Characterisation of promoter sequences

in a Capripoxvirus genome

by

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A thesis submitted in partial fulfilment
of the requirements for the degree of
Master of Science (Medical Microbiology)
in the Faculty of Medicine,
University of Cape Town.

Pretoria June 1992

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dedicated to my husband, Kobie

and children Kobus and Helenè

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation and thanks to:

Dr. A. A. van Dijk, for her guidance, valuable criticism and encouragement throughout this study and the writing of the thesis.

Prof. K.R. Dumbell, for his continual interest, advice and encouragement.

Dr. G. J. Viljoen, for his valuable advice and support.

Dr. D. W. Verwoerd, Director of the Onderstepoort Veterinary Institute, for permission granted to carry out this study and to use the results for this thesis.

Dr. D.H. du Plessis, for proofreading parts of the thesis.

All my colleagues at the Biochemistry and Molecular Biology Sections of OVI, for their interest.

The staff of the Photography Section at OVI, for assistance with preparation of the figures.

My family and friends, for their constant encouragement.

My husband, Kobie, for his support, encouragement and help with our children, Kobus and Helene.

I acknowledge a bursary from the Department of Agriculture, Pretoria.

SUMMARY

CHARACTERISATION OF PROMOTER SEQUENCES IN A CAPRIPOXVIRUS GENOME

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Capripoxviruses are of particular interest as live recombinant vectors for use in the veterinary field, since their host-range is restricted to cattle, goats and sheep. The work presented in this thesis is a preliminary study undertaken on the South African Neethling vaccine strain of lumpy skin disease virus (LSDV).

As a departure point towards the eventual identification of strong promoter areas in the 143 kb genome of LSDV, a portion of its genome was cloned. Three methods for purification of LSDV DNA were compared, to determine which yielded the best quality DNA for cloning. DNA extracted directly from infected cells was excessively contaminated with bovine host-DNA, complicating the cloning of LSDV DNA. The use of pulsed field gel electrophoresis solved the contamination problem, by separating viral DNA from bovine DNA. However, insufficient amounts of viral DNA for cloning purposes, could be recovered from the gel. Sufficient amounts of good quality LSDV DNA was

obtained by extraction from purified virions. Purified LSDV DNA was digested with various restriction enzymes to identify those which yielded several 4-10 kb fragments, for cloning into the Bluescribe plasmid transcription vector. Enrichment for large fragments (8-10 kb) was achieved by sucrose density centrifugation. Cloned fragments were analysed by Southern blot hybridisation to verify their viral origin. Hybridisation studies indicated that several unique regions of the LSDV genome were cloned as Pst I and Bam HI fragments respectively, i.e. the cloned fragments contained no overlapping regions. In total, 71.25 kb of the DNA of the LSDV Neethling vaccine strain has been cloned, representing approximately 50% of the viral genome. The availability of these clones now paves the way for further molecular investigations of the LSDV Neethling genome, including identification of promoter regions.

A trial gene, which will be cloned and expressed in LSDV, namely the cloned VP5-gene of bluetongue virus serotype 4, was prepared and its nucleotide sequence determined. Homopolymer sequences present at the terminal ends of the gene as a result of the original cloning strategy, are known to interfere with expression and were removed by means of the polymerase chain reaction (PCR). The nucleotide sequence of the resulting PCR-tailored BTV4 VP5-gene was determined and used to deduce the amino acid sequence of the protein. The gene is 1638 bp in length and encodes a protein of 526 aa. Conserved sequences, 6 bp in length and unique to the 5'- and 3'-terminal ends of all BTV genes, were detected at the termini of the tailored gene, confirming that the original clone was a full-length copy of the gene. Amplification by PCR did not mutate the open reading frame (ORF) of the gene, since it was of similar length to that reported for 5 other BTV serotypes.

