks in cattle with the consequent possibility of transmission to contact animals or handlers. Although they prevented overt infection in challenged cattle, subclinical replication of the challenge occurred, especially in animals that received the F recombinant. Cattle immunised with an attenuated dual recombinant vaccinia vaccine that expresses both HA and F were found to be completely protective against a lethal dose of RPV (Giavedoni at al., 1991). All animals

vaccinated with the recombinants produced neutralising antibodies to RPV whereas pock lesions were limited to the site and healed completely within 2 weeks (WHO meeting, 1989).

1.5 HOST-RANGE RESTRICTED POXVIRUSES USED AS VECTORS

Vaccinia has a wide host range that could be an advantage for a veterinary vector. However, it is capable of infecting animals of many different species in addition to the target species. Extensive use of vaccinia-based recombinant vaccines may therefore lead to a wide dissemination of vaccinia with the possibility of recombination occurring with other orthopoxviruses present in non-target animals (Romero *et al.*, 1993). This means that transmission to and safety in contact species, including humans, have to be considered as well as morbidity in target species. Not all strains of vaccinia virus were used as smallpox vaccine, and some that were used were discontinued for safety reasons (Baxby, 1993). In light of this view, the basic technologies used to construct vaccinia virus recombinants have been modified and extended to other members of poxviruses with host-range that are restricted to particular target animal groups.

Avipox viruses:

The natural host of fowlpox virus (FPV) is limited to avian species. The virus is the prototype species of the *Avipoxvirus* genus of the family *Poxviridae* (Taylor *et al.*, 1988a). FPV, possessing many of the biological properties of vaccinia virus such as stability and ease of production, has clear utility for the development of a species-specific recombinant viral vector. A fowlpox virus recombinant expressing the haemagglutinin molecule from a highly virulent avian influenza virus was developed by Taylor *et al.* (1988b). Immunised chickens and turkeys were protected by the recombinant when challenged with either the homologous or heterologous influenza virus strain. In the same way the haemagglutinin-neuraminidase (HN) gene from the Beaudette C strain of Newcastle disease virus (NDV) has been expressed in a recombinant fowlpox virus vector (Boursnell *et al.*, 1990). Chickens vaccinated with the fowlpox/HN recombinant were protected against challenge with a virulent strain of NDV despite the low levels of induced antibodies.

The use of fowlpox virus as a live vector that would not productively infect non-avian species presents an interesting approach in the development of safe and effective vaccines. A

fowlpox virus was engineered to express glycoprotein of the rabies virus (Taylor *et al.*, 1988a). On inoculation of the fowlpox virus recombinant into either avian or non-avian cells the rabies glycoprotein was expressed as a membrane-associated antigen. The inoculation of six different mammal species with the recombinant resulted in specific immune responses to both fowlpox antigens and to rabies glycoprotein. When challenged with a live rabies virus the immune response obtained in mice, cats and dogs was sufficient to protect them. These results demonstrated that although infection leads to incomplete replication outside the host-range of fowlpox virus, the expression of the foreign gene is sufficient to induce a protective immune response (Taylor *et al.*, 1988a). The ability to utilise this recombinant vector to induce an immune response without the production of infectious progeny virus provides a built in safety feature in vaccination procedures, since the potential for transmission to other species or to non-vaccinated individuals would be reduced.

The positive results obtained with the fowlpox virus recombinant as potential vaccine vector were confirmed and extended by construction of a canarypox vector which expresses the rabies glycoprotein (Baxby, 1993). Like fowlpox, canarypox is a member of the *Avipoxvirus* genus and its natural host limited to avian species. The efficiency of the canarypox recombinant was compared with that of two other pox-rabies recombinants. When canarypox virus was used as the virus vector, a 100-fold lower dose of inoculum virus was required to achieve the same level of protection gained with the fowlpox virus recombinant and similar protection to that induced by a replicating vaccinia vector (Taylor *et al.*, 1991). Because avian poxviruses do not cause productive infection in mammalian cells their use as vaccine vectors would seem to bypass most if not all of the objections to the use of vaccinia virus as a vaccine vector, whilst remaining the advantages of using an established vaccine.

Swinepox viruses:

Swinepox virus (SPV) is the only member of the genus *Suipoxvirus*, belonging to the family *Poxviridae*. SPV's, another poxvirus with limited host-range, natural host is the pig. The virus causes a mild, generalised infection with lesions detected only in the skin and regional lymph nodes. The natural characteristics of SPV infection make it well suited for the development of recombinant vaccines and its use to vaccinate against other diseases has been considered (Tuboly *et al.*, 1993; Van der Leek *et al.*, 1994).

Evaluation of a swinepox virus as a vaccine vector in pigs, using an Aujeszky's disease (pseudorabies) virus gene insert coding for glycoproteins GP50 and GP63, showed that pigs vaccinated with the swinepox recombinant developed serum neutralising antibodies to Aujeszky's disease virus. Upon challenge with virulent virus, significantly fewer pigs developed clinical Aujeszky's disease (Van der Leek *et al.*, 1994).

A recombinant SPV expressing β-galactosidase (β-gal) was constructed and characterised by Barcéna and Blasco (1998). The recombinant SPV expressing β-gal was used to characterise the host-range of the virus in different cell lines. Surprisingly, the recombinant SPV was able to infect and replicate in several cell lines of non-swine origin. Upon infection with the recombinant SPV, there was a significant level of viral replication and spread in certain non-porcine cell lines. The data indicated that although SPV grows more efficiently in porcine-derived cell lines, the virus exhibits a relatively broad range in cell culture (Barcéna and Blasco, 1998).

Results obtained with the Avipox viruses as possible vaccine vectors initiated the development of capripoxviruses, which naturally infect ungulates, as host-restricted vaccine vectors for the expression of important immunising antigens.

Capripoxviruses:

Recombinant sheeppox viruses, expressing either the fushion (F) or haemagglutinin (HA) protein genes of rinderpest virus, were developed as possible vaccines against rinderpest in cattle. In both recombinant capripoxviruses, a cDNA copy of the coding sequence of either the F or HA genes of RPV was inserted in the thymidine kinase gene of the capripox genome under control of the VV late promoter p11 together with the *Escherichia coli gpt* gene in the opposite orientation under control of the vaccinia early/late promoter p7.5K. In both cases the vaccine, prepared from the recombinant viruses, protected cattle against a lethal challenge with virulent rinderpest as well as lumpy skin disease. Protection with the recombinant capripox containing the HA-gene, was achieved using lower doses of vaccine than those used with a similar recombinant expressing the fusion protein gene of rinderpest (Romero *et al.*, 1994).

Peste des petits ruminants (PPR) is a morbillivirus infection of sheep and goats and some species of deer. The virus of PPR is antigenically closely related to that of rinderpest (RP) and small ruminants can be protected against PPR using a RP vaccine (Taylor, 1979). Following vaccination with a recombinant capripoxvirus containing either the fusion (F) or haemagglutinin (HA) – gene of rinderpest virus protected goats against a lethal challenge of peste des petits ruminants virus. Neither recombinant produced detectable levels of specific antibodies to PPR virus.

Bluetongue is an economically important noncontagious disease of sheep and cattle. The causative agent, bluetongue virus (BTV) is the prototype member of the orbivirus genus within the family Reoviridae. A recombinant capripox virus was constructed containing a cDNA copy of genome segment 7 of BTV (Wade-Evans *et al.*, 1996). VP7 is the major serogroup-specific antigen of BTV and was immunodominant during monoclonal antibody production using *in vitro* priming techniques. Serotype cross-reactive T- cell epitopes have also been identified within VP7 (Angove, 1995). Sheep vaccinated with this recombinant capripox virus developed antibodies to VP7 but no neutralising antibodies to either the homologous or heterologous BTV serotype. Following challenge, the vaccinated sheep were partially protected against the lethal effects of challenge with a virulent, heterologous serotype of BTV demonstrating that the vaccine provided cross-serotype protection (Wade-Evans *et al.*, 1996).

1.6 CONSTRUCTION OF RECOMBINANT POXVIRUSES

The term "recombinant poxvirus" is used to describe a poxvirus that expresses a foreign gene that has been inserted into the poxvirus genome by *in-vivo* recombination. Such recombinants retain their infectivity, and those expressing immunising antigens can be assessed as potential vaccines. The use of poxviruses as live expression vectors of foreign genes has been well documented since it was demonstrated in 1982 that foreign antigens could be expressed using vaccinia virus (Mackett et al., 1982; Panicali & Paoletti, 1982).

Certain features of poxvirus structure and replication promote or constrain the usefulness of poxviruses as recombinant vectors. The large size of the genome make the construction of recombinant genomes *in vitro* particularly difficult. Even if this was possible, isolated poxvirus DNA is not infectious because the virion contains enzymes, including RNA polymerase and

other enzymes essential for the production of fully functional mRNA. Therefore, it would be difficult to produce infectious recombinants from recombinant genomes constructed *in vitro*. Although it is possible to construct recombinants by inserting foreign DNA into available restriction enzyme sites within non-essential regions of the VV genome (Panicali & Paoletti, 1982), the procedure limits the promoters used. Foreign DNA under control of the non-essential gene promoters may result in lower expression of the genes. The preferred method to construct recombinants is *via* homologous recombination between plasmids containing virus sequences and the homologous sequence in the virus genome.

Vaccinia virus recombinants, expressing foreign genes, were constructed by Mackett *et al.* (1985), using a two stage process. Firstly, recombinant DNA techniques were used for the construction of a plasmid (transfer / insertion vector) containing a chimeric gene flanking by vaccinia virus DNA. The chimeric gene consists of a vaccinia virus transcriptional start site and upstream regulatory sequences adjacent to the promoter coding sequence for the foreign gene and genetically engineered restriction endonuclease sites for introduction of foreign DNA. The poxvirus RNA polymerase recognises only its own transcriptional control sequences (promoters) and therefore it is essential that any foreign gene inserted into the poxvirus genome is downstream of an adjacent poxvirus promoter. In some instances the foreign gene is inserted downstream of an existing vaccinia promoter (Panicali & Paoletti, 1982) and in other cases a particular vaccinia promoter is specifically inserted into the construct (Mackett *et al.*, 1982).

The next stage is the insertion of the chimeric gene into the vaccinia virus genome. This can be done by transfecting wild-type virus infected cells with the transfer / insertion vector. Homologous recombination occurs between the vaccinia sequences flanking the chimeric gene in the plasmid and the virus genome, thus producing a recombinant virus. The virus DNA that flanks the chimeric gene determines the site at which the foreign gene is incorporated into the vaccinia genome.

By definition the foreign gene has to be inserted into a region of the poxvirus genome not essential for replication and should contain its own translational start and stop signals to avoid problems with improper reading frames (Baxby, 1993). Several non-essential regions of the vaccinia virus have been identified (Smith and Mackett, 1992). Although any of these regions could be used for insertion of foreign DNA, the chimeric gene is most often inserted

into the virus thymidine kinase (TK) gene that is thereby inactivated. Recombinants that are TK^- can be selected by growth in TK^- cells in the presence of 5-bromodioxyuridine (BUDR). Foreign genes inserted into other non-essential regions of the virus genome do not have the advantage of providing a phenotypic marker that can be selected (Mackett *et al.*, 1985). Unless there is some phenotypic marker, it is difficult or tedious to distinguish rare plaques containing recombinant virus from the much larger number of plaques composed of parental virus. Due to the increasing importance of recombinant viruses as expression vectors, several different selection and screening methods have been developed. These include selection for TK-negative and -positive phenotypes, β-galactosidase screening, dominant selective markers for neomycin or mycophenolic acid resistance, screening for haemagglutinin-negative phenotype, reversal of plaque size, host range mutations and transient selection of a marker gene (reviewed by Falkner & Moss, 1990).

Selection for recombinant Capripoxviruses

Different isolates of capripoxvirus have similar DNA genomes of about 145 Kb in length with similar patterns of distribution of restriction enzyme recognition sites (Gershon & Black, 1987; 1988). Like the other poxviruses, capripoxvirus isolates contain terminal loops that covalently close the ends of the DNA genome; the sequences next to the termini are also repeated at both ends. Four non-essential regions in the capripoxvirus genome, the thymidine kinase (TK) gene, the CT3C region, the CT4 region and the Q2 region, have been published (Bostock, 1990).

For the construction of recombinant capripoxviruses, intergenic regions of the LSDV genome (e.g. the region between the A2L and A3L gene analogs of VV) can be targeted to allow homologous recombination in conjunction with a dominant selective marker such as the *Escherichia coli* xanthine-guanine phosphoribosyl transferase (gpt) gene. Expression of the gpt-gene allows recombinant LSDV (containing the gpt-gene under control of a VV promoter) to grow in the presence of mycophenolic acid (MPA) - medium. Mycophenolic acid blocks the *de novo* pathway for synthesis of guanine monophosphate (GMP). Expression of the gpt-gene allows cells to produce GMP from xanthine, allowing growth on medium that contains xanthine but no guanine. The expressed product of gpt is a bacterial enzyme that does not have a mammalian homologue, therefore it can be used as a dominant selectable marker gene for the isolation of LSDV recombinants against wt LSDV (Mulligan and Berg, 1981).

