CHAPTER THREE

GENERATION OF LSDV RECOMBINANTS EXPRESSING IMMUNOLOGICAL IMORTANT 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 immunological 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 primary 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 ^{35}S-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/*Lac*E and pHSWF/*Lac*L 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 °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 °C. The viral cores were resuspended in 100 µl DMEM. One volume of lysis buffer (1 % SDS; 20 mM βME; 20 mM EDTA) and 1.4 µl proteinase K (final concentration 100 µg/ml) were added and the virus mixture was incubated at 37 °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 µg tRNA and 2.5 volumes 100 % EtOH and left O/N at -20 °C. The DNA was concentrated by centrifugation at 12 000 rpm for 30 min at 4 °C and washed with ice-cold 70 % EtOH followed by another centrifugation as described above. The DNA pellet was air-dried, resuspended in 30 µl ddH_2O and stored at -20 °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 µl 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 µl 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 Tm 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	Tm $4(GC)_n + 2(AT)_n$	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(GATAAACCACAGAAATGC)	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 µCi/mMol ^{32}P dATP (10 µCi/µl 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 °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 °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 °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 °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 ^{32}P-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 cm^2 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 % CO_2-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 % CO_2 - 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:

ffu/ml = A x B x 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 µl 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 µl 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 µl 0.1 % diethylpyrocarbonate (DEPC) H_2O. During the whole procedure precautions were taken to ensure that solutions and apparatus were free of ribonucleases by using DEPC H_2O 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 ^{32}P-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 ^{32}P-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 Eeagle'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 (^{35}S)-methionine (60 µCi/1 x 10^6 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 µl N,N,N',N'-Tetra-methylethylendiamin (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 µl 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 βME, 4 % SDS, 20 % glyserol 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 cm^2 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 µl 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 cm^2 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 KH_2PO_4/Na_2HPO_4-buffer pH 7.4. The fixated sample was filtered onto a 0.2 µm nylon filter, washed 3 times with 0.075 M KH_2PO_4/Na_2HPO_4-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 spatter 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). Eletrophoretic 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.

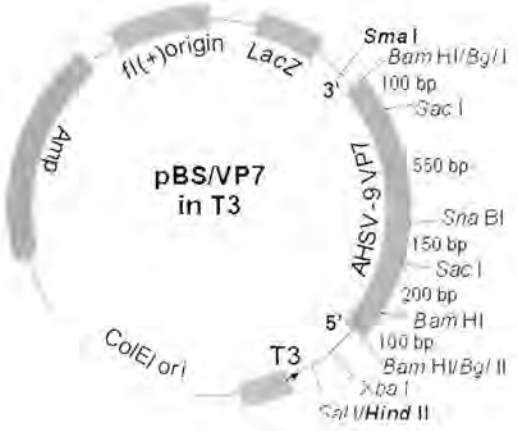

Fig. 3.1: Plasmid pBS/VP7 in T3

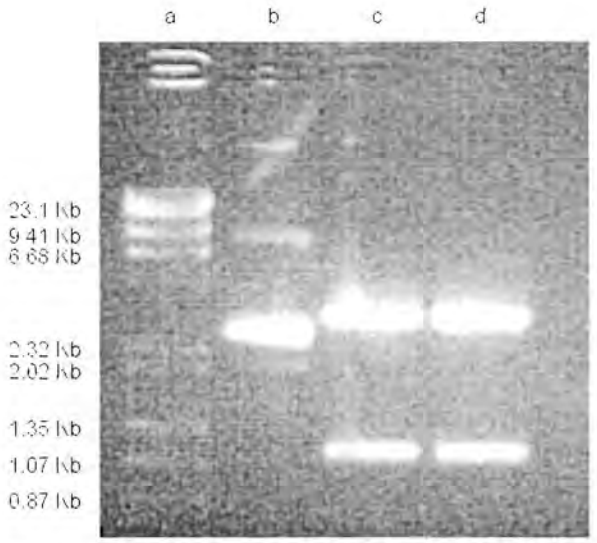

Fig. 3.2: Agarose gel electrophoretic analysis of pBS/VP7
(a) Size markers SMII and ϕX
(b) Undigested pBS/VP7
(c)-(d) pPS/VP7 digested with *Sma* I and *Hind* II

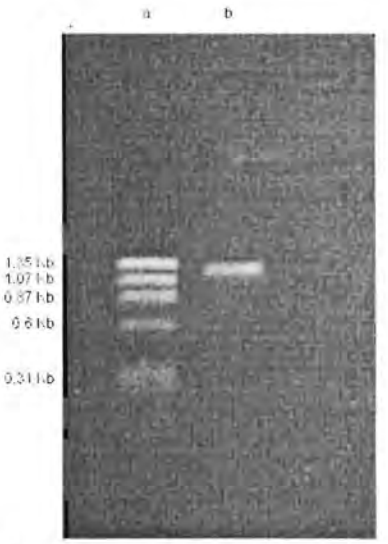

Fig. 3.3: Agarose gel electrophoretic analysis of genecleaned recovered VP7-DNA
(a) Size marker, ϕX
(b) Approximately 350 ng DNA

Fig. 3.4: Agarose gel electrophoretic analysis of linearised vector, pHSWF
(a) Size markers, SMII and ϕX
(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 genecleaned recovered vector, pHSWF
(a) Size marker, SMII
(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.