With a view to future investigations, including the identification of promoter sequences in the LSDV genome, a preliminary investigation of LSDV protein synthesis was undertaken, to acquire some knowledge of the growth cycle of the virus. Eighteen putative virus-specific proteins were identified by radio-labelling infected cells with [^{35}S]-methionine. By pulse-labelling infected cells with [^{35}S]-methionine at various times post infection (p.i.), viral proteins were first detected at 16 hr p.i. It is, however, unlikely that the early phase of viral replication commences as late as 16 hr p.i. and these results might be attributed to various problems, such as the low multiplicity of infection used and that host protein shut-down was inefficient, thus masking the presence viral proteins.

In conclusion, this investigation resulted in the cloning of 71,25 kb of the LSDV genome, the tailoring and sequencing of the BTV4 VP5 gene and the identification of 18 putative LSDV proteins. This now paves the way for further research to develop LSDV as a vaccine vector.

ABBREVIATIONS

aa	-amino acid
amp	-ampicillin
ATP	-adenosine triphosphate
BCG	-Bacillus Calmette-Guerin
bp	-base pairs
BTV	-bluetongue virus
BUdR	-5-bromo-deoxi-uridine
β-ME	-beta-mercaptoethanol
CAR	-cytosine arabinoside
ccc	-covalently closed circular
cDNA	-complementary deoxiribonucleic acid
CFK	-calf fetal kidney
cm	-centimeter
CTP	-cytidine triphosphate
°C	-degrees Celsius
dATP	-2'-deoxyadenosine-5'-triphosphate
dCTP	-2'-deoxycytidine-5'-triphosphate
dGTP	-2'-deoxyguanosine-5'-triphosphate
dTTP	-2'-deoxythymidine-5'-triphosphate
DTT	-dithiothreitol
DNA	-deoxyribonucleic acid
DNAse	-deoxyribonuclease
ds	-double stranded
EDTA	-ethylenediaminetetra-acetic acid
EHDV	-epizootic haemorrhagic disease virus
EtBr	-ethidium bromide
FCS	-fetal calf serum
ffu	-focus forming unit
Fig.	-figure
FPV	-fowlpox virus
g	-gravitational force
gpt	-xanthine-guanine phosphoribosyl transferase
GTP	-guanosine triphosphate

h	-hour
HBsAg	-hepatitis B virus surface antigens
HIV	-human immunodifficiecy virus
HSV	-herpes simplex virus
IPTG	-isopropyl-3-D-galactopyranosid
ITR	-inverted terminal repetition
K	-kiloDalton
kb	-kilobase pairs
LB	-Luria broth
LSD	-lumpy skin disease
LSDV	-lumpy skin disease virus
M	-molar
mA	-milliAmpere
mCi	-milliCurie
MCS	-multiple cloning site
MDBK	-Madin Darby bovine kidney
mg	-milligram
min	-minute
ml	-millilitre
mM	-millimolar
m.o.i.	-multiplicity of infection
MPA	-mycophenolic acid
mRNA	-messenger ribonucleic acid
NLS	-N-lauryl sarcosinate
nm	-nanometer
nt	-nucleotide
OD_{590}	-optical density at 590 nanometer
ORF	-open reading frame
PAGE	-polyacrylamide gel electrophoresis
PCR	-Polymerase Chain Reaction
PEG	-polyethylene glycol
pfu	-plaque forming units
p.i.	-post infection
RB	-roller bottles
r.e.	-restriction endonuclease

R_f	-relative migration distance
RNA	-ribonucleic acid
RNAse	-ribonuclease
rpm	-revolutions per minute
RPV	-rinderpest virus
s	-second
SDS	-sodium dodecyl sulphate
SPV	-swinepox virus
tet	-tetracycline hydrochloride
TK	-thymidine kinase
Tris	-Tris-(hydroximethyl)-aminoethane
ts	-temperature sensitive
TX-100	-Triton X-100 (polyethyleneglycol (9-10)-p-t-octylphenol
μCi	-microCurie
μg	-microgram
μl	-microlitre
UTP	-uridine triphosphate
UV	-ultraviolet
V	-volt
VEE	-Venezuelan equine encephalitis virus
VV	-vaccinia virus
WHO	-World Health Organization
X-gal	-5-Bromo-4-chlor-3-indolyl-3-D-galactopyranoside

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CHAPTER ONE

LITERATURE REVIEW

1.1 INTRODUCTION

Recent advances in recombinant DNA technology have allowed vaccinologists to enter a new era in vaccine development. For many human and veterinary diseases, a vaccine (or an improved one), is still needed and may only be developed by the application of the newer biotechnology. These diseases include those for which an *in vivo* produced reagent is undesirable (e.g. infant mouse brain extracts), those for which the etiological agent cannot be cultured *in vitro* and human acquired immuno-deficiency syndrome (AIDS). Possible future developments include the following:

- the development of biosynthetic vaccines, defined as formulations containing non-infectious protective subunit immunogens that are produced by the use of a biological system.
- the use of non-infectious immunogens in the form of synthetic peptides in vaccines
- employing different micro-organisms as vectors for the delivery and expression of foreign antigens to a variety of systems

Various recombinant subunit and live vaccines are being investigated to supplement or replace vaccines currently being used in the fight against disease. One of the main advantages of live versus subunit or dead vaccines is that they may elicit life-long immunity with a single dose. The acceptance of recombinant vaccines will depend on evidence of their efficacy and safety. Especially if such a vaccine is intended for use in man, then adverse effects must be kept to a minimum. Well documented background information on recombinant vaccines is thus essential.

Lumpy skin disease virus (LSDV), a member of the Poxviridae family, has the potential to be developed as a recombinant live vector for veterinary vaccine purposes. At the Onderstepoort Veterinary Institute, improved vaccines for the Orbiviruses, including African Horsesickness virus (AHSV) and Bluetongue virus (BTV), are being investigated. These are economically important pathogens of sheep and horses in many parts of the world. The expression of relevent Orbivirus genes in a LSDV vector may offer possible solutions.

BTV is the prototype of the Orbiviruses, which are members of the *Reoviridae* family. The genome of BTV consists of 10 double stranded RNA segments (Els and Verwoerd, 1969), each of which encodes a single viral protein (Mertens *et al.*, 1984; Van Dijk and Huismans, 1988). The virion consists of a core of icosahedral symmetry composed of two major proteins (VP3 and VP7) enclosing three minor proteins (VP1,VP4 and VP6), together with the 10 RNA segments (Verwoerd and Huismans, 1972). This core particle is enclosed by a diffused outer layer, the capsid, which consists of polypeptides VP2 and VP5. Three non-structural proteins are also synthesized in infected cells, but these are not incorporated into the virion (Huismans, 1979).

1.2 POTENTIAL ADVANTAGES OF A LIVE RECOMBINANT VACCINE

The development of live recombinant vaccines which involve viral or bacterial vectors containing foreign genes have many potential advantages which make them preferable to subunit vaccines. General advantages of a live vaccine include the following:

- it may mobilize different aspects of the immune system (humoral as well as cell-mediated immunity).
- it may provide long-term (even life-long) immunity with a single dose.
- there is no need for the addition of carriers, activators or adjuvants.
- since only the essential part of the pathogen is used in the creation of a live recombinant vaccine, growth of the pathogen in the host can easily be avoided.
- several genes from various pathogens could be combined in the same vector, making vaccination against several diseases possible with a single immunisation.
- once a suitable vector has been developed, the same technology could be applied to other vaccines.
- the possible development of heat stable vectors could lengthen the shelf-life of such a vaccine.

Each of these attributes needs to be addressed in research to develop vectors for vaccine purposes.

1.3 PROFILE OF AN IDEAL LIVE VECTOR

Any effective vaccine must stimulate a protective immune response, antibody or cell-mediated, against the appropriate antigens and offer complete protection for the lifetime of the animal or human. An ideal vector used in the preparation of recombinant vaccines must be safe, efficacious and easy to manipulate. Furthermore, the host's immune response to the vector must be fully understood.