Promoters used in recombinant poxviruses

The successful expression of immunogenic antigens in poxviruses is dependant upon the availability of cis-acting transcriptional control elements – promoters. Both the level and the temporal regulation of foreign gene expression appear to have effects upon the successful use of poxvirus recombinants for vaccine vectors (Boyle, 1992).

Many poxvirus recombinants described to date have utilised strong vaccinia virus (VV) promoters to express foreign genes, for example:

(i) A fowlpox virus recombinant expressing the haemagglutin molecule from avian influenza virus under control of the VV early/late promoter H6, provided protection for chickens against challenge with a virulent influenza virus strain (Taylor *et al.*, 1988b). Chickens inoculated with a fowlpox virus recombinant expressing the fusion protein of Newcastle Disease virus (NDV) under the VV early/late promoter H6, also protected chickens against lethal challenge with NDV (Taylor *et al.*, 1990).

Another example using the VV early/late promoter H6 was with the canarypox virus recombinant expressing the rabies glycoprotein gene. Upon inoculation into non-avian species the canarypox-rabies recombinant was shown to protect these animals against lethal challenge (Taylor *et al.*, 1991).

(ii) Great success was also achieved using the VV major late promoter p11 together with the VV early/late promoter p7.5K in constructing poxvirus recombinants. A recombinant capripoxvirus has been constructed containing the fusion gene of rinderpest virus under control of the VV p11 promoter with the *E.coli* gpt gene in the opposite direction under control of the p7.5K promoter. A vaccine prepared from this recombinant virus protected cattle against clinical rinderpest when challenged with a virulent virus isolate.

The same principles were used for constructing a recombinant capripoxvirus expressing the major core structural protein (VP7) of bluetongue virus (VP7 under control of the p11 promoter and *E.coli* gpt under control of the p7.5K promoter in the opposite direction). Sheep

vaccinated with the recombinant capripox virus were partially protected against the lethal effects of a challenge with virulent BTV (Wade-Evans *et al.*, 1996).

The dependence of poxviruses on its own promoters, and their separation into those which function early (pre-replicative), late (post-replicative) and through-out the replication cycle (constitutive) allow construction of recombinants where the foreign gene can be expressed at different times during the replication cycle.

Regulation of the expression of influenza haemagglutinin (HA) by the VV promoters pF (early), p7.5K (early/late) and p11 (late) has been demonstrated by Coupar et al. (1986), using HA-vaccinia recombinant viruses. Levels of HA obtained on the surface of infected cells were lower with the pF promoter than with either the p7.5K and p11 promoters. HA expressed under control of either the early promoters (pF and p7.5K) was recognised by cytotoxic T lymphocytes (CTL) when the different recombinant vaccinia viruses were used to infect target cells. In contrast, HA expressed under control of the late promoter (p11) failed to prime for a CTL response. These results contributed important knowledge for the development of recombinant viral vectors as vaccines. It suggests that an antigen important for eliciting a humoral response should preferentially be expressed from a late promoter while another that is important for eliciting cellular immunity should be expressed from an early promoter.

Boyle (1992) compared the efficiency of poxvirus promoters in vaccinia and fowlpox virus (FPV) recombinants respectively and found higher levels of marker gene expression in the case of homologous virus-promoter recombinants when compared to the levels obtained with heterologous virus-promoter recombinants. These results motivated the identification, characterisation and use of an authentic LSDV promoter (Fick and Viljoen, 1999).

1.7 AIMS OF THIS INVESTIGATION

This study is part of an investigation to develop lumpy skin disease virus (LSDV) as a live recombinant system for expressing immunogenic important genes cloned under control of the authentic bi-directional LSDV promoter. This promoter has previously been identified and characterised (Fick and Viljoen, 1999). Transient transcription assays using a reporter gene *LacZ*, verified the temporally regulated nature of the promoter and revealed both early and late transcriptional activities. To date the LSDV promoter element has not been analysed for its ability to drive the expression of foreign genes in live LSDV recombinants.

Previous attempts to generate recombinants using a transfer vector (pTKsLR) containing the SA-LSDV thymidine kinase (TK)-gene were unsuccessful (Fick W.C., personal communication, U.P.). A new transfer vector pHSWF, specific for LSDV, was constructed by Dr. W.C. Fick (Department of Genetics, UP). The vector contains the LSDV bi-directional promoter pA7LA8R, 2 flanking regions of LSDV to allow homologous recombination with the wild-type LSDV genome, and the *E.coli* xanthine-guanine phosphoribosyl transferase gene under control of the vaccinia early/late p7.5K promoter as a positive selectable marker. The vector directs the insertion of a foreign gene into an intergenic region of the LSDV genome, between the A2L and A3L gene analogs of VV instead of targeting the TK-gene. The *E. coli* gpt dominant marker gene allows recombinants to grow in MPA-medium that is used for selection.

The main goal of this study was to evaluate the use of the new transfer vector for generating LSDV recombinants. To accomplish this, the following aims were envisaged:

(1) (a) To confirm the activity of the bi-directional promoter in the newly constructed vector by transient expression assays using the *LacZ* reporter gene under control of either the early or late promoter.
(b) Generation of LSDV recombinants that express the *LacZ* gene (Chapter 2).

(2) (a) To generate a single LSDV recombinant that expresses an immunological important antigen encoded by the VP7 gene of AHSV-9, under control of the early promoter.

(b) To generate dual LSDV recombinants that express two immunological important antigens simultaneously. The AHSV-9 VP7 gene will be expressed under control of the early - and AHSV-9 VP2 under control of the late promoter (Chapter 3).

CHAPTER TWO

EVALUATION OF A LSDV BI-DIRECTIONAL PROMOTER ELEMENT FOR EXPRESSING A REPORTER GENE IN LSDV RECOMBINANTS

2.1 INTRODUCTION

The success of recombinant poxviruses depends greatly on the poxvirus promoter used to drive the expression of the foreign gene (Mackett *et al.*, 1982). The preference for authentic promoters, the limited host-range of LSDV and the lack of information on features of capripoxvirus regulatory elements initiated the investigation of possible LSDV promoters.

An early/late bi-directional promoter element of LSDV was identified and characterised by Fick and Viljoen (1999). The 56 bp element shows substantial structural similarities with other poxvirus promoters and provided further evidence that transcriptional elements are conserved within the *Poxviridae*. The predicted critical regions of the early and late LSDV promoters overlap. The LSDV element was designated pA7LA8R where the early promoter pA8R, drives expression of an open reading frame (ORF) in a rightward direction while the late promoter pA7L, drives expression of an ORF in a leftward direction (Fick and Viljoen, 1999).

When comparing the transcriptional activities of the bi-directional promoter with well characterised VV promoters, results of transient expression assays confirmed that the expression levels from the LSDV promoter elements were in the same range as for other poxvirus promoters. The LSDV late promoter (pA7L) induced higher levels of β-galactosidase expression than the VV p7.5K promoter but was not as efficient as the VV p11 promoter. The comparative strength of the LSDV late promoter suggested that it should successfully drive the expression of foreign antigens in LSDV recombinants (Fick and Viljoen, 1999). The LSDV early promoter (pA8R) produced levels of β-galactosidase expression in the same order of magnitude than the p7.5K early promoter (Fick and Viljoen, 1999).

In this part of the study, the pHSWF vector was evaluated for its ability to generate stable recombinants that could express foreign genes from both the early and late promoters. The *LacZ*-reporter gene would be used for the analyses. By placing the *LacZ*-gene

downstream of the early and late promoters respectively, it would be possible to evaluate the efficiency of the 2 promoter elements to drive the expression of a reporter-gene, and hence their potential to drive foreign genes of immunological importance. The reporter-gene was cloned either into the unique *Eco* RI-site or into the unique *Bam* HI-site of the vector. When cloned into the *Eco* RI-site, the *LacZ*-gene is placed under control of the early promoter (pA8R), and when cloned into the *Bam* HI-site it is placed under control of the late promoter (pA7L).

The two constructs pHSWF/*Lac*E and pHSWF/*Lac*L would be used to transfect FBT-cells infected with wt LSDV and firstly tested by transient expression studies where the blue coloration of cells would confirm expression of the *LacZ*-gene. The constructs would then be used to generate LSDV recombinants by allowing homologous recombination to take place. The *E.coli* gpt gene included in the transfer vector would enable the selection of recombinants in the presence of MPA-selection medium. An intense blue coloration of foci obtained with X-gal staining, would be an indication that the X-gal substrate had been converted by the enzymatic activity of β-galactosidase, the gene-product of *LacZ*. The presence of the *LacZ*-gene in the LSDV genome would be confirmed by Southern blot analysis.

2.2 MATERIALS AND METHODS

2.2.1 Construction of plasmids containing the *LacZ*-gene

Information about the construction of the pHSWF vector has not yet been documented. For the sake of completeness a schematic presentation is included (see Appendix 1, p 110-112).

The pHSWF vector has a bi-directional LSDV promoter, pA7LA8R. The pA7LA8R-promoter has on the one side a unique *Eco* RI-site and on the other a unique *Bam* HI-site. If cloned into the *Eco* RI-site, a gene is placed under control of the early promoter (pA8R), while a gene that is cloned into the *Bam* HI-site is placed under control of the late promoter (pA7L). A reporter gene *LacZ*, was excised as a 3737 bp *Hind* III - *Bam* HI fragment from the commercial available pSV-β-Galactosidase vector (Promega), see fig. 2.1, and cloned under control of either the early or late LSDV pA7LA8R-promoter. The two plasmids were named pHSWF/*Lac*E and pHSWF/*Lac*L, see fig. 2.9a and 2.9b (p 44-45).

2.2.2 Restriction enzyme digestion

Restriction endonucleases (r.e.) are enzymes that recognise and cleave double stranded (ds) DNA in a sequence dependent manner at specific sites within or adjacent to the sequences. The efficiency of the digestion reaction is in part dependent upon the purity of the DNA. Different restriction enzymes are effective at different conditions and buffer-types are used to create the specific optimal conditions. Each buffer has an unique salt- and ion-concentration and a pH where a specific enzyme functions optimally.

To obtain the insert of interest, double digestions were carried out in a total volume of 50 µl, containing 15 µg DNA and 2.5 units of each enzyme. The transfer vector was linearised in a single digestion reaction in a total volume of 20 µl, containing 3 µg DNA and 1.5 units of enzyme. All the digestions were done overnight at the recommended temperature according to the manufacture's conditions for the enzyme concerned. The digested products were analysed on 1 % agarose gels.

2.2.3 Agarose gel electrophoresis

Restriction enzyme (r.e.) digested DNA was analysed by electrophoresis on horisontal 1 % agarose gels using a minisub DNA (7 x 10 cm) electrophoresis equipment with opposite polarised electrodes at the two ends. The electrophoresis buffer contained 1 X TAE (40 mM Tris base, 2 mM $Na_2EDTA.2H_2O$, 1.14 mM Glacid acetic acid) and gels were made according to the method described by Sambrook *et al.* (1989). DNA-molecules of different sizes are separated when moving in an electric field from the negative to positive pole due to the constant negative charge of DNA. 0.5 μg/ml Ethidium bromide (a carcinogenic DNA intercalating agent that is fluorescent in ultra violet light) was added to the solution before pouring the gel. Prior to loading the gel, the DNA samples were mixed with an equal volume of 6 x loading buffer (0.25 % bromo-phenol blue, 40 % sucrose in water) to monitor movement of DNA fragments. The loading buffer gives DNA a higher density and ensures that the DNA moves down to the bottom of the well. Electrophoresis was performed at 67 V and 35 mA for 3.5 hr after which the DNA was visualised by UV fluorescence.

Restriction enzyme digested viral DNA was analysed by electrophoresis on a horisontal 0.6 % agarose slab gel using a custom-built electrophoresis equipment. The electrophoresis buffer contained 1 x TBE (89 mM Tris-base, 89 mM boric acid, 1 mM EDTA) and the gel was made as described above except that it was stored at 4 °C for 45 min, after the gel has settled, before loading the DNA-samples. Electrophoresis was performed at 43 V and 31 mA for 16 hr, after which the gel was stained with ethidium bromide (0.5 μg/ml) in 1 x TBE-buffer for 30 min and visualised by UV fluorescence.

2.2.4 Purification of DNA excised from gels.

The correct DNA fragments and linearised vector were obtained by excising the specific bands from 1 % agarose gels, making use of standard molecular size markers in the presence of ethidium bromide (EtBr) under a UV-light (Sambrook *et al.*, 1989). The size markers were PhiX174/*Hae* III Marker (φX) from Promega and DNA molecular weight marker II (SMII) from Boehringer Mannheim.

Gene fragments were purified with Glassmilk methodology by making use of the Geneclean™ kit (BIO 101) according to the manufacture's instructions. The excised fragments were mixed with 2.5 volumes of saturated NaI solution. NaI gives a high salt-

concentration that ensures DNA-binding to the silica. The gel was dissolved at 55 °C for 5 min followed by the addition of 5 µl Glassmilk™ suspension per 5 µg (or less) of DNA with an additional 1 µl for each 0.5 µg of DNA above 5 µg. Glassmilk is a silica-matrix that allow binding of single and double stranded DNA without binding any contaminants. After 15 min on ice, the silica-matrix with bound DNA was pelleted by centrifugation at 12 000 rpm for 1 min and washed three times with ice-cold NEW WASH (NaCl, ethanol, water). NEW WASH, with the optimal salt-concentration, ensures DNA silica-matrix binding while all contaminants are washed away. The DNA was finally eluted from the silica by resuspending in 10 µl double distilled water (ddH_2O) and incubating at 55 °C for 3 minutes. The elution step was repeated to yield a final volume of 20 µl purified DNA.

2.2.5 Generation of blunt-ended fragments

Purified gene fragments were blunt-ended in a reaction containing 5 mM dNTP's and 10 U Klenow enzyme (Boehringer Mannheim) for 30 min at 37 °C to fill the 5' sticky end. To inactivate the Klenow enzyme, the reaction was incubated at 65 °C for 3 min followed by Glassmilk purification.

2.2.6 Dephosphorylation of vector

The linearised vector was dephosphorylated with phosphatase alkaline enzyme (Boehringer Mannheim) in the 10 x dephosphorylation buffer (0.5 M Tris-HCl, 1 mM EDTA, pH 8.5) at 37 °C for 30 min. The enzyme removes the 5'-terminal phosphatase residues in order to reduce the possibility of recircularisation of the vector. The phosphatase alkaline enzyme was inactivated by incubating at 65 °C for 3 min before vector DNA was recovered by Glassmilk purification.

2.2.7 Ligation of DNA fragments

Within a ratio of approximately 4:1 the purified insert to vector was ligated in a 10 µl reaction containing 10 x ligase buffer (660 mM Tris-HCl, 50 mM $MgCl_2$, 10 mM dithioerythritol, 10 mM ATP, pH 7.5) and 1 U T4 DNA ligase (Boehringer Mannheim) for 18 hr at 16 °C. T4 DNA ligase catalyses the formation of phosphodiester bonds between neighbouring 3'-hydroxyl- and 5'-phosphate ends in double-stranded DNA. The linearised dephosphorylated vectors were self-ligated under the same conditions as ligation controls.

2.2.8 Preparation of competent *Eschericia coli* cells

The calcium chloride method of preparing competent Top10F-cells was used. The use of $CaCl_2$ to enhance foreign DNA uptake by bacterial cells was first demonstrated by Mandel and Higa (1970). The procedure is recommended due to the fact that the bacterial cells are rendered more susceptible to the uptake of foreign DNA by exposure to calcium ions at 0 °C. The plasmid DNA that expresses an antibiotic-resistance gene allows the transformed bacterial cells to survive in the presence of the appropriate antibiotic.

A 2 ml overnight culture of *E.coli* Top10F-cells was used to inoculate 100 ml sterile Luria-Bertani medium (1 % bactotryptone, 0.5 % bacto-yeast and 1 % NaCl adjusted to a pH of 7.5) pre-warmed to 37 °C. The culture was grown to log phase (OD_{550} = 0.471), with shaking at 37 °C and placed on ice for 10 min to inhibit mitosis as described by Sambrook *et al.* (1989). The cells were harvested by centrifugation at 4000 rpm for 5 min at 4 °C and resuspended in half of the original volume ice cold 50 mM $CaCl_2$. After 30 min on ice, the cells were collected by centrifugation at 4000 rpm for 5 min at 4 °C and carefully resuspended in 2 ml of ice cold 50 mM $CaCl_2$. Before using in transformation the cells had to be incubated on ice for 1 hour.

Sterile glycerol, to a final concentration of 15 %, was added to cells that were not used for transformation on the same day. These cells were aliquoted into 350 µl fractions and stored at -70 °C for later use.

2.2.9 Transformation procedure

The respective annealed insert/vector ligation mixtures (5 µl) were added to 300 µl of Top10F competent *E.coli* cells and placed on ice for 30 min. The DNA uptake was heat-induced (Sambrook *et al.*, 1989) by incubating the cells at 42 °C for 90 seconds followed by a 2 min cooling down on ice. Eight-hundred µl sterile Luria-Bertani medium (LB broth), pre-warmed to 37°C, was added and the cells were incubated at 37 °C for 1 hr with shaking to allow expression of the antibiotic resistance gene encoded by the vector. The transformed cells were plated onto 1.2 % LB broth agar plates containing 100 µg/ml ampicillin and 12.5 µg/ml tetracyclin, using a spreading technique as described by Sambrook *et al.* (1989), and incubated for 24 hr at 37 °C. Test transformations with supercoiled pHSWF plasmid and self-ligated linearised dephosphorylated pHSWF vector

were included to confirm competency of the cells and that the dephosphorylation procedure was successful.

2.2.10 Purification of plasmid DNA

The alkaline lysis method (Sambrook *et al.*, 1989) was used to isolate plasmid DNA from bacterial cells. The method is based upon the fact that a narrow pH range (12-12.5) exists within which linear DNA, but not covalently closed circular (ccc) DNA, is denatured. Plasmid DNA was first purified on a small scale and if characterised successfully, purification followed on large scale using the "Nucleobond Ax kit" from Macherey - Nagel (based on the same principles as for the alkaline lysis method, described below).

Two-hundred ml (large-scale) or 5 ml (small-scale) Sterile LB broth containing 100 μg/ml ampicillin and 12.5 μg/ml tetracycline was inoculated with a single colony obtained after transformation and incubated at 37 °C overnight with shaking. Colonies were replica-plated on similar plates using the tooth-pick method.

On small scale the bacterial cells were harvested by centrifugation at 5000 rpm for 1 min at room temperature (RT) and the cell walls were weakened by resuspending in 100 μl of a solution containing 50 mM glucose, 10 mM EDTA and 25 mM Tris pH 8 and incubating at RT for 5 min followed by ice for 1 minute. Controlled lysis of the cells was achieved by adding 200 μl of the alkaline sodium dodecyl sulphate (SDS) buffer containing 0.2 M NaOH, 1 % SDS, and incubating on ice for less than 5 minutes. Chromosomal DNA, high molecular mass RNA and SDS denatured proteins were precipitated by the addition of 150 μl 3 M sodium acetate (NaAc) pH 4.8. After 5 min on ice, the chromosomal DNA and insoluble proteins were pelleted by centrifugation at 12 000 rpm for 10 min at RT while plasmid DNA remained in the supernatant together with small RNA fragments. The plasmid DNA was precipitated by the addition of 2 volumes of 96 % ethanol to the supernatant followed by incubation at -20 °C for 30 min and recovered by centrifugation as described above. The DNA pellet was resuspended in 100 μl ddH_20 and low mass RNA was removed by adding half a volume of 7.5 M NaAc followed by incubation on ice for 15 min and centrifugation. The purified plasmid DNA was finally recovered by precipitation with 2.5 volumes of 96 % ethanol leaving it at -20 °C for 60 min and centrifugation at 12 000 rpm for 10 min. After washing with 70 % ethanol the DNA pellet was recovered by centrifugation, vacuum-dried for 5 min and resuspended in 30 μl ddH_2O after which it was stored at -20 °C.

2.2.11 Characterisation of transfer DNA

The miniprep plasmid DNA was analysed by electrophoresis in horisontal 1 % agarose gels as described in 2.2.3. Plasmid DNA of larger size than the vector DNA was analysed further by r.e. digestions. For both constructs single restriction enzyme digestions were performed to determine whether the foreign gene was cloned in the correct orientation and double digestions to ascertain that the full length gene was retained.

The restriction enzyme digested DNA was analysed on 1 % agarose gels and the recombinants containing the full length gene in the correct orientation were selected for large scale purification using the "Nucleobond Ax kit" (Macherey - Nagel) followed by a 1% agarose gel. The recombinants were digested again to confirm that the full length gene was cloned in the correct orientation and named pHSWF/*Lac*E and pHSWF/*Lac*L respectively.

2.2.12 Sterile conditions for cell culture work

A laminar flow hood (Labotec) was used at all times when working with culture cells or related mediums (e.g. growth medium) and chemicals (e.g. antibiotics). The following sterile techniques were adopted:

When not in use, the hood was kept free of any items and the circulator fan was switched on at all times (100 kPa). Before the hood was to be used the interior was irradiated with ultra-violet (UV) light and the fan speed increased to approximately 400 kPa for at least 20 minutes. The working surface inside the hood was cleaned thoroughly with Bromocide from Notio Technologies followed with 70 % ethanol (EtOH). All laboratory equipment to be used inside the hood were cleaned with 70 % EtOH before being returned to the hood. The operator's hands and arms were washed with liquid soap and water, dried with paper-towel and sprayed with 70 % EtOH. After working in the hood all the items were removed and the working surface was washed with 70 % EtOH and irradiated with UV light for approximately 20 minutes.

2.2.13 Primary cell culture preparation

For obtaining a primary foetal bovine testis (FBT) cell line, fresh foetal calf testis tissue was obtained from the Pyramid Abattoir and placed in ice-cold phosphate buffered saline (13.7 mM NaCl, 0.27 mM KCl, 0.43 mM $Na_2HPO_4.2H_2O$, 0.14 mM KH_2PO_4, pH 7.3)

without calcium and magnesium containing 0.2 % gentamycin (PBS(A)). After rinsing the testis with fresh PBS(A), the outer connective tissue was removed using scissors according to the above mentioned sterile technique. The testis tissue was washed in PBS(A) and left on ice for approximately 10 min after which it was transferred to a sterile small conical flask containing a magnetic bar and just enough PBS(A) to cover the tissue. This flask was placed on a magnetic stirrer (Fried electric, Haifa, Israel) at 200 revolutions per minute (rpm) at RT for 20 minutes. The PBS(A) medium was discarded, replaced with 0.25 % activated trypsin versene (4 % NaCl, 0.2 % KCl, 0.29 % sodium bicarbonate, 0.5 % dextrose, 0.25 % trypsin and 0.1 % EDTA) and the stirring cycle repeated. After 20 min the trypsinised tissue was removed and fresh 0.25 % activated trypsin versene (ATV) was added to the remaining tissue. The tripsinised tissue was diluted 1:1 with 10 % foetal calf-serum growth-medium (45 % Dubelco's Modified Eagle's Medium (DMEM) and 45 % Ham's F12 supplemented with 10 % foetal calf-serum and the appropriate antibiotics, i.e. penicillin G (120 μg/ml), streptomycin sulphate (120 μg/ml) and fungizone (0.3 μg/ml)). The cells were pelleted in a Sigma 301K benchtop centrifuge (Sigma, Germany) at 1500 rpm for 10 minute. The cell pellet was resuspended in 25 ml 10 % foetal calf-serum growth-medium where 5 ml was seeded per 75 cm^2 culture flask (Sarstedt, Highveld Biological). Fifteen ml of the 10 % foetal calf-serum growth medium was added per 75 cm^2 flask and placed in a 5 % CO_2 incubator at 37 °C for 48 hours before being examined for cell growth. Once the cells had formed a monolayer they were passaged as described in 2.2.14.

The FBT-cells recovered from the next two trypsinised cycles were frozen away in liquid nitrogen. The same procedure as described above was used except that the cell pellet was resuspended in medium consisting of Cryoprotective medium (Basal Eagle's medium with Hank's BSS and 15 % Dimethylsulfoxide without L-glutamine) obtained from Bio Whittaker and 30 % foetal calf-serum growth medium in a ratio of 2:1. Approximately 1.8 ml of the resuspended cells was aliquoted into Nunc Tubes. The tubes were slowly cooled by wrapping it with cotton wool and tin foil and placing at -70 °C. After 2 days at -70 °C the cells were put in liquid nitrogen where it stays viable for approximately 2 years.

2.2.14 Passaging culture cells

The 10 % foetal calf-serum growth medium was removed from a flask containing a confluent cell monolayer. The cell sheet was washed with 0.1 % activated trypsin versene (1.6 % NaCl, 0.08 % KCl, 0.116 % sodium bicarbonate, 0.2 % dextrose, 0.1 % trypsin and

0.04 % EDTA) preheated to 37 °C, by swirling the ATV over the cells after which it was removed and replaced with fresh 0.1 % ATV. The flask was placed in a 5 % CO_2 - incubator at 37 °C. Once the cells started detaching from the bottom of the flask, it was removed from the incubator and the contents made up to a predetermined final volume with 10 % foetal calf-serum growth medium, preheated to 37°C. This volume is depended on the split ratio of the cells where the split ratio refers to the number of confluent cell monolayers that can be obtained within 3-4 days after subculturing the cells from the parental flask. For a split ratio of 1:3, one flask of confluent cells is divided into three flasks of the same size.

The cells were gently pipetted up and down using a 10 ml syringe (Promex) with an 18 G needle (Promex) until no clumps were visible. The cell suspension was then divided into the predetermined amount of flasks containing 15 ml of preheated 10 % foetal calf-serum growth medium each. The flasks were swirled gently to ensure even spread of cells and placed in a 5 % CO_2 - incubator at 37 °C until the cells formed a confluent monolayer. After approximately 4 days incubation, the monolayers were used for either another division or infection with viruses.

2.2.15 Transfection procedures

Monolayers of 90 % confluent FBT-cells, approximately 3 X 10^5 cells/well of a 6-well Nunc-plate, were washed with DMEM supplemented with antibiotics (120 μg/ml penicillin G, 120 μg/ml streptomycin sulphate and 0.3 μg/ml fungizone) and covered with 1 ml growth-medium without foetal calf serum. The cells were infected with purified lumpy skin disease virus stock (Neethling strain) at a multiplicity of infection (M.O.I.) of 0.1 focus forming units per cell (ffu)/cell. The transfection of plasmids pHSWF/*Lac*E and pHSWF/*Lac*L were performed 90 min post infection by means of the Fugene-mediated transfer method as follows:

Ninety-min post infection the infected FBT-cells were washed with DMEM supplemented with the appropriate antibiotics and covered with 2 ml of 2 % foetal calf serum growth-medium. The respective "Fugene-mixtures" (1.5 μg plasmid DNA, 3 μl Fugene and 97 μl DMEM) were added dropwise onto the cells followed by incubation for 3 to 4 days in the 5 % CO_2 -incubater at 37 °C.

2.2.16 X-gal staining

After a transfection period of 96 hours, the medium was aspirated and the transfected cells washed in phosphate buffered saline (1 x PBS). The cells were fixed in 2 ml -20 °C 100 % methanol for 5 min at room temperature. After 2 washes with 1 x PBS, the cells were covered with X-gal stain (1 mg/ml X-gal in DMSO, 5 mM K-ferricyanide, 5 mM K-ferrocyanide, 2 mM $MgCl_2$) and incubated at 37 °C for 16 hours. Cells transfected with either pHSWF/*Lac*E or pHSWF/*Lac*L were identified by the intense blue coloration of X-gal, indicating that the X-gal substrate had been converted by the enzymatic activity of β-galactosidase (the gene-product of *LacZ*).

2.2.17 Generation of LSDV recombinants

Monolayers of 90 % confluent FBT-cells in six-well plates were transfected with either pHSWF/*Lac*E or pHSWF/*Lac*L using the method as described in 2.2.15. After an incubation time of 96 hours, the transfected cells were harvested after which the progeny of the *in vivo* homologous recombination event were released from the cells by three cycles of freeze-thawing and stored at -20 °C to be used for infection of FBT-cells for the generation of recombinant LSDV/*Lac*E and LSDV/*Lac*L. Cell debris was removed by low speed centrifugation (2000 rpm) for 5 min and the supernatant (1.2 ml) was used to infect monolayers of 90 % confluent FBT-cells in six-well plates, pre-incubated with mycophenolic acid (MPA)-selection medium 24 hours prior infection. MPA-selection medium consists of 30 μg/ml mycophenolic acid, 250 μg/ml xanthine, 200 μg/ml HAT from Highveld Biological (13.6 μg/ml hypoxanthine, 0.16 μg/ml aminopterin and 3.84 μg/ml thymidin) and 2.5 % foetal calf serum in DMEM supplemented with 120 μg/ml penicillin G, 120 μg/ml streptomycin sulphate and 0.3 μg/ml fungizone.

At 90 min post infection (p.i.) the infected FBT-cells were washed with DMEM supplemented with the appropriate antibiotics and covered with 2 ml fresh MPA - selection medium. During the infection period, the MPA-selection medium has to be replaced every 72 hours with fresh selection medium to keep the conditions consistent. At 10 days p.i., the supernatants were harvested and used in a dilution series (100 ffu, 50 ffu, 25 ffu, 10 ffu, 5 ffu and 1 ffu) to inoculate fresh FBT monolayers in 24-well plates that had been pre-incubated (24 hours) with MPA- selection medium. Prior to infection with the dilution series the selection medium was replaced with fresh MPA-selection medium. The supernatants of wells containing single foci were harvested after approximately 10 to 12

days. The selected foci were subjected to two further rounds of purification by making use of a dilution series during each round in the presence of selective media.

One third of the selected foci (supernatant) were used to evaluate it's status as a LSDV recombinant expressing the reporter gene by infecting monolayers of 90 % confluent FBT-cells in six-well plates in the presence of MPA-selection medium. Five days post infection, when CPE developed, the selection medium was aspirated and the infected cells washed with 1 x PBS. The cells were fixed and stained using the X-gal staining method as described in 2.2.16. After an incubation time of 16 hours an intense blue coloration of foci would be an indication that LSDV recombinants were probably present. Foci were selected according to the intense blue coloration obtained with X-gal staining and used to infect monolayers of 90 % confluent FBT-cells in 25 cm^2 flasks (Sarstedt, Highveld Biological) in the presence of MPA-selection medium. When CPE was observed, the supernatants were collected for large-scale infection of monolayers of 90 % confluent FBT-cells in 75 cm^2 flasks.

2.2.18 Purification of LSDV DNA

Viral DNA was purified according to a procedure described for orthopox viruses (Esposito *et al.*, 1981), as follows:

The infected FBT-cells of 3 x 75 cm^2 flasks were harvested using 0.1 % ATV. The cells were collected by centrifugation at 1500 rpm for 20 min, resuspended in 4.5 ml McIlvain's buffer (0.18 mM Citric acid, 3.63 mM $Na_2HPO_4.12H_2O$, pH 7.4) and placed on ice for 90 minutes. Virus contained in the supernatant was pelleted by centrifugation at 13 000 rpm for 90 min at 4 °C through a cushion of 36 % sucrose in 1 X TE buffer (1 mM EDTA, 10 mM Tris-HCl pH 7.5) in a Beckman SW28 rotor. The virus pellet was resuspended in 4.5 ml McIlvain's buffer and pooled with the already resuspended cell pellet after which it was placed on ice for a further 10 min. Twenty-six µl βeta-mercapto-ethanol (βME) and 1 ml 10 % (w/v) Triton X-100 were added and incubation continued for another 10 min at 0 °C. This treatment solubilised the plasma membranes and released viral cores, the cell nuclei and cytoplasmic organelles. After the 10 min incubation period the cells were dounced 10 times and the cellular debris removed by centrifugation at 2000 rpm for 10 min. The supernatant was collected and the cell pellet resuspended in 9 ml McIlvain's buffer to be centrifuged again under the same conditions. The supernatant was collected and pooled with the previously collected supernatant and

centrifuged at 13 000 rpm for 90 min at 4 °C through a cushion of 36 % sucrose in 1 X TE-buffer in a Beckman SW28 rotor to pellet the viral cores. The cores were resuspended in 300 µl 1 X TE-buffer and mixed with an equal volume of solution containing 4 % (w/v) N-lauryl sarcosinate (NLS), 1 µl βME and 6 µl Proteinase K (60 µg) and incubated at 40 °C overnight. The NLS lysed the cores and released viral DNA. DNA was purified from the core digest by a phenol:chloroform:iso-amylalcohol (25:24:1) extraction. The aqueous phase was removed and extracted with an equal volume of chloroform:iso-amylalcohol (24:1). DNA was precipitated by adding one-tenth volume of sodium-acetate and 2.5 volumes 100 % ethanol after which it was stored at -20 °C overnight. The DNA was collected by centrifugation at 6000 rpm for 30 min at 4° C and washed in 70 % ethanol that was followed by another centrifugation under the same conditions for 25 min. The virus DNA was air-dried for approximately 25 min and resuspended, very gently, in 1 X TE-buffer. DNA was stored at 4 °C.

2.2.19 Restriction enzyme digestions of virus DNA

The digestion procedures of the viral DNA were based on the same principles as described in 2.2.2. The reaction mixtures, for the recombinant LSDV DNA, contained approximately 5-6 µg viral DNA in a total volume of 30 µl with 0.5 units of enzyme per µg of DNA. Two units of the appropriate enzyme were added after 3.5 hours of incubation at 37 °C and the total volume was increased to 40 µl followed by incubation at 37 °C overnight. The two control reactions namely, wt LSDV-DNA and DNA of uninfected FBT-cells, consisted each of 5 µg DNA in a total volume of 30 µl with 3 units of enzyme and were incubated at 37 °C overnight. The digestion products were analysed on agarose gels.

2.2.20 Non-radioactive labeling of DNA to be used as probes

Plasmid DNA, purified *LacZ*-insert and SMII-size marker were labelled using the DIG DNA Labeling and Detection Kit (Boehringer Mannheim). The non-radioactive DIG system uses digoxigenin (DIG), a steroid hapten, to label DNA for hybridisation and subsequent luminescence detection. For DNA labeling, DIG is coupled to dUTP via an alkali-labile ester-bond. DIG-labelled DNA probes are generated enzymatically by making use of random primed labeling (Feinberg and Vogelstein, 1983) which is based on the hybridisation of random oligonucleotides, caused by the hexanucleotides, to the denatured DNA template. The complementary DNA strand is synthesised by the Klenow

enzyme which uses the 3'OH termini of the random oligonucleotides as primers and a mixture of deoxyribonucleosides containing DIG-II-dUTP, alkali labile, that results in the incorporation of digoxigenin into the new synthesised DNA strand.

For DNA labeling, 0.5-3.0 µg DNA template was diluted to a final volume of 15 µl and denatured by boiling the diluted DNA for 10 min and quickly chilling on ice. Two µl of the hexanucleotide mix (10 x concentrated hexanucleotide reaction mix), 2 µl of the dNTP-mix (10x concentrated dNTP labeling mixture containing 1 mM dATP; 1 mM dCTP; 1 mM dGTP; 0.65 mM dTTP; 0.35 mM DIG-dUTP pH 7.5) and 1 µl Klenow enzyme (labeling grade, 2 units/µl) was added to the single stranded DNA followed by incubation at 37 °C overnight (O/N). The reaction was terminated by placing the mixture on ice and adding of 2 µl 0.2 M EDTA. To purify the probe, 2.5 µl 4M LiCl and 75 µl 100% EtOH was added to precipitate the labelled DNA followed by incubation at -20 °C O/N. The purified labelled DNA was collected by centrifugation for 20 min at 13 000 rpm after which the pellet was washed with 50 µl 70 % EtOH. The labelled DNA was finally recovered with centrifugation, as described above, vacuum-dried for 5 min and resuspended in 50 µl 1 x TE-buffer after which it could be stored at -20 °C for at least one year.

2.2.21 Southern blot analysis

Electrophoretic separated r.e. digested DNA was transferred to a Hybond N^+ -nylon membrane (Amersham) by a procedure developed by Southern (1975). The 0.6 % agarose gel was soaked for 20 min with gentle agitation in 400 ml depurination solution (0.2 M HCl), after which it was rinsed three times with distilled water (dH_2O). The DNA fragments were then denatured by gentle agitation in 200 ml denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 20 minutes. This step was repeated by changing the denaturation solution once. The gel was neutralised by soaking it twice with gentle agitation in 200 ml neutralisation solution (1 M Tris-HCl, 1.5 M NaCl) for 20 min each. The single stranded DNA fragments were now ready to be transferred to a Hybond N^+-nylon membrane (Amersham).

The membrane was put in dH_2O until the membrane was uniformly wet. Six pieces of Whatman 3 MMChr filter paper were wet with 20 x SSC (3 M NaCl, 0.3 M Na_3citrate.$2H_2O$) and placed between 4 pieces of plastic on a perspex plate on top of a perspex container. The filter paper at the bottom of the stack stayed in contact with 20 x SSC (in the perspex container) during the whole transfer-process. The agarose gel

was then placed onto the filters followed by the nylon membrane, pre-wetted in 2 x SSC (300 mM NaCl, 30 mM Na_3citrate$2H_2O$). Another 2 filters, prewetted with 2 x SSC, were put on top of the nylon membrane, followed with 2 dry filter papers and precut paper towels. On top of the stack another perspex plate was layed followed by weight. The blotting stack was left overnight at RT to assure successful DNA-transfer to the membrane after which the membrane was removed. The transferred DNA was fixed onto the membrane by UV-light exposure for 5 min on each side. The membrane was sealed in a plastic bag and stored at RT until use.

Hybridisation of probes with transferred DNA

Hybridisation of digoxigenin-labelled probes (e.g. to target DNA) was carried out according to the standard protocols, (see below), except that a special blocking reagent was used to eliminate background. The signal on the nucleic acid blot is detected according to the methods developed for western blots. An anti-digoxigenin antibody-alkaline phosphatase conjugate is allowed to bind to the hybridised probe and is then visualised with the chemiluminescent alkaline phosphatase substrate where the signal can be detected on an X-ray film.

The membrane with fixed nucleic acids were pre-hybridised (5 x SSC; 50 % formamide, deionized; 0.1 % (w/v) N-lauroylsarcosine; 0.02 % (w/v) SDS; 2 % blocking reagent (supplied in the DIG kit) for at least 4 hr at 42 °C before the hybridisation was carried out. The DIG-labelled probes were denatured by boiling for 5 min and rapidly cooling on ice before it was added to the pre-warmed (42 °C) hybridisation solution, the same composition used for pre-hybridisation. Hybridisation was allowed to proceed for at least 16 hr at 42 °C after which the membrane was removed and washed at the required stringency as prescribed by the kit-manual. Low stringency washes were performed at RT with 2 changes of 2 x SSC buffer; 0.1 % SDS after 10 min each. The membrane was washed once with a higher stringency by using 0.5 x SSC buffer, 0.1 % SDS at 68 °C for 20 min under constant agitation. The last wash was performed with a high stringency for 20 min under constant agitation, by making use of 0.1 x SSC buffer, 0.1 % SDS at 68 °C.

After the post-hybridisation washes, the membrane was equilibrated in washing buffer (0.3% (w/v) Tween^R 20 in Maleic acid buffer (0.1 M maleic acid; 0.15 M NaCl; pH 7.5)) for 5 min. The membrane was blocked by gentle agitation in blocking solution (1 x concentrated working solution is prepared by diluting the 10 x stock solution 1:10 in

Maleic acid buffer, while the 10 x stock solution is composed of blocking reagent, 10 % (w/v) in Maleic acid buffer) for 60 min at RT after which it was replaced with the antibody solution (Anti-Digoxigenin-AP diluted 1:10 000 in blocking solution) to be incubated for another 30 min at RT with gentle agitation. The membrane was washed twice, 15 min per wash in washing buffer and equilibrated in detection buffer (0.1 M Tris-HCl; 0.1 M NaCl; 50 mM $MgCl_2$; pH9.5) for 2 min. The chemiluminescent alkaline phosphatase substrate was diluted 1:100 in detection buffer just prior to use to the membrane and incubated for 5 min at RT. The semi-dry membrane was sealed in a plastic bag and incubated at 37 °C for 20 min to reach a steady state reaction before it was loaded in an X-ray cassette. The membrane was exposed to a Cronex MRF31 X-ray film for approximately 16 hr at RT after which the X-ray film could be developed to observe results on the autoradiograph.

2.3 RESULTS

2.3.1 Construction of plasmids containing the *LacZ*-gene

The reporter gene *LacZ* was cloned under control of either the early or late LSDV pA7LA8R promoter in order to evaluate the bi-directional promoter in vector pHSWF in a live LSDV system. The *LacZ*-gene was respectively cloned into the unique *Eco* RI or *Bam* HI-site of pHSWF as presented in (fig. 2.9a p 44 and 2.9b p 45). In the *Eco* RI-site, the *LacZ*-gene is placed under control of the early promoter pA8R, and in the *Bam* HI-site under the late promoter pA7L. The two constructs were named pHSWF/*Lac*E and pHSWF/*Lac*L respectively.

The reporter gene *LacZ* was excised as a 3737 bp fragment from the commercially available pSV-β-Galactosidase vector (fig. 2.1) using the restriction enzymes *Hind* III and *Bam* HI in a double digestion reaction (section 2.2.2). Electrophoretic analysis of the digested product is shown in (fig. 2.2). The upper band (fig. 2.2 lane c) was excised from the 1 % agarose gel and purified with Glassmilk methodology (2.2.4). The lower band of 3084 bp represents the rest of the pSV-β-Galactosidase vector (fig. 2.2 lane c). A small amount of the recovered DNA fragment was analysed on a 1 % agarose gel (fig. 2.3).

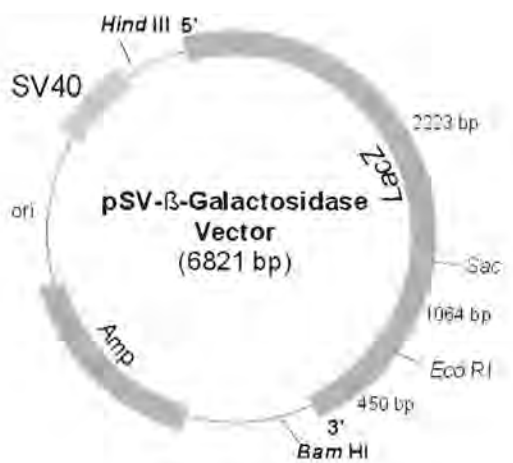

Fig. 2.1: pSV-β-Galactosidase Vector circle map

The transfer vector pHSWF was either linearised with *Eco* RI or *Bam* HI in two separate single digestion reactions followed by electrophoresis (fig. 2.4) and Glassmilk purification. Recovery of the vector was confirmed on a 1 % agarose gel (fig. 2.5). For cloning purposes blunt-end ligation was performed on both the insert and the respective linearised vectors using a Klenow reaction (section 2.2.5). To prevent self-ligating of the transfer vectors the vectors were dephosphorilated (2.2.6). The DNA of the gene fragment and the transfer vectors was recovered by Glassmilk purification (fig. 2.6) and ligated.

The respective insert/vector ligation mixtures were transformed to Top10F competent *E.coli* cells. Putative transfer vectors that contained the *LacZ*-gene, according to larger plasmid size in relation to the intact vector (fig. 2.7 lanes d, f and i and fig. 2.8 lanes b, h, j and k), were investigated by r.e. digestions to determine the orientation of the full length *LacZ*-gene. In both cases the restriction enzyme *Sac* I was used to determine the orientation of the full-length *LacZ*-gene. The correct transcriptional orientations of the full-length gene in the 2 constructs are shown in (fig. 2.9a and 2.9b).

Fig. 2.2: Agarose gel electrophoretic analysis of vector pSV-β-Galactosidase
(a) Size marker SMII
(b) Undigested pSV-β-Galactosidase
(c) pSV-β-Galactosidase digested with *Hind* III and *Bam* HI.

Fig. 2.3: Agarose gel electrophoretic analysis of genecleaned recovered DNA
(a) Approximately 500 ng *Lac* Z DNA - sample 1
(b) Approximately 500 ng *Lac* Z DNA - sample 2

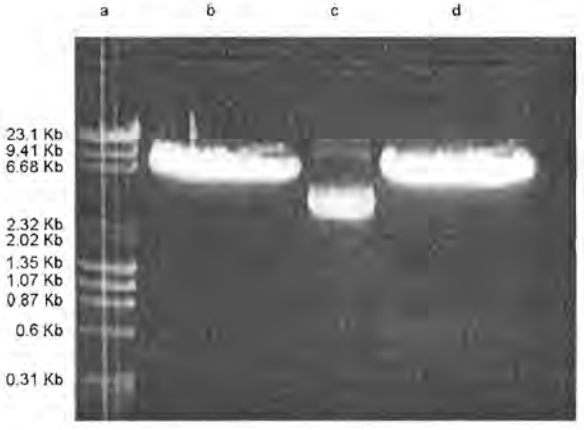

Fig. 2.4: Agarose gel electrophoretic analysis of vector pHSWF
(a) Size markers SMII and ϕX
(b) pHSWF digested with *Eco* RI
(c) Undigested pHSWF
(d) pHSWF digested with *Bam* HI

Fig. 2.5: Agarose gel electrophoretic analysis of genecleaned recovered linearised vector, pHSWF
(a) Approximately 100 ng DNA, pHSWF digested with *Eco* RI
(b) Approximately 150 ng DNA, pHSWF digested with *Bam* HI

Fig. 2.6: Agarose gel electrophoretic analysis of genecleaned recovered DNA following dephosphorilation
(a) Size markers SMII and ϕX
(b) Approximately 400 ng *LacZ* DNA
(c) Approximately 500 ng DNA, pHSWF digested with *Eco* RI
(d) Approximately 500 ng DNA, pHSWF digested with *Bam* HI

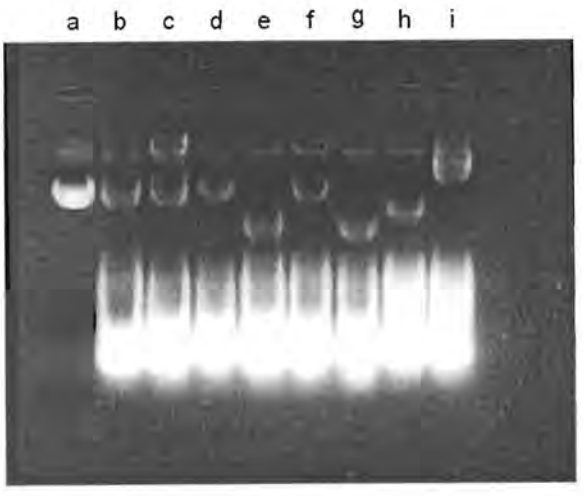

Fig. 2.7: Agarose gel electrophoretic analysis of putative *LacZ* recombinants with the *LacZ*-gene cloned downstream of the early promoter, pA8R
(a) Intact vector, pHSWF, as control
(b)-(i) Undigested putative plasmids containing the *LacZ*-gene under control of the early promoter, pA8R

Fig. 2.8: Agarose gel electrophoretic analysis of putative *LacZ* recombinants with the *LacZ*-gene cloned downstream of the late promoter, pA7L
(a) Intact vector, pHSWF, as control
(b)-(k) Undigested putative plasmids containing the *LacZ*-gene under control of the late promoter, pA7L

Orientation of pHSWF/*Lac*E

If *LacZ* was cloned in the correct orientation under control of the early LSDV promoter (pA8R) using the pHSWF-plasmid, 2 fragments of sizes 2.3 Kb and 7.5 Kb were expected with *Sac* I-digestion (fig. 2.9a). In the correct transcriptional orientation *Sac* I digests at the one end of the pA7LA8R-promoter and asymmetrically within the *LacZ*-gene, 2.2 Kb from it's 5' end. An electrophoretic analysis of one clone in the correct orientation is shown in (fig. 2.10 lane e). In the incorrect orientation 2 fragments of sizes 1.6 Kb and 8.2 Kb each would be generated. From (fig. 2.10) there was no recombinant containing the *LacZ*-gene in the incorrect orientation, the other two vectors in (fig. 2.10 lane c and d) didn't contain cloned product and were discarded.

Orientation of pHSWF/*Lac*L

If *LacZ* was cloned in the correct orientation downstream from the late LSDV promoter (pA7L) using the pHSWF-plasmid, 2 fragments of sizes 1.5 Kb and 8.3 Kb were expected with *Sac* I-digestion (fig. 2.9b). *Sac* I digests down-stream from the 3'end of the *LacZ*-gene and asymmetrically within the gene, 1.5 Kb from it's 3' end. In the incorrect orientation 2 fragments of sizes 2.2 Kb and 7.6 Kb would be expected (fig. 2.9b). An electrophoretic analysis of one clone in the correct orientation is shown in (fig. 2.11 lane d) and two clones in the incorrect orientation (fig. 2.11 lanes e and f).

As judged from the fragment sizes obtained following electrophoresis, one clone for each plasmid was selected, propagated (fig. 2.10 lane e for pHSWF/*Lac*E and fig. 2.11 lane d for pHSWF/*Lac*L) and used for large-scale plasmid extraction. *Sac* I was used again to confirm that *LacZ* was cloned in the correct orientation (fig. 2.12) and a double digestion with *Sma* I and *Sal* I was performed to assure that the full length gene was present (fig. 2.13). In both cases *Sma* I and *Sal* I digest outside the cloned *LacZ*-gene and pA7LA8R-promoter, therefore 2 fragments of sizes 3.8 Kb and 6 Kb were expected (fig. 2.9a and 2.9b). From (fig. 2.13 lane c and d) the 2 fragments were obtained in both cases. The plasmids shown in (fig. 2.13 lane c and d) were selected for further studies and designated pHSWF/*Lac*E and pHSWF/*Lac*L.

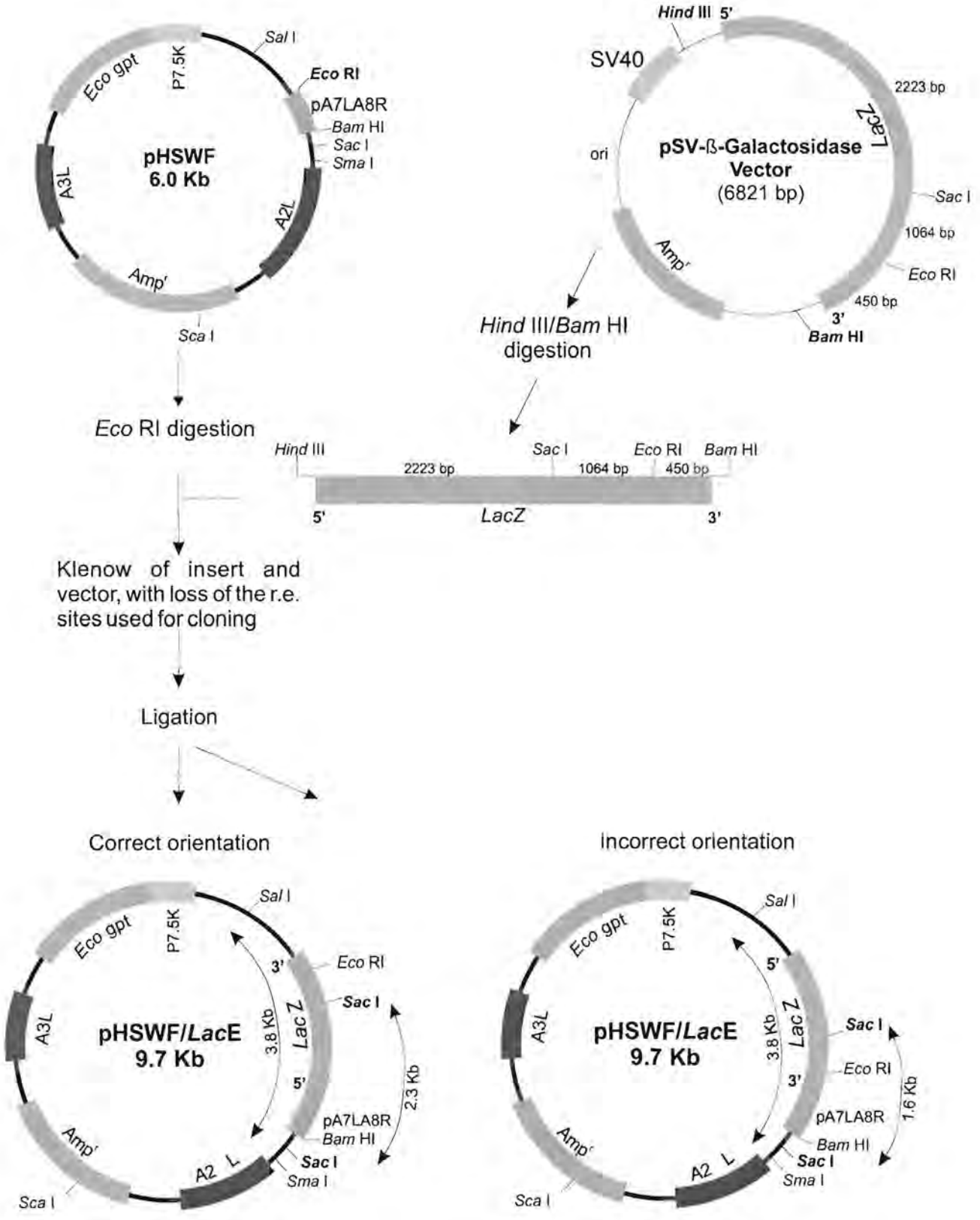

Fig. 2.9a: Schematic representation of the cloning of *LacZ* into the transfer vector pHSWF under control of the early LSDV promoter (pA8R). A partial r.e. map of *LacZ* is shown, as well as the correct and incorrect transcriptional orientations of the gene after cloning.

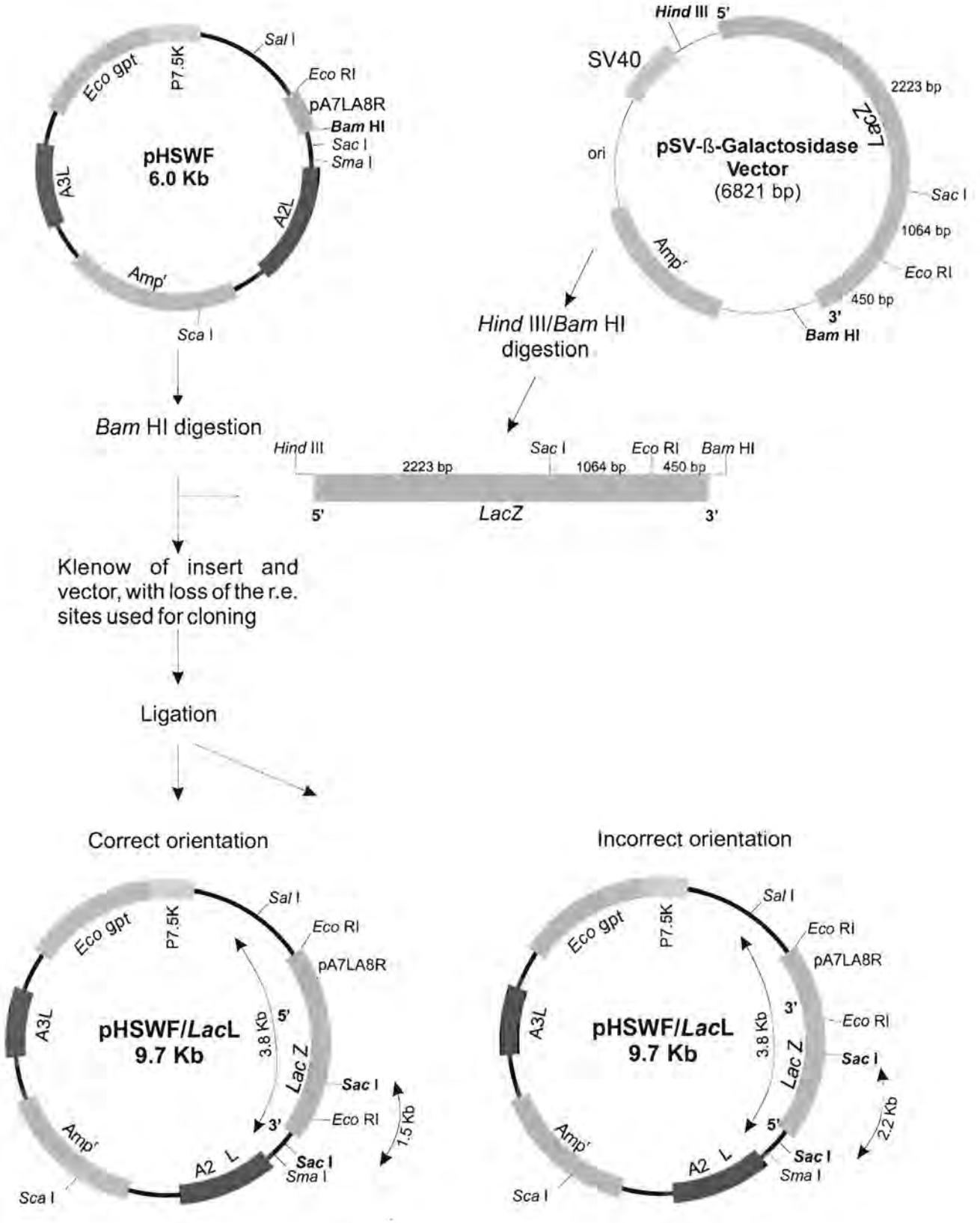

Fig. 2.9b: Schematic representation of the cloning of *LacZ* into the transfer vector pHSWF under control of the late LSDV promoter (pA7L). A partial r.e. map of *LacZ* is shown, as well as the correct and incorrect transcriptional orientations of the gene after cloning.

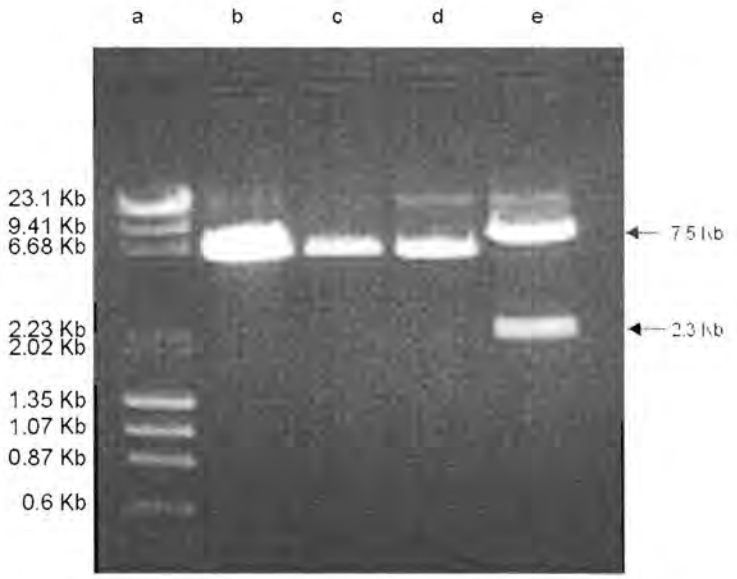

Fig. 2.10: Agarose gel electrophoretic analysis of putative plasmids containing the *Lac*Z-gene, pHSWF/*Lac*E, to establish the correct transcriptional orientation

(a) Size markers SMII and ϕX

(b) pHSWF digested with *Sac* I as control

(c)-(e) Putative plasmids containing the *Lac*Z-gene, pHSWF/*Lac*E, digested with *Sac* I

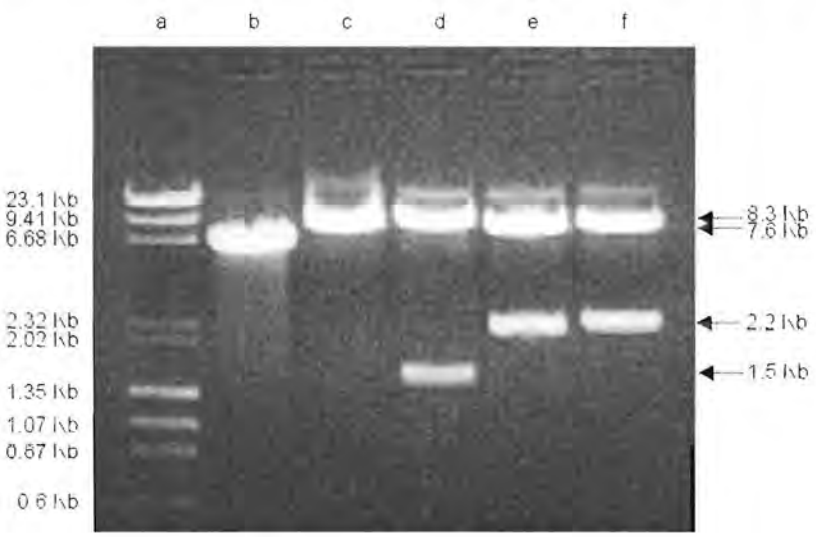

Fig. 2.11: Agarose gel electrophoretic analysis of putative plasmids containing the *Lac*Z-gene, pHSWF/*Lac*L, to establish the correct transcriptional orientation

(a) Size markers SMII and ϕX

(b) pHSWF digested with *Sac* I as control

(c)-(f) Putative plasmids containing the *Lac*Z-gene, pHSWF/*Lac*L, digested with *Sac* I

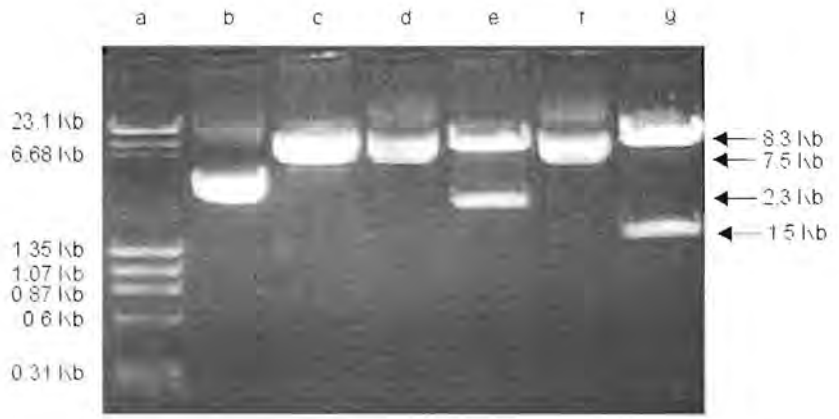

Fig. 2.12: Agarose gel electrophoretic analysis of plasmids pHSWF/*Lac*E and pHSWF/*Lac*L to confirm the correct orientation

(a) Size markers SMII and ϕX

(b) Undigsted pHSWF

(c) pHSWF digested with *Sac* I as control

(d) Undigested pHSWF/*Lac*E

(e) pHSWF/*Lac*E digested with *Sac* I

(f) Undigested pHSWF/*Lac*L

(g) pHSWF/*Lac*L digested with *Sac* I

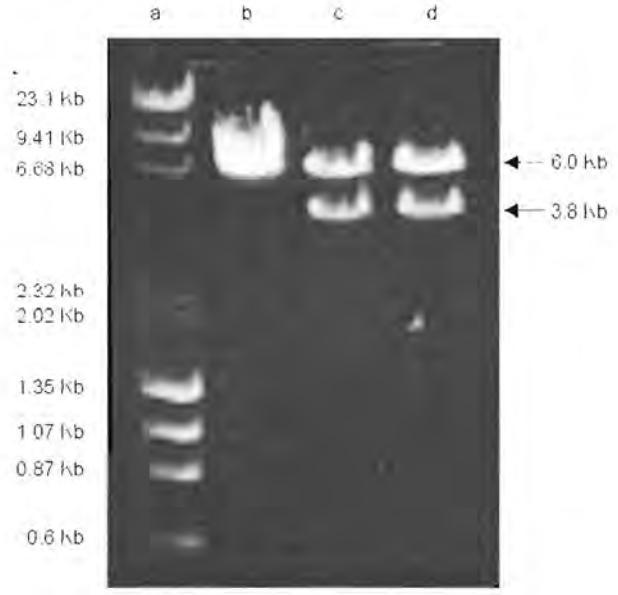

Fig. 2.13: Agarose gel electrophoretic analysis of plasmids pHSWF/*Lac*E and pHSWF/*Lac*L

(a) Size markers SMII and ϕX

(b) pHSWF digested with *Sma* I and *Sal* I as control

(c) pHSWF/*Lac*E digested with *Sma* I and *Sal* I

(d) pHSWF/*Lac*L digested with *Sma* I and *Sal* I

2.3.2 Generation of LSDV recombinants

2.3.2.1 Transient expression of the *Lac*Z-gene

When the appropriate cis-acting regulatory signals are associated with the transfected genes, recombinant DNA introduced into eukaryotic cells by transfection, may be transiently expressed in an unintegrated state (Gorman *et al.*, 1982; An *et al.*, 1982; Selden *et al.*, 1986). A heterologous gene will similarly be expressed in eukaryotic cells if under control of an upstream poxvirus regulatory signal (Cochran *et al.*, 1985; Chakrabarti *et al.*, 1985; Panicali *et al.*, 1986). With poxviruses, expression of the transfected gene depends on cells being infected with the wt virus to provide the correct trans-acting transcription factors. The reason for using the *E.coli LacZ*-gene, encoding the β-galactosidase (β-gal) enzyme, is as follows: (1) most cells have low endogenous β-gal activity, (2) β-gal is very stable and resistant to proteolytic degradation in cellular lysates and (3) β-gal activity can easily be assayed using X-gal substrate.

Before studies on the generation of LSDV recombinants expressing the *Lac*Z-gene could be attempted it was necessary to verify that the LSDV bi-directional promoter pA7LA8R, acted as a functional promoter element in transient expression assays. FBT-cells, infected with wt LSDV, were transfected with the plasmids pHSWF/*Lac*E and pHSWF/*Lac*L respectively. After an infection period of 96 hours the cells were covered with X-gal stain and in both instances a blue colour was observed in the cells within 16 hours after staining. The blue coloured cells were an indication that the X-gal substrate had been converted by the enzymatic activity of β-gal (fig. 2.14 C and D). Uninfected FBT-cells and infected FBT-cells, without donor DNA, were included as controls and failed to produce a blue coloured product (fig. 2.14 A and B).

Infected FBT-cells transfected with pHSWF/*Lac*L, showed a higher quantity of intensively blue coloured cells when compared to cells transfected with pHSWF/*Lac*E. The results may be explained by the fact that during the growth cycle of capripoxviruses at molecular level, two aspects of viral development are of importance: firstly the mRNA transcriptional switch from early to late and secondly, DNA replication. The synthesis of early mRNA is initiated immediately after infection and continue for approximately 9 hr whereas the late-gene transcription require first DNA replication and occur approximately 10 hr post-infection continuing much longer (Fick and Viljoen, 1994). In the transfer plasmid pHSWF/*Lac*E, *LacZ* is cloned under control of the early LSDV promoter pA8R, and

transcription occur for a shorter time (9hr) compared to transcription of *LacZ* in pHSWF/*Lac*L, cloned under control of the late LSDV promoter pA7L. The blue coloured single cells obtained with transient expression of the *LacZ*-gene indicated that both the early and late LSDV promoters were able to drive the transcription of the reporter gene upon transfection.

2.3.2.2 Generation of LSDV recombinants

Recombinant lumpy skin disease viruses containing the *E.coli LacZ*-gene were generated based on similar procedures and principles developed for a capripoxvirus recombinant vaccine for protection of cattle against rinderpest and lumpy skin disease (Romero *et al.*, 1993).

FBT-cells were transfected with either pHSWF/*Lac*E or pHSWF/*Lac*L as described in section 2.2.15. The supernatant of the transfected cells was used to infect FBT-cells, pre-incubated with MPA to select against wt LSDV. After three rounds of purification, as described in section 2.2.17, the supernatants of infected cells with single foci were harvested for further studies. Single foci most often formed in the wells where FBT-cells were infected with the highest dilutions, 5 or 1 focus forming units in total.

Selected foci (supernatants) were used to infect FBT-cells to confirm their status as LSDV recombinants expressing the *LacZ*-gene in a recombinant LSDV system. Five days post infection the infected cells were stained with X-gal (section 2.2.16). After an incubation time of 16 hr it was observed that not only single cells were stained as in the transient expression studies, but also foci of cells that represent the progeny from a single virus, stained blue (fig.2.15 C and D). FBT-cells infected with recombinant LSDV, expressing the *LacZ*-gene under control of the early LSDV promoter pA8R, exhibited the same quantity of intensely blue coloured foci when compared to FBT-cells infected with recombinant LSDV, expressing the *LacZ*-gene under control of the late LSDV promoter pA7L.

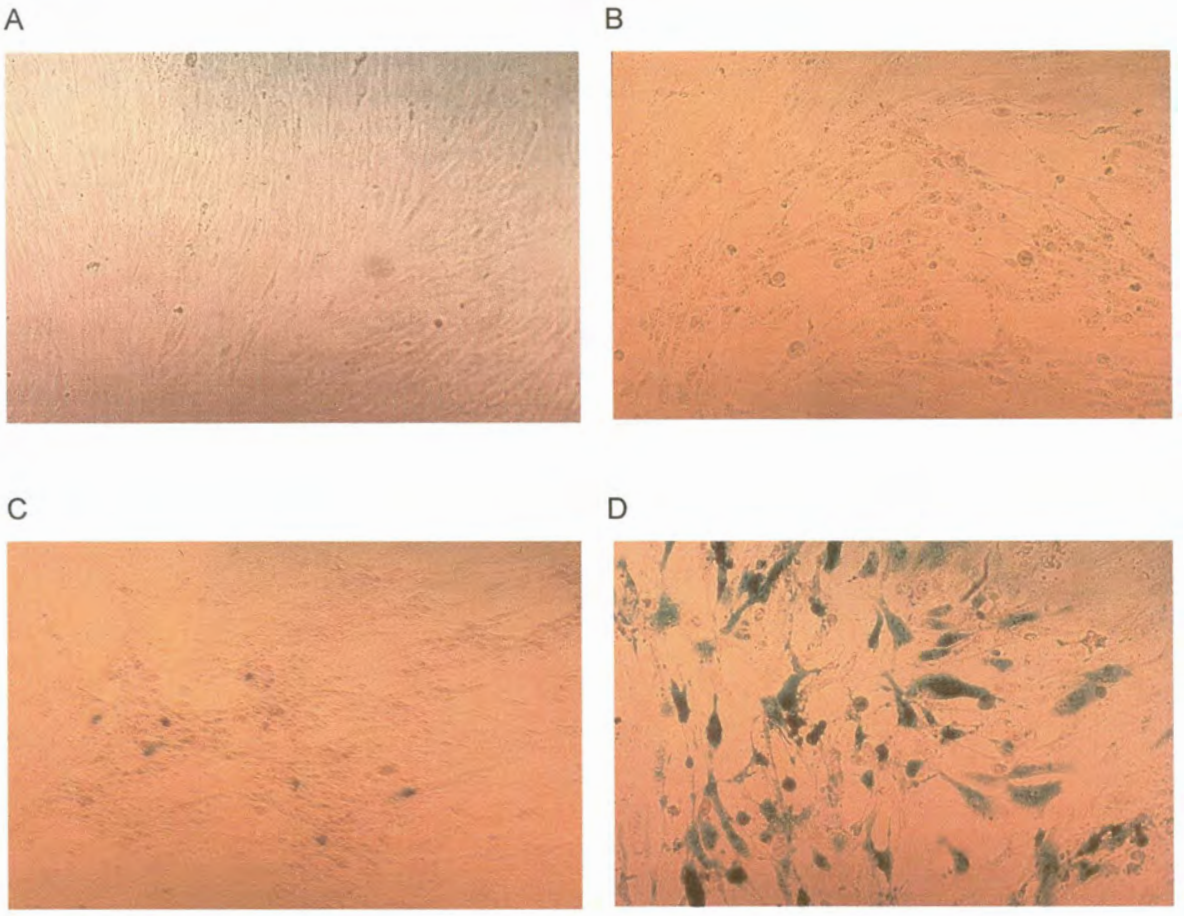

Fig. 2.14: Transient expression of enzyme β-Galactosidase in LSDV-infected cells

(A) Uninfected FBT-cells (as control)

(B)-(D) Monolayers of FBT-cells infected with LSDV at a M.O.I. of 0.1 ffu/cell and transfected with:

(B) No plasmid (as control)

(C) Plasmid pHSWF/*Lac*E containing *LacZ* under control of the early LSDV promoter (pA8R) and

(D) Plasmid pHSWF/*Lac*L containing *LacZ* under control of the late LSDV promoter (pA7L).

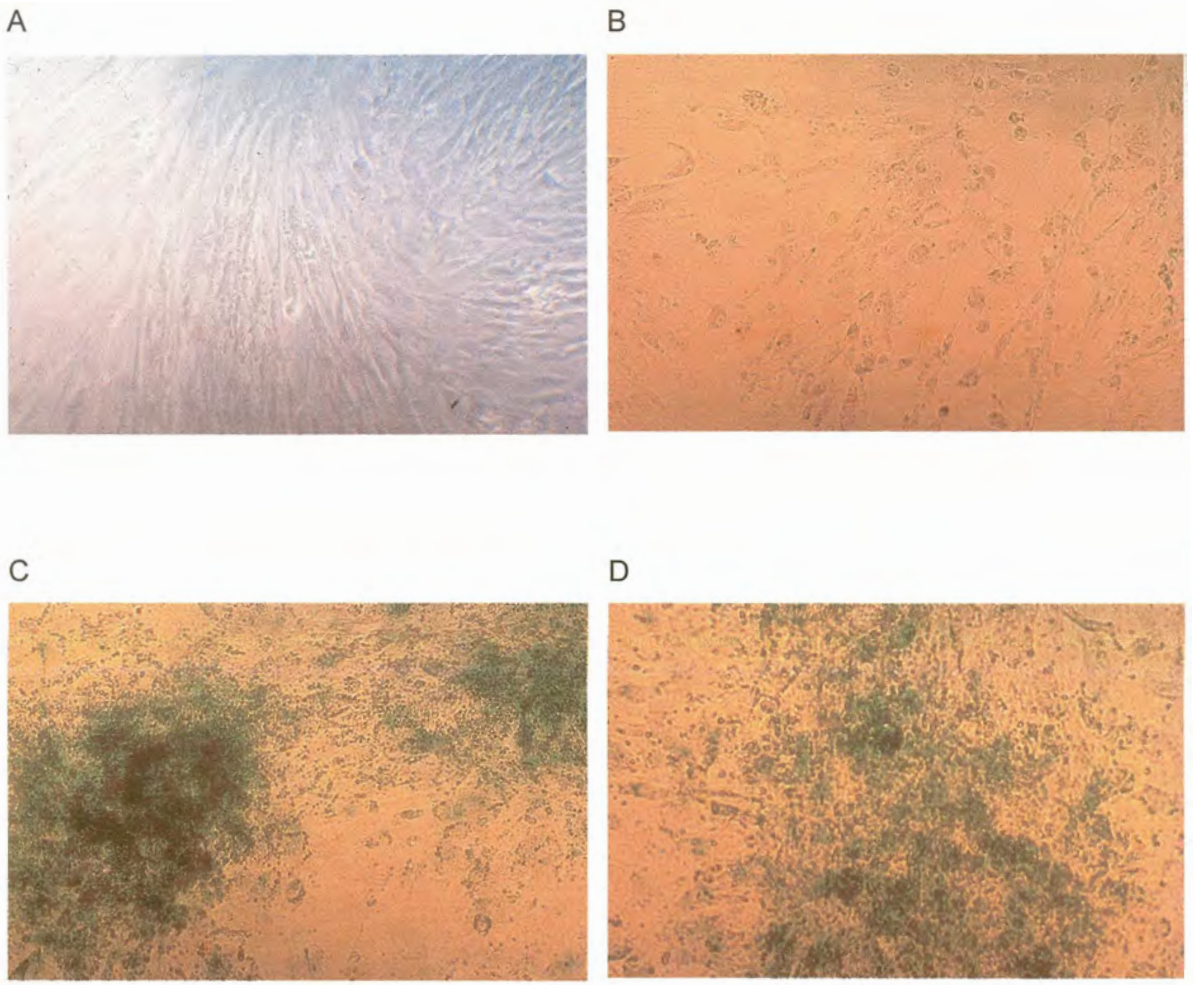

Fig. 2.15: Expression of the enzyme β-Galactosidase in FBT-cells infected with recombinant LSDV

(A) Uninfected FBT-cells (as control)

(B) Monolayers of FBT-cells infected with wt LSDV (as control)

(C) FBT-cells infected with recombinant LSDV expressing the *LacZ*-gene under control of the early LSDV promoter (pA8R)

(D) FBT-cells infected with recombinant LSDV expressing the *LacZ*-gene under control of the late LSDV promoter (pA7L)

2.3.3 Confirmation of LSDV recombinants

Selected foci were analysed for integration of the *LacZ*-gene by Southern blot analysis (section 2.2.21). The separated DNA restriction fragments were transferred to a Hybond N^+-membrane and probed for the presence of the *LacZ*-gene. Three DIG-labelled probes were used for hybridisation, i.e. the purified *LacZ*-gene (insert), pHSWF (plasmid) and SMII-size marker. All three probes were included in the hybridisation reaction. The following fragments were expected following digestion with *Bam* HI:

(1) In case of recombinant LSDV DNA where the *LacZ*-gene is cloned under control of the early promoter pA8R, 2 fragments of sizes 6.9 Kb and 9.9 Kb each, if no parental viruses reside in the recombinant. The inserted fragment has an internal *Bam* HI-site. If parental LSDV resides in the recombinant an extra fragment of 11.5 Kb will be obtained. (Information supplied by D.B. Wallace, OVI. The A2L (Harry) and A3L (Sally) region of wt LSDV is contained in a *Bam* HI-fragment of 11.5 Kb when wt LSDV DNA is digested with *Bam* HI). For the expected fragment sizes see Appendix 2A, p 113.

(2) In case of recombinant LSDV DNA where the *LacZ*-gene is cloned under control of the late promoter pA7L, 1 fragment of size 16.8 Kb, if no parental viruses reside in the recombinant. The inserted fragment has no internal *Bam* HI-site. The *Bam* HI-site, used for cloning of the *LacZ*-gene into pHSWF, is lost due to the blunt-end cloning procedure. As previously discussed, if parental LSDV resides in the recombinant an extra fragment of 11.5 Kb will be obtained. For the expected fragment sizes see Appendix 2B p 114.

According the autoradiograph (fig. 2.16 B lane c), 2 fragments of sizes 9.9 Kb and 6.9 Kb were obtained, although light due to the low concentration of the digested transferred recombinant LSDV DNA (fig. 2.16 A lane c). These results were an indication that the *LacZ*-gene was cloned under control of the early LSDV promoter pA8R and integrated into the LSDV genome. A clearly visible band (fig. 2.16 B lane d) of 16.8 Kb confirmed the presence of the *LacZ*-gene under control of the late LSDV promoter pA7L and integration of the gene into the LSDV genome.

In both cases a fragment of 11.5 Kb was observed; an indication that the selected foci of the recombinant LSDV still have residing wt LSDV. Since the purpose of these recombinants were simply to confirm the authenticity of the promoter-elements and the

ability of the vector to yield recombinant viruses, no further attempt was made to eliminate the residing wt LSDV. Another few rounds of foci purification by making use of dilution series in the presence of MPA-selection medium, will be needed to eliminate the residing wt LSDV from the recombinant cultures.

The recombinant LSDV was designated as LSDV/*Lac*E (*LacZ* under control of the early LSDV promoter pA8R) and LSDV/*LacL* (*LacZ* under control of the late LSDV promoter pA7L) respectively.

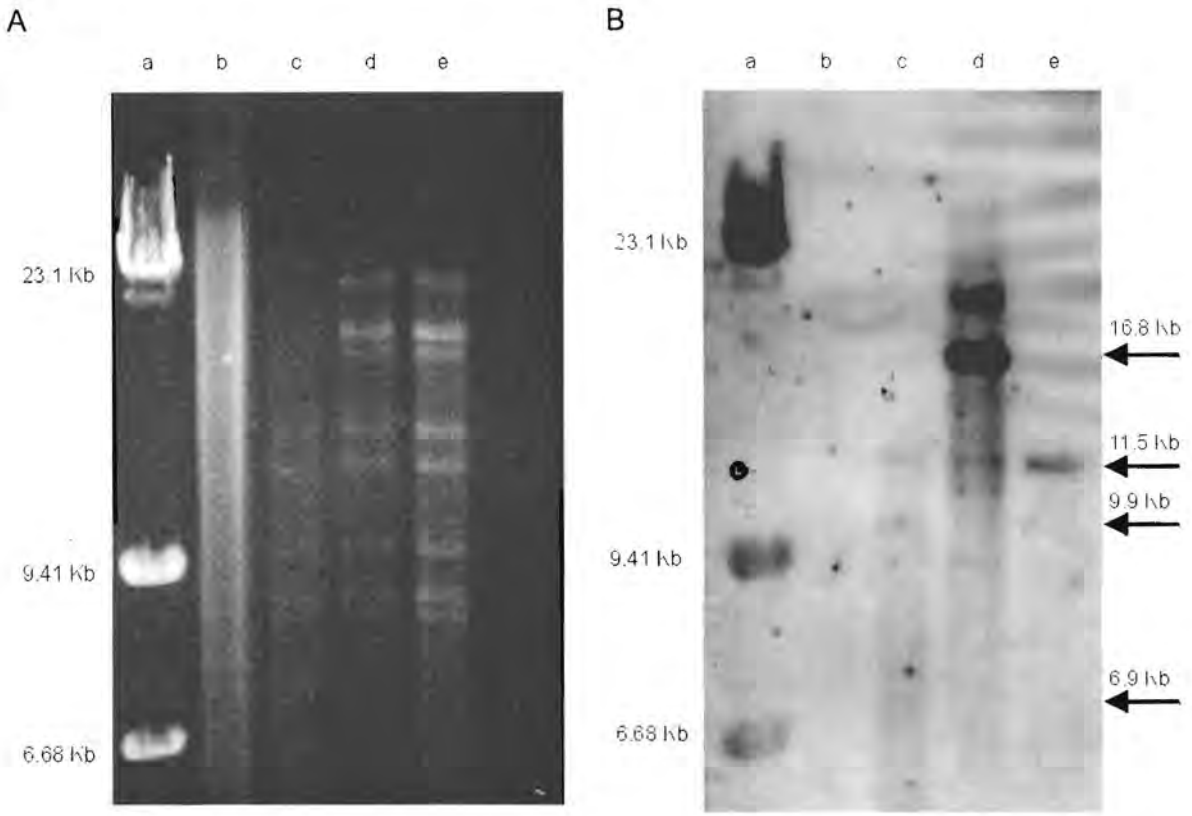

Fig.2.16 A and B: Genomic analysis of parental and recombinant LSDV DNA. After *Bam* HI digestion the DNA restriction fragments were separated by means of 0.6 % agarose gel electrophoresis (A)

(a) Size marker SMII

(b) DNA from uninfected FBT-cells digested with *Bam* HI (as control)

(c) Recombinant LSDV/*Lac*E DNA digested with *Bam* HI

(d) Recombinant LSDV/*Lac*L DNA digested with *Bam* HI

(e) wt-LSDV DNA digested with *Bam* HI (as control)

An autoradiograph of the gel in (A) after hybridisation with the purified *LacZ*-gene (insert), pHSWF (plasmid) and SMII-size marker DIG-labelled probes is shown in (B)

2.4 DISCUSSION

Capripoxviruses do not infect man and have a very limited host-range that make them attractive candidates for live recombinant vaccine purposes in the veterinary field. The success of recombinant poxviruses to express immunological important antigens, depends greatly on the poxvirus promoter to be used (Mackett *et al.*, 1982). According to Boyle (1992), FPV recombinants illustrated higher levels of marker gene expression in homologous virus-promoter recombinants when compared to heterologous virus-promoter recombinants that can presumably be caused by the fact that promoter recognition may not be uniform in different systems.

An early/late bi-directional LSDV promoter was identified and characterised by Fick and Viljoen (1999). Transient transcription assays done by Fick and Viljoen (1999) verified the temporally regulated nature of the promoter and revealed both early and late transcriptional activities. The transient expression studies were performed using the transfer vector pTKsLR that targets the TK-gene of LSDV, therefore allowing TK^- recombinants to grow in the presence of 5-Bromodeoxyuridine (BudR) on a TK^-cell line. In brief the vector contained the TK-gene of LSDV and a bi-directional promoter. Although the temporally regulated nature of the early/late LSDV bi-directional promoter was confirmed (Fick and Viljoen, 1999), attempts to generate stable recombinants expressing the VP2-gene of AHSV-9 were unsuccessful using this vector.

In this chapter, the identified LSDV elements have been analysed for expressing the *Lac*Z-reporter gene in a recombinant LSDV system by making use of a new transfer vector specifically designed for a LSDV system by Dr. W.C. Fick (Department of Genetics, UP). The characteristics of the new transfer vector pHSWF have been summarised in the literature survey. Two transfer plasmids, pHSWF/*Lac*E and pHSWF/*Lac*L were constructed to transfect infected FBT-cells respectively. In pHSWF/*Lac*E, *Lac*Z is placed under control of the early LSDV promoter pA8R, and in pHSWF/*Lac*L under control of the late LSDV promoter pA7L. The results obtained with the transient expression experiments confirmed the temporarily nature of both the early and late controlling elements of pA7LA8R and correlated with the assays done by Fick and Viljoen (1999). Blue coloured cells observed after X-gal staining of the transfected cells were an indication of the enzymatic activity of β-galactosidase. In case of the infected FBT-cells transfected with pHSWF/*Lac*L, the quantity of intensely blue coloured cells was much higher when compared to the quantity obtained with pHSWF/*Lac*E. These results don't necessarily confirm a higher expression level of the late pA7L promoter when compared

to the early pA8R promoter due to the fact that early transcription occurs for approximately 9 hr in comparison to the much longer late transcription (Fick and Viljoen, 1994).

The status of the two LSDV recombinants (LSDV/*Lac*E and LSDV/*Lac*L) expressing the *LacZ*-gene in a recombinant LSDV system was evaluated by infecting FBT-cells with each recombinant respectively. The results obtained with X-gal staining of the infected FBT-cells revealed that the same quantity of intensely blue coloured foci was obtained with LSDV/*Lac*E when compared to that of LSDV/*Lac*L. This was an indication that high levels of expression was achieved from the early promoter. In comparison to the transient expression studies, not only single cells stained blue but also foci of cells that represent the progeny from a single virus and proved the presence of LSDV recombinants. The results from the Southern blot analysis confirmed stable integration of the *LacZ*-gene into the LSDV genome. The Southern blot analysis and X-gal staining of cells infected with the 2 recombinants respectively showed that the new transfer vector pHSWF, could be used for the generating of stable LSDV recombinants. Both controlling elements of the bi-directional promoter drove expression of the *LacZ*-gene in a LSDV system. The apparent high levels of expression from the early promoter was encouraging in light of possible use of this transfer vector for generating recombinants in non-host species. In non-host species, foreign proteins would probably not be expressed from late promoters, seeing that late expression relies on viral DNA replication.