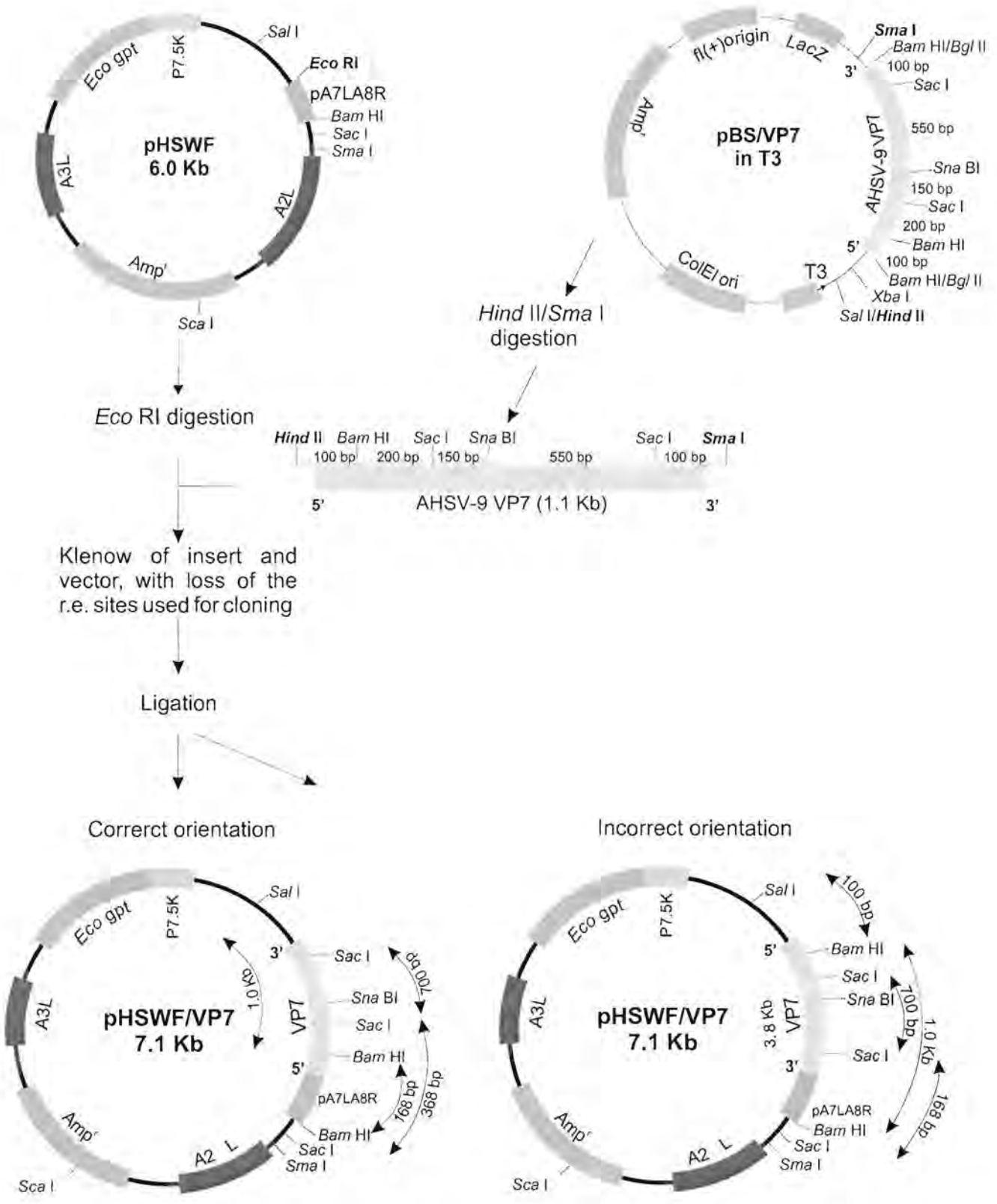

Fig. 3.6: Schematic representation of the cloning of AHSV-9 VP7 into the transfer vector pHSWF, under control of the early LSDV promoter (pA8R). A partial r.e. map of VP7 is shown, as well as the correct and incorrect transcriptional orientations of the gene after cloning.

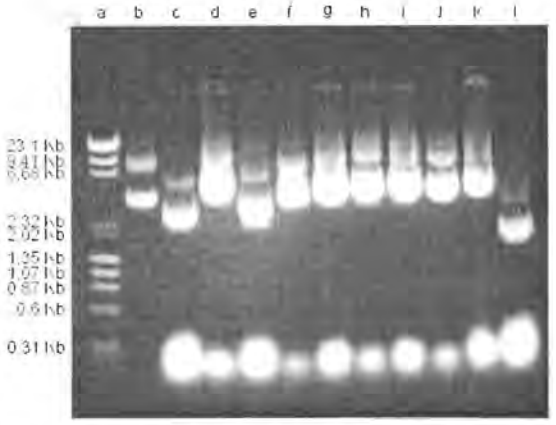

Fig. 3.7: Agarose gel eletrophoretic analysis of putative plasmids containing VP7
(a) Size marker, SMII
(b) Intact vector, pHSWF, as control
(c)-(l) Putative pHSWF containing VP7 cloned downstream of the early promoter

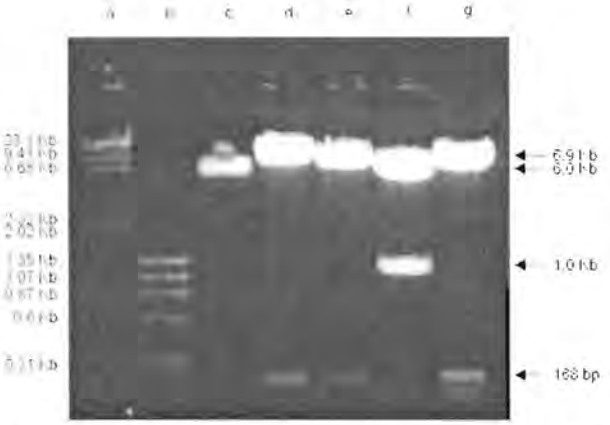

Fig. 3.8: Agarose gel electrophoretic analysis of plasmids digested with *Bam* HI to confirm orientation
(a) Size marker, SMII
(b) Size marker, ϕX
(c) pHSWF digested with *Bam* HI
(d)-(g) Putative pHSWF containing VP7 digested with *Bam* HI

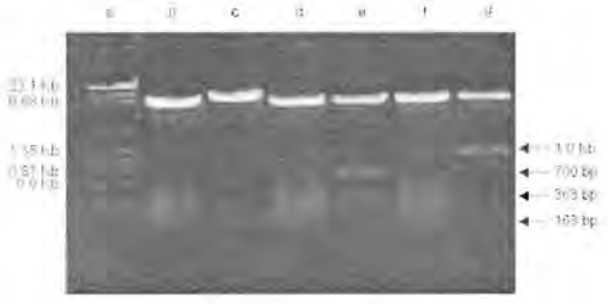

Fig. 3.9: Agarose gel electrophoretic analysis of a selected plasmid to confirm cloning of the full-length VP7-gene in the correct orientation
(a) Size markers, SMII and ϕX
(b) pHSWF digested with *Bam* HI
(c) pHSWF containing VP7 digested with *Bam* HI
(d) pHSWF digested with *Sac* I
(e) pHSWF containing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

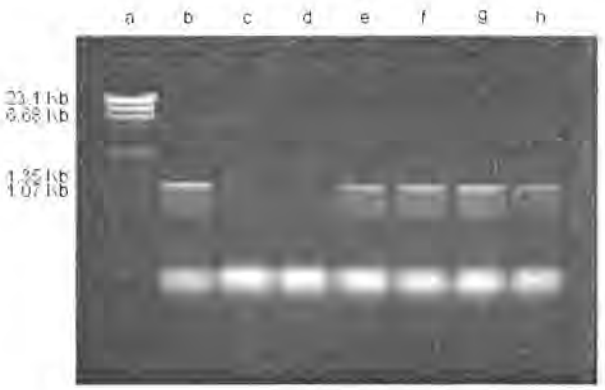

Fig. 3.10: Agarose gel electrophoretic analysis of putative LSDV/VP7 recombinants. PCR amplification using the VP7-specific primer-set, SON2a and SON2b was performed
(a) Size markers, SMII and ϕX
(b) pHSWF/VP7 plasmid as positive control
(c) wt LSDV DNA
(d) uninfected FBT-cells
(e)-(h) Putative LSDV/VP7 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 cm^2 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-^{32}P labelling. Three ^{32}P-labeled probes were used for hybridisation, i.e. the purified VP7-gene (insert), pHSWF (plasmid) and SMII-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 eletrophoretic separated DNA and probes of the purified VP7-gene (insert), pHSWF (plasmid) and SMII-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 an 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 excepted 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.

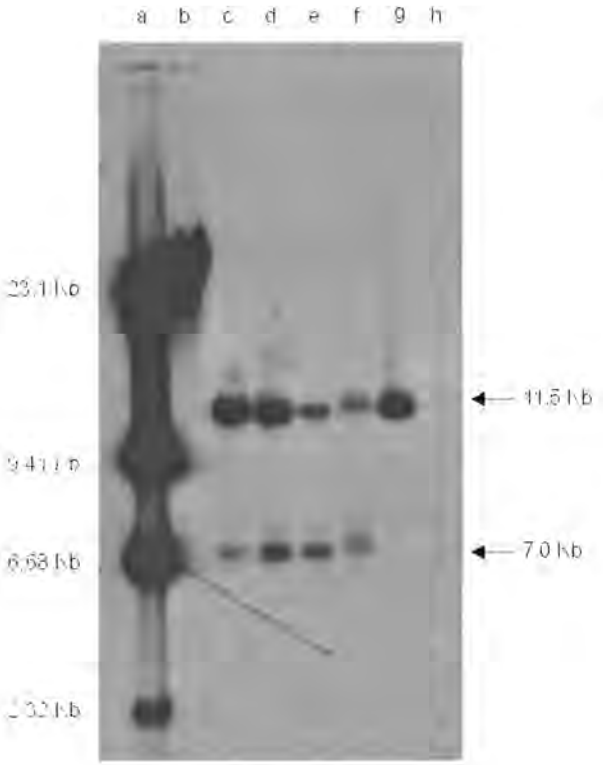

Fig. 3.11: An autoradiograph of a Southern blot hybridisation between *Bam* HI digested LSDV/VP7 DNA and α-^{32}P probes of the VP7-insert and pHSWF.
(a) Size marker, SMII
(b) Undigested LSDV/VP7 DNA
(c)-(f) LSDV/VP7 DNA digested with *Bam* HI
(g) wt LSDV DNA digested with *Bam* HI
(h) Uninfected FBT-cells 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 75cm^2 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 9^{th} 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:

8 ↘
average number of foci: 7
6 ↗

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

$= 7 \times 10^{7} \times 10$

$= 7 \times 10^{8}$ 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-^{32}P 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.

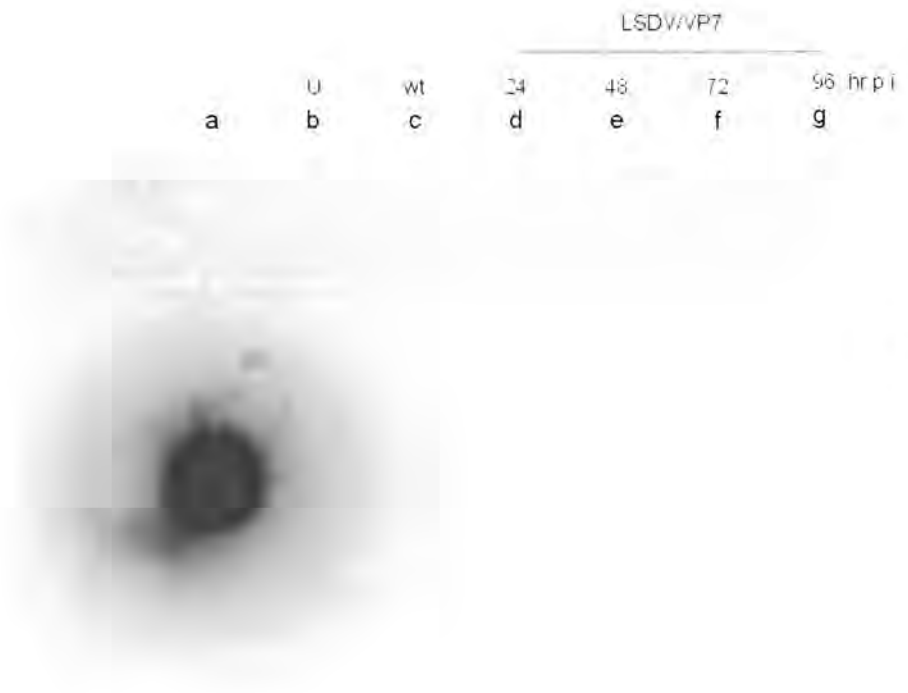

Fig. 3.12: Autoradiograph of total RNA isolated at different times from cells infected with LSDV/VP7 and hybridised to the purified VP7-insert
(a) pHSWF/VP7 as positive control
(b) RNA isolated from uninfected FBT-cells 48 hr p.i.
(c) RNA isolated from wt LSDV 48hr p.i.
(d)-(g) RNA isolated from LSDV/VP7 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.

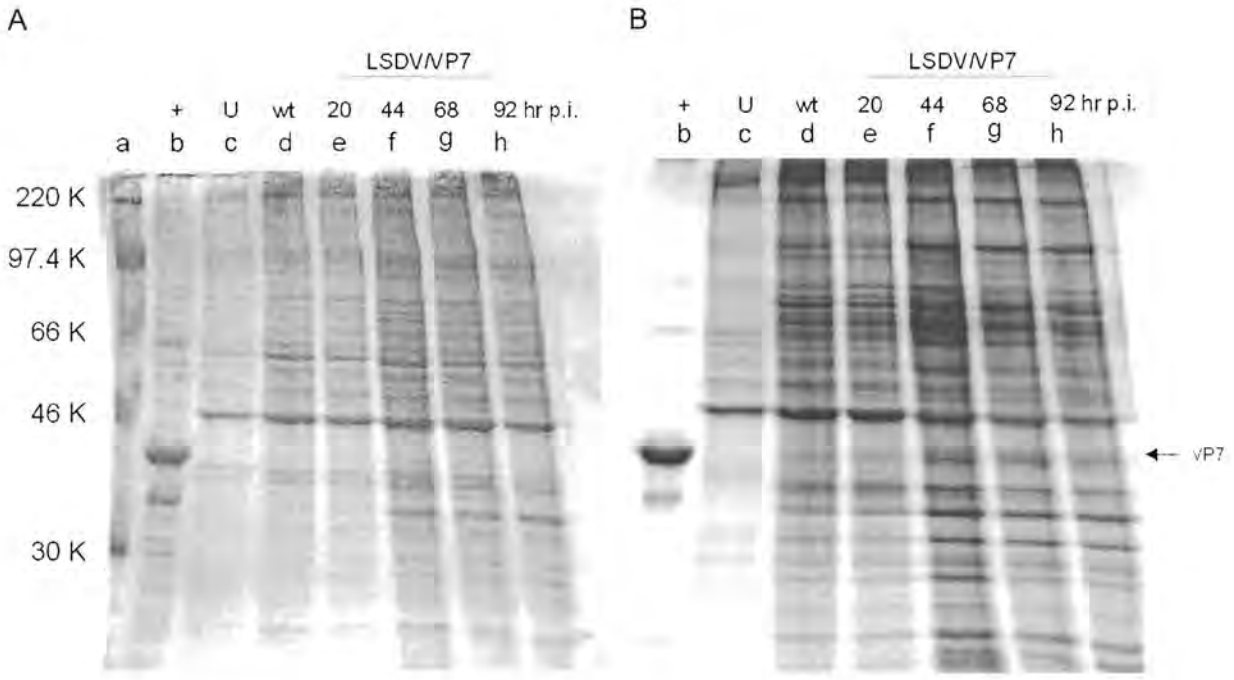

Fig. 3.13 A and B: (A) 15% Polyacrylamide gel of LSDV infected FBT-cells. At the different times indicated, the uninfected and infected cells were labelled for 10 hr using ^{35}S-methionine. The molecular weights (kilodaltons) are shown next to the gels as an indication of the protein-sizes.

(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).

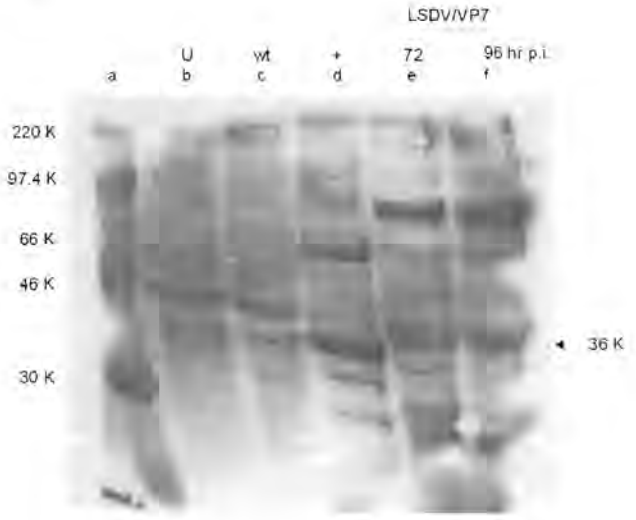

Fig. 3.14: Expression of VP7 analysed by Western blotting. At the different times shown, the infected cells were harvested. The molecular weights (kilodaltons) are an indication of the protein-sizes.

(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.

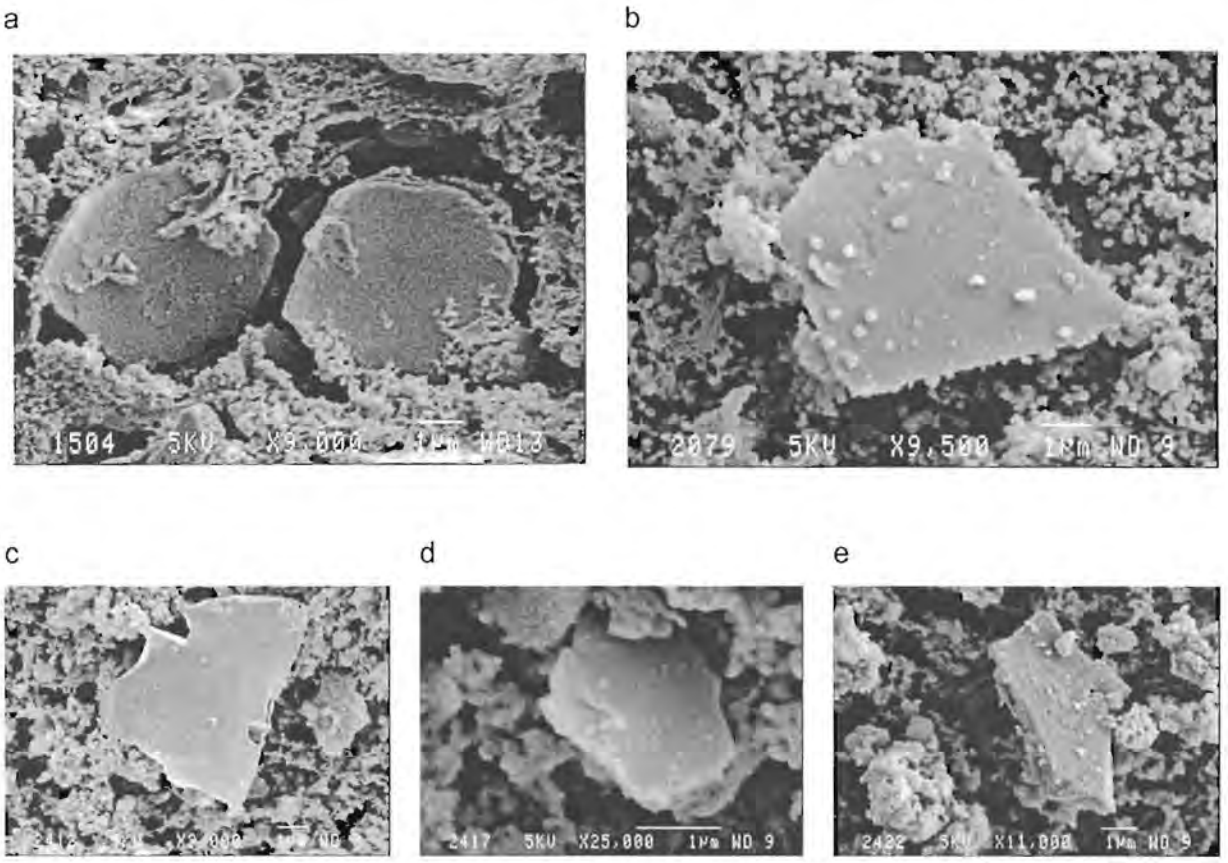

Fig.3.15: VP7 crystal formation as analysed by Jeol scanning electron microscopy.

(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, ϕX
(b) Uninfected FBT-cells
(c) wt LSDV
(d)-(i) LSDV/VP7 recombinants

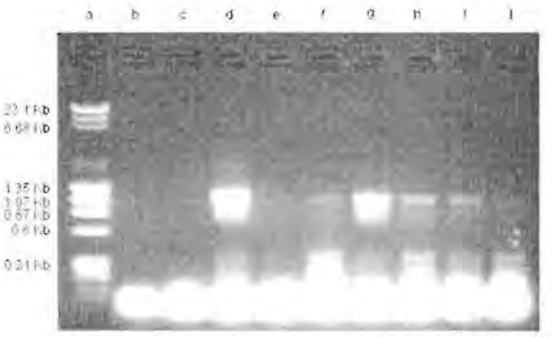

Fig. 3.17: PCR amplification of LSDV/VP7 recombinants using the primers SON2a and SON2b
(a) Size-marker, ϕX
(b) Uninfected FBT-cells
(c) wt LSDV
(d) pHSWF/VP7 plasmid
(e)-(j) LSDV/VP7 recombinants

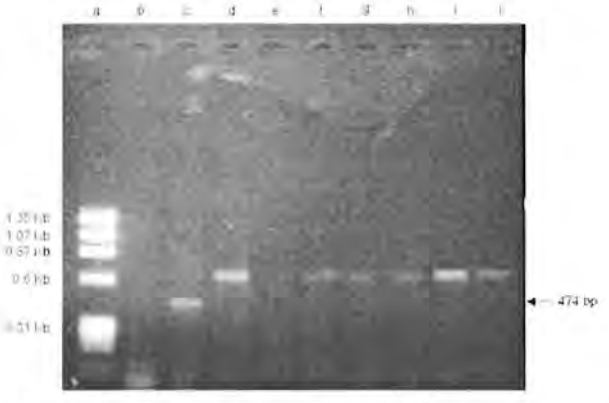

Fig. 3.18: Identification of LSDV recombinants with residing wt LSDV. PCR amplification was performed using the primer-set Harry-WF and Sally-WF
(a) Size marker, ϕX
(b) Uninfected FBT-cells
(c) wt LSDV
(d) pHSWF plasmid
(e)-(j) LSDV/VP7 recombinants

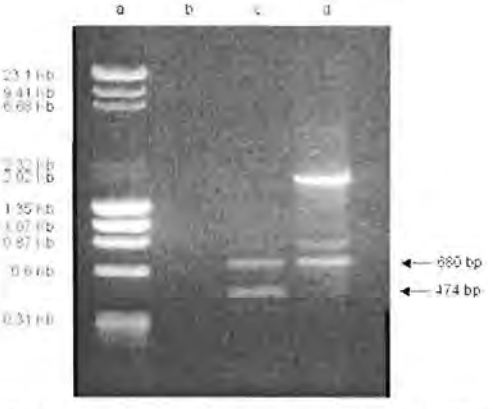

Fig. 3.19: Analysis of non-specific amplification using the primer-set Harry-WF and Sally-WF
(a) Size-markers, SMII and ϕX
(b) Uninfected FBT-cells
(c) wt LSDV
(d) pHSWF plasmid

(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 SMII-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 been 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.

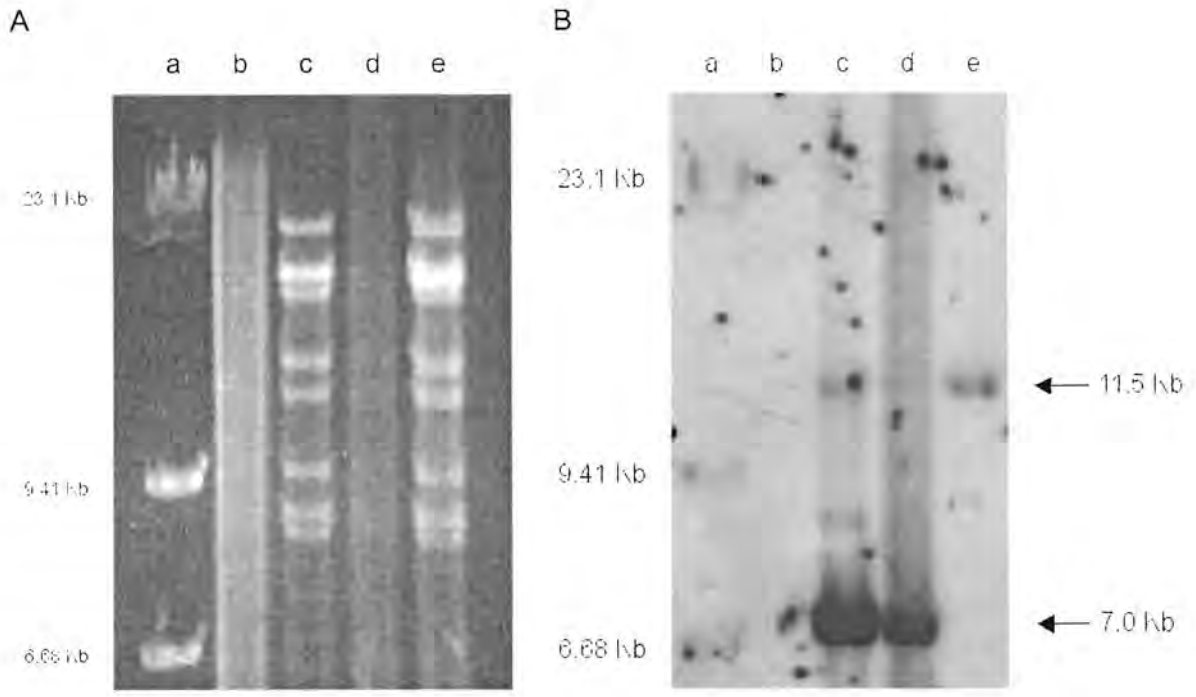

Fig. 3.20 A and B: LSDV/VP7 recombinants digested with *Bam* HI analysed by 0.6% agarose gel electrophoresis (A) and Southern blot hybridisation (B). An autoradiograph of the gel in (A), after hybridisation with a VP7-specific probe labelled with the DIG-system, is shown in (B). The size marker (SMII) 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 genecleaned 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 ASVH-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 an 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 dephosphorilated 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. 32.6 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.

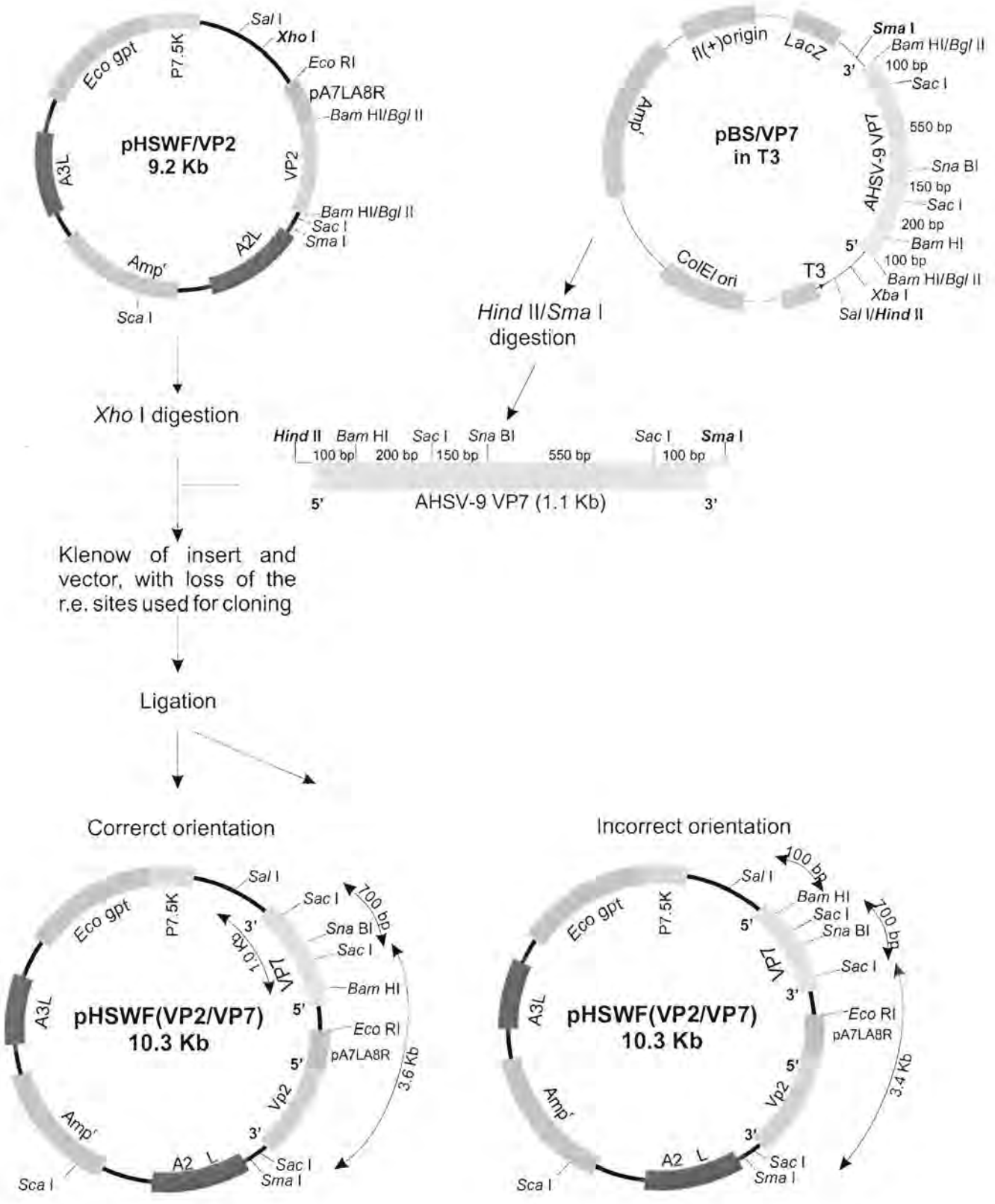

Fig. 3.21: Schematic representation of the cloning of AHSV-9 VP7 into the transfer vector pHSWF/VP2 under control of the early LSDV promoter (pA8R). A partial r.e. map of VP7 is shown, as well as the correct and incorrect transcriptional orientations of the gene after cloning.

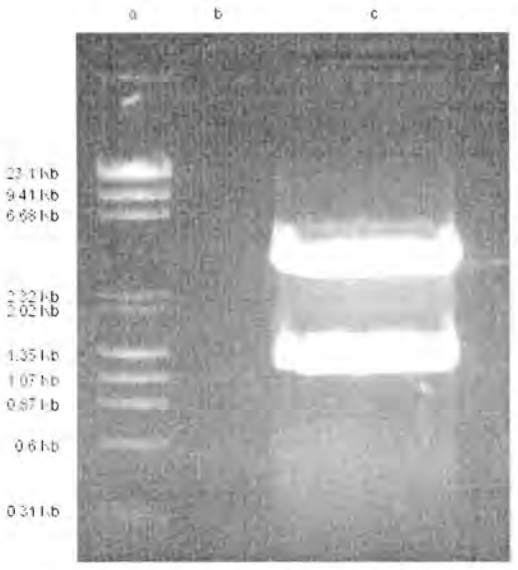

Fig. 3.22: Agarose gel electrophoretic analysis of pBS/VP7
(a) Size markers SMII and ϕX
(b) pBS/VP7 digested with *Sma* I and *Sal* I

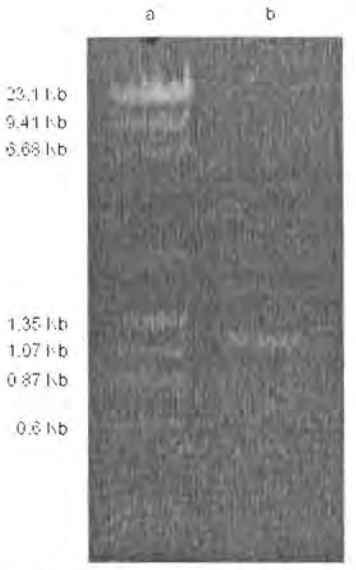

Fig. 3:23 Agarose gel electrophoretic analysis of genecleaned recovered VP7-DNA
(a) Size markers SMII and ϕX
(b) Approximately 150 ng DNA

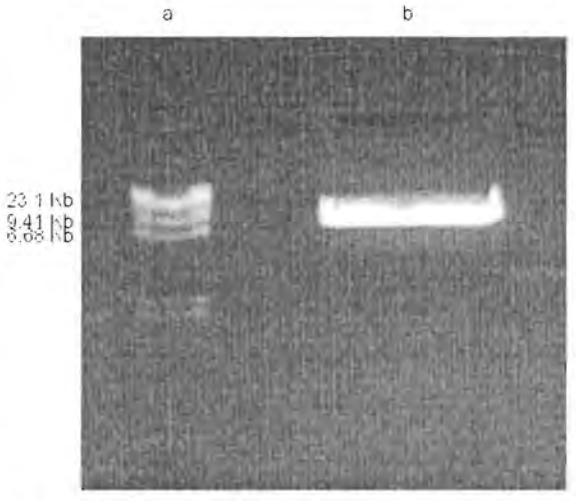

Fig. 3.24: Agarose gel electrophoretic analysis of linearised vector, pHSWF/VP2
(a) Size marker SMII
(b) pHSWF/VP2 digested with *Xho* I

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

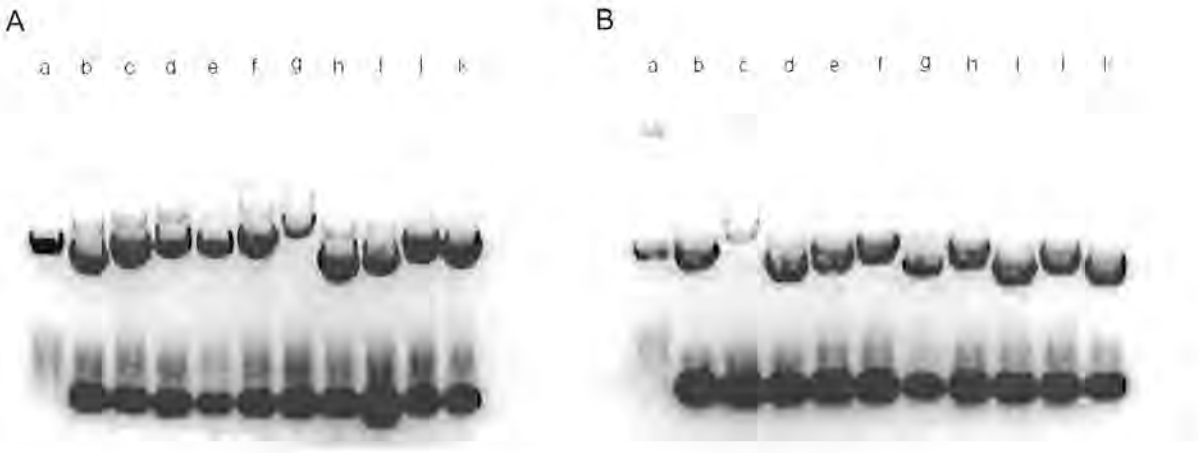

Fig. 3.26 A and B: Agarose gel electrophoretic analysis of putative dual plasmids.

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 an 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.

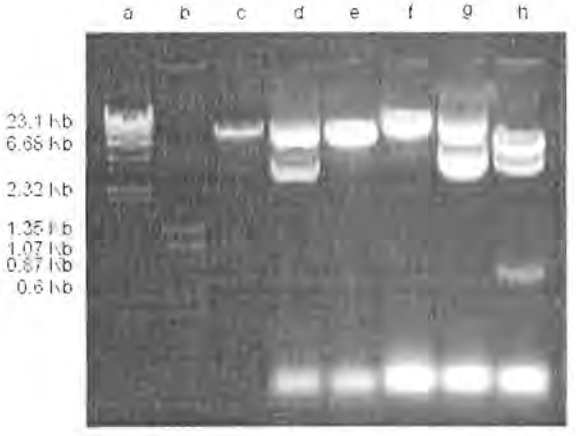

Fig. 3.27: Agarose gel electrophoretic analysis of the dual plasmids digested with *Sac* I to confirm the correct orientation of VP7
(a) Size marker, SMII
(b) Size marker, ϕX
(c)-(h) Selected clones of putative dual pHSWF(VP2/VP7) plasmids digested with *Sac* I

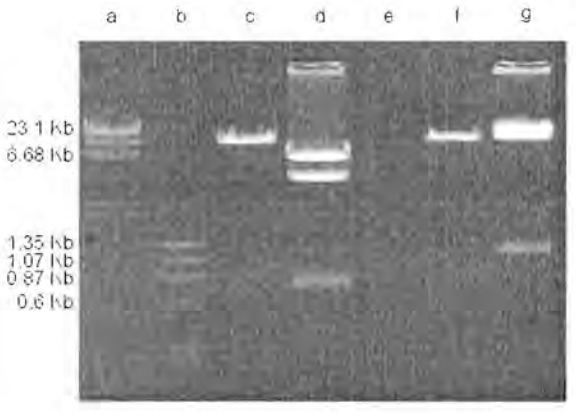

Fig.3.28: Agarose gel electrophoretic analysis of a putative dual plasmid to confirm cloning of the full-length VP7 gene in the correct orientation
(a) Size marker, SMII
(b) Size marker, ϕX
(c) pHSWF/VP2 digested with *Sac* I
(d) pHSWF(VP2/VP7) digested with *Sac* I
(e) Unloaded
(f) pHSWF/VP2 digested 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	Tm $4(GC)_n + 2(AT)_n$	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

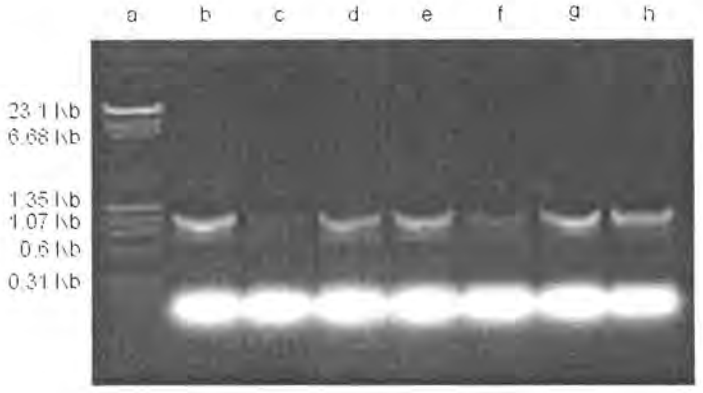

Fig. 3.29: Agarose gel electrophoretic analysis of putative dual LSDV(VP2/VP7) recombinants. PCR amplification using the VP7-specific primer-set, SON2a and SON2b

(a) Size markers, SMII and ϕX

(b)-(h) Putative LSDV(VP2/VP7) containing VP7

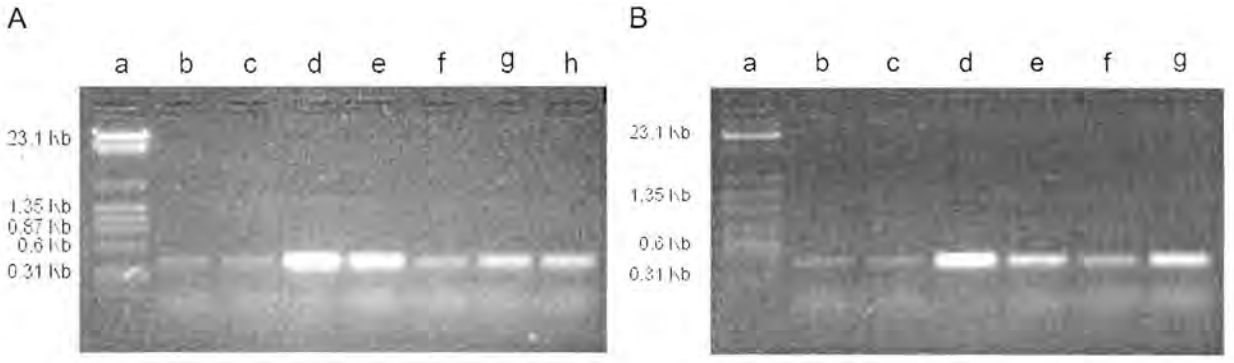

Fig. 3.30 A and B: Agarose gel electrophoretic analysis of putative dual LSDV(VP2/VP7) recombinants. PCR amplification using the VP2-specific primer-set. The following apply for fig. 3.30 A and B:

(a) Size markers, SMII and ϕX

(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)

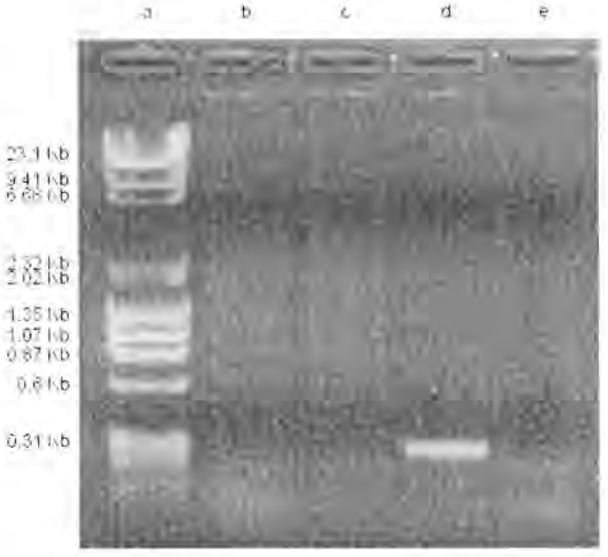

Fig. 3.31: Amplification of the specific VP2-fragment to determine the external contamination in the DNA of the uninfected cells and wt LSDV
(a) Size markers, SMII and ϕX
(b) Uninfected FBT-cells
(c) wt LSDV DNA
(d) pHSWF/VP2 as positive control
(e) PCR-reaction using all the PCR-reagents without any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

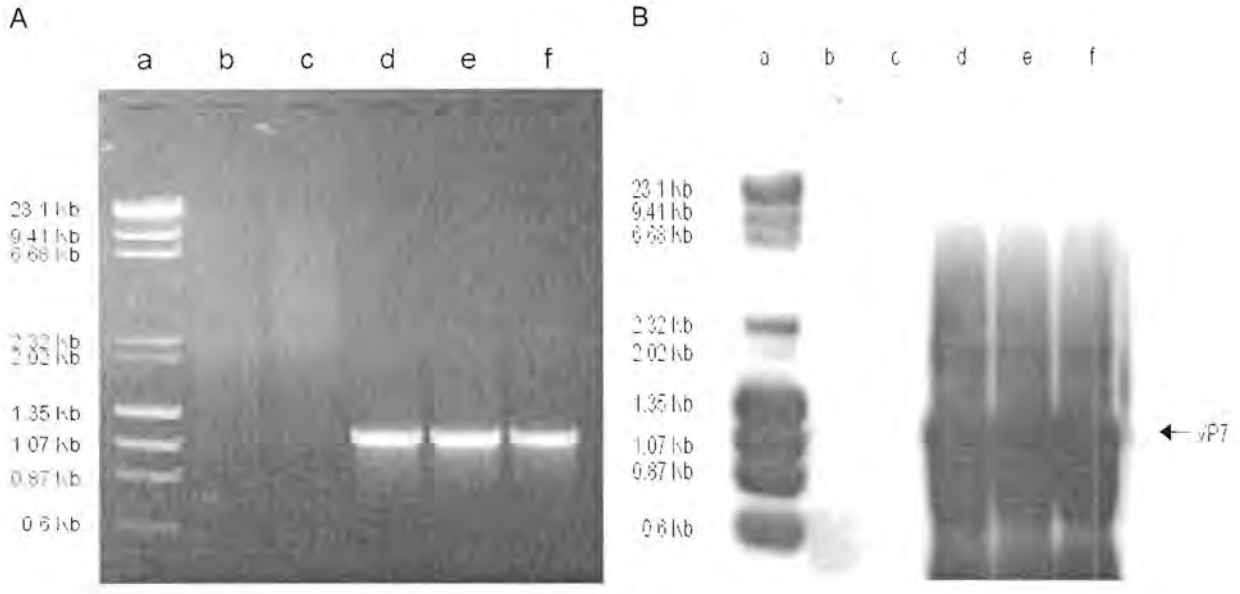

Fig. 3.32 A and B: Confirmation of the presence of the AHSV-9 VP7 gene in the selected putative dual LSDV recombinants. The primer-set (SON2a and SON2b) was used for amplification of the VP7-gene. The PCR products were analysed on a 1% agarose gel (A) followed by Southern blot hybridisation (B).

The following apply for both figures

(a) Size markers, SMII and ϕX

(b) Uninfected cells

(c) wt LSDV DNA

(d) pHSWF/VP7 as positive control

(e)-(f) Putative dual LSDV(VP2/VP7) recombinants

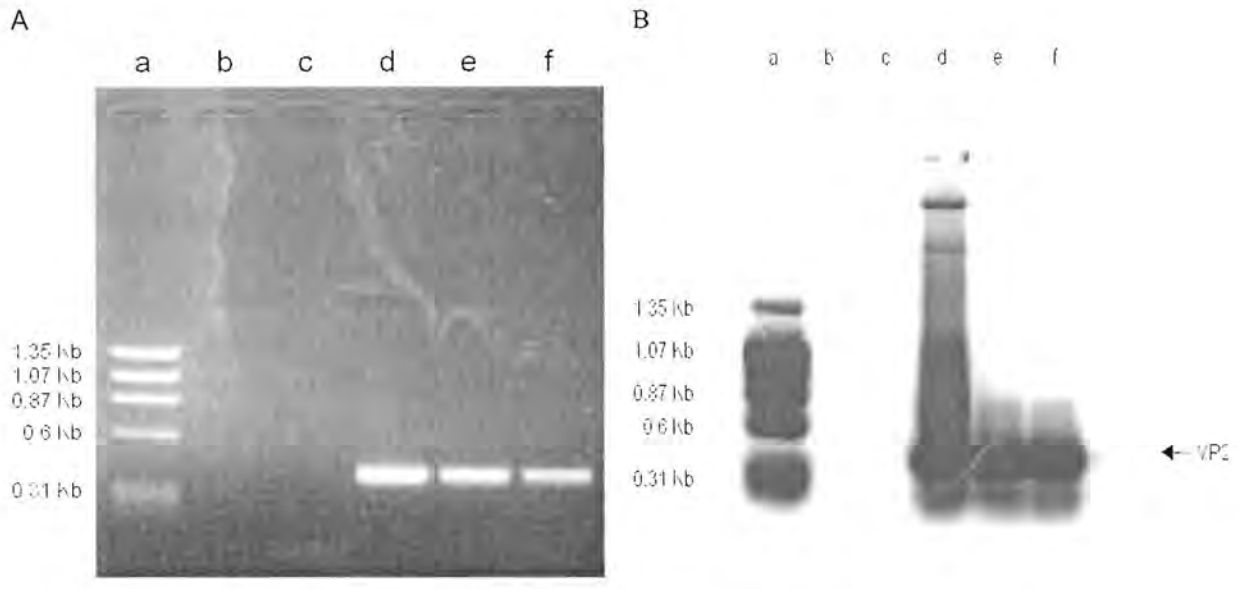

Fig. 3.33 A and B: Confirmation of the presence of the AHSV-9 VP2 gene in the selected putative dual LSDV recombinants. The primer-set AHSV-9 VP2 (for and rev) was used to amplify the specific VP2-fragment. The PCR products were analysed on a 1% agarose gel (A) followed by Southern blot hybridisation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

The following apply for both figures

(a) Size marker, ϕX

(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 ^{35}S-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/*Lac*E (*LacZ* placed under control of the early promoter pA8R) and pHSWF/*Lac*L (*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/*Lac*E and LSDV/*Lac*L) 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 ^{35}S 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 ^{35}S 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 ^{35}S 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 ^{35}S-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.