Major safety issues, especially when considering human vaccines, include the nature of the side effects and the risk associated with the immunisation of immuno-difficient or immuno-compromised individuals. The live vector should not integrate into the DNA of the host genome and should be infectious for the target population only. As far as efficacy is concerned, the vaccine should offer long-term protection and induce an effective immunity which would include stimulation of both specific T- and B-cell responses. It should also generate as large a pool of memory cells as possible. The optimal route of administration should be explored and evaluation done in the most appropriate animal model.

In constructing stable live vectors, aspects such as high level expression promoters, the most convenient insertion sites for stability and DNA-carrying capacity of the vector are to be considered. Finally, in order for a new vaccine to be utilized in the field, it would have to be incorporated into existing immunisation programmes at low cost and should not interfere with existing vaccines.

1.4 AIMS OF THIS INVESTIGATION

In order to develop a virus as a possible live recombinant vector, considerable knowledge of its molecular aspects has to be acquired. Most of the advances in this field have been based upon extensive knowledge gained on vaccinia virus (VV) over many years. Because of several drawbacks associated with the use of VV as a vector (see section 1.6.2), LSDV is considered as a possible alternative.

Very little is however known about the molecular aspects of LSDV. The little that is known, is based upon the Kenyan cattle strain which differs from the South African vaccine strain in severity of the disease and restriction enzyme sites (see section 1.10.2 and 1.10.7). The South African vaccine strain has been in use for over 30 years and field experience has proven it to be both safe and effective.

This is a report of preliminary studies on the molecular biology of the S.A. vaccine strain of LSDV. Several lines of investigation have however been undertaken in parallel, rather than pursuing one to completion, partly to gain wide methodological experience.

The aims of this investigation were:

(a) the preparation and preliminary analysis of LSDV DNA.

(b) cloning of parts of the genome for future analysis, mapping with restriction enzymes and eventual sequencing of regions of importance.

(c) investigating the time course of viral protein production and viewing the size and time of expression of the most abundantly expressed proteins.

(d) preparation and sequencing of a "trial" gene for insertion into LSDV from a preliminary clone of Bluetongue virus serotype 4 segment 5 (BTV4VP5), that encodes the outer capsid protein VP5.

1.5 GENERAL CHARACTERISTICS OF POXVIRUSES

These characteristics are based on vaccinia virus as prototypical member of the poxvirus group.

Table 1.1 Classification of the family Poxviridae *

Subfamilies	**Genera**	**Some members**
Chordopoxviridae (vertebrate poxviruses)	*Orthopoxvirus*	Vaccinia, variola
	Parapoxvirus	Orf
	Avipoxvirus	Fowlpox, canary pox
	Capripoxvirus	Goatpox, sheeppox, lumpy skin disease
	Leporipoxvirus	rabbit (Shope) fibroma
	Suipoxvirus	Swinepox
	Molluscipoxvirus	Molluscum contagiosum
	Yatapoxvirus	Tanapox
Entomopoxviridae (insect poxviruses)	A	*Melontha melontha*
	B	*Amsacta moori*
	C	*Chironimus luridus*

* Taken from Moss, 1990.

1.5.1 Virion structure

Poxvirus virions are the largest (approximately 300 x 240 x 100 nm in size) and structurally the most complex of all animal viruses. Virions have 4 major components : a dumbell-shaped core that contains the DNA genome, lateral bodies lying in the concavity of the core, an outer membrane and a surrounding envelope (Dales and Pogo, 1981, Fenner *et al.*, 1987, 1988) .

1.5.2 The poxvirus genome

The genomic DNA of poxviruses is a very large linear double stranded (ds) molecule which ranges in size from 110 kb (parapoxvirus) to 145 kb (capripoxvirus), 165 kb to 210 kb (orthopoxviruses) and 280 kb (avipoxviruses). Both ends of poxvirus DNA are covalently closed (Geshelin and Berns, 1974) forming incompletely base-paired hairpin loops at each end of the genome (Baroudy *et al.*, 1982). The sequence at the two ends of the genome are identical for about 10 000 bp (Garon *et al.*, 1978), forming inverted terminal repetitions (ITRs). Within vaccinia virus ITRs, 200 bp from each end, are tandem repetitions arrayed in adjacent sets (about 1 kb each) that are separated by short intervening sequences of about 400 bp each (Wittek *et al.*, 1978). Hypervariability of the ITRs suggests that this region contains most non-essential genes.

It has been found that the length of the VV genome can vary considerably, whilst retaining the ability to be replicated or packaged. For example, a viable 9 kb deletion mutant of VV has been isolated (Panicali *et al.*, 1983) as well as rabbitpox mutants with even larger deletions (Moyer and Roth, 1980). Intact VV DNA molecules can be isolated, but the DNA is not infectious (Sam and Dumbell, 1981).

1.5.3 Replication

DNA replication takes place in the cytoplasm of the host cell, which is unique to poxviruses. The poxvirus virion adsorbs and penetrates a cell directly via plasma membrane fusion or indirectly via endophagocytosis (Esposito and Murphy, 1989). Once inside the cell, the virion uncoats, which causes the core to be released into the cytoplasm.

Viral cores carry a complete transcription system, that produces early messenger RNA (mRNA)

during early stages of infection. This includes the viral DNA polymerase and transcription enzymes. Soon after infection a shutdown of host-cell protein synthesis then occurs. Poxvirus replication can be divided into distinct early and late stages, that flank the peak of DNA replication. During the later stages, (when viral DNA has started to accumulate), virion structural protein synthesis takes place upon which new virions are assembled. The complete DNA sequence of the VV genome and the open reading frames it contains, has been determined (Goebel *et al.*, 1990). A total of 263 potential protein-coding regions were then identified.

1.5.4 Expression of the genome

Both DNA strands are transcribed via a distinctive RNA polymerase and other RNA-processing enzymes (for capping, polyadenylation, etc.) soon after VV cores are released into the cytoplasm (Esposito and Murphy, 1989). Transcripts can be classified as being early, late and early + late relative to viral DNA replication (Moss, 1985). RNA from opposite DNA strands anneal very late in infection.

About half of the genome is expressed before replication starts (Paoletti and Grady, 1977, Boone and Moss, 1978) and there appears to be about 100 of these early genes distributed throughout the length of the DNA (Belle Isle *et al.*, 1981). Most, if not all VV mRNAs are polyadenylated (Nevins and Joklik, 1975) and all genes examined thus far have continuous coding segments, with no evidence of RNA splicing.

Following the onset of DNA replication, the late class of genes is expressed and many of the early genes are no longer functionally active. Early mRNAs are of relatively uniform size, but the 3' ends of late mRNAs are extremely heterogeneous and thus they vary in size. For example, the mRNAs for cowpox virus A-type inclusion protein and VV structural II kDa protein have variable A-rich leader sequences (Bertholet *et al.*, 1987, Schwer *et al.*, 1987, Patel and Pickup 1987). Although late genes are also distributed throughout the genome, they appear to be concentrated in the central region (Belle Isle *et al.*, 1981).

Signals for the termination of transcripts at the 3' end of poxvirus open reading frames appear to be unevenly spaced tandem TTTTTNT repeats (Rohrmann *et al.*, 1986). Transcriptional signals (promoters) for RNA polymerases are AT-rich sequences located immediately upstream of coding sequences. Consensus TATA and AATA promoter sequences have been identified for several early and late VV genes (Plucienniczak *et al.*, 1985).

1.6 VACCINIA VIRUS AS A VIRAL VECTOR

1.6.1 Advantages

As early as 1796, Jenner discovered that immunisation against the deadly disease smallpox (caused by variola virus), could be performed by inoculating uninfected individuals with pustular fluid from pox lesions, originating from the teats of cows (Jenner, E., 1798). Successful repetition of this practice, originally called cowpoxing, caused the practice to continue for almost 200 years. The term vaccination was then proposed and the inoculum became kown as vaccinia virus. Vaccinia virus was subsequently used extensively as a vaccine during the WHO Smallpox Eradication programme, and as such smallpox became the first and only viral disease to be eradicated worldwide. This programme has lead to much experience on the clinical, laboratory and epidemiological aspects of vaccine usage as well as gaining extensive knowledge of the virus. The vaccine is administered as a single dose which induces relatively long-lived immunity (Henderson and Arita, 1985), is simple to manufacture and inexpensive to test and the vaccine is very stable (the freeze-dried vaccine can withstand 37°C for 30 days).

The potential advantages of recombinant vaccinia virus vectors include the fact that they represent a live virus infection, which stimulates both humoral and cellular immunity. The virus has a large genome (approximately 180 kb) of which significant portions may be deleted without loss of viral infectivity. It has been found that at least 25 kb of exogenous DNA can be inserted into vaccinia virus (Smith and Moss, 1983). Theoretically it thus has the potential to carry several heterologous genes, originating from various disease-causing agents. Such a vaccine would approach the ultimate ideal vaccine both for human and veterinary purposes, being able to immunise against multiple diseases with a single immunisation, offering life-long protection.

1.6.2 Disadvantages

Primary vaccination of humans with vaccinia virus causes a more severe local and general reaction than any other live virus vaccine currently in use. In individuals with severe T-cell - or combined immuno-deficiencies, progressive vaccinia which is fatal, can occur (Arita and Fenner, 1985). Vaccinia virus is also associated with central nervous system complications, notably vaccinia encephalitis, which occurs very rarely, but has a high mortality rate. Vaccination of persons with

eczema can lead to eczema vaccinatum, but this can be treated with vaccinia- immunoglobulin (Arita and Fenner, 1985).

If vectored vaccinia virus vaccines are to be introduced for human use, a substantial reduction of adverse effects could be achieved with appropriate prevaccination screening (Esposito and Murphy, 1989). The vector strains may well be less virulent than those originally used for vaccination. Veterinary use of vectored vaccinia virus vaccines would involve a minimal risk of adverse effects in humans. The principal risk would be the accidental inoculation of vaccinators and other animal handlers. Still, this would be of little consequence if vaccinators were prescreened and vaccinated accordingly.

1.7 POXVIRUS VECTOR CONSTRUCTION

1.7.1 Gene transfer by marker rescue

Because of the large size of the poxvirus genome, genetic recombination using DNA ligation is not possible. The non-infectious nature of the DNA also prevents its propagation by transfection of uninfected cells. These difficulties had to be overcome in order to develop VV as an infectious recombinant vectored virus. Genetic recombination studies, which involved gene transfer by marker rescue (Sam and Dumbell, 1981), led directly to the discovery that foreign genes can be inserted in the poxvirus genome by homologous DNA recombination (Mackett *et al.*, 1982).

Genetic recombination studies were first done by co-inoculation of two inactivated poxviruses into animals or cell cultures (Fenner, 1970, 1979). This involved rescue of a protein- inactivated poxvirus by a poxvirus in which the DNA was inactivated (see Fig. 1.1 A).

In 1981, Sam and Dumbell reported the recovery of rabbitpox virus when its DNA was transfected into ectromelia virus-infected cells. Also, when they transfected DNA restriction fragments of the rabbitpox strain of VV into cells already infected with a phenotypically different VV, recombinants with both rabbitpox and VV phenotypic markers were recovered (see Fig. 1.1 B and C). Several reports which confirmed and extended this concept, followed when Condit *et al.*, 1983, Nakano *et al.*, 1982 and Weir *et al.*, 1982 identified physical loci of temperature sensitive (ts) mutations, reconstituted a VV deletion mutant and mapped the VV TK locus.

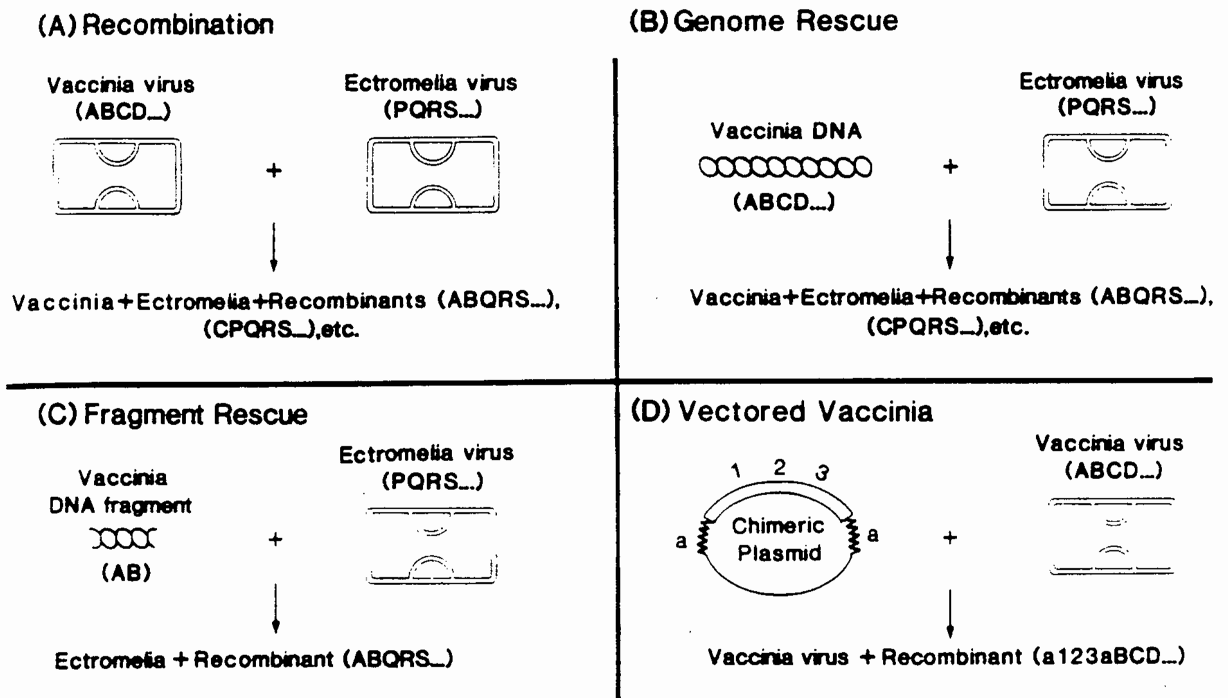


Figure 1.1
Steps in the historical development of the use of vaccinia virus as an expression vector for heterologous DNA sequences.

(A) Demonstration of recombination between vaccinia and ectromelia viruses by co-infecting cells with both viruses producing vaccinia, ectromelia and recombinant viruses. (B) Rescue of vaccinia virus genome in cells transfected with intact VV DNA and infected with ectromelia virus or temperature-sensitive mutants of VV to produce vaccinia, ectromelia and recombinant viruses. (C) Marker rescue of fragment of VV DNA by homologous DNA recombination in cells transfected with VV fragment (marker$^+$ DNA) and infected with marker$^-$ virus. (D) Targeted insertion of heterologous coding sequences into VV genome by homologous DNA recombination. Chimeric plasmid DNA (a) that contains VV DNA flanking the heterologous DNA (2) is transfected into cells already infected with VV. VV regulatory elements (1), VV transcript termination elements (3). (Taken from Esposito and Murphy, 1989.)

The first infectious vectored VV recombinants were subsequently produced by Panicali and Paoletti, (1982) and Mackett *et al.*, 1982 when they developed methods for inserting heterologous coding sequences into VV by homologous recombination.

1.7.2 Construction of a recombinant vaccinia virus vector

Two approaches for the expression of foreign genes in VV have been developed. The first consists of inserting foreign DNA into available r.e. sites within non-essential regions of the VV genome (*Panicali* and Paoletti, 1982). Although the method is technically simple, the procedure limits the promoters used, since the foreign DNA is under control of the non-essential genes promoter. This promoter might not necessarily result in optimal expