

**Evaluation of an authentic bi-directional
promoter in a new transfer vector for generating
lumpy skin disease virus recombinants**

BY

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**Evaluation of an authentic bi-directional promoter in a new transfer vector
for generating LSDV recombinants**

by

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dedicated to my beloved husband, Nico

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EVALUATION OF AN AUTHENTIC BI- DIRECTIONAL PROMOTER IN A NEW TRANSFER VECTOR FOR GENERATING LSDV RECOMBINANTS

ABSTRACT

Lumpy skin disease virus (LSDV), a member of the *Capripoxvirus* genus, is one of the poxviruses currently being investigated as a live recombinant system for expressing foreign genes. The strict tropism of the virus, limited to ungulates, renders it very promising as a vector for veterinary vaccine purposes. Poxvirus recombinants are generated by a process of homologous recombination, mediated by using specific transfer vectors. In this study a new transfer vector pHSWF was evaluated for its ability to generate stable LSDV recombinants that would express foreign genes from an authentic bi-directional LSDV promoter.

A reporter gene, the *E. coli LacZ*-gene encoding the β -galactosidase (β -gal) enzyme, was used in initial studies. It was placed under control of either the early or the late promoter by constructing two different transfer plasmids pHSWF/*LacE* and pHSWF/*LacL*. These were used to generate LSDV/*LacE* and LSDV/*LacL* recombinants in fetal bovine testis (FBT) cells. Southern blot analysis confirmed that the *LacZ*-gene was stably integrated into an intergenic region of the LSDV genome. Expression of the *LacZ*-gene from both the early and late promoters was confirmed by monitoring the β -gal enzyme activity in infected cells. Similar intensities of blue stained foci, indicative of β -gal activity, was observed in FBT-cells infected with the respective recombinants. This suggested that similar high levels of expression were induced by both the early and the late promoters. The results on the *LacZ* recombinants confirmed that the new transfer vector may indeed be used to generate stable recombinants and that foreign genes are expressed at high levels.

The transfer vector was then used to generate LSDV recombinants that express an immunological important gene, the VP7 gene of AHSV from the LSDV early promoter. The VP7 protein encodes the major group-specific antigen of AHSV and is a possible candidate for use in recombinant vaccines. Expression of the VP7 protein in the recombinant LSDV system was confirmed by SDS-PAGE analysis and radioactive labelling of proteins in infected cells. Characteristically, high levels of VP7 expression leads to its assembly in flat hexagonal crystals in infected cells. This property was analysed by cryoelectron microscopy. Crystal formation was indeed observed, confirming

that high levels of VP7 expression occurred in the LSDV recombinant. These results further supported the fact that the LSDV early promoter has the ability to induce high levels of foreign gene expression in the LSDV system.

The new transfer vector was also tested for its ability to generate dual LSDV recombinants that would express foreign genes from both the early and late promoter in the same recombinant. An existing plasmid vector in which the VP2 gene of AHSV was cloned under control of the late promoter was modified by placing the VP7 gene under control of the early promoter. The construct was used to generate dual recombinant viruses. PCR results confirmed that both genes were integrated into the LSDV genome, however no expression of VP2 could be observed.

In summary, this study has contributed to our knowledge of using LSDV as a live expression system. The new transfer vector allows the integration of foreign genes into a non-essential site within the LSDV genome. This procedure, whereby no LSDV genes are disrupted, should not debilitate the already attenuated LSDV virus any further. Foreign genes may be expressed from both an early and / or late promoter. The high levels of expression from the early promoter is promising for using LSDV recombinants in non-host species where the recombinants are unlikely to complete their viral replication cycle.

ABBREVIATIONS

| | |
|--------------------|----------------------------------------|
| AHS | african horsesickness |
| AHSV-9 | african horsesickness virus serotype 9 |
| ATG | adenosine thymidine guanosine |
| ATP | adenosine triphosphate |
| ATV | activated trypsin versene |
| bp | base pairs |
| BTV | bluetongue virus |
| BudR | bromodioxyuridine |
| β-gal | β-galactosidase |
| βME | beta-mercapto-ethanol |
| CaCl ₂ | calcium chloride |
| ccc | covalently closed circular |
| cDNA | complementary deoxyribonucleic acid |
| Ci | Curie |
| cm | centimeter |
| cm ² | centimeter square |
| CO ₂ | carbon dioxide |
| CPE | cytopathic effect |
| cpm | counts per minute |
| CTL | cytotoxic T lymphocytes |
| °C | degrees Celcius |
| dATP | 2'-deoxyadenosine-5'-triphosphate |
| dCTP | 2'-deoxycytidine-5'-triphosphate |
| dGTP | 2'-deoxyguanosine-5'-triphosphate |
| dTTP | 2'-deoxythymidine-5'-triphosphate |
| dUTP | 2'-deoxy-uracil-5'-triphosphate |
| ddH ₂ O | double distilled water |
| DEPC | diethylpyrocarbonate |
| dH ₂ O | distilled water |
| DIG | digoxigenin |
| DMEM | Dubelco's Modified Eagle's Medium |
| DMSO | dimethyl sulphoxide |
| DNA | deoxyribonucleic acid |
| DNAse | deoxyribonuclease |

| | |
|-------------------|---------------------------------------------------------------------|
| dNTP | 2'-deoxy-nucleotide-5'-triphosphate |
| ds | double stranded |
| dsDNA | double stranded ribonucleic acid |
| DTT | dithiothreitol |
| <i>E.coli</i> | <i>Escherichia coli</i> |
| EDTA | ethylenediaminetetra-acetic acid |
| EtBr | ethidium bromide |
| EtOH | ethanol |
| F | fusion gene of rinderpest |
| FBT | foetal bovine testis |
| ffu | focus forming units |
| Fig | figure |
| FPV | fowlpox virus |
| GMP | guanine monophosphate |
| gpt | <i>Escherichia coli</i> xanthine-guanine phosphoribosyl transferase |
| HA | haemagglutinin gene |
| HAT | hypoxanthine, aminopterin and thymidine |
| HCl | hydroxyl chloride |
| HN | haemagglutinin-neuraminidase |
| H ₂ O | water |
| hr | hour |
| ITR's | inverted terminal repetitions |
| K | kiloDalton |
| Kb | kilobase pairs |
| kPa | kiloPascal |
| kV | kiloVolt |
| LB broth | Luria-Bertani medium |
| LiCl | litium chloride |
| LSDV | lumpy skin disease virus |
| M | molar |
| mA | miliAmpere |
| MgCl ₂ | magnesium chloride |
| min | minute |
| ml | milliliter |
| MM/mMol | millimolar |
| M.O.I. | multiplicity of infection |

| | |
|-------------------|-------------------------------------------------------------------------------------|
| MPA | mycophenolic acid |
| mrRNA | messenger ribonucleic acid |
| MgSO ₄ | magnesium sulphate |
| MVA | modified vaccinia Ankara |
| NaAc | sodium acetate |
| NaCl | sodium chloride |
| Nal | sodium iodine |
| NaOH | sodium hydroxide |
| NDV | newcastle disease virus |
| ng | nanogram |
| NLS | N-lauryl sarcosinate |
| nm | nanometer |
| OD ₅₅₀ | optical density at 550 nanometer |
| OH | hydroxyl |
| O/N | overnight |
| OVI | Onderstepoort veterinary institute |
| φX | PhiX174/ <i>Hae</i> Marker |
| PBS | phosphate buffered saline |
| PBS(A) | phosphate buffered saline without calcium and magnesium containing 0.2 % gentamycin |
| PCR | polymerase chain reaction |
| p.i. | post infection |
| pmol | picomole |
| PPR | peste des petits ruminants |
| PSB | protein solvent buffer |
| r.e. | restriction endonuclease |
| RNA | ribonucleic acid |
| rpm | revolutions per minute |
| RP | rinderpest |
| RPV | rinderpest virus |
| RT | room temperature |
| SA | South-Africa |
| SDS | sodium dodecyl sulphate |
| SDS-PAGE | sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| sec | second |
| Sf9 | <i>Spodoptera frugiperda</i> |

| | |
|------------|-------------------------------------------------|
| SMII | DNA molecular weight marker II |
| SPV | swinepox virus |
| ST | sodium chloride Tris HCl |
| TAE | Tris acetate EDTA |
| <i>Taq</i> | <i>Thermus aquaticus</i> |
| TBE | Tris-boric acid EDTA buffer |
| TBST | Tris-buffered saline Tween |
| TE | Tris EDTA |
| TEMED | N,N,N',N'-Tetra-methylethylenediamin |
| TGS | Tris glycine sodium dodecyl sulphate buffer |
| TK | thymidine kinase |
| tRNA | transfer ribonucleic acid |
| Tris | Tris-(hydroxymethyl)-aminomethane |
| U | unit |
| UP | University of Pretoria |
| USA | United States of America |
| UV | ultraviolet |
| V | volt |
| VV | vaccinia virus |
| WHO | World Health Organization |
| wt | wild-type |
| w/v | weight per volume ratio |
| X-Gal | 5-bromo-4-chlor-3-indolyl-3-D-galactopyranoside |
| μCi | microCurie |
| μg | microgram |
| μl | microliter |

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

(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.

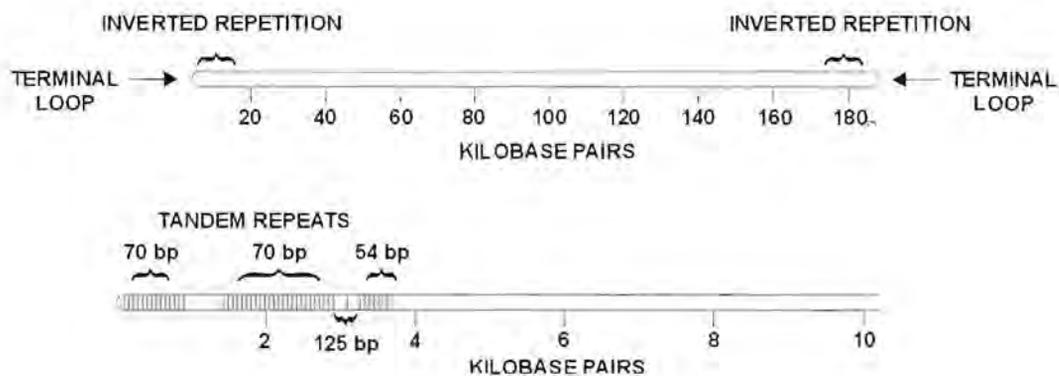


Fig. 1.1 Structural features of vaccinia viral DNA. A representation of the entire double-stranded DNA genome and an expansion of the 10 000 bp inverted terminal repetition (Moss, 1996).

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 live 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 *Stomoxys 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 simplex 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 pox 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 fusion (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-bromodioxuridine (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 haemagglutinin 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/*LacE* and pHSWF/*LacL* 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/*LacE* and pHSWF/*LacL*, 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 μ l, containing 15 μ g DNA and 2.5 units of each enzyme. The transfer vector was linearised in a single digestion reaction in a total volume of 20 μ l, containing 3 μ g 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 horizontal 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 Na₂EDTA.2H₂O, 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 horizontal 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 (ddH₂O) 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 MgCl₂, 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 *Escherichia 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 ($\text{OD}_{550} = 0.471$), 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 $\mu\text{g/ml}$ ampicillin and 12.5 $\mu\text{g/ml}$ 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 ddH₂O 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 ddH₂O after which it was stored at -20 °C.

2.2.11 Characterisation of transfer DNA

The miniprep plasmid DNA was analysed by electrophoresis in horizontal 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/LacE and pHSWF/LacL 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 Na₂HPO₄·2H₂O, 0.14 mM KH₂PO₄, 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 trypsinised 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 cm² culture flask (Sarstedt, Highveld Biological). Fifteen ml of the 10 % foetal calf-serum growth medium was added per 75 cm² flask and placed in a 5 % CO₂ 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 % CO₂ - 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 % CO₂ - 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 3×10^5 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/LacE and pHSWF/LacL 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 % CO₂-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 MgCl₂) and incubated at 37 °C for 16 hours. Cells transfected with either pHSWF/LacE or pHSWF/LacL 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/LacE or pHSWF/LacL 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/LacE and LSDV/LacL. 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 μ g/ml mycophenolic acid, 250 μ g/ml xanthine, 200 μ g/ml HAT from Highveld Biological (13.6 μ g/ml hypoxanthine, 0.16 μ g/ml aminopterin and 3.84 μ g/ml thymidin) and 2.5 % foetal calf serum in DMEM supplemented with 120 μ g/ml penicillin G, 120 μ g/ml streptomycin sulphate and 0.3 μ g/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 its 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 cm² 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 cm² 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 cm² 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 Na₂HPO₄.12H₂O, 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 beta-mercaptoethanol (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 (dH₂O). 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 dH₂O 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 Na₃citrate.2H₂O) 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 Na₃citrate2H₂O). Another 2 filters, prewetted with 2 x SSC, were put on top of the nylon membrane, followed with 2 dry filter papers and pre-cut paper towels. On top of the stack another perspex plate was laid 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 MgCl₂; 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/*LacE* and pHSWF/*LacL* 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).

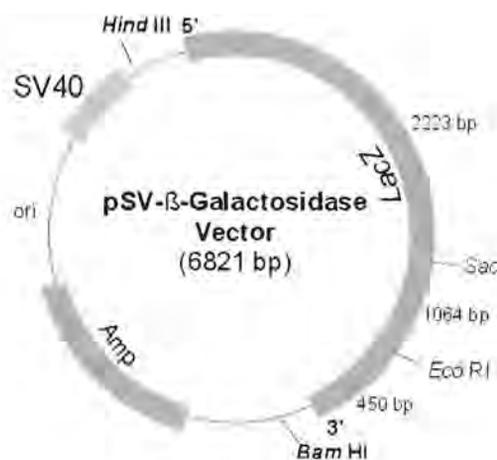


Fig. 2.1: pSV- β -Galactosidase Vector circle map

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 SmaI
(b) Undigested pSV-β-Galactosidase
(c) pSV-β-Galactosidase digested with *Hind* III and *Bam* HI.



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

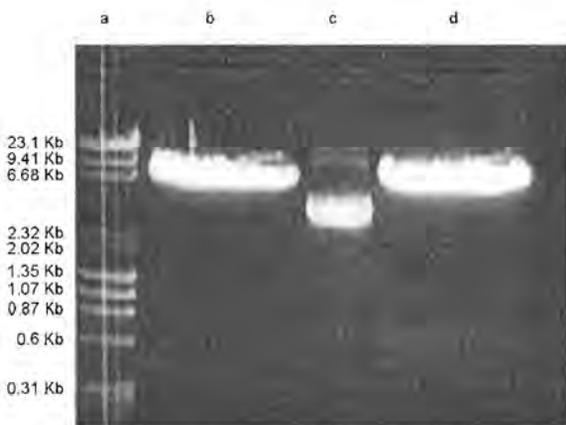


Fig. 2.4: Agarose gel electrophoretic analysis of vector pHSWF
(a) Size markers SmaI and ϕ X
(b) pHSWF digested with *Eco* RI
(c) Undigested pHSWF
(d) pHSWF digested with *Bam* HI



Fig. 2.5: Agarose gel electrophoretic analysis of gene-cleaned 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 gene-cleaned recovered DNA following dephosphorylation
 (a) Size markers *S*MII and ϕ X
 (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

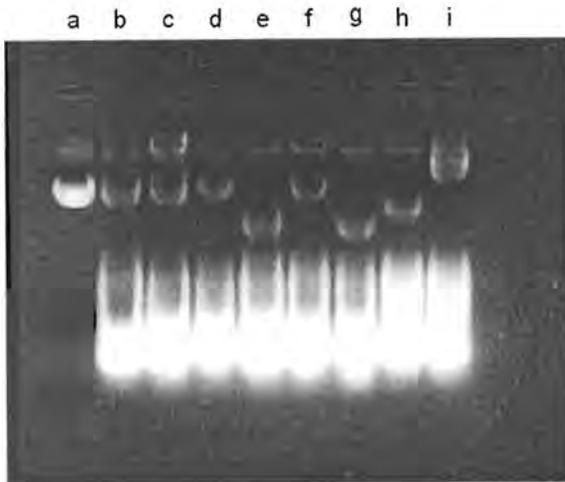


Fig. 2.7: Agarose gel electrophoretic analysis of putative *LacZ* recombinants with the *LacZ*-gene cloned downstream of the early promoter, pA8R
 (a) Intact vector, pHSWF, as control
 (b)-(i) Undigested putative plasmids containing the *LacZ*-gene under control of the early promoter, pA8R

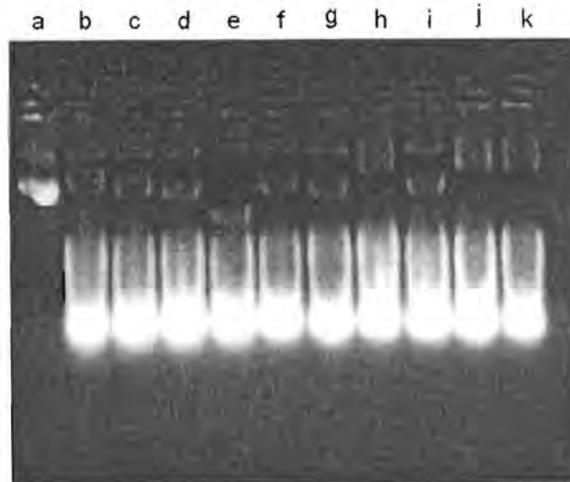


Fig. 2.8: Agarose gel electrophoretic analysis of putative *LacZ* recombinants with the *LacZ*-gene cloned downstream of the late promoter, pA7L
 (a) Intact vector, pHSWF, as control
 (b)-(k) Undigested putative plasmids containing the *LacZ*-gene under control of the late promoter, pA7L

Orientation of pHSWF/*LacE*

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 its 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/*LacL*

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 its 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/*LacE* and fig. 2.11 lane d for pHSWF/*LacL*) 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/*LacE* and pHSWF/*LacL*.

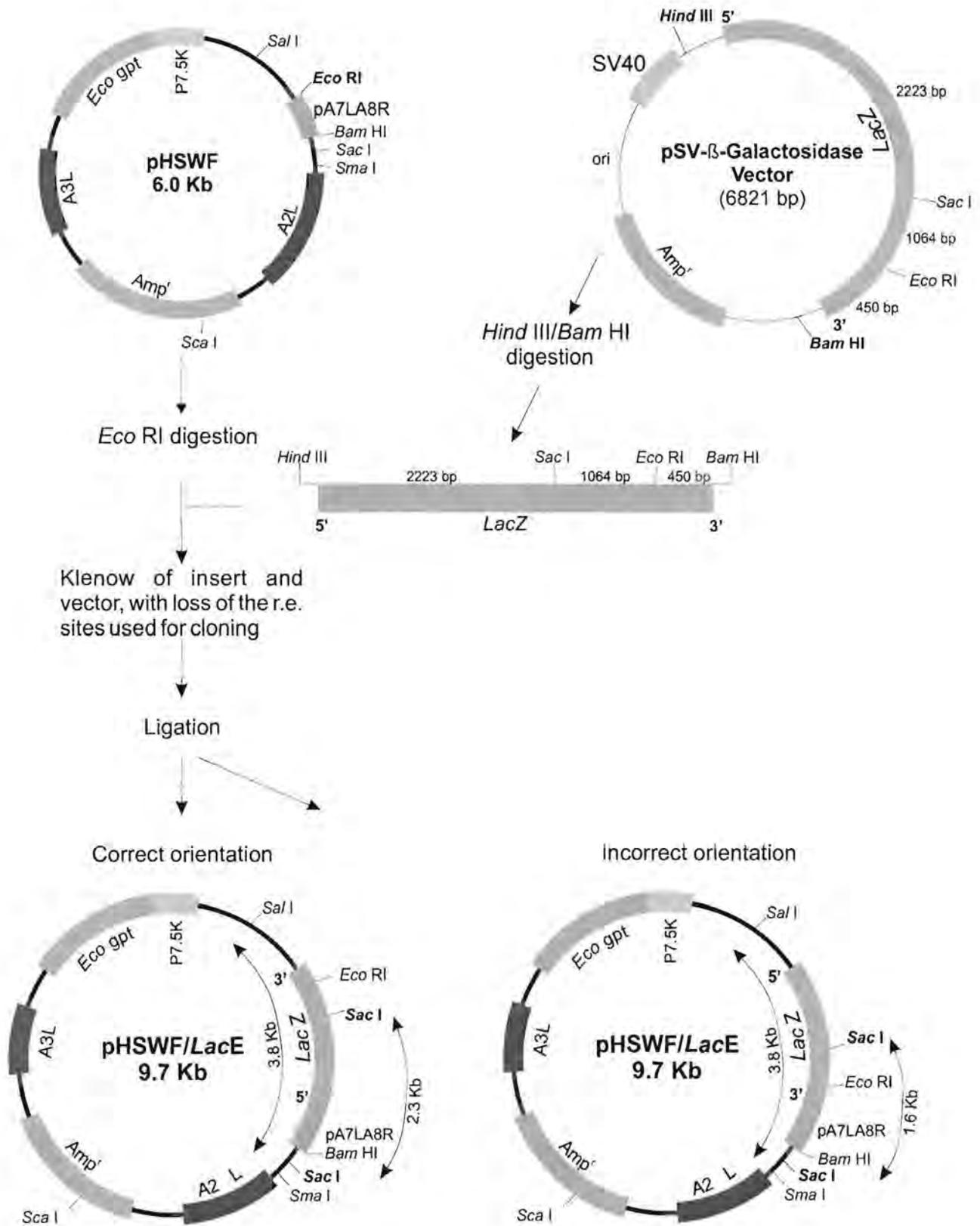


Fig. 2.9a: Schematic representation of the cloning of *LacZ* into the transfer vector pHSWF under control of the early LSDV promoter (pA8R). A partial r.e. map of *LacZ* is shown, as well as the correct and incorrect transcriptional orientations of the gene after cloning.

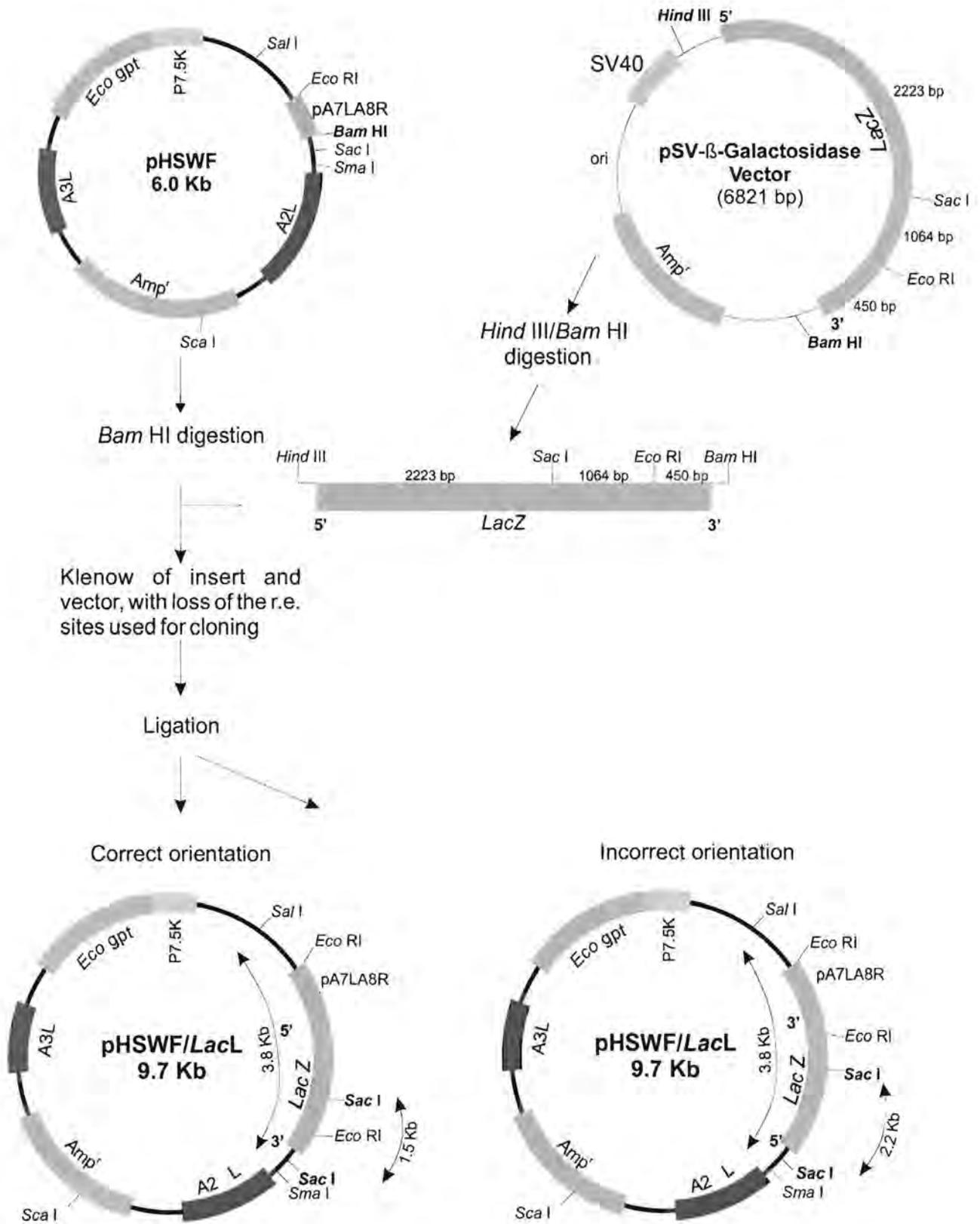


Fig. 2.9b: Schematic representation of the cloning of *LacZ* into the transfer vector pHSWF under control of the late LSDV promoter (pA7L). A partial r.e. map of *LacZ* is shown, as well as the correct and incorrect transcriptional orientations of the gene after cloning.

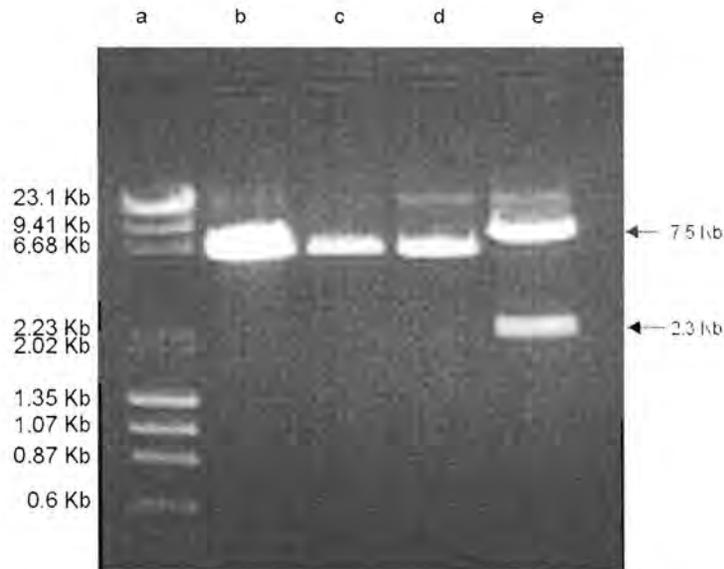


Fig. 2.10: Agarose gel electrophoretic analysis of putative plasmids containing the *LacZ*-gene, pHSWF/*LacE*, to establish the correct transcriptional orientation
 (a) Size markers *S*MII and ϕ X
 (b) pHSWF digested with *Sac* I as control
 (c)-(e) Putative plasmids containing the *LacZ*-gene, pHSWF/*LacE*, digested with *Sac* I



Fig. 2.11: Agarose gel electrophoretic analysis of putative plasmids containing the *LacZ*-gene, pHSWF/*LacL*, to establish the correct transcriptional orientation
 (a) Size markers *S*MII and ϕ X
 (b) pHSWF digested with *Sac* I as control
 (c)-(f) Putative plasmids containing the *LacZ*-gene, pHSWF/*LacL*, digested with *Sac* I

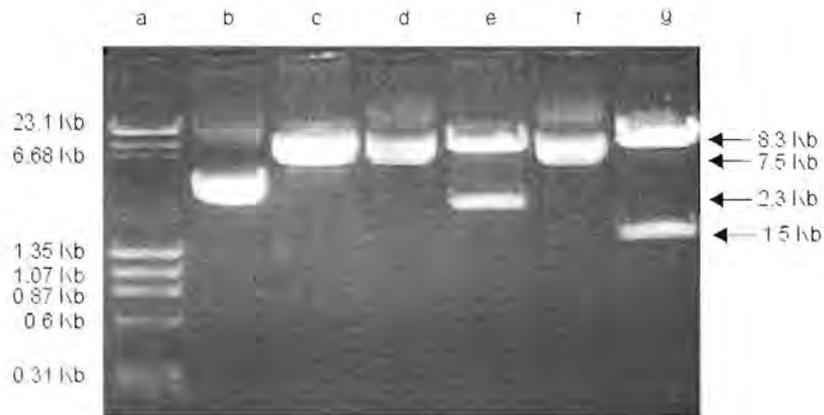


Fig. 2.12: Agarose gel electrophoretic analysis of plasmids pHSWF/*LacE* and pHSWF/*LacL* to confirm the correct orientation

- (a) Size markers *S*MII and ϕ X
- (b) Undigested pHSWF
- (c) pHSWF digested with *Sac* I as control
- (d) Undigested pHSWF/*LacE*
- (e) pHSWF/*LacE* digested with *Sac* I
- (f) Undigested pHSWF/*LacL*
- (g) pHSWF/*LacL* digested with *Sac* I

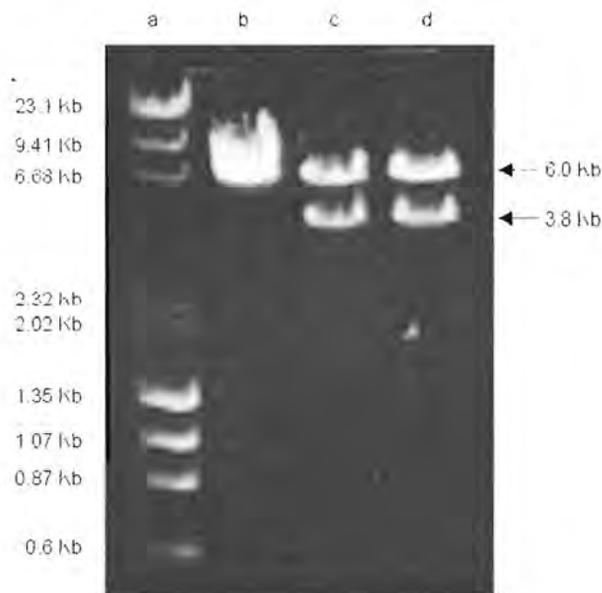


Fig. 2.13: Agarose gel electrophoretic analysis of plasmids pHSWF/*LacE* and pHSWF/*LacL*

- (a) Size markers *S*MII and ϕ X
- (b) pHSWF digested with *Sma* I and *Sal* I as control
- (c) pHSWF/*LacE* digested with *Sma* I and *Sal* I
- (d) pHSWF/*LacL* digested with *Sma* I and *Sal* I

2.3.2 Generation of LSDV recombinants

2.3.2.1 Transient expression of the *LacZ*-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 *LacZ*-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/*LacE* and pHSWF/*LacL* 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/*LacL*, showed a higher quantity of intensively blue coloured cells when compared to cells transfected with pHSWF/*LacE*. 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/*LacE*, *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/*LacL*, 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/*LacE* or pHSWF/*LacL* 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.

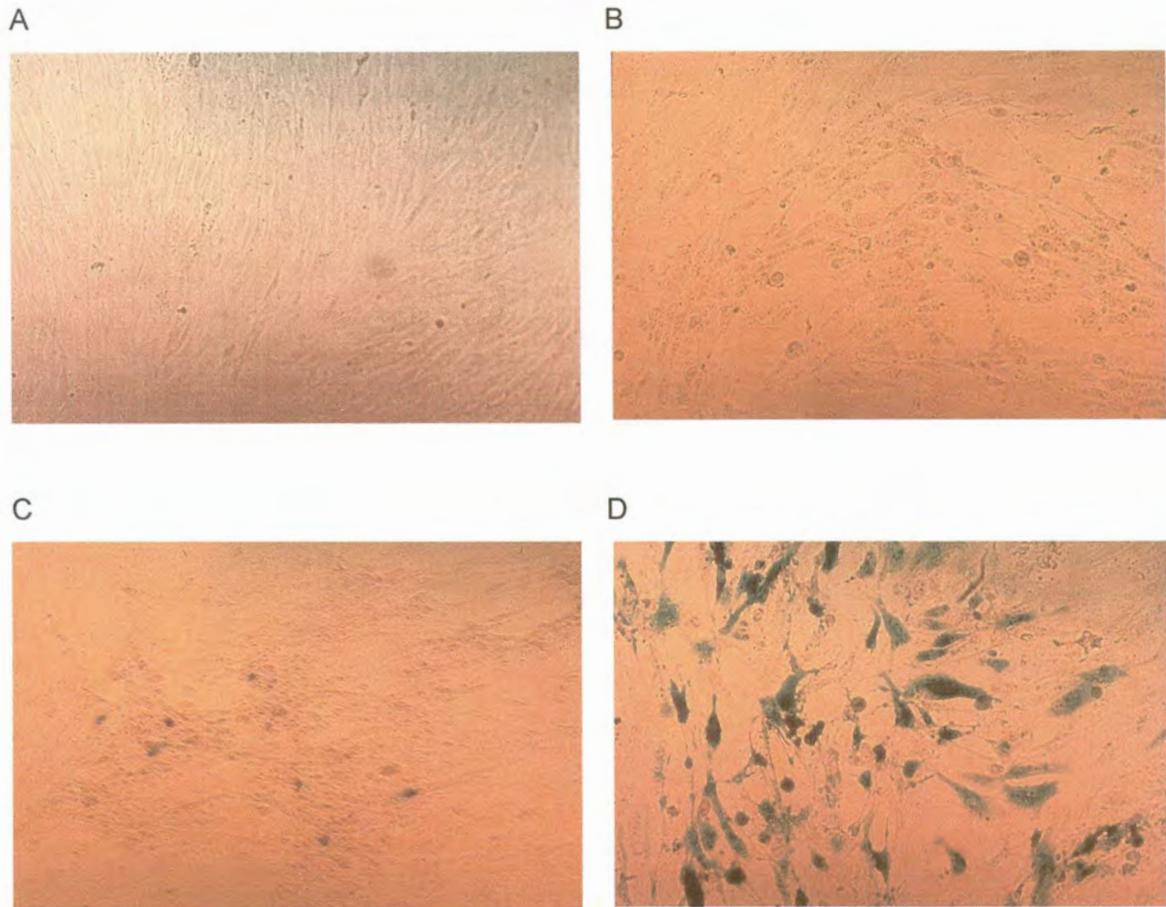


Fig. 2.14: Transient expression of enzyme β -Galactosidase in LSDV-infected cells
(A) Uninfected FBT-cells (as control)
(B)-(D) Monolayers of FBT-cells infected with LSDV at a M.O.I. of 0.1 ffu/cell and transfected with:
(B) No plasmid (as control)
(C) Plasmid pHSWF/LacE containing *LacZ* under control of the early LSDV promoter (pA8R) and
(D) Plasmid pHSWF/LacL containing *LacZ* under control of the late LSDV promoter (pA7L).

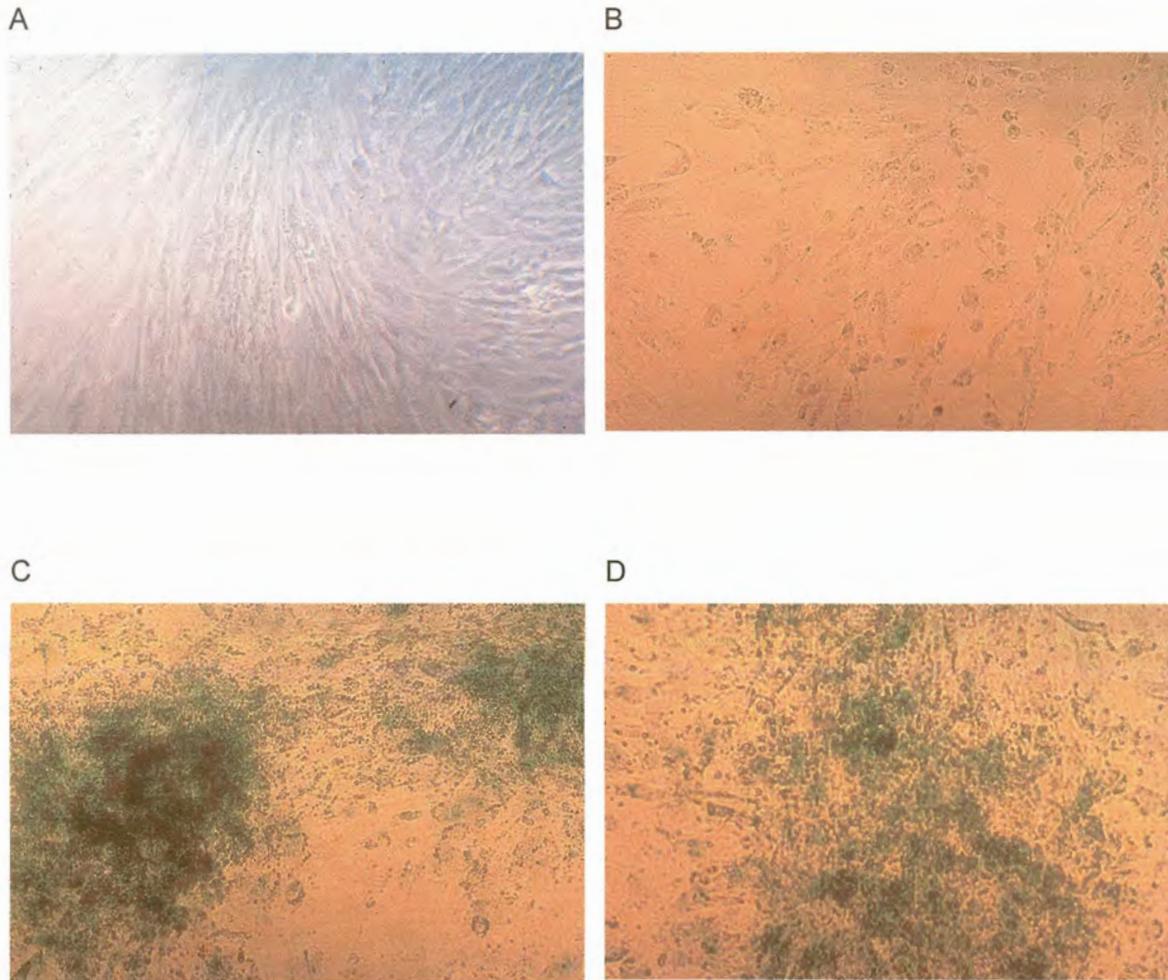


Fig. 2.15: Expression of the enzyme β -Galactosidase in FBT-cells infected with recombinant LSDV

- (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 SMI-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 to 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/*LacE* (*LacZ* under control of the early LSDV promoter pA8R) and LSDV/*LacL* (*LacZ* under control of the late LSDV promoter pA7L) respectively.

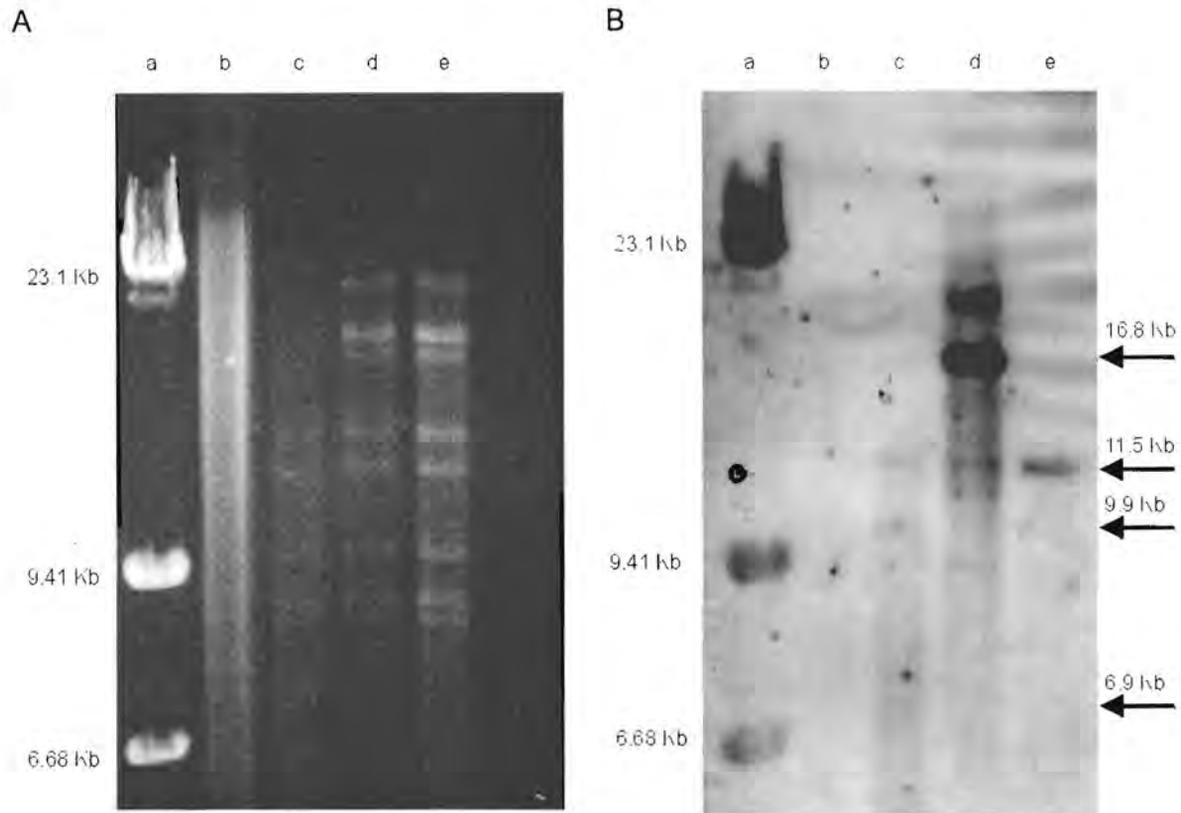


Fig.2.16 A and B: Genomic analysis of parental and recombinant LSDV DNA. After *Bam* HI digestion the DNA restriction fragments were separated by means of 0.6 % agarose gel electrophoresis (A)

(a) Size marker SMII

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

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

(d) Recombinant LSDV/*LacL* 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 *LacZ*-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/*LacE* and pHSWF/*LacL* were constructed to transfect infected FBT-cells respectively. In pHSWF/*LacE*, *LacZ* is placed under control of the early LSDV promoter pA8R, and in pHSWF/*LacL* 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/*LacL*, the quantity of intensely blue coloured cells was much higher when compared to the quantity obtained with pHSWF/*LacE*. 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/*LacE* and LSDV/*LacL*) 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/*LacE* when compared to that of LSDV/*LacL*. 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.

CHAPTER THREE

GENERATION OF LSDV RECOMBINANTS EXPRESSING IMMUNOLOGICAL IMPORTANT ANTIGENS

3.1 INTRODUCTION

The encouraging results obtained with the *LacZ* recombinants initiated an investigation concerning the ability of the early promoter to drive the expression of an immunological important gene. The gene encoding the major group-specific antigen that is located on the outer surface of the core, VP7 of African horsesickness virus serotype 9 (AHSV-9), was chosen as a model gene.

African horsesickness (AHS) is an infectious, but non-contagious orthropod-borne disease, that affects all species of the *Equidae* (horses, mules and donkeys). The causative agent, African horsesickness virus (AHSV), is a dsRNA virus classified as a distinct species in the genus *Orbivirus*, within the family *Reoviridae* (Holmes, 1991). Nine different serotypes of AHSV (AHSV-1 to AHSV-9) have been identified with little, if any, cross-neutralisation between them (McIntosh, 1958; Howell, 1962). AHSV is enzootic in Africa but serious epizootics have also occurred at infrequent intervals in Egypt, the Middle East, Yemen and Spain (reviewed by Bremer *et al.*, 1990).

AHSV is closely related to bluetongue virus (BTV), the prototype member of the *Orbiviruses* (Holmes, 1991). Like BTV, AHSV exhibits typical orbivirus morphology. The icosahedral virion is composed of a double-shelled protein capsid that encloses ten double-stranded RNA (dsRNA) segments (Oellerman *et al.*, 1970; Huismans, 1979). Each genome segment encodes a specific viral protein. The seven structural proteins (VP1-VP7) and three non-structural proteins (NS1-NS3) are encoded by these genome segments (Grubman and Lewis, 1992). The major outer capsid layer of the virus consists of two of the seven structural proteins, VP2 and VP5, and surrounds an icosahedral core particle, which is composed primarily of VP3 and VP7, with a lesser amount of three minor structural proteins VP1, VP4 and VP6 (Vreede and Huismans, 1994). According to Roy *et al.* (1994), the four non-structural (NS) proteins, NS1, NS2, NS3 and NS3a, are believed to be involved in virus morphogenesis.

At present, AHS is controlled by annual vaccinations with polyvalent live attenuated vaccines (House *et al.*, 1992). A major potential problem in the use of such vaccines is

that reassortment between serotypes can result in the generation of progeny viruses with novel phenotypes, in terms of serological and virulence characteristics. Furthermore, AHSV has been implicated as causing neurotropic infection in workers involved with large-scale preparation of live, attenuated AHSV vaccines (Wade-Evans *et al.*, 1997). Therefore there is a need for a safe economical vaccine that is effective against multiple serotypes of AHSV.

VP7, the major group-specific antigen, is located on the outer surface of the core. It is an extremely hydrophobic protein and characteristically forms trimers when expressed in a recombinant system (Roy, 1992). The VP7 protein of BTV (equivalent to the VP7 of AHSV) has been shown to contain immunodominant, serotype-cross-reactive T-cell epitopes but does not generate neutralising antibodies to intact virus particles (Wade-Evans *et al.*, 1997). VP7 was therefore considered as a possible candidate for use in recombinant vaccines that might induce a protective and serotype-cross-reactive immune response to AHSV (possibly via a cell-mediated mechanism).

We also investigated the use of the new transfer vector pHSWF to generate dual LSDV recombinants, and the ability of the LSDV bi-directional promoter to drive the expression of two immunologically important antigens from both the early and late promoter simultaneously. The purpose was to express the AHSV-9 VP7-gene from the early promoter and VP2 from the late promoter by making use of one construct.

It has been shown that VP2, the outer capsid protein of AHSV, bears the major neutralising epitopes and thus is responsible for inducing serotype-specific responses (Burrage *et al.*, 1993; Vreede, F.T., and Huismans, H., 1994). M.A. Stone-Marchat *et al.* (1996) demonstrated that AHSV VP2 alone is sufficient to induce a protective immune response in horses when challenged with a homologous highly virulent AHSV where the protection was presumably mediated by neutralising antibodies. Observations that the outer-capsid protein VP2 is the main determinant of the AHSV neutralisation-specific immune response were confirmed when vaccination, using baculovirus-expressed AHSV-4 VP2 protein, provided protection against death caused by virulent AHSV-4 challenge (Stone-Marschat *et al.*, 1996).

As mentioned previously, foreign genes expressed from late promoters elicit mainly humoral immune responses (Romero *et al.*, 1994), while proteins expressed from early promoters primarily lead to cell-mediated immune responses (Coupar *et al.*, 1986). By using the LSDV bi-directional promoter to construct LSDV recombinants, an antigen that

is important for eliciting a humoral response could in theory be expressed from the late promoter, while another that is important for eliciting cellular immunity could be expressed from the early promoter. Due to the fact that VP2 induce a humoral- and VP7 a cell-mediated immune response, VP2 was cloned under control of the late, and VP7 under the early LSDV promoter.

In the first part of this chapter the generation of a LSDV/VP7 recombinant is described. The AHSV-9 VP7-gene would be cloned into the *Eco* RI-site of the pHSWF transfer vector, thereby placing it under control of the LSDV early promoter. Successful integration of the VP7-gene into the LSDV genome would be evaluated by PCR and Southern blot analysis. Transcription of the gene would be analysed by dot-blot analysis of total RNA. Expression of VP7 in the recombinant LSDV system would be evaluated by ³⁵S-methionine labelling of synthesised proteins in infected cells at different times post infection, followed by SDS-PAGE analysis and autoradiography. Electron microscopy studies would also be performed to observe VP7 crystal formation, a feature of high levels of VP7 protein expression.

The second part of this chapter involves the generation of dual LSDV(VP2/VP7) recombinants by transfecting FBT-cells with the dual plasmid pHSWF(VP2/VP7). After a few rounds of single focus purification, selected foci would be used to evaluate their status as dual recombinants by PCR and Southern blot analysis.

3.2 MATERIALS AND METHODS

3.2.1 Construction of transfer plasmids

The plasmids pHSWF/VP7 and pHSWF(VP2/VP7) were constructed using the same strategy as for pHSWF/LacE and pHSWF/LacL described in Chapter 2, section 2.2.1. Similar methods, i.e. restriction enzyme digestions, agarose gel electrophoresis, purification of DNA excised from gels, generation of blunt-ended fragments, dephosphorylation of the vector, ligation of vector and insert, transformation of competent *E.coli* cells and characterisation of large-scale purified plasmids were used as described previously in section 2.2.2 to 2.2.11.

3.2.2. Transfection procedure

A monolayer of 90 % confluent FBT-cells in a six-well plate (Nunc) was infected with purified LSDV stock at a M.O.I. of 0.1 ffu/cell. Transfection of plasmids pHSWF/VP7 and pHSWF(VP2/VP7) was performed by means of the liposome-mediated transfer method, 90 min post infection. For liposome-mediated transfer the following conditions were used per one well of a six-well plate: 5 µg plasmid DNA diluted with OPTI-MEM (Life Technologies) to give a total volume of 100 µl and in a separate reaction tube, 5 µl of Lipofectamine (Life Technologies) added to 95 µl OPTI-MEM. The solution containing the plasmid DNA was transferred to the diluted liposome mixture, mixed very gently and incubated for 60 min at RT prior to infection.

After the 90 min infection period, the virus inoculum was removed and the cells washed with 1 ml OPTI-MEM. Eight-hundred µl OPTI-MEM was added to the Lipofectamine/DNA solution and gently dripped onto the infected monolayer. The cells were incubated for 7 hr at 37 °C after which 1 ml of 2 % foetal calf-serum growth medium without any antibiotics was added for a further 17 hours. The transfection-medium was then discarded, the cells washed with 0 % foetal-calf serum growth medium and incubated for another 96 hr at 37 °C in the presence of 10 % foetal calf-serum growth medium.

3.2.3 Generation of LSDV recombinants

After an incubation time of 96 hr, the transfected cells were harvested after which the progeny of the *in vivo* homologues recombinant event were released from the cells by

three cycles of freeze-thawing. The cell debris was removed by low speed centrifugation (2000 rpm) for 5 min and the supernatant (1 ml) was used to infect monolayers of 90 % confluent FBT-cells in a six-well plate pre-incubated with MPA-selection medium. The same principles as for generating LSDV recombinants expressing the *LacZ*-gene, were used as described in Chapter 2, section 2.2.17.

Approximately 5 days post infection the infected cells were harvested followed by three cycles of freeze-thawing and low speed centrifugation to remove the cell debris. The supernatant was diluted (10^{-1} 10^{-2} 10^{-3}) and plated onto FBT monolayers, pre-incubated in the presence of MPA. Single foci were picked at approximately 7-9 days p.i. and subjected to another two rounds of focus purification under selective conditions in six well plates. After the third round of focus purification, one third of the material from a selected focus was used to evaluate its status as a LSDV recombinant by PCR. The rest of the infected cells were stored at $-20\text{ }^{\circ}\text{C}$ for further propagation as described in section 2.2.17.

3.2.4 Purification of LSDV DNA for analysis by PCR

The cell debris of each selected focus was removed by low speed centrifugation (2000 rpm) for 5 min at RT. Virus contained in the supernatant was pelleted by centrifugation at 13 000 rpm for 90 min at $4\text{ }^{\circ}\text{C}$. The viral cores were resuspended in $100\text{ }\mu\text{l}$ DMEM. One volume of lysis buffer (1 % SDS; 20 mM β ME; 20 mM EDTA) and $1.4\text{ }\mu\text{l}$ proteinase K (final concentration $100\text{ }\mu\text{g/ml}$) were added and the virus mixture was incubated at $37\text{ }^{\circ}\text{C}$ for 30 minutes.

To remove extraneous proteins, viral DNA was purified 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). The DNA was precipitated by adding sodium acetate (final concentration = 0.3 M), $5\text{ }\mu\text{g}$ tRNA and 2.5 volumes 100 % EtOH and left O/N at $-20\text{ }^{\circ}\text{C}$. The DNA was concentrated by centrifugation at 12 000 rpm for 30 min at $4\text{ }^{\circ}\text{C}$ and washed with ice-cold 70 % EtOH followed by another centrifugation as described above. The DNA pellet was air-dried, resuspended in $30\text{ }\mu\text{l}$ ddH₂O and stored at $-20\text{ }^{\circ}\text{C}$.

3.2.5 Polymerase chain reaction

In a polymerase chain reaction (PCR) a specific region is amplified of which the flanking regions are known. DNA is amplified *in vitro* by a series of polymerisation cycles consisting of three temperature-dependant steps: DNA denaturation, primer-template annealing, and DNA synthesis by a thermostable DNA polymerase isolated from *Thermus aquaticus* (*Taq* DNA polymerase).

Combinations of oligonucleotide primers, either AHSV-9 VP7 specific (Son2a and Son2b), LSDV TK specific (OP48 and OP49), or LSDV specific for the A2L-gene (Harry) and A3L-gene (Sally) (Table 3.1) were used in a PCR reaction to yield specific amplification products (as described in Table 3.2). The 50 μ l PCR reaction mixture consisted of approximately 50 ng genomic DNA, 100 pmol of each primer involved, 1.25 mM of each dNTP, 1.5 mM $MgCl_2$, 5 μ l 10 x *Taq* polymerase buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0 at 25 °C, 15 mM $MgCl_2$ and 1 % Triton^R X-100) and 1 U *Taq* DNA polymerase enzyme. Reactions were incubated in a Perkin Elmer Cetus Gene Amp PCR system 9600. The DNA template was denatured at 95 °C for 5 min. This was followed by 30 cycles of denaturation (95 °C for 30 sec), primer annealing (30 sec at a temperature approximately 5 °C below the lowest T_m of the primer set, Table 3.1) and elongation (72 °C between 1 and 3 min, depending on the size of the expected product, Table 3.2). The PCR products were analysed electrophoretically on a 1 % agarose gel.

Table 3.1 List of primers

| Primer | Sequence | T_m $4(GC)_n + 2(AT)_n$ | Target |
|----------|------------------------------------------|------------------------------|-----------------------------|
| SON2a | 5'd(CACAGATCTTTCGGTTAGGAT GGACGC-G) | 66.8 °C | AHSV-9 VP7 (8-26bp) |
| SON2b | 5'd(CACAGATCTGTAAGTGTATTC GGTATTGA-C) | 56.9 °C | AHSV-9 VP7 (1147-1167bp) |
| OP48 | 5'd(CCATGTATCTGCCATATCAAC) | 60 °C | LSDV TK (688-708bp) |
| OP49 | 5'd(GTGCTATCTAGTCGAGCTAT) | 58 °C | LSDV TK (835-854bp) |
| Harry-WF | 5'd(GCATCAACATCTTCAGAAG) | 52.4 °C | LSDV A2L- specific |
| Sally-WF | 5'd(GATAAACACAGAAATGC) | 49.1 °C | LSDV A3L- specific |

Table 3.2 Sets of primers used and the sizes of expected specific amplification products from a given source of DNA

| Primer set | DNA template | Annealing temp | Elongation time (min) | PCR product (bp) |
|-----------------------|-----------------------------------------------------------|-------------------|--------------------------|---------------------|
| SON2a and SON2b | LSDV/AHSV-7 | 56 °C | 2 | 1159 |
| OP48 and OP49 | wt LSDV TK | 48 °C | 0.5 | 166 |
| Harry-WF and Sally-WF | wt LSDV (A2L and A3L analogs of VV) | 45 °C | 2 | 474 |
| Harry-WF and Sally-WF | Recombinant LSDV/VP7 (A2L and A3L analogs of VV) | 45 °C | 2 | 3042 |

3.2.6 Nick - translation

Relevant DNA was labelled with ^{32}P in a nick-translation reaction using the Promega Reagent Kit. The *E.coli* DNA Polymerase I enzyme adds nucleotides to the 3' OH-terminus that is created when one strand of a dsDNA molecule is nicked by DNase I. The enzyme removes nucleotides from the 5'-side of the nick by its 5'- to 3'-exonuclease activity and fills in gaps with a 5' - 3' polymerase function resulting in movement of the nick (nick translation) along the DNA. By replacing the pre-existing nucleotides with radioactive nucleotides, it is possible to prepare ^{32}P -labeled DNA (Sambrook et al., 1989).

The nick-translation mixture comprised of 1 μg DNA, 0.1 mM dCTP, 0.1 mM dGTP, 0.1 mM dTTP, 10 $\mu\text{Ci}/\text{mMol}$ ^{32}P dATP (10 $\mu\text{Ci}/\mu\text{l}$ with a specific activity of 800 Ci/mMol), nick translation buffer (50 mM Tris-HCl pH 7.2, 10 mM MgSO_4 , 0.01mM DTT) and enzyme mix (1 U DNA polymerase I and 0.1 ng DNase I) in a total volume of 20 μl . The reaction was carried out for 60 min at 15 $^\circ\text{C}$ after which the reaction was terminated by adding stop solution (50 mM EDTA, pH 8) and placing the mixture on ice. The labelled DNA was separated from unincorporated nucleotides using a small Sephadex G75 column.

The Sephadex G75 column was packed in a siliconized Pasteur Pipette and rinsed 3 - 4 times with 1 x TE-buffer containing 0.5 % SDS. The labelled probe (made up to a final volume of 100 μl with 1 x TE-buffer containing 0.5 % SDS) was loaded onto the column and eluted using the same buffer. A first fraction of 500 μl was collected followed by 100 μl samples thereafter. All the fractions were counted for the presence of ^{32}P -cpm using a scintillation counter. The relevant fractions were pooled and stored at -20 $^\circ\text{C}$ if not used (for not more than 14 days).

3.2.7 Hybridisation of ^{32}P -labeled probes with transferred DNA

The electrophoretic separated DNA was transferred to a Hybond N⁺-nylon membrane (Amersham) by a procedure described in Chapter 2, section 2.2.21. Hybridisation was performed after the membrane was pre-hybridised in a solution (50 % formamide, 5 x SSPE, 0.1 % fat free milk, 0.2 % SDS) for at least 6 hr at 42 $^\circ\text{C}$. The pre-hybridisation buffer was replaced with fresh hybridisation buffer (the same composition) after which the labelled probe was added. Before adding the labelled probe, it was denatured by heating at 95 $^\circ\text{C}$ for 5 min and rapidly cooled on ice. Hybridisation proceeded for at least 16 hr at

42 °C after which the membrane was removed and washed at the required stringency choosing the appropriate temperature and buffer. The membrane was rinsed thoroughly in 2 x SSC to remove the most unbound probe followed by low stringency washes at RT with 2 changes of 2 x SSC buffer; 0.1 % SDS after 10 min each. For higher stringency 2 washes were performed in 1 x SSC buffer, 0.1 % SDS at 65 °C for 20 min each, followed by one wash with 0.1 x SSC buffer, 0.1 % SDS at 65 °C for 15 min. All the washes were performed under constant agitation.

The membrane was removed from the washing solution and sealed in a plastic bag before loading in an X-ray cassette. An X-ray film (Cronex MRF31) and a ³²P-intensifying screen were placed over the membrane and the cassette kept at -70 °C for approximately 3-4 days. Upon development of the X-ray film, the results could be observed on the autoradiograph.

3.2.8 Virus purification for viral stocks

Nine 75 cm² cell culture flasks (Nunc, Denmark) were seeded with primary FBT-cells. Once the cells reached 90 % confluency, they were infected with selected virus in the presence of MPA-selective media. When the infected FBT cells exhibited cytopathic effects (CPE), intracellular and extracellular viruses were harvested as follows:

The selective medium containing detached cells and extracellular virions was removed and the remaining cells attached to the floor of the flask, harvested with mild trypsin digestion (2.2.14). The harvested cells were pooled with the selective medium and centrifuged at 1500 rpm for 10 min at 4 °C in a Beckman JS-21 centrifuge. The supernatant was removed and kept on ice. The cell pellet was resuspended in 2 ml McIlvain's buffer and incubated on ice for 10 min after which the cell suspension (containing released virus) was centrifuged at 2000 rpm for 5 min at 4 °C. The 2 ml supernatant fluid was collected and the cell debris resuspended in another 2 ml McIlvain's buffer followed by centrifugation as described above. The 2 ml supernatant was pooled with the first supernatant fluid (2 ml) and the cell debris resuspended in 1 ml DMEM to be stored at -20 °C as viral stock back-up.

The medium supernatant and the 4 ml pooled supernatant (obtained from the cell solution) were separately centrifuged at 11 000 rpm for 90 min at 4 °C through a cushion of 36 % sucrose in 1 x TE-buffer in a Beckman JS-21 centrifuge to concentrate the virus

particles. The viral pellet obtained from the medium supernatant was resuspended in 2 ml DMEM and the pellet from the cell solution supernatant in 1 ml DMEM after which it was stored at -20 °C.

3.2.9 Virus titration

FBT-cells were seeded into 6-well titration plates and incubated in a 5 % CO₂-incubator at 37 °C. When the cells reached 90 % confluency, the culture medium was removed and replaced with MPA-selection medium, 24 hr prior infection. A 10-fold dilution series of the stock of LSDV was prepared in DMEM and the appropriate antibiotics. One well of each plate was left uninfected as a control, and the remaining wells were infected with the various dilutions. A 0.1 ml aliquot of each dilution was added per well. After a 90 min adsorption period, the viral inoculum was removed, the cells washed with DMEM and the DMEM replaced with MPA-selection medium, using the same principles as described in Chapter 2, section 2.2.17. Incubation continued in the 5 % CO₂ - incubator at 37 °C.

3.2.10 Calculation of virus titer

The number of foci was counted in wells where between one and ten foci were present. The average number of foci was determined for at least 2 duplications of the same dilution. The concentration of the original virus stock could then accurately be determined as follows:

The virus concentration was calculated as focus forming units per milliliter (ffu/ml) by making the assumption that one focus in a cell monolayer represents one infectious viral particle, therefore:

$$\text{ffu/ml} = A \times B \times C$$

where A = the reciprocal of the dilution where the number of foci averaged between one and ten per well

B = the average number of foci per well, inoculated with the dilution described in A

C = the reciprocal of the inoculum volume

e.g. 0.1 ml of each virus dilution was used to inoculate the cell sheet of a well, therefore C = 10

3.2.11 Total RNA isolation

Monolayers of 90 % confluent FBT-cells in six-well plates were infected with virus stock at a M.O.I. of 0.1 ffu/cell in the presence of selective media. After a 90 min adsorption period, the virus inoculum was removed and replaced with fresh medium. Total RNA was isolated from wells at different times post infection using Trizol Reagent (Life Technologies, GibcoBrl).

Trizol Reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is a ready-to-use reagent for the isolation of total RNA from cells and tissues. During sample homogenization or lysis, the reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. The infected FBT-cells were directly lysed by adding 1 ml of Trizol reagent to one 3.5 cm diameter well and passing the cell lysate several times through a pipette. The homogenised samples were incubated for 5 min at RT to permit the complete dissociation of nucleoprotein complexes. Two-hundred μ l chloroform was added, incubated for 3 min at RT and centrifuged at 12 000 rpm for 15 min at 4 °C. The addition of chloroform followed by centrifugation separates the solution into an aqueous phase and an organic phase. The RNA remains exclusively in the aqueous phase. Following transfer of the aqueous phase, the RNA is recovered by using 500 μ l isopropyl alcohol per 1 ml of Trizol Reagent used for the initial homogenization. The samples were incubated for 10 min at RT followed by centrifugation for 10 min at 4 °C after which the gel-like pellet was washed with 1 ml 75 % EtOH and stored as an ethanol precipitate at -70 °C. For analysis, RNA was centrifuged at 7500 rpm for 5 min at 4 °C, the pellet briefly air-dried and resuspended in 50 μ l 0.1 % diethylpyrocarbonate (DEPC) H₂O. During the whole procedure precautions were taken to ensure that solutions and apparatus were free of ribonucleases by using DEPC H₂O and wearing gloves.

3.2.12 Dot blot analysis of RNA

A Biorad vacuum blotting apparatus was used to immobilise RNA onto a Hybond N⁺ (nylon) membrane. Prior to blotting, secondary structures in RNA were dissolved by incubating samples at 55 °C for 10 min followed by rapidly cooling on ice. After blotting the membrane was air-dried and exposed to UV-light for fixation, 5 min on each side.

The membrane was pre-hybridised O/N in a solution consisting of 50 % deionized formamide, 2 x Denhardt's solution, 5 x SSPE, 0.1 % SDS, 100 µg/ml denatured herring sperm DNA at 42 °C. The pre-hybridisation buffer was replaced with fresh hybridisation buffer (the same composition without any sperm DNA) after which the ³²P-probe of the gene of interest, was added. Before adding the labelled probe, it was denatured by incubating at 95 °C for 5 min and rapidly cooled on ice. Hybridisation was performed for approximately 16 hr at 42 °C after which the membrane was washed at the following stringency conditions:

- (i) 2 x SSC buffer at RT to remove the most unbound probe
- (ii) 2 x SSC buffer, 0.1 % SDS at RT for 20 min – a low stringency wash
- (iii) 2 x SSC buffer, 0.1 % SDS at 65 °C for 20 min – higher stringency wash
- (iv) 1 x SSC buffer, 0.1 % SDS at 65 °C for 20 min – a high stringency wash

All the washes were done under constant agitation. Before loading in an X-ray cassette, the membrane was sealed in a plastic bag. A Cronex MRF31 X-ray film and a ³²P-intensifying screen were placed over the membrane and the cassette kept at -70 °C for approximately 4-5 days before being autoradiographed.

3.2.13 Radio-labelling of proteins

Primary FBT-cells in six-well plates were infected with virus stock at a M.O.I. of 0.1 ffu/cell in the presence of selective media as described previously (section 3.2.11). For labelling purposes, the infected FBT-cells were washed with 37 °C methionine-free Eagle's medium and the cells starved for 1 hr at 37 °C. The proteins were then labelled by replacing the medium with fresh methionine-free medium (1 ml/3.5 cm diameter well) and the addition of (³⁵S)-methionine (60 µCi/1 x 10⁶ infected cells). Labelling commenced at different times post infection (20, 44, 68 or 92 hr p.i.) and each sample was labelled for 10 hr at 37 °C. After incubation, the dislodged cells were recovered from the medium by centrifugation at 3000 rpm for 5 min at RT. The infected cells from each well were harvested with 1 x PBS by passing the cell lysate several times through a pipette. Harvested cell material was pooled, centrifuged under the same conditions and washed twice in 1 x PBS. The pellet obtained after the last wash-step was resuspended in 30 µl 1 x PBS and stored at -20 °C prior electrophoresis.

3.2.14 Protein electrophoresis and autoradiography

Protein electrophoresis was carried out in polyacrylamide gels under denaturing conditions, allowing separation of proteins based only on their molecular sizes. The ratio of acrylamide:bisacrylamide used for both the 15 % polyacrylamide separating - and 5 % stacking gels was 30:0,8. The 15 % separating gels were polymerised at RT in a buffer containing 0.375 M Tris pH 8,8, 0.1 % SDS, 0.06 % ammonium persulphate and 10 μ l N,N,N',N'-Tetra-methylethylenediamin (TEMED) per 15 ml of gel. The 5 % stacking gels were polymerised at RT in a buffer containing 0.125 M Tris pH 6,8, 0.1 % SDS, 0.16 % ammonium persulphate and 10 μ l TEMED per 6 ml of gel. The running buffer (1 x TGS) consisted of 24.7 mM Tris pH 8,9, 191.8 mM Glycine and 0.1 % SDS. Before electrophoresis, protein samples were mixed with an equal volume of 2 x protein solvent buffer (0.125 M Tris pH 8,9, 1.4 M β ME, 4 % SDS, 20 % glycerol and 0.1 % Bromophenol blue as dye marker) and denatured by incubation at 95 °C for 5 min. Electrophoresis was performed using the mini-gel Hoefer electrophoresis system, applying a current of 125 V for approximately 6.5 hr. After electrophoresis, the gels were stained in Coomassie brilliant blue staining solution (0.125 % Coomassie blue, 50 % methanol, 10 % acetic acid) for 20 min at RT and destained in 5 % EtOH, 5 % acetic acid at RT overnight. The gels were dried in a vacuum-drier at 80 °C for 60 min before it was placed in a cassette and stored at RT for 3 days before being autoradiographed.

3.2.15 Western blot analysis

Monolayers of 90 % confluent FBT-cells in 75 cm² cell culture flasks were infected with virus stock at a M.O.I. of 1 ffu/cell in the presence of selective media. At different times p.i. the medium was removed and the cells harvested using ATV as described in section 2.2.14. The harvested cells were pooled with the medium and centrifuged at 3000 rpm for 5 min. The cell pellet was washed twice in 1 x PBS and resuspended in 100 μ l 1 x PBS to be stored at -20 °C

To separate the proteins by their molecular sizes, 12 % polyacrylamide gels were used instead of the 15 % polyacrylamide gels as described in section 3.2.14. The 12 % polyacrylamide gels allow a better transfer of proteins to the PVDF membrane (Millipore Corporation, Bedford) when compared to the 15 % gels. Before electrophoresis, protein samples were mixed with an equal amount of 2 x protein solvent buffer (PSB) and denatured by incubation at 95 °C for 5 min followed by sonification for 10 minutes.

Electrophoresis was carried out at 110 V for 2.5 hours. The first three lanes of the gel (duplicate samples) were stained with Coomassie brilliant blue for 20 min at RT and destained with 5 % EtOH, 5 % acetic acid to serve as control. The rest of the gel was put in transfer buffer (50 mM Tris, 40 mM Glycine, 20 % methanol) 30 min prior the transfer process. The PVDF-membrane was first soaked in -20 °C methanol after which it was assembled with 2 filter papers in transfer buffer for 15 min. The transfer of separated proteins to the nitrocellulose membrane was done in the presence of transfer buffer using the EC140 Mini Blot Module apparatus. The transfer process was performed at 12 V, 0.1 A for 15 min; 16 V, 0.1 A for 45 min and 24 V, 0.1 A for another 45 minutes.

After transfer, the membrane was incubated in 5 % milk powder in Tris-buffered saline Tween (20 mM Tris base, 137 mM sodium chloride, 3.8 ml of 1M HCl and 0.1 % Tween 20) for one hour at RT to block the non-specific binding sites on the blot. The membrane was briefly rinsed using two changes of Tris-buffered saline Tween washing buffer (TBST) followed by one wash for 15 min and twice for 5 min with fresh changes of the washing buffer at RT under constant agitation. The membrane was sealed in a plastic bag and incubated in primary antibody (guineapig antibody against AHSV-9 diluted in TBST 1: 100) overnight at RT with agitation. Before adding the secondary antibody (peroxidase-conjugated protein A diluted in TBST 1: 100), labelled with horseradish peroxidase, the membrane was washed using the same conditions prior the incubation with the primary antibody. The membrane was incubated for 1 hr at RT in the diluted secondary antibody, washed for 15 min and thereafter 4 times for 5 min each, with fresh changes of washing buffer to minimise the background when autoradiographed.

For detection of the proteins, ECL™ Western blotting detection reagents (Amersham Pharmacia Biotech UK) were used. ECL™ Western blotting is a light emitting non-radioactive method for the detection of immobilised specific antigens, conjugated directly or indirectly with horseradish peroxidase-labelled antibodies. An equal volume of detection solution 1 was mixed with detection solution 2 (ECL™ Western blotting from Amersham Pharmacia Biotech) to be sufficient to cover the membrane. The detection reagent was added to the protein side of the membrane so that the reagents could be held by surface tension on the surface of the membrane for exactly 1 minute. The membrane was then placed between two see-through transparencies and put, protein side-up, in the film cassette. An X-ray film (Cronex MR31) was placed on top of the membrane and exposed for 5 to 7 minutes before developing and kept as a permanent copy.

3.2.16 Separation of cytoplasmic - and nucleus fractions in cells

At least two 75 cm² cell culture flasks of 90 % confluent FBT cells were infected with virus stock at a M.O.I. of 1 ffu/cell in the presence of MPA-selection medium. When CPE was visible, the infected cells were harvested and pooled with dislodged cells contained in the selective medium. The harvested solution was then centrifuged in a Beckman JS-21 at 3000 rpm for 5 min, the cell pellet resuspended in 1 x PBS and kept on ice. Viruses in the supernatant were recovered by centrifugation at 11 000 rpm for 90 min at 4 °C, resuspended in 200 µl lysis buffer (150 mM NaCl, 50 mM Tris HCl pH 8.0, 0.1 or 0.5 % Nonident P40) and kept on ice. The original cellular material was centrifuged at 3000 rpm for 5 min, resuspended in 500 µl lysis buffer, pooled with the 200 µl resuspended solution and incubated on ice for 30 minutes. To ensure that all cells were lysed, a mechanical dounce step was included.

The cytoplasmic and nucleus fractions were separated by centrifugation at 1500 rpm for 5 min and the supernatant (cytoplasmic fraction) removed to a fresh tube. The pellet was washed 3 times with 500 µl lysis buffer, centrifuged again under the same conditions and the supernatants pooled with the cytoplasmic fraction. To concentrate the VP7 crystals in the cytoplasmic fraction, the pooled supernatants were centrifuged through a 40 % sucrose cushion in ST-buffer (150 mM NaCl, 50 mM Tris HCl pH 8.0) at 30 000 rpm for 90 min at 4°C in a Beckman L-70 ultracentrifuge. The cytoplasmic pellet was gently resuspended in 100 µl ST-buffer and stored at 4 °C. All fractions were analysed on a 12 % SDS-PAGE gel.

3.2.17 Electron microscopy

Half of the cytoplasmic sample was fixated with an equal volume of 2.5 % Glutaraldehyde in 0.075 M KH₂PO₄/Na₂HPO₄-buffer pH 7.4. The fixated sample was filtered onto a 0.2 µm nylon filter, washed 3 times with 0.075 M KH₂PO₄/Na₂HPO₄-buffer and then dehydrated by successive treatment with 50 %, 70 %, 90 % and 100 % EtOH. The treatment with 100 % ethanol was repeated three times after which the filter was air-dried, mounted onto a stub and sputter coated with gold-beladium, a few atoms thick followed by a layer carbon. The stub was viewed at 5.0 kV in a Jeol scanning electron microscope.

3.2.18 Generation of LSDV recombinants without residing wt LSDV

A dilution series of the recombinant virus stock (100 ffu, 50 ffu, 25 ffu, 10 ffu, 5 ffu, 1 ffu) was used to inoculate 90 % confluent FBT monolayers in 24-well plates (Nunc) in the presence of MPA-selection medium. The supernatants of those wells containing a single focus were harvested to be used in the next dilution series instead of picking single foci (section 3.2.3). On recommendation of the group at OVI, only viruses contained in the supernatant were used for the next round of selection. The hypothesis is that single viruses are released into the supernatant, whereas cell-associated viruses tend to clump together during the freeze-thaw procedures after picking foci. In theory therefore, residual wild viruses could clump together with recombinants. Upon infection of a single cell, the presence of a recombinant virus may allow clumped wt virus to replicate. A single focus may hence represent the progeny of both recombinant and wt viruses. By using only viruses that are present in the supernatant, one should ensure that cells are infected by a single recombinant virus. The selected foci were subjected to another 2 rounds of purification after which a few foci were tested to reconfirm the presence of the foreign gene in the LSDV genome and to establish whether any residual wt LSDV was still associated with the recombinant LSDV.

3.3 RESULTS OF THE SINGLE RECOMBINANT

3.3.1 Construction of a plasmid containing the AHSV-9 VP7-gene

The AHSV-9 VP7-gene was excised as a *Hind* II - *Sma* I 1.1 Kb fragment from the pBS/VP7 plasmid (fig. 3.1). Both enzymes generate blunt end products. A number of additional nucleotides (non-VP7) are included in the excised fragment. The DNA sequence of the region 5' to the VP7-gene in pBS/VP7 was analysed to confirm that the first ATG to be encountered after cloning into the transfer vector, would be the initiation codon of the VP7-gene. The lower band (fig. 3.2 lane c and d) was excised from the 1 % agarose gel and purified using the Glassmilk methodology (2.2.4). Electrophoretic analysis of a small amount of the recovered DNA fragment is shown in (fig. 3.3 lane b).

To clone the VP7-gene under control of the early promoter pA8R, the transfer vector pHSWF was linearised with *Eco* RI (sticky-end enzyme) followed by electrophoresis (fig. 3.4) and Glassmilk purification (fig. 3.5). The *Eco* RI - linearised vector was blunt-ended with a Klenow reaction and dephosphorylated followed by a phenol:chlorophorm:iso-amylalcohol (25:24:1) extraction. A schematic representation of the cloning strategy is shown in fig. 3.6.

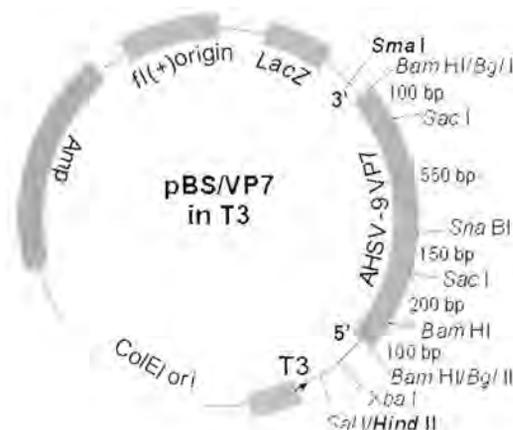


Fig. 3.1: Plasmid pBS/VP7 in T3

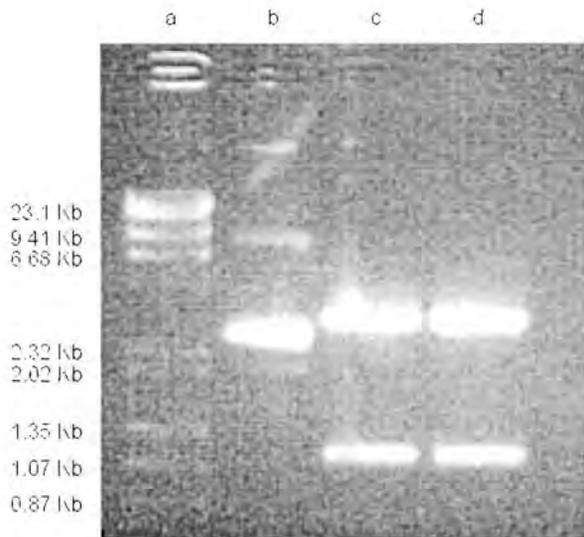


Fig. 3.2: Agarose gel electrophoretic analysis of pBS/VP7
 (a) Size markers Sma I and ϕ X
 (b) Undigested pBS/VP7
 (c)-(d) pBS/VP7 digested with *Sma* I and *Hind* II

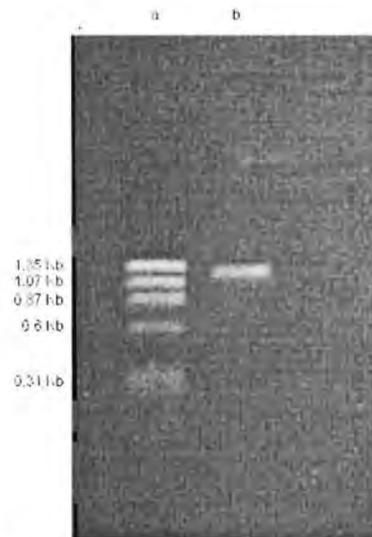


Fig. 3.3: Agarose gel electrophoretic analysis of gene-cleaned recovered VP7-DNA
 (a) Size marker, ϕ X
 (b) Approximately 350 ng DNA

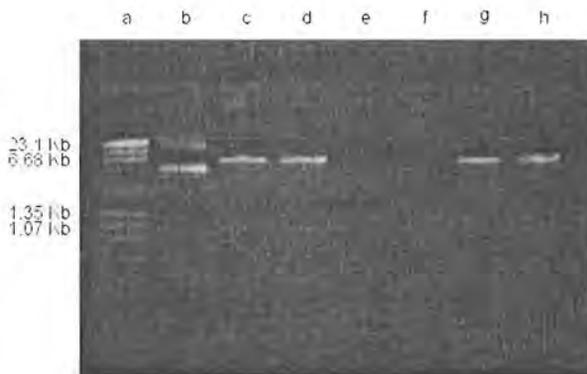


Fig. 3.4: Agarose gel electrophoretic analysis of linearised vector, pHSWF
 (a) Size markers, Sma I and ϕ X
 (b) Undigested pHSWF as control
 (c), (d), (g), (h) pHSWF digested with *Eco* RI
 (e), (f) Open lanes



Fig. 3.5: Agarose gel electrophoretic analysis of gene-cleaned recovered vector, pHSWF
 (a) Size marker, Sma I
 (b) Approximately 250 ng DNA

Following transformation of Top10F competent *E. coli* cells with the insert/vector ligation mixtures, putative recombinants were picked to make overnight cultures followed by mini-prep plasmid DNA-extraction and electrophoresis. A representative analysis of some of the isolated plasmids is shown in (fig. 3.7 lanes c-l). From the larger plasmid sizes in relation to the intact vector, samples were investigated by r.e. digestions to determine the correct orientation of the full-length VP7-gene.

The restriction enzyme *Bam* HI digests asymmetrically at the one end of the pA7LA8R-promoter and inside the VP7-gene, 100bp from its 5' end (fig. 3.6). From a digestion with *Bam* HI, 2 DNA fragments of sizes 6.9 Kb and 168 bp were expected if the full-length VP7-gene was cloned in the correct orientation (fig. 3.8 lanes d and g). Two fragments with sizes 6.0 Kb and 1.0 Kb were expected when cloned in the incorrect orientation (fig. 3.8 lane f). Although in the correct orientation, it seemed that (fig. 3.8 lane e) was smaller, probably due to a deletion, when compared to (fig. 3.8 lanes d and g) and was not analysed further. Following electrophoresis one clone (fig. 3.8 lane d) was selected to grow an overnight culture using the "Nucleobond Ax kit" for DNA purification.

To confirm the correct orientation and ascertain that the full-length VP7-gene was cloned under control of the pA8R promoter, the large-scale preparation was analysed further. *Sac* I digests asymmetrically inside the VP7-gene; 300 bp and 1000 bp downstream from its 5' end. Another *Sac* I-site is present at the one end of the LSDV promoter (fig. 3.6). Therefore 3 bands with sizes 368 bp, 700 bp and 6.1 Kb respectively were expected if cloned in the correct orientation (fig. 3.9 lane e). In the incorrect orientation fragments with sizes 168 bp, 700 bp and 6.3 Kb could be expected (fig. 3.6). A double digestion with *Bam* HI and *Sal* I was also included where *Sal* I digests the pHSWF-transfer vector outside the VP7-gene (fig. 3.6). Three bands with sizes 168 bp, 1.0 Kb and 6.0 Kb were expected with the full-length gene cloned in the correct orientation (fig. 3.9 lane g). If cloned in the incorrect orientation fragments with sizes 100 bp, 1.0 Kb and 6.0 Kb could be expected (fig. 3.6). Electrophoretic analysis obtained from the r.e. digestions (fig. 3.9), confirmed that the plasmid contained the full-length VP7-gene in the correct transcriptional orientation. The plasmid was named pHSWF/VP7 and used for the generation of recombinant LSDV/VP7.

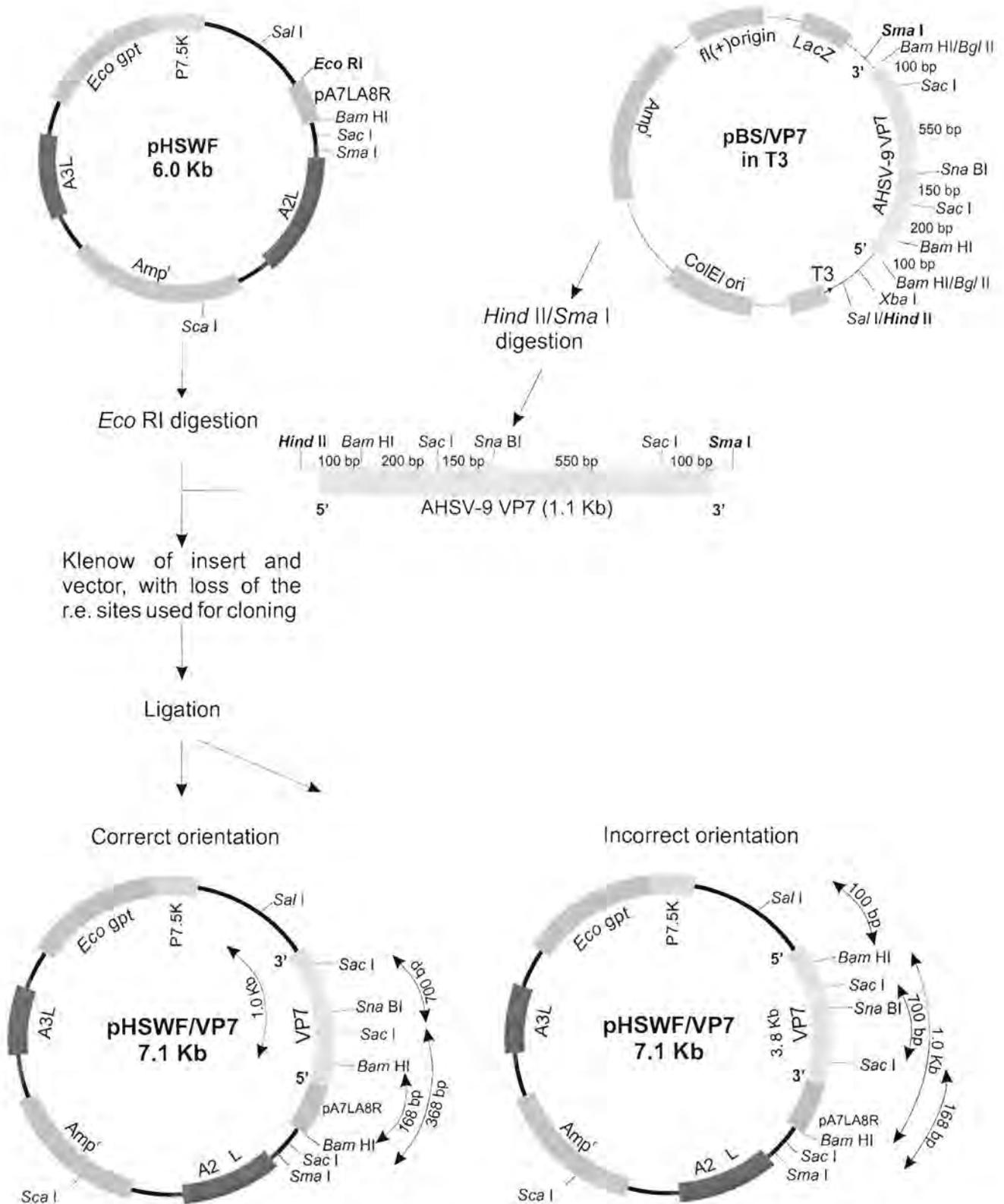


Fig. 3.6: Schematic representation of the cloning of AHSV-9 VP7 into the transfer vector pHSWF, under control of the early LSDV promoter (pA8R). A partial r.e. map of VP7 is shown, as well as the correct and incorrect transcriptional orientations of the gene after cloning.

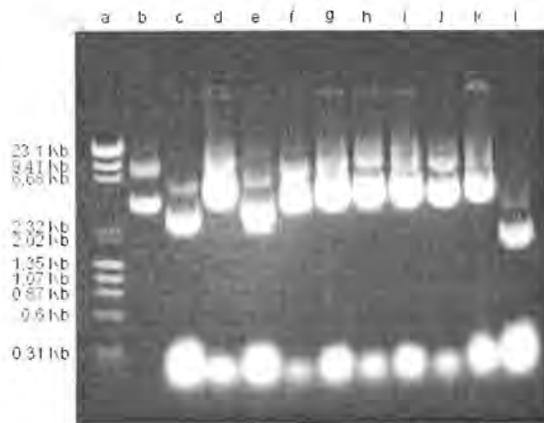


Fig. 3.7: Agarose gel electrophoretic analysis of putative plasmids containing VP7
(a) Size marker, SMI I
(b) Intact vector, pHSWF, as control
(c)-(l) Putative pHSWF containing VP7 cloned downstream of the early promoter

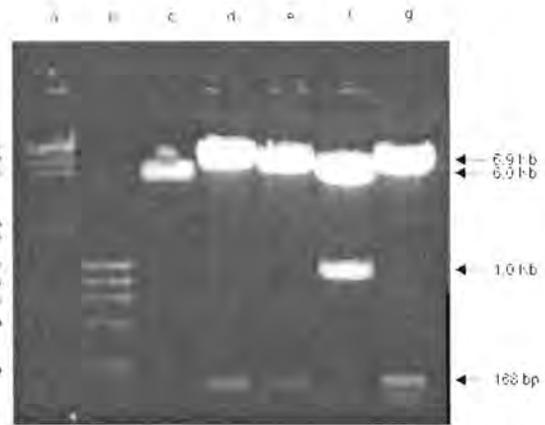


Fig. 3.8: Agarose gel electrophoretic analysis of plasmids digested with *Bam* HI to confirm orientation
(a) Size marker, SMI I
(b) Size marker, ϕ X
(c) pHSWF digested with *Bam* HI
(d)-(g) Putative pHSWF containing VP7 digested with *Bam* HI

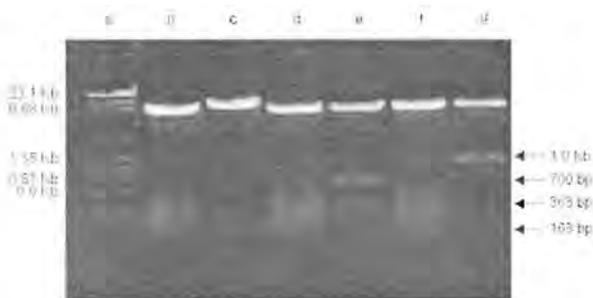


Fig. 3.9: Agarose gel electrophoretic analysis of a selected plasmid to confirm cloning of the full-length VP7-gene in the correct orientation
(a) Size markers, SMI I and ϕ X
(b) pHSWF digested with *Bam* HI
(c) pHSWF containing VP7 digested with *Bam* HI
(d) pHSWF digested with *Sac* I
(e) pHSWF containing VP7 digested with *Sac* I
(f) pHSWF digested with *Bam* HI and *Sal* I
(g) pHSWF containing VP7 digested with *Bam* HI and *Sal* I

3.3.2 Generation of LSDV/VP7 recombinants

FBT-cells, transfected with plasmid pHSWF/VP7 (section 3.2.2), were used to infect FBT-cells in the presence of selective medium. After approximately 5 days a good CPE was visible and the infected cells were harvested followed by three cycles of freeze-thawing and centrifugation. The supernatant was diluted and plated onto FBT monolayers. Single foci could be picked at approximately the 9th day p.i. and was subjected to another 2 rounds of focus purification (section 3.2.3).

After the third round of single focus selection, foci were randomly picked for characterisation by PCR to establish their status as LSDV/VP7 recombinants. Most foci yielded the expected PCR amplicon of 1.1 Kb when the VP7-specific primer set (SON2a and SON2b) was used (fig. 3.10 lanes e-h). No VP7-specific PCR products were detected from wt LSDV DNA or uninfected FBT-cells that were included as negative controls (fig. 3.10 lane c and d). The pHSWF/VP7 plasmid was included as positive control and yielded an amplified product of 1.1 Kb (fig. 3.10 lane b).

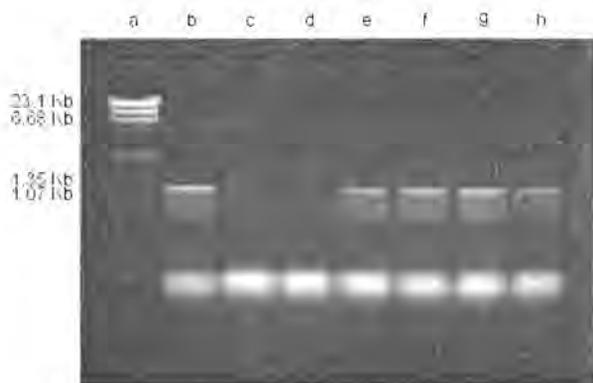


Fig. 3.10: Agarose gel electrophoretic analysis of putative LSDV/VP7 recombinants. PCR amplification using the VP7-specific primer-set, SON2a and SON2b was performed

- (a) Size markers, SMII and ϕ X
- (b) pHSWF/VP7 plasmid as positive control
- (c) wt LSDV DNA
- (d) uninfected FBT-cells
- (e)-(h) Putative LSDV/VP7 recombinants

3.3.3 Confirmation of LSDV recombinants

Since the PCR-results of VP7 could be ascribed to plasmid contamination, the presence of VP7 in the LSDV genome was confirmed by Southern blot analysis (section 2.2.21). Viruses from 4 selected foci, identified by the PCR-reactions, were used to infect FBT-cells in the presence of selective media. Virus from each of the foci was used to infect 3 x 75 cm² culture flasks. After 7 days, DNA was purified from the infected cells and digested with *Bam* HI followed by 0.6 % agarose gel electrophoresis. The electrophoretic separated DNA was transferred to a Hybond N⁺-nylon membrane (Amersham) and selectively probed for the AHSV-9 VP7 gene by using α -³²P labelling. Three ³²P-labeled probes were used for hybridisation, i.e. the purified VP7-gene (insert), pHSWF (plasmid) and SMI-size marker. The latter two were included as controls. The pHSWF plasmid contains the A2L and A3L regions that will hybridise with the wt LSDV genome, giving an indication of the *Bam* HI-fragment containing the A2L and A3L region in the wt genome, and hence also give an indication of the presence of remaining wt viruses in the recombinant cultures.

The following fragments were expected after hybridisation between the electrophoretic separated DNA and probes of the purified VP7-gene (insert), pHSWF (plasmid) and SMI-size marker used in the same hybridisation reaction:

- In case of a complete *Bam* HI - digestion and if a double cross-over had occurred between the wt LSDV genome and the pHSWF/VP7 plasmid, 3 fragments with sizes 168 bp, 6.9 Kb and 7.1 Kb if no parental LSDV resides in the recombinant. The inserted fragment has 2 internal *Bam* HI-sites. The pA7LA8R-promoter has a unique *Bam* HI-site and there is a *Bam* HI-site in the VP7-gene, 100 bp from its 5' end. If parental LSDV resides in the recombinant a fragment of 11.5 Kb will be observed, i.e. the size of the *Bam* HI-fragment that contains the A2L/A3L region in the wt genome (see section 2.3.3). For the expected fragment sizes see Appendix 3 p 115.

From the autoradiograph, (fig. 3.11 lanes c-f), all four selected foci showed fragments of approximately 7 Kb; confirming integration of the VP7-gene into the LSDV genome. This confirmed that the positive PCR-results were not a result of remaining pHSWF/VP7 plasmid. To ensure good separation of the digested DNA the 0.6 % agarose gel was run overnight, hence the 168 bp-fragment could not be detected on the autoradiograph. It was impossible to distinguish between the 6.9 Kb and 7.1 Kb fragments; only one

fragment of approximately 7 Kb was visible. It was expected that both fragments were present, although not visible. Undigested recombinant LSDV DNA, digested wt LSDV DNA and digested uninfected FBT-cells were included as controls (fig. 3.11 lanes b, g and h). The extra fragment of 11.5 Kb was an indication that all 4 selected foci contained residing wt LSDV (fig. 3.11 lanes c-f). The recombinant LSDV was designated as LSDV/VP7.

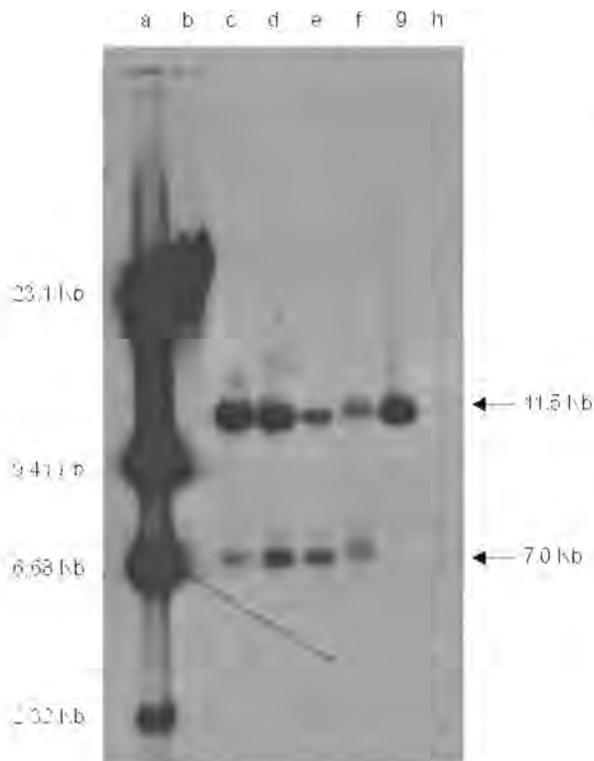


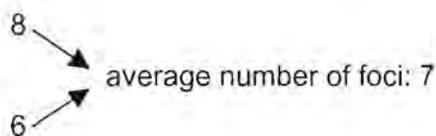
Fig. 3.11: An autoradiograph of a Southern blot hybridisation between *Bam* HI digested LSDV/VP7 DNA and α -³²P probes of the VP7-insert and pHSWF. (a) Size marker, SMI (b) Undigested LSDV/VP7 DNA (c)-(f) LSDV/VP7 DNA digested with *Bam* HI (g) wt LSDV DNA digested with *Bam* HI (h) Uninfected FBT-cells digested with *Bam* HI

From the Southern blot analysis viruses from one recombinant LSDV focus (fig. 3.11 lane f) was chosen for a large-scale infection of 9 x 75cm² culture flasks to prepare a virus stock (section 3.2.8).

3.3.4 The titer of LSDV/VP7

FBT-cells were seeded into 6-well plates. When the cells reached 90% confluency, the cells were infected with a 10-fold dilution series (section 3.2.9). At the 9th day p.i. single foci could be easily distinguished and counted. In the wells with the 10⁻⁷ dilution, the number of foci was less than ten and could easily be determined. The number of foci correlated well with between 60 and 80 foci on the 10⁻⁶ dilutions. The titration of the recombinant LSDV stock was calculated as follows:

Number of foci present in the wells with a 10⁻⁷ dilution of the recombinant LSDV:



Therefore: Infectious recombinant LSDV/VP7 = A x B x C (section 3.2.10)

$$= 7 \times 10^7 \times 10$$

$$= 7 \times 10^8 \text{ ffu/ml}$$

3.3.5 Analysis of VP7-RNA transcription.

Monolayers of 90% confluent FBT-cells in 6-well plates were infected with LSDV/VP7 at a M.O.I. of 0.1 ffu/cell. Total RNA was isolated from wells at times 24 hr, 48 hr, 72 hr and 96 hr p.i. using Trizol Reagent. Isolated RNA from uninfected cells and cells infected with wt LSDV at a M.O.I. of 0.1 ffu/cell were included as negative controls. Plasmid pHSWF/VP7 DNA was used as positive control. To identify mRNA transcription of VP7 in LSDV/VP7, all the isolated RNA from one well was used for dot-spot hybridisation studies and probed with the purified VP7-insert labelled with α -³²P radio-isotope.

From the autoradiograph (fig. 3.12), the highest degree of mRNA transcription occurred at 48 and 72 hr p.i. (fig. 3.12 lanes e and f) after which it decreased at 96 hr (fig. 3.12 lane g). The relatively late times at which mRNA was first detected was probably due to the fact that such a low virus-concentration (0.1 ffu/cell) was used for infection and that it did not represent a synchronous infection. At 96 hr p.i. some of the infected cells were already lysed and floated in the selective medium, possibly explaining the lower transcriptional level of VP7.

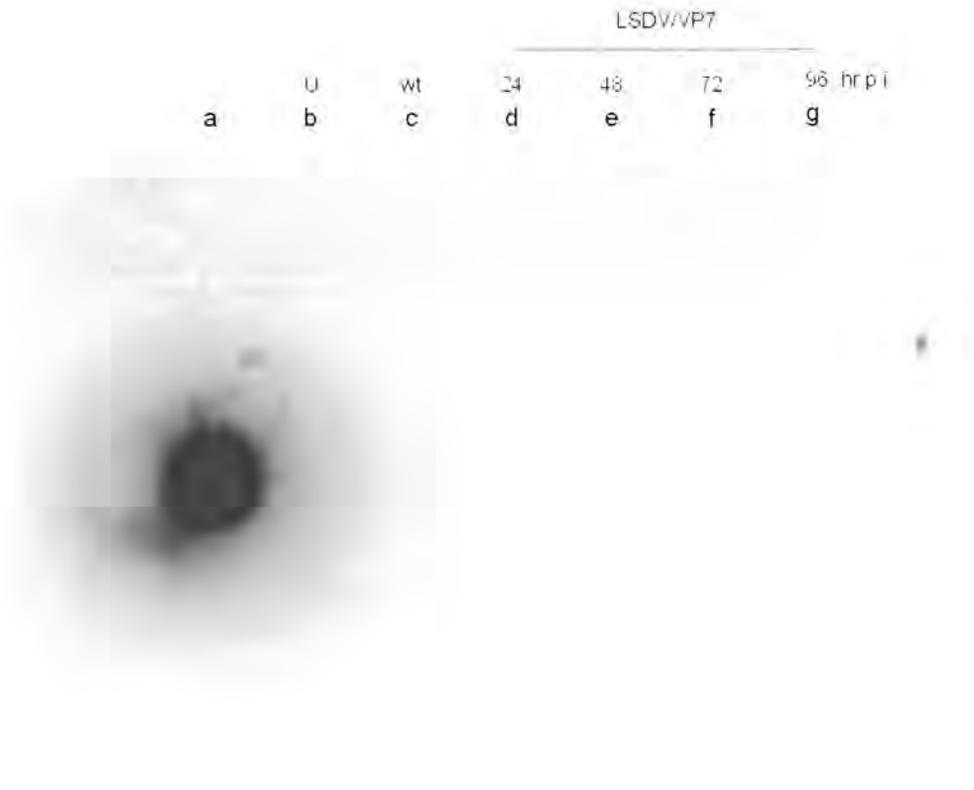


Fig. 3.12: Autoradiograph of total RNA isolated at different times from cells infected with LSDV/VP7 and hybridised to the purified VP7-insert

- (a) pHSWF/VP7 as positive control
- (b) RNA isolated from uninfected FBT-cells 48 hr p.i.
- (c) RNA isolated from wt LSDV 48hr p.i.
- (d)-(g) RNA isolated from LSDV/VP7 24 hr, 48 hr, 72 hr and 96 hr p.i.

3.3.6 Expression of VP7 in the LSDV/VP7 recombinant

(a) Radio-labelling of VP7 proteins and analysis by SDS PAGE

VP7-gene expression was analysed by ^{35}S radio-labelling of expressed proteins at different times post infection. Monolayers of FBT-cells in 6-well plates were infected with LSDV/VP7 stock at a M.O.I. of 0.1 ffu/cell when the cells were 90 % confluent. Four different times post infection, namely 20 hr, 44 hr, 68 hr and 92 hr were analysed for the expression of VP7. Labelling commenced at the indicated times and proceeded for 10 hr before the cells were harvested for analysis on a 15% polyacrylamide gel (fig. 3.13 A) and autoradiographed (fig. 3.13 B). The gel was run at 125 V for 6.5 hours.

Uninfected FBT-cells and FBT-cells infected with wt LSDV were labelled at 44 hr p.i. as negative controls. As positive control *Spodoptera frugiperda* (Sf9) cells were infected with

a recombinant baculovirus containing the AHSV-9 VP7 gene. After an infection time of 33 hr the infected cells were labelled using ^{35}S -methionine for 3 hr and harvested using the same method as described in section 3.2.13.

No unique band was visible from the SDS-PAGE gel after staining with Coomassie brilliant blue (fig. 3.13 A). The results obtained from the autoradiograph revealed that the LSDV/VP7 recombinant virus synthesised an unique protein with molecular size of 36 K that was absent from the uninfected and wt LSDV infected cells (fig. 3.13 B lanes f-h). Although very light, the protein correlates with the size of the VP7-protein in the positive control (fig. 3.13 B lane b). The expression of VP7 increased from 20 hr p.i. to 68 hr p.i. after which it decrease at 92 hr p.i. (fig. 3.13 B). Due to the low M.O.I. of 0.1 ffu/cell, many host-specific proteins were also labelled.

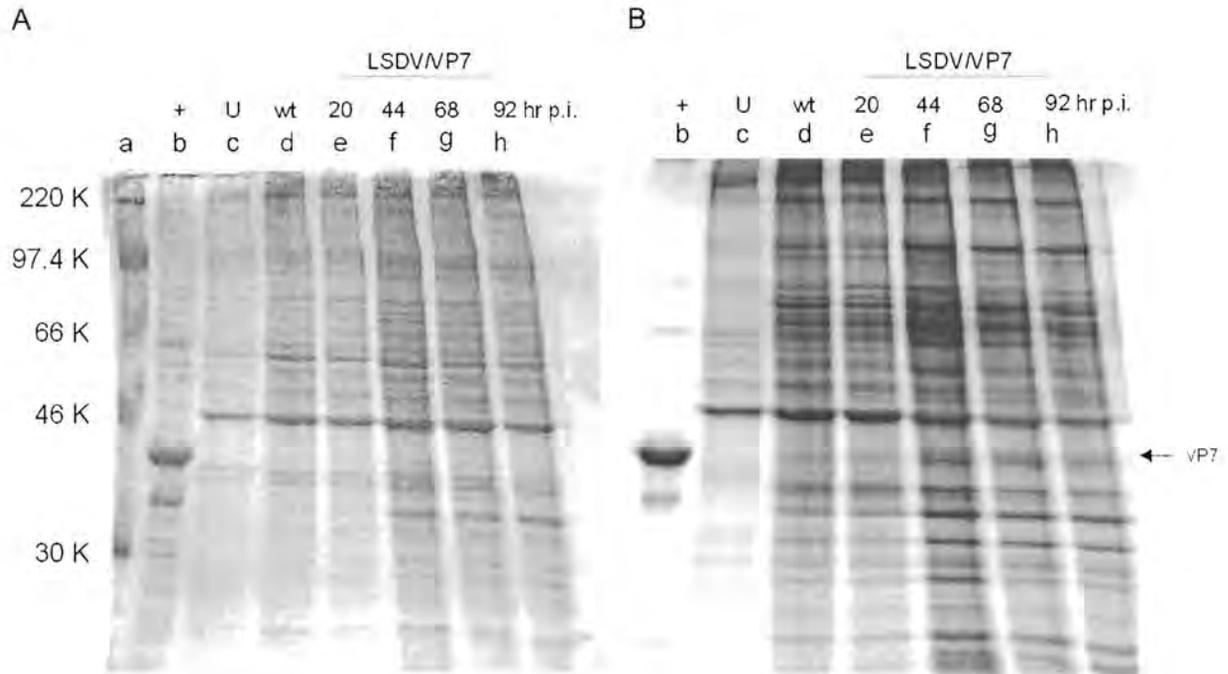


Fig. 3.13 A and B: (A) 15% Polyacrylamide gel of LSDV infected FBT-cells. At the different times indicated, the uninfected and infected cells were labelled for 10 hr using ^{35}S -methionine. The molecular weights (kilodaltons) are shown next to the gels as an indication of the protein-sizes.

(a) Rainbow-marker

(b) Sf9-cells infected with the recombinant baculovirus containing AHSV-9 VP7

(c) Uninfected FBT-cells labelled after 44 hr p.i.

(d) FBT-cells infected with wt LSDV, labelled after 44 hr p.i.

(e)-(h) FBT-cells infected with LSDV/VP7, labelled at different times post infection as indicated

(B) Autoradiograph of the 15 % polyacrylamide gel in (A)

(b) Western blot analysis

Both standard and ECL™ Western blotting detection reagents were used. Despite numerous attempts, we were unable to detect the VP7-protein expressed from LSDV/VP7 (fig. 3.14 lanes e and f). The technique was successful, indicated by the positive results obtained from a recombinant baculovirus expressing VP7 (fig. 3.14 lane d). The results may be due to a much higher level of VP7 expressed from the recombinant baculovirus when compared to the LSDV/VP7 system. Due to the high sensitivity of the ECL™ Western blotting detection reagents, two non-specific bands in case of the negative controls (uninfected FBT-cells and FBT-cells infected with wt LSDV) were also visible (fig. 3.14 lanes b and c).

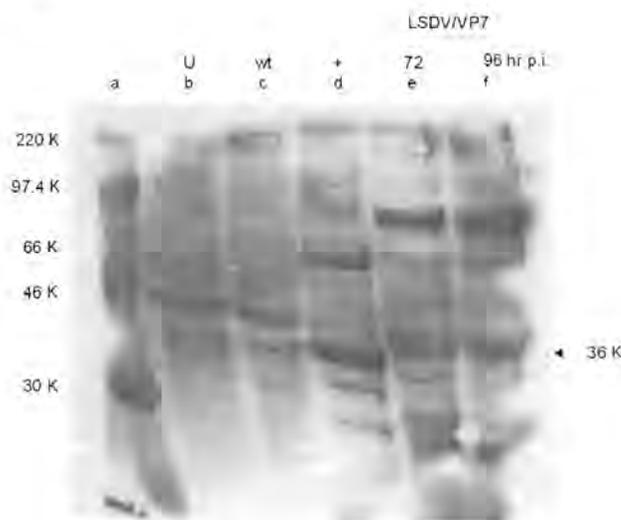


Fig. 3.14: Expression of VP7 analysed by Western blotting. At the different times shown, the infected cells were harvested. The molecular weights (kilodaltons) are an indication of the protein-sizes.

- (a) Rainbow-marker as protein-size marker
- (b) Uninfected FBT-cells harvested 72 hr p.i.
- (c) FBT-cells infected with wt LSDV harvested 72 hr p.i.
- (d) Sf9-cells infected with recombinant Baculovirus containing AHSV-9 VP7
- (e)-(f) FBT-cells infected with LSDV/VP7 harvested at different times p.i. as indicated

(c) Electron Microscopy

If VP7 of AHSV is expressed as a baculovirus recombinant, the major core protein results in the spontaneous assembly of unique crystals (Burroughs *et al.*, 1994). The distinctive VP7 hexagonal crystals are a characteristic of AHSV and are composed entirely of an

ordered array of VP7 trimers (Basak *et al.*, 1996). The ability of VP7, expressed by recombinant LSDV/VP7 in FBT-cells to form crystals, was analysed by means of scanning electron microscopy. Protein extracts were prepared from FBT-cells infected with the recombinant virus by differential fractionation. The cytoplasmic-and nucleus fractions were separated and the cytoplasmic fraction used as sample for electron microscopy. The VP7-crystals appear in the cytoplasmic-fraction (F. Maree, a student from the Department of Genetics, UP). The different fractions were analysed on a 12 % SDS-PAGE gel (section 3.2.14).

The AHSV-9 VP7 crystals, expressed by a recombinant Baculovirus in Sf9-cells, were used as positive control to analyse crystal formation in the LSDV/VP7 recombinant (fig. 3.15 a). Crystals were observed when VP7 were expressed with LSDV/VP7. The crystals exhibited a slightly different structure when compared to the perfect hexagonal crystal obtained with baculovirus-expressed VP7 (fig. 3.15 a, kindly supplied by F. Maree, a student from the Department of Genetics, UP). Crystals formed by the baculovirus-expressed VP7-protein fall in the size-range between approximately 6 and 8 μm in diameter that correlate with the size obtained with the crystals formed by the VP7-expressed protein from LSDV/VP7. Although different in shape, large quantities of these crystals were observed, especially where 0.1 % Nonident P40 was used in the lysis buffer (fig. 3.15 c, d and e). One of the few crystals where 0.5 % Nonident P40 was used in the lysis buffer, is shown in (fig. 3.15 b). From these surprising results it was clear that the AHSV-9 VP7 protein was expressed at high levels from the early pA8R-promoter in the recombinant LSDV/VP7 system.

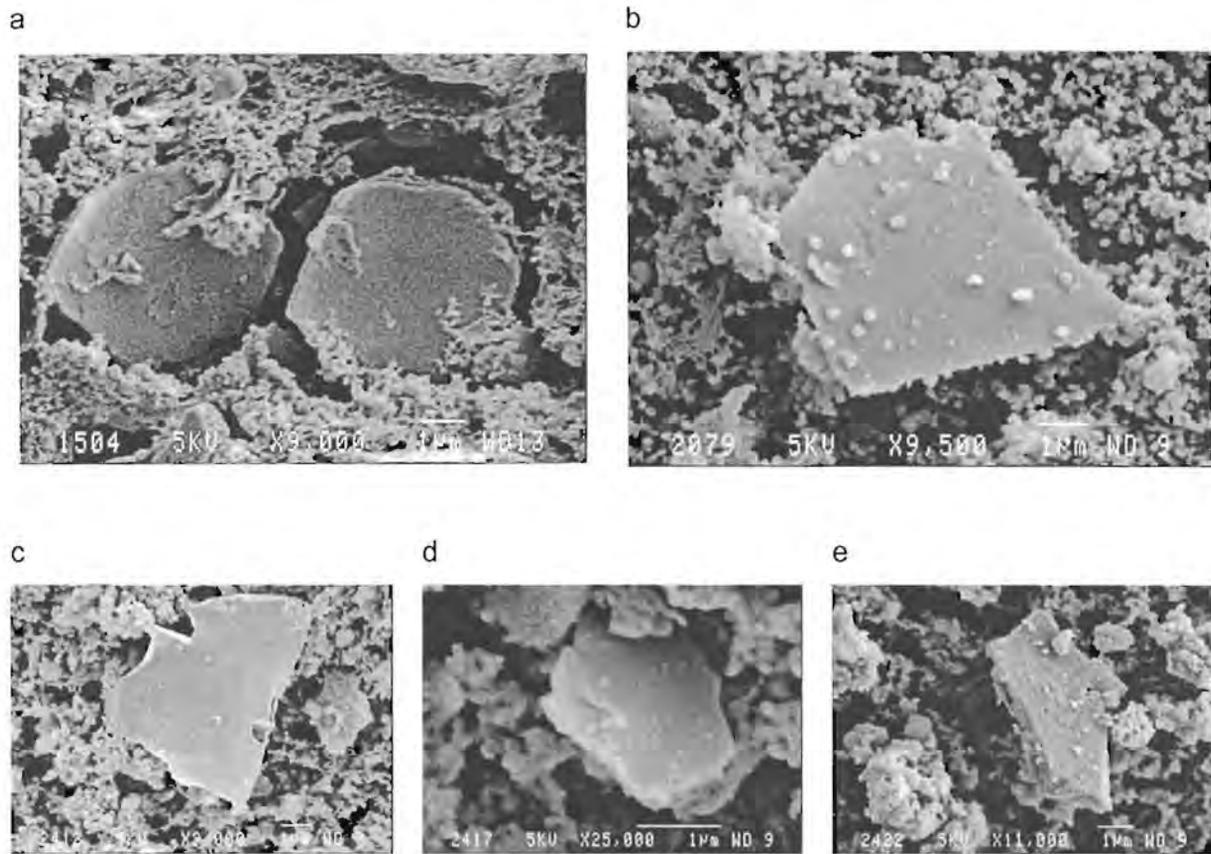


Fig.3.15: VP7 crystal formation as analysed by Jeol scanning electron microscopy.

(a) Crystals formed by AHSV-9 VP7 expressed in the recombinant baculovirus system as control

(b) Crystal formed by AHSV-9 VP7 expressed in the recombinant LSDV/VP7 system, 0.5 % Nonident P40 was used in the lysis buffer

(c)-(e) Crystals formed by AHSV-9 VP7 expressed in the recombinant LSDV/VP7 system, 0.1 % Nonident P40 was used in the lysis buffer

3.3.7 Generation of LSDV recombinants without residing wt LSDV

An effort was made to generate a LSDV recombinant without any residing parental viruses following the positive results that were obtained after analysing the VP7 expression from the LSDV/VP7 recombinant

(a) PCR-analysis

A dilution series of the virus stock was used to inoculate 90 % confluent FBT monolayers in the presence of MPA-selection medium. Instead of picking single foci to present a recombinant for using in the next purification series (section 3.2.3) the supernatants of those wells containing a single focus were harvested to be used in the next dilution series. Another two rounds of focus purification were included to generate a recombinant without any residing parental viruses.

After the third round of focus purification (2.2.17) foci were randomly picked to be further analysed. The viral DNA isolated from the foci were first amplified using the LSDV-specific primer set OP48 and OP49. The expected 166 bp PCR amplicon indicated that LSDV DNA, irrespective of whether it is wt or recombinant, was present in the samples (fig. 3.16 lanes c-i).

To reconfirm integration of VP7 in the LSDV genome, PCR-reactions were performed using the primer-set (SON2a and SON2b) and the expected 1.1 Kb amplified product was obtained (fig. 3.17 lanes f-j). To analyse for the presence of wt viral DNA, the primer-set Harry-WF and Sally-WF was used. If parental LSDV still resided in the recombinant a fragment of 474 bp (Table 3.2) would be amplified, similar to the amplicon from the wt LSDV that was used as control (fig. 3.18 lane c). In none of the selected recombinants the 474 bp amplified product could be detected (fig. 3.18 lanes e-j). A non-specific PCR-product (~680 bp) is however visible in all the LSDV/VP7 recombinants. The fact that the 680 bp product is also amplified from the pHSWF plasmid, confirmed that the amplicon is non-specific. The non-specificity may be explained by the low annealing temperatures of the primers. Fig. 3 19 (lane b), shows that the same non-specific amplification product (~680 bp) is also amplified in wt LSDV-DNA although the expected size of 474 bp is clearly observed. The results obtained from the PCR-reactions seemed to indicate that the LSDV/VP7 recombinants did not contain any residing wt LSDV.



Fig. 3.16: Agarose gel electrophoretic analysis of LSDV/VP7 recombinants by PCR. The LSDV-specific primer-set OP48 and OP49 was used
(a) Size-marker, ϕ X
(b) Uninfected FBT-cells
(c) wt LSDV
(d)-(i) LSDV/VP7 recombinants

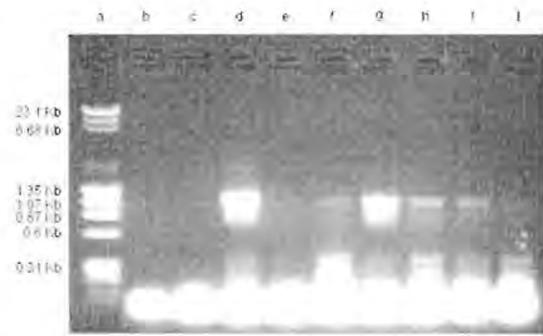


Fig. 3.17: PCR amplification of LSDV/VP7 recombinants using the primers SON2a and SON2b
(a) Size-marker, ϕ X
(b) Uninfected FBT-cells
(c) wt LSDV
(d) pHSWF/VP7 plasmid
(e)-(j) LSDV/VP7 recombinants

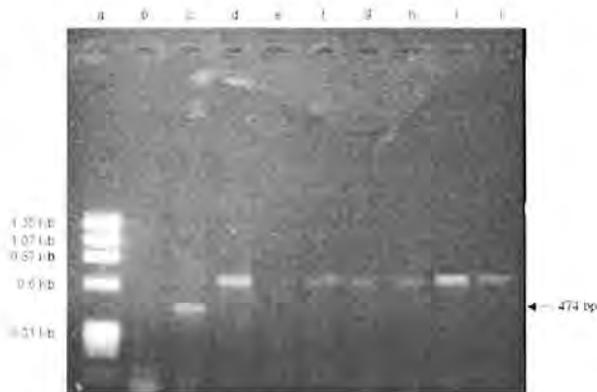


Fig. 3.18: Identification of LSDV recombinants with residing wt LSDV. PCR amplification was performed using the primer-set Harry-WF and Sally-WF
(a) Size marker, ϕ X
(b) Uninfected FBT-cells
(c) wt LSDV
(d) pHSWF plasmid
(e)-(j) LSDV/VP7 recombinants

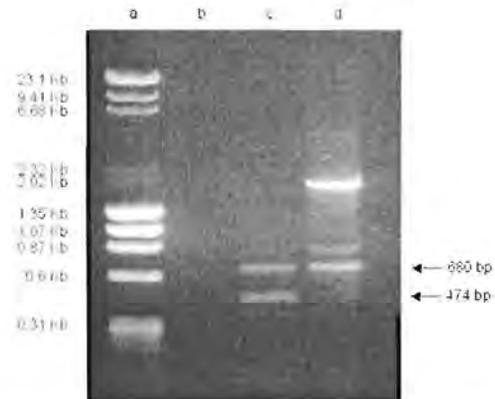


Fig. 3.19: Analysis of non-specific amplification using the primer-set Harry-WF and Sally-WF
(a) Size-markers, SMI1 and ϕ X
(b) Uninfected FBT-cells
(c) wt LSDV
(d) pHSWF plasmid

(b) Southern blot analysis

Two of the selected foci were analysed by Southern blotting as previously described (section 2.2.21). Purified LSDV/VP7 viral DNA was digested with *Bam* HI, separated on an 0.6% agarose gel (fig. 3.20 A), transferred to a Hybond N⁺-nylon membrane and probed for the presence of the VP7-gene. Three probes namely the purified VP7-gene (insert), pHSWF (plasmid) and SMI-size marker were labelled using the DIG DNA Labelling and Detection Kit. All three probes were included in the hybridisation reaction. The following fragments were expected upon *Bam* HI digestion:

Three fragments with sizes 168 bp, 6.9 Kb and 7.1 Kb indicative of a double cross-over event and correct integration into the LSDV genome. A fragment of 11.5 Kb would be visible if wt LSDV still resided in the LSDV/VP7 recombinant.

Results from the autoradiograph (fig. 3.20 B lanes c and d) showed that both foci contained a fragment of approximately 7.0 Kb, confirming the presence of the VP7-gene in the LSDV/VP7 recombinant genome. Only one fragment of 7.0 Kb was visible since the expected 7.1 Kb and 6.9 Kb fragments could not be distinguished. In sample 1 (fig. 3.20 B lane c) the fragment of 11.5 Kb is still visible, indicating that despite the stringent selection conditions, this LSDV/VP7 recombinant still contains residing parental viruses. Analysis of the other sample (fig. 3.20 B lane d) indicates that this recombinant may be without any wt LSDV. No definite fragment that correlates with the 11.5 Kb fragment, indicative of the presence of wt LSDV, is visible. A final proof that this specific LSDV/VP7 recombinant is without any residing wt LSDV would be to subject the recombinant to another few cycles of infecting FBT-cells in the presence of selective medium followed by a few cycles without using MPA-selection medium. There-after the purity has to be reconfirmed by PCR and Southern blot analysis.

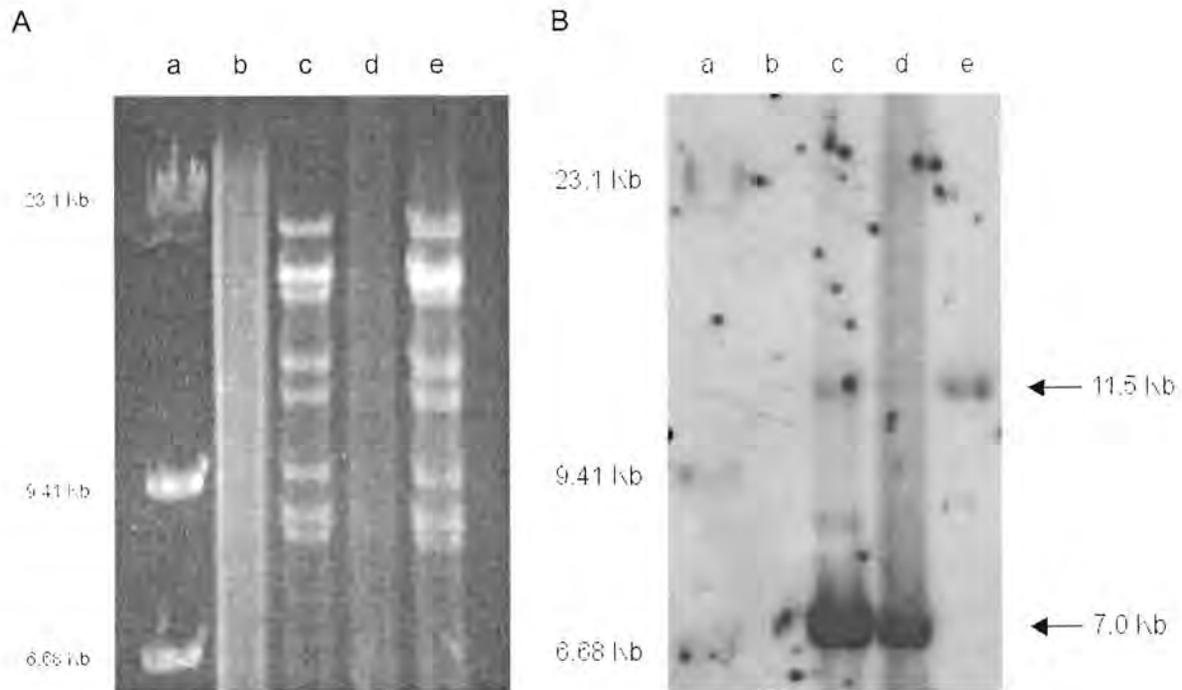


Fig. 3.20 A and B: LSDV/VP7 recombinants digested with *Bam* HI analysed by 0.6% agarose gel electrophoresis (A) and Southern blot hybridisation (B). An autoradiograph of the gel in (A), after hybridisation with a VP7-specific probe labelled with the DIG-system, is shown in (B). The size marker (SMII) and the transfer vector (pHSWF) were also labelled with the DIG DNA Labelling and Detection Kit and included as probes. The following applies to both figures:

- (a) Size marker, SMII
- (b) Uninfected FBT-cells
- (c) Recombinant LSDV/VP7 – focus 1 – digested with *Bam* HI
- (d) Recombinant LSDV/VP7 – focus 2 – digested with *Bam* HI
- (e) wt LSDV digested with *Bam* HI

3.4 RESULTS OF THE DUAL RECOMBINANT

3.4.1 Construction of a dual plasmid containing both the VP2- and VP7-genes of AHSV-9.

The AHSV-9 VP7 gene was excised as a *Sma* I - *Sal* I 1.1 Kb fragment from the plasmid pBS/VP7 (fig. 3.1). The lower band (fig. 3.22 lane b) was excised from the 1 % agarose gel and purified using Glassmilk methodology (section 2.2.4). Recovery of the gene-cleaned DNA was confirmed on a 1 % agarose gel (fig. 3.23 lane b).

The transfer plasmid pHSWF/VP2 was linearised with *Xho* I to enable cloning of the AHSV-9 VP7 gene under control of the early promoter, pA8R. Additional nucleotides (non-VP7) were included in the excised VP7-fragment as well as between the pA8R promoter and the VP7-gene after cloning in the pHSWF/VP2 plasmid. The DNA sequence of the region 5' to the VP7-gene in pBS/VP7, and the region between the unique *Eco* RI-site of the pA8R promoter and the *Xho* I-site in the pHSWF/VP2 plasmid were analysed to confirm that the first ATG to be encountered after cloning, would be the initiation codon of the VP7-gene. Although a *Eco* RI-site was engineered for cloning purposes, placing a foreign-gene under control of the early promoter, it was not possible to use this site since the AHSV-9 VP2 gene (already present in pHSWF/VP2) contains three *Eco* RI-sites. The linearised vector was electrophoretically analysed on a 1% agarose gel (fig. 3.24 lane b) followed by Glassmilk methodology. Electrophoretic analysis confirmed that purified vector DNA was recovered (fig. 3.25 lane b). Both the insert and vector were blunt-ended with a Klenow reaction after which the linearised vector was dephosphorylated with the phosphatase alkaline enzyme. To inactivate the different enzymes a phenol:chloroform:iso-amylalcohol (25:24:1) extraction was performed. A schematic representation of the cloning strategy is shown in (fig. 3.21).

Putative dual plasmids were picked after transforming competent Top10F *E.coli* cells with the ligation mixtures and used to make O/N cultures followed by mini-prep plasmid DNA extraction and electrophoresis (fig. 3.26 A and B lanes b-k). When compared to the supercoiled vector pHSWF/VP2, larger size samples were chosen for further investigation (fig. 3.26 A lanes b, d, g, j and k and fig. 3.26 B lane c). Restriction enzyme digestion studies were performed to confirm that the full-length VP7-gene was cloned in the correct orientation under control of the early pA8R promoter.

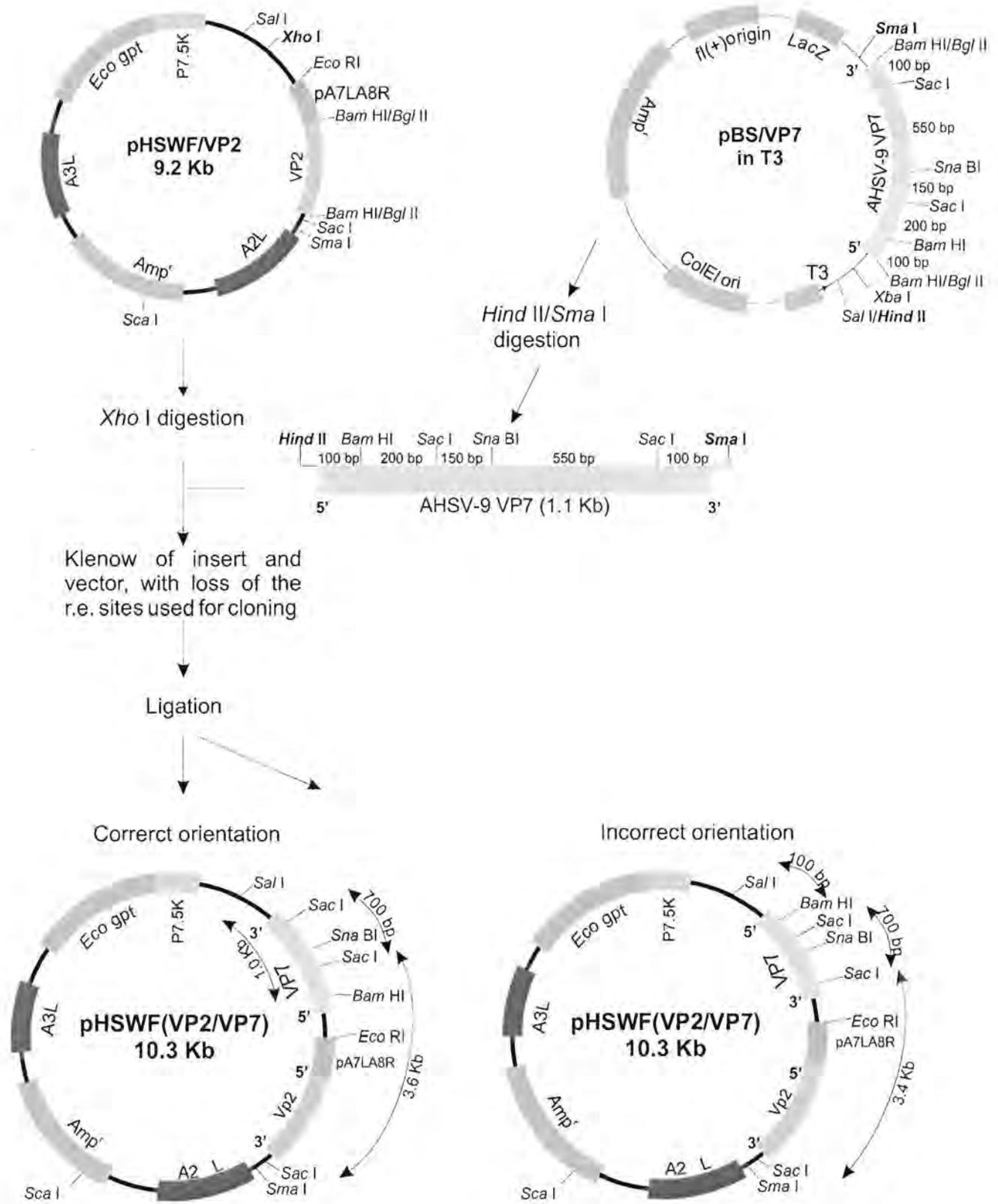


Fig. 3.21: Schematic representation of the cloning of AHSV-9 VP7 into the transfer vector pHSWF/VP2 under control of the early LSDV promoter (pA8R). A partial r.e. map of VP7 is shown, as well as the correct and incorrect transcriptional orientations of the gene after cloning.



Fig. 3.22: Agarose gel electrophoretic analysis of pBS/VP7
(a) Size markers SmaII and ϕ X
(b) pBS/VP7 digested with *Sma* I and *Sal* I



Fig. 3.23 Agarose gel electrophoretic analysis of gene-cleaned recovered VP7-DNA
(a) Size markers SmaII and ϕ X
(b) Approximately 150 ng DNA

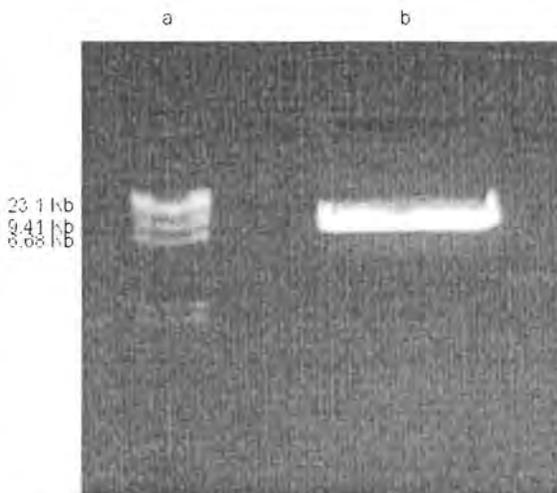


Fig. 3.24: Agarose gel electrophoretic analysis of linearised vector, pHSWF/VP2
(a) Size marker SmaII
(b) pHSWF/VP2 digested with *Xho* I



Fig. 3.25: Agarose gel electrophoretic analysis of gene-cleaned recovered linearised vector, pHSWF/VP2
(a) Size marker, SmaII
(b) Approximately 1000 ng DNA

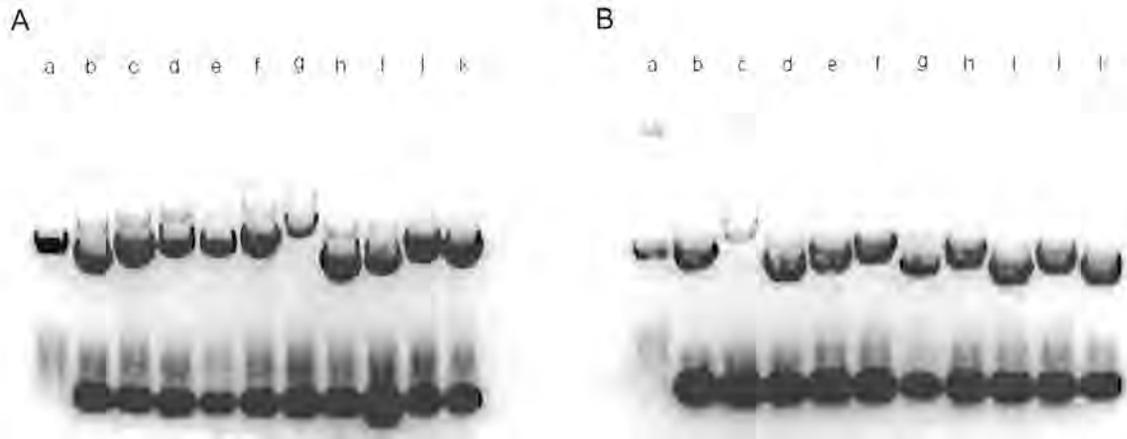


Fig. 3.26 A and B: Agarose gel electrophoretic analysis of putative dual plasmids.

The following apply for both figures

(a) Supercoiled vector pHSWF/VP2

(b)-(k) Putative dual pHSWF(VP2/VP7) plasmids

The correct orientation of the VP7-gene could be determined by a single digestion using the restriction enzyme *Sac* I. The VP7-gene contains 2 *Sac* I-sites, one 300 bp and the other 1000 bp from its 5' end. The internal VP7-fragment would therefore yield a fragment of 700 bp. Another *Sac* I-site is present at the 3' end of the cloned VP2 gene. Three fragments of sizes 700 bp, 3.6 Kb and 6.0 Kb would therefore be expected (for fragment sizes see the restriction enzyme map of VP7, fig. 3.21). If cloned in the incorrect orientation, fragments with sizes 700 bp, 3.4 Kb and 6.2 Kb could be expected (fig. 3.21). From the results observed on the 1 % agarose gel, one clone was selected (fig. 3.27 lane h). Incorrect orientation of the VP7 gene occurred in two selected clones (fig. 3.27 lanes d and g), the clones in (fig. 3.27 lanes e and f) gave unknown sizes – all four clones were discarded.

To confirm that the full-length VP7-gene was cloned in the correct orientation, double digestions were performed using *Bam* HI and *Sal* I. *Bam* HI digests asymmetrically within the VP7-gene, 100 bp from its 5' end. The pHSWF/VP2 vector contains a unique *Sal* I-site (fig. 3.21), therefore 2 fragments of approximately 1.0 Kb and 9.3 Kb were expected (fig. 3.28 lane g). If cloned in the incorrect orientation 2 fragments with sizes 100 bp and 10.2 Kb could be expected (fig. 3.21). Digestion of the "Nucleobond" purified DNA using *Sac* I was also included (fig. 3.28 lane d). Results obtained with the restriction enzyme digestions confirmed that the dual transfer vector contains the full-length VP7-gene in the

correct transcriptional orientation. The plasmid was named pHSWF(VP2/VP7) and used to generate a dual LSDV recombinant.

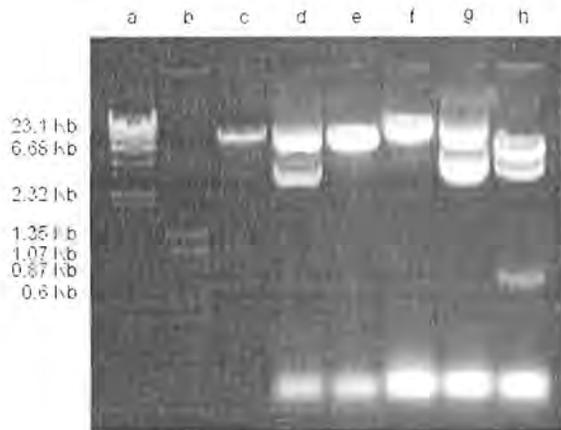


Fig. 3.27: Agarose gel electrophoretic analysis of the dual plasmids digested with *Sac* I to confirm the correct orientation of VP7
(a) Size marker, *S*MII
(b) Size marker, ϕ X
(c)-(h) Selected clones of putative dual pHSWF(VP2/VP7) plasmids digested with *Sac* I



Fig.3.28: Agarose gel electrophoretic analysis of a putative dual plasmid to confirm cloning of the full-length VP7 gene in the correct orientation
(a) Size marker, *S*MII
(b) Size marker, ϕ X
(c) pHSWF/VP2 digested with *Sac* I
(d) pHSWF(VP2/VP7) digested with *Sac* I
(e) Unloaded
(f) pHSWF/VP2 digested with *Bam* HI and *Sal* I
(g) pHSWF(VP2/VP7) digested with *Bam* HI and *Sal* I

3.4.2 Generation of dual LSDV recombinants

Dual recombinant lumpy skin disease viruses containing the AHSV-9 VP2 and VP7-genes were generated based on the same principles as for the LSDV/VP7 recombinant in section 3.2.3.

After the third round of single focus selection in the presence of MPA-selection medium, foci were randomly picked and further characterised by PCR analysis. All the selected foci yielded the expected 1.1 Kb amplified product (fig. 3.29) using the VP7 primer-set (SON2a and SON2b). In some cases a better amplification was obtained that could be due to a higher DNA concentration achieved during the viral DNA purification procedure (fig. 3.29 lanes b-h). When using the AHSV-9 VP2 specific primer-set (see table 3.3) all foci yielded the expected PCR amplicon of 401 bp (Table 3.4). As the case with amplification of the VP7-gene, some samples showed better amplification when compared to the others (fig. 3.30 A and B). The results suggested that all putative LSDV recombinants contained both the VP2- and VP7-gene of AHSV-9. The 401 bp amplicon detected in both control lanes (fig. 3.30 A and B lanes b and c) was probably due to external contamination. The problem was solved by repeating the purification of DNA with new uninfected and wild-type samples and used in further analysis (see fig. 3.31).

Table 3.3 List of primers

| Primer | Sequence | T _m 4(GC) _n + 2(AT) _n | Target |
|---------------|-------------------------|-----------------------------------------------------------|------------------------------|
| AHSV-9 VP2for | 5'd(TGGACGAATTACGGTGAC) | 53.7°C | AHSV-9 VP2 (1702-1719 bp) |
| AHSV-9 VP2rev | 5'd(GCCCGCTAATAAATCCTG) | 53.7°C | AHSV-9 VP2 (2086-2103 bp) |

Table 3.4 AHSV-9 VP2 primer-set used and the size of the expected specific amplified product

| Primer-set | DNA template | Annealing temp | Elongation Time (min) | PCR product (bp) |
|---------------------------------|-------------------|----------------|-----------------------|------------------|
| AHSV-9 VP2for and AHSV-9 VP2rev | LSDV/AHSV-VP2/VP7 | 49°C | 1 | 401 |



Fig. 3.29: Agarose gel electrophoretic analysis of putative dual LSDV(VP2/VP7) recombinants. PCR amplification using the VP7-specific primer-set, SON2a and SON2b
(a) Size markers, SMI and ϕ X
(b)-(h) Putative LSDV(VP2/VP7) containing VP7

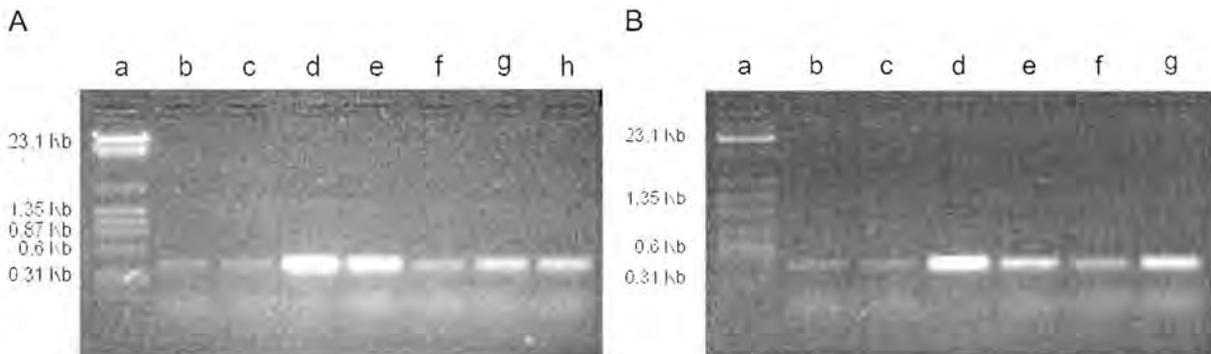


Fig. 3.30 A and B: Agarose gel electrophoretic analysis of putative dual LSDV(VP2/VP7) recombinants. PCR amplification using the VP2-specific primer-set. The following apply for fig. 3.30 A and B:
(a) Size markers, SMI and ϕ X
(b) Uninfected FBT-cells
(c) wt LSDV DNA
(d) pHSWF/VP2 as positive control
(e)-(h) The first four putative dual LSDV(VP2/VP7) recombinants containing VP2 (fig. 3.30 A)
(e)-(g) The last three putative dual LSDV(VP2/VP7) recombinants containing VP2 (fig. 3.30 B)

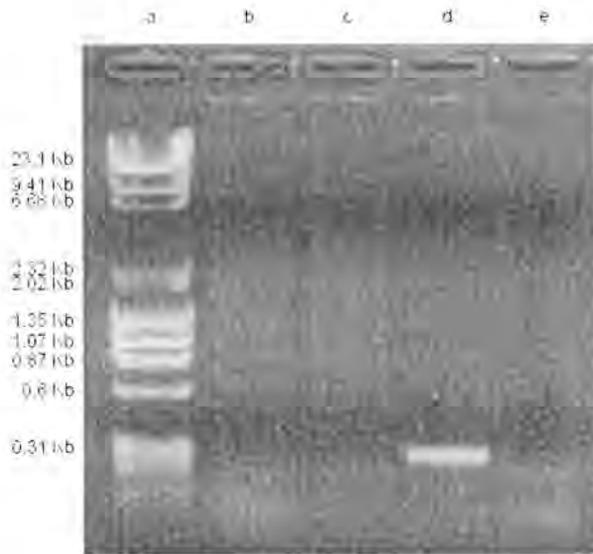


Fig. 3.31: Amplification of the specific VP2-fragment to determine the external contamination in the DNA of the uninfected cells and wt LSDV
 (a) Size markers, SMI and ϕ X
 (b) Uninfected FBT-cells
 (c) wt LSDV DNA
 (d) pHSWF/VP2 as positive control
 (e) PCR-reaction using all the PCR-reagents without any DNA-template to identify reagent contamination.

From the results obtained with PCR analysis foci were selected for large-scale infection of FBT-cells in the presence of MPA-selection medium. After 5 days p.i. the infected cells were harvested followed by viral DNA purification and PCR analysis. Positive results were obtained with PCR-amplification of both genes (amplification of the VP7-gene, fig. 3.32 A and amplification of the specific VP2-fragment, fig. 3.33 A). Southern blot hybridisation of the amplified material with AHSV-9 VP7- and VP2-specific probes were performed to confirm the authenticity of both the VP7- and VP2-amplified products (fig. 3.32 B and 3.33 B).

At this stage further investigation was discontinued due to unexpected difficulties in showing expression with the single LSDV/VP2 recombinant as analysed by Dr. W. C. Fick (Department of Genetics, UP). Although it was shown that LSDV/VP2 recombinants were generated no expression of the VP2-gene could be detected; therefore it seemed unlikely that VP2 expression would be detected in the dual recombinant. The dual recombinant viruses are, nevertheless, available for further analysis.

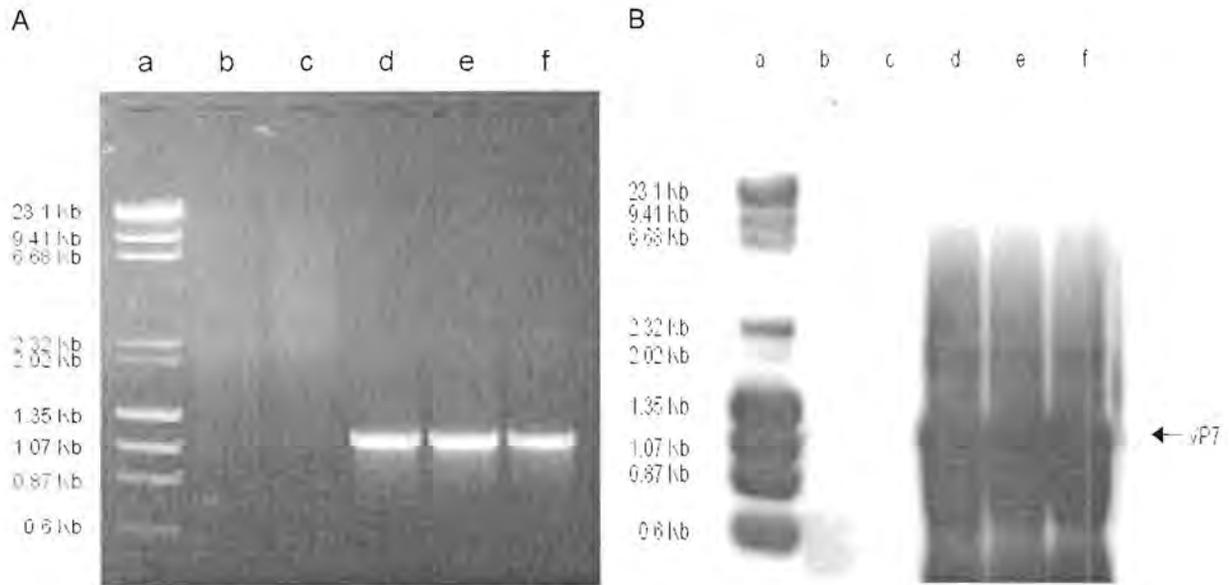


Fig. 3.32 A and B: Confirmation of the presence of the AHSV-9 VP7 gene in the selected putative dual LSDV recombinants. The primer-set (SON2a and SON2b) was used for amplification of the VP7-gene. The PCR products were analysed on a 1% agarose gel (A) followed by Southern blot hybridisation (B).

The following apply for both figures

- (a) Size markers, SMI and ϕ X
- (b) Uninfected cells
- (c) wt LSDV DNA
- (d) pHSWF/VP7 as positive control
- (e)-(f) Putative dual LSDV(VP2/VP7) recombinants

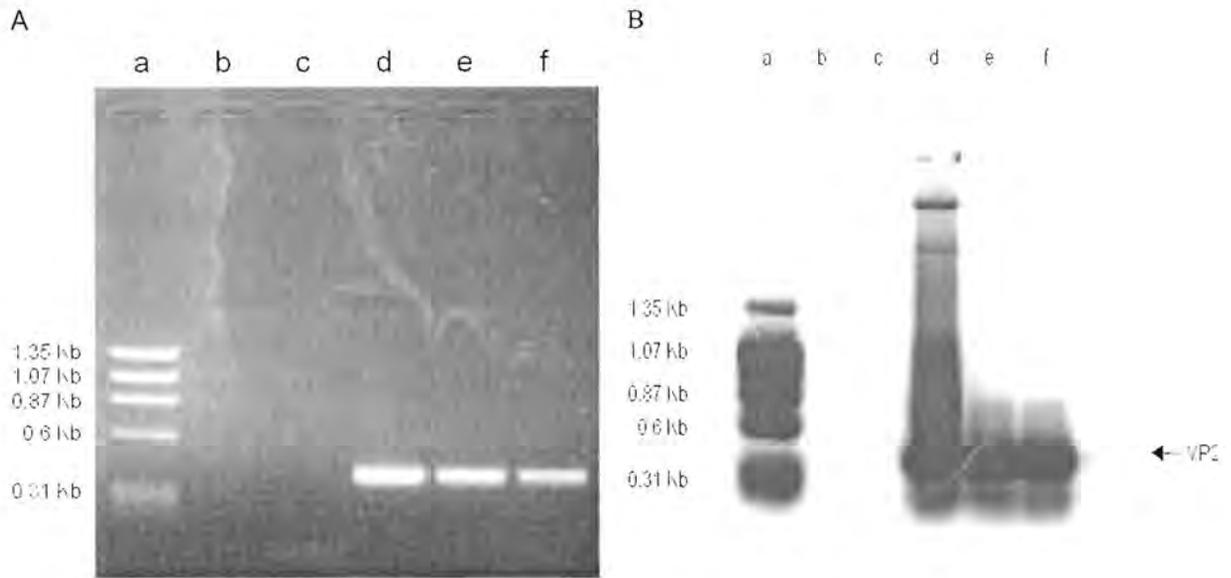


Fig. 3.33 A and B: Confirmation of the presence of the AHSV-9 VP2 gene in the selected putative dual LSDV recombinants. The primer-set AHSV-9 VP2 (for and rev) was used to amplify the specific VP2-fragment. The PCR products were analysed on a 1% agarose gel (A) followed by Southern blot hybridisation (B).

The following apply for both figures

- (a) Size marker, ϕ X
- (b) Uninfected cells
- (c) wt LSDV DNA
- (d) pHSWF/VP2 as positive control
- (e)-(f) Putative dual LSDV(VP2/VP7) recombinants

3.5 DISCUSSION

The high expression level of the *LacZ*-gene from the early promoter described previously initiated an investigation concerning the ability of the early promoter to drive expression of an immunologically important gene. An important approach for making vaccines lies in the possibility of using recombinants not only in host-, but also non-host species. Although infection of host-restricted poxviruses leads to incomplete replication outside their host-range, it was demonstrated in case of the fowlpox- and canarypox virus that foreign genes were expressed outside their host-range and appeared to be sufficient to elicit a protective immune response in non-host species (Taylor *et al.*, 1998a; Taylor *et al.*, 1991). By placing an immunologically important gene under control of the early pA8R promoter, the gene can be expressed during the early phase of the growth cycle of LSDV, although in a non-host species.

Previously a recombinant capripox virus was constructed containing the major core structural VP7-gene of bluetongue virus (Wade-Evans *et al.*, 1997). Sheep vaccinated with the recombinant virus developed antibodies to VP7, but no neutralising antibodies to either the homologous or heterologous bluetongue virus (BTV) serotype prior to challenge. A significant level of serotype and cross-serotype protection against the lethal effects of a challenge with virulent BTV was, nevertheless, observed. These results confirm that a single viral protein, VP7, can play an important role in protection, possibly via a cell-mediated mechanism (Wade-Evans *et al.*, 1996; Wade-Evans *et al.*, 1997). These data led us consider VP7 as a possible candidate for vaccine purposes against AHSV by expressing the gene from the early LSDV promoter.

For the generation of LSDV/VP7 recombinants, the plasmid pHSWF/VP7 was constructed and used to transfect FBT-cells. PCR and Southern blot analysis were performed to confirm stable integration of the AHSV-9 VP7 gene into the LSDV genome. Positive results were obtained with the mRNA transcription of the AHSV-9 VP7 gene when analysed by dot blot analysis. Expression of VP7 was analysed by ³⁵S-radioactive labelling of proteins and the analysis of LSDV/VP7 by electron microscopy. Despite the fact that the SDS-PAGE gel didn't show expression of VP7 at a high level (due to the faint band), we were surprised to observe such a high quantity of crystals when analysed by electron microscopy. Crystal formation is a unique feature of AHSV VP7 that it is an extremely hydrophobic protein and has been demonstrated by its ability to form trimers (Roy, 1992). It has been illustrated that the VP7 trimers spontaneously aggregate in infected cells as large, flat, hexagonal crystals of about 6 µm when expressed by a

recombinant baculovirus (Burroughs *et al.*, 1994). The structure of the crystals formed by the VP7-protein when expressed in LSDV/VP7 differed slightly when compared to the perfect hexagonal crystals formed by the VP7-protein, expressed by a recombinant baculovirus containing the AHSV-9 VP7 gene. The reason for the different structure could be explained by the different expression system been used. Although different in shape, the size of the crystals formed by the LSDV/VP7 expressed VP7 protein, correlates well with the size of the crystals formed by the VP7-protein expressed by the recombinant baculovirus. The high levels of expression from the early promoter have important implications for the generation of vaccines in non-host species by placing immunological important antigens under control of the early promoter.

The last part of our study was to investigate the ability of the bi-directional promoter to drive the expression of foreign genes from the early and late promoter in a dual LSDV recombinant by making use of the new transfer vector. Studies done by Coupar *et al.* (1986), showed that antigens expressed by recombinant poxviruses early in infection were recognised by both B and T cells while products expressed after DNA replication were not generally recognised by T cells. According to Boyle (1992), high levels of expression, as is expected from a late promoter, may not necessarily be a prerequisite for the stimulation of a protective cell-mediated immune response. These results seem to suggest that expression from early poxvirus promoters may be better suited for eliciting cellular immunity, whereas expression from late promoters is necessary for eliciting a strong humoral immune response. Therefore it was decided to place the AHSV-9 VP7 gene that could elicit a cell-mediated response under control of the early promoter, whereas the VP2-gene would be placed under control of the late promoter for inducing a humoral immune response.

Although the natural productive host range of LSDV is limited to cattle and outside their host-range infection leads to incomplete replication of the virus, it was shown that host-restricted poxviruses (e.g. fowlpox and canarypox) could induce a specific immune response in non-host species as stated above. Another example to support the idea of placing an immunological important gene under control of a late promoter in a host-restricted virus, to be used as a vaccine outside its host-range, is the modified vaccinia Ankara. Modified vaccinia Ankara (MVA), a highly attenuated vaccinia virus strain, has multiple genomic deletions and is severely host-cell restricted. It grows in avian cells but is unable to multiply in human and most other mammalian cells. Nevertheless, it was found that replication of viral DNA appeared normal and that both early and late viral

proteins were synthesised in human cells, however only immature virus particles were detected by electron microscopy (Sutter and Moss, 1992).

The results on the dual recombinant indicated that it is possible to generate a dual LSDV recombinant using the new transfer vector. The presence of both foreign genes, AHSV-9 VP7 and VP2, was demonstrated by the correct amplified products that were obtained during PCR-amplification using the specific primer-sets respectively. Due to the fact that expression of the AHSV-9 VP2 gene could not be detected from the single LSDV/VP2 recombinant it was decided to delay any further studies concerning the dual LSDV recombinant.

CHAPTER FOUR

CONCLUDING DISCUSSION

Lumpy skin disease virus (LSDV), a member of the *Capripoxvirus* genus, is one of the poxviruses being investigated as a live recombinant system for expressing foreign genes. The strict tropism of the virus renders it very promising as a vector for veterinary purposes. An important attraction in developing vaccines lies in the possibility of using recombinants not only on host-, but also non-host species. In case of fowlpox (Taylor *et al.*, 1988a) and canarypox virus (Taylor *et al.*, 1991), it has been demonstrated that foreign genes may be expressed in both avian and mammalian species. Although infection leads to incomplete replication outside their host-range, the level of expression was sufficient to induce a specific immune response.

Studies done by Boyle (1992) also highlighted the importance of using authentic virus promoters for the optimal expression of foreign genes when he compared the efficiency of poxvirus promoters in vaccinia and fowlpox virus recombinants. The results illustrated 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 and may presumably be caused by the fact that promoter recognition may not be uniform in different systems. These results motivated the identification and characterisation of a strong authentic bi-directional LSDV promoter, pA7LA8R (Fick and Viljoen, 1999). Transient expression assays using a reporter gene *LacZ*, verified the temporarily regulated nature of the promoter and revealed both early and late transcriptional activities. Previous attempts to generate recombinants using a transfer vector pTKsLR containing the bi-directional LSDV promoter and TK-gene of LSDV were unsuccessful.

This study is part of an investigation to develop lumpy skin disease virus (LSDV) as a live recombinant system for expressing immunogenic important antigens, cloned under control of the bi-directional LSDV promoter by using the new transfer vector pHSWF as base. The vector contains the bi-directional LSDV promoter, 2 flanking regions of LSDV to allow homologous recombination with wt LSDV and the *E. coli* *gpt* gene under control of the vaccinia early/late p7.5K promoter as positive selectable marker. The value of the new transfer vector lies in the fact that it directs the integration of foreign genes into an intergenic

region into the LSDV genome, between the vaccinia virus A2L and A3L gene analogs. The vector also contains an authentic bi-directional LSDV promoter whereby expression of foreign genes could be obtained in the early and late phases of viral replication. To date the LSDV promoter has not been analysed for its ability to drive the expression of foreign genes in a live recombinant LSDV system by using the new transfer vector pHSWF as base.

The main goal of this study was to evaluate the use of the new transfer vector for generating LSDV recombinants. To accomplish this we had to confirm the activity of the bi-directional promoter in the newly constructed vector by transient expression assays with the *LacZ*-reporter gene under control of either the early or late promoter. Two transfer plasmids pHSWF/*LacE* (*LacZ* placed under control of the early promoter pA8R) and pHSWF/*LacL* (*LacZ* cloned under control of the late promoter pA7L) were constructed and used to transfect wt LSDV infected FBT-cells. The results of the transient expression experiments confirmed the temporary nature of both the early and late controlling elements of pA7LA8R and correlated with the assays done by Fick and Viljoen (1999). Blue colouration observed after X-gal staining of the transfected cells were an indication of the enzymatic activity of β -galactosidase, the gene-product of *LacZ*. The same constructs were used for the generation of LSDV recombinants. Following at least 3 rounds of focus purification, under selective conditions, the status of the putative recombinants was evaluated by infecting FBT-cells with each recombinant followed by X-gal staining. After an incubation time of 16 hr, not only single cells were stained as with the transient expression studies, but also foci of cells representing the progeny of a single virus. The same quantity of intensely blue coloured foci obtained in both cases revealed that high levels of expression was achieved from the early promoter. The apparent high levels of expression from the early promoter is encouraging in light of the possible use of this transfer vector for generating recombinants for vaccine purposes in non-host species, since the expression of early proteins can occur in the absence of viral DNA replication. Selected foci were analysed for integration of the *LacZ*-gene into the LSDV genome by Southern blot analysis. Results obtained from the autoradiograph indicated that the *LacZ*-gene was stably integrated into the LSDV-genome in both cases. All the LSDV recombinants had residing parental viruses despite the stringent selection conditions that were included during the developing procedures to generate the recombinants.

The *LacZ* recombinants could be used to investigate the host-range of LSDV after eliminating the residing parental viruses from recombinant cultures. Although the multiplication of LSDV in non-host species have been investigated previously, the extend of early and late protein expression is not known. In the case of modified vaccinia Ankara, a severely host-cell restricted virus, it was shown that the virus grows in avian cells but is unable to multiply in human and most other mammalian cells. Nevertheless the replication of viral DNA still appeared normal and both early and late proteins were synthesised in human cells while only immature virus particles were detected by electron microscopy (Sutter and Moss, 1992). The two constructs (LSDV/*LacE* and LSDV/*LacL*) could be used to compare expression of the *LacZ*-reporter gene in different non-host cell-lines. The *LacZ* recombinant expressing the β -gal enzyme from the LSDV early promoter would be expressed during the early phase of virus replication in non-host species; while the *LacZ* recombinant expressing the β -gal enzyme from the late promoter would depend on whether DNA replication actually occur. By infecting different cell-lines with the two recombinants respectively, early and late gene expression may be easily analysed with X-gal staining to determine in which non-host cell-lines expression of late proteins do occur. Results should have implications for extending the use of the LSDV system in non-host species.

The encouraging high levels of expression of *LacZ* from the early promoter in our initial studies led to the investigation concerning the use of the new transfer vector to generate LSDV recombinants that may be important for vaccine purposes in non-host species by expressing an immunological important antigen from the early promoter. The VP7-gene encoding the major group-specific antigen of African horsesickness virus serotype 9 was chosen as model gene. For the generation of LSDV/VP7 recombinants, the plasmid pHSWF/VP7 was constructed and used to transfect FBT-cells. Following at least 3 rounds of single foci picking under selective conditions, the putative recombinants were analysed by PCR and Southern blot analysis that confirmed stable integration of the VP7-gene into the LSDV genome. All the recombinant cultures still contained residing parental viruses. Positive results were obtained with the mRNA transcription of the AHSV-9 VP7-gene when analysed by dot blot analysis. The expression of VP7 in the LSDV/VP7 recombinant system was analysed by ³⁵S radiolabelling, Western blot analysis and electron microscopy. Despite numerous attempts, we were unable to detect the VP7-protein expressed from LSDV/VP7 by means of Western blotting. The expression studies of VP7 in the LSDV/VP7 recombinant by making use of ³⁵S radiolabelling of proteins and the analysis of LSDV/VP7 by electron

microscopy, were the first to show expression of a foreign gene in this specific LSDV recombinant system. VP7 crystal formation is a feature of high levels of VP7 expression. The structure of the crystals formed by the VP7-protein when expressed in LSDV/VP7 differed slightly when compared to the flat hexagonal crystals formed by the VP7-proteins expressed by a recombinant baculovirus that was included as control. The size of the crystals however correlates well with the size of the crystals formed in the baculovirus system.

The unique feature of AHSV VP7 to spontaneously aggregate as large, flat hexagonal crystals when expressed by a recombinant baculovirus (Burroughs *et al.*, 1994) has initiated studies on using the VP7 itself as a vaccine vector for displaying epitopes. The top domain of VP7, composed of amino acids 121-249, is exposed on the surface of these crystalline particles. Three cloning sites have already been constructed in the hydrophylic domains of the top domain of AHSV VP7 (Department of Genetics, UP) that enable the insert of different peptides/epitopes simultaneously. Some inserts increased VP7 solubility about 6 fold and changed the shape of the crystals to more rounded tennisball structures (Maree *et al.*, submitted for publication) while other modifications left the solubility unchanged and the VP7 crystal structure intact. Studies are currently under way in the Department of Genetics (UP) to investigate the use of VP7 as an epitope delivery system.

An effort was made to eliminate the residing parental viruses from the LSDV/VP7 recombinants following the positive results gained with the VP7-expressed protein from LSDV/VP7. Despite all the stringent selection conditions some of the recombinant cultures still contain residing wt LSDV although it seemed as if one LSDV/VP7 recombinant was without any residing parental viruses. The final proof would be to propagate this specific recombinant another few times in the presence of MPA-selection medium, followed by a few cycles without any selective medium, and reconfirm the purity by PCR and Southern blot analysis. Our results suggest that when trying to generate a LSDV recombinant, the supernatant of infected FBT-cells transfected with the transfer vector has to be harvested instead of harvesting all the transfected cells containing a high quantity of wt LSDV. The hypothesis is that single viruses are released into the supernatant, whereas viruses tend to clump together in the isolated transfected cells (D.B. Wallace, OVI.). By using only viruses that are present in the supernatant, the possibility would be increased to infect cells with a single recombinant virus instead of a virus clump containing residing parental viruses. In our

experience it would be easier to generate a LSDV recombinant without residing wt LSDV from transfected cells instead of trying to eliminate residing parental viruses from recombinant cultures.

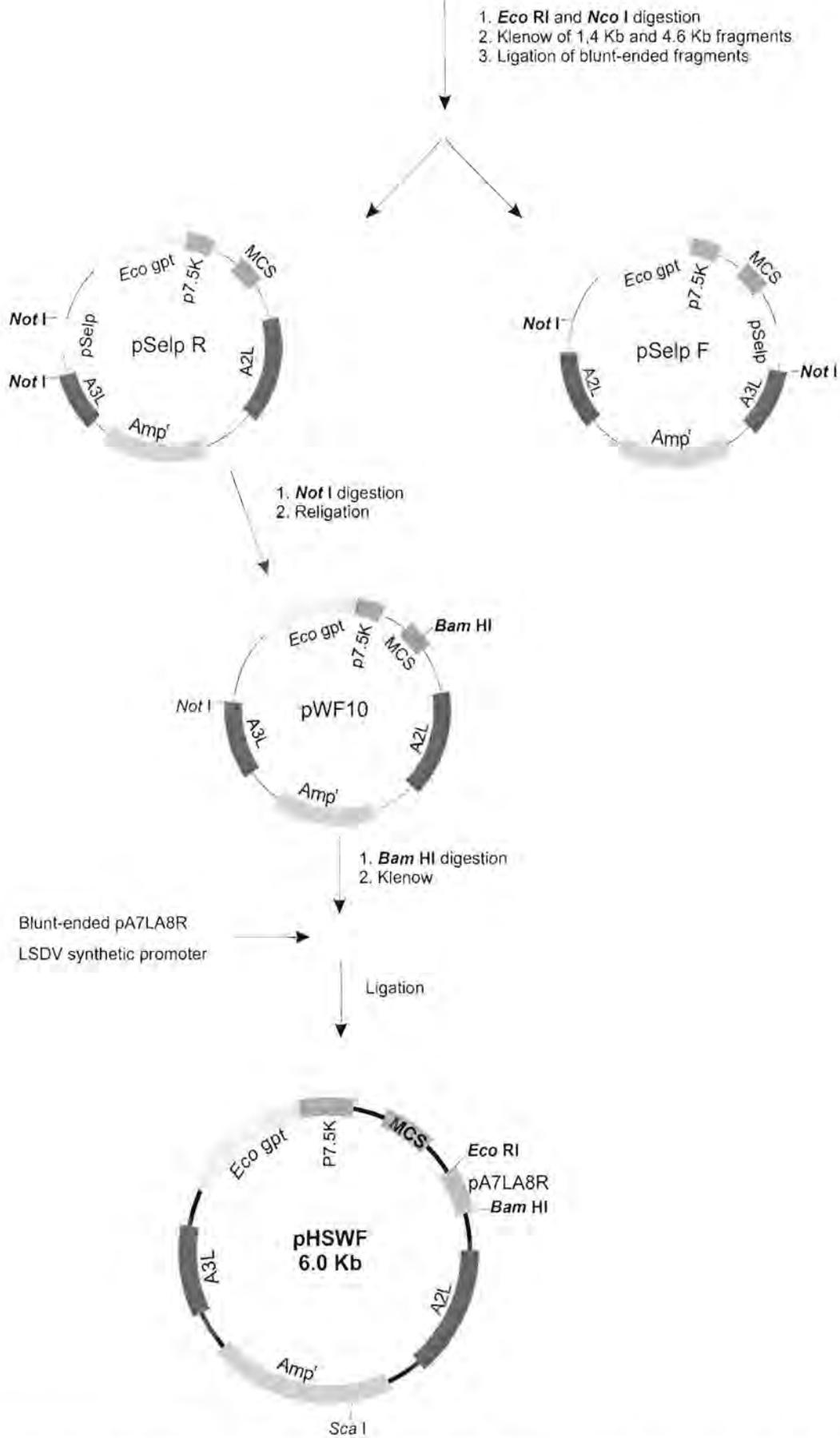
A second LSDV/VP7 recombinant, containing the AHSV-9 VP7-gene under control of the late pA7L promoter, is in the process of being developed (Department of Genetics, UP). After confirming stable integration of the VP7-gene into the LSDV genome, expression of the VP7-gene under control of the late promoter, will be analysed by ³⁵S radiolabelling of infected cells and electron microscopy. The expression level of the VP7-gene could then be compared to the recombinant described in this study.

The last part of our study involved the generation of a dual LSDV recombinant to express two immunological important antigens simultaneously by making use of one construct. In the transfer vector pHSWF(VP2/VP7) the AHSV-9 VP7-gene was cloned under control of the early pA8R promoter and the AHSV-9 VP2-gene under control of the late pA7L promoter. Following at least 3 rounds of single foci picking under selective conditions, putative dual recombinants were analysed by PCR analysis. Preliminary results indicated that it is possible to generate dual LSDV recombinants using the new transfer vector pHSWF. At this stage, further investigation concerning the dual LSDV recombinant was discontinued, due to the unexpected difficulties in showing expression of the AHSV-9 VP2-gene with the single LSDV/VP2 recombinant (Fick W.C., personal communication). No expression of the VP2-gene could be observed by either ³⁵S-methionine labelling, Western blotting or immunoprecipitation analysis, although it was shown that LSDV/VP2 recombinants were generated by using the transfer vector pHSWF. It is suspected that there may be an intrinsic problem associated with the immunogenicity of the VP2-protein or the quality of the anti-sera (personal communication). The detection of VP2 expression merits further investigation. The dual LSDV recombinant containing both the VP7 and VP2 genes of AHSV-9 is nevertheless available for further analysis as soon as detection of the VP2-protein can be resolved.

Appendix 1

Construction of the pHSWF transfer vector

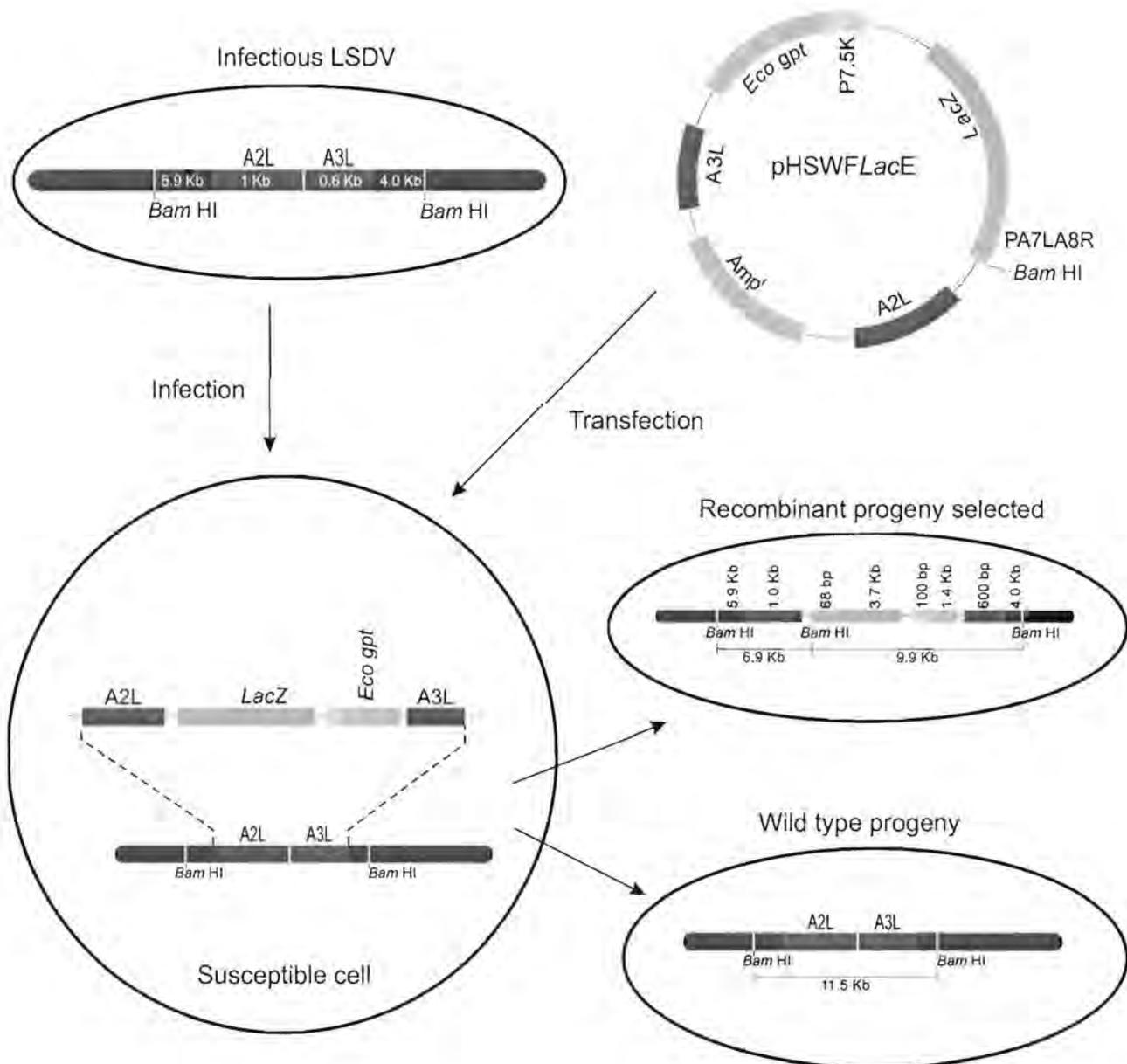
The plasmids, pMTL25 and pSelp-gptL2(G₁G₂), were used as basis for the construction of the pHSWF transfer vector. The A2L and A3L gene analogs of VV (Harry and Sally) was cloned into pMTL25 as a *Pst* I-fragment and named pMTL25 (E⁻)HS. The pSelp-gptL2(G₁G₂) plasmid (constructed by a co-worker at OVI) was linearised with *Not* I to give a fragment that contains the *E.coli* xanthine-guanine phosphoribosyl transferase (*Eco* gpt) gene under control of the VV early/late p7.5K promoter. The pMTL25(E⁻)HS was cut with *Not* I and ligated with the above mentioned fragment to give rise to the pSelp-HS-(G₁G₂) vector. (The vector was constructed by Dr. A.L. Williamson and A.S. Cohen in the Virology Department of the Medical School at the University of Cape Town and used with permission). Three fragments with sizes 1.4 Kb, 3.1 Kb and 4.6 Kb were obtained with digestion of the pSelp-HS-(G₁G₂) vector using *Eco* RI and *Nco* I. The 1.4kb and 4.6kb fragments were blunt-ended and ligated to obtain 2 vectors named pSelpR and pSelpF. The transfer vector pHSWF was constructed by using the pSelpR vector. The pSelpR vector was digested with *Not* I, to remove the pSelp promoter, after which the vector was allowed to religate and named pWF10. pWF10 was linearised with *Bam* HI and blunt-ended to facilitate cloning of the bi-directional pA7LA8R promoter. The pA7LA8R promoter is also a blunt-ended product, obtained by annealing two complementary 68bp oligonucleotides representing the promoter.



Construction of the transfer vector pHSWF. The pWF10 plasmid was linearized with *Bam* HI and blunt-ended to facilitate cloning of the blunt-ended bi-directional pA7LA8R LSDV synthetic promoter.

Appendix 2A

Homologous recombination and the results of double cross-over events between wt LSDV-DNA and pHSWF/LacE

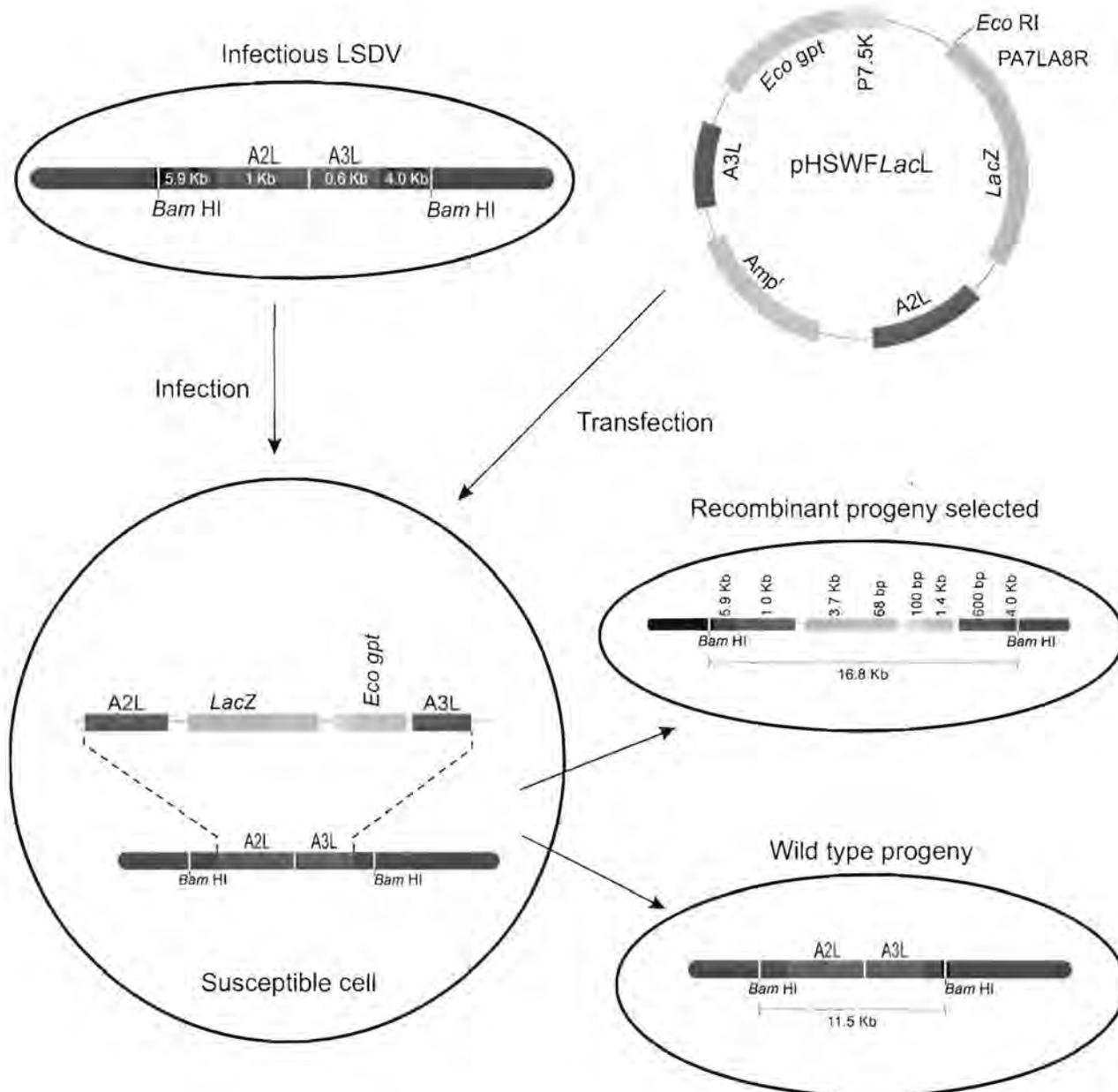


Fragments expected with *Bam* HI digested recombinant LSDV/LacE DNA, hybridized with *LacZ*- and pHSWF-DIG probes:

- Fragments with sizes 6.9 Kb and 9.9 Kb each, if no residing wt LSDV is still present in the recombinant.
- If parental LSDV resides in recombinant, an extra fragment of 11.5 Kb will be obtained.

Appendix 2B

Homologous recombination and the results of double cross-over events between wt LSDV-DNA and pHSWF/LacL

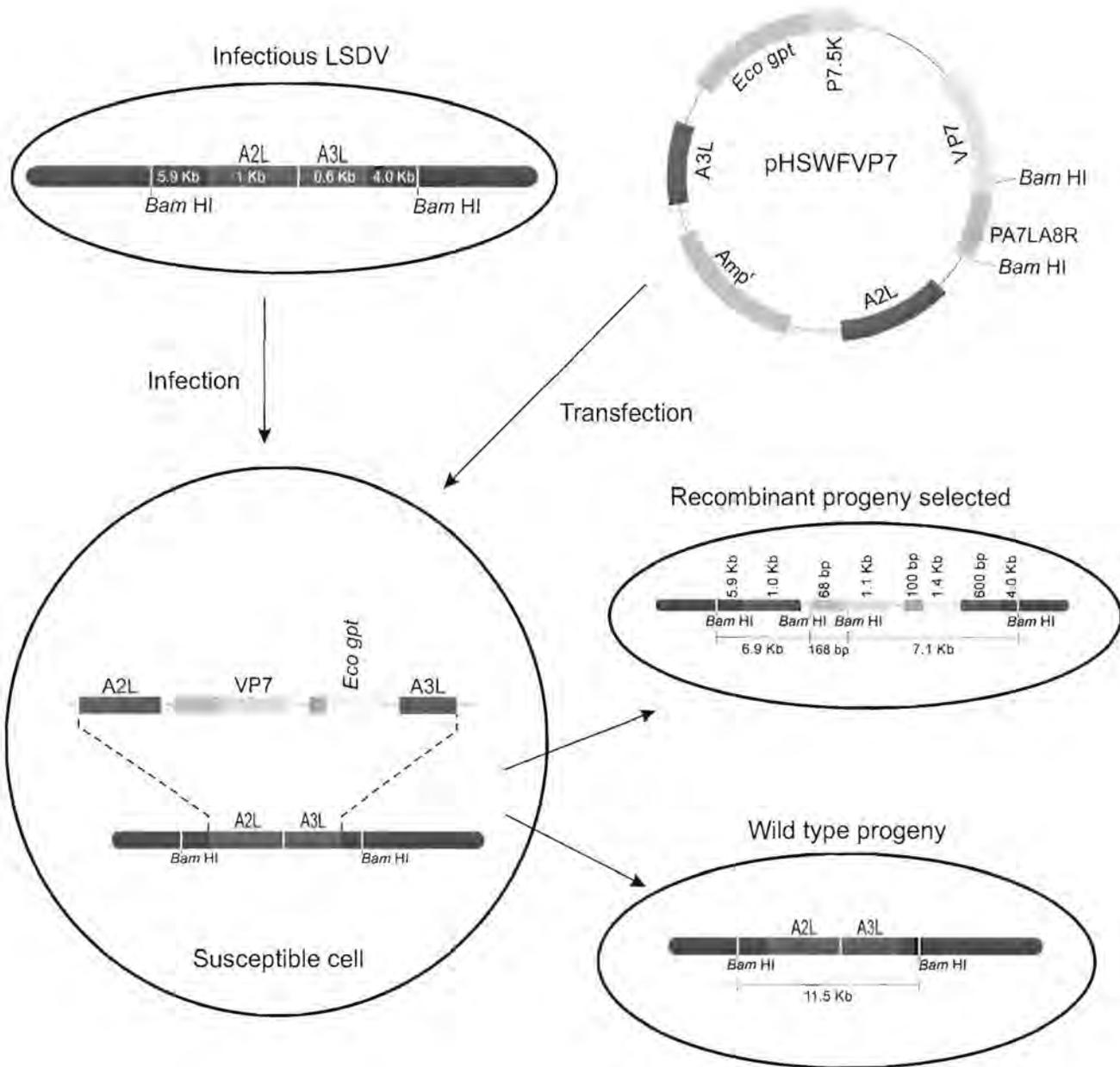


Fragments expected with *Bam* HI digested recombinant LSDV/LacL DNA, hybridized with *LacZ*- and pHSWF-DIG probes:

- A fragment with size 16.8 Kb, if no residing wt LSDV is still present in the recombinant.
- If parental LSDV resides in recombinant, an extra fragment of 11.5 Kb will be obtained.

Appendix 3

Homologous recombination and the results of double cross-over events between wt LSDV-DNA and pHSWF/VP7



Fragments expected with *Bam* HI digested recombinant LSDV/VP7 DNA, hybridized with VP7- and pHSWF-DIG probes:

- Fragments with sizes 168 bp, 6.9 Kb and 7.1 Kb each, if no residing wt LSDV is still present in the recombinant.
- If parental LSDV resides in recombinant, an extra fragment of 11.5 Kb will be obtained.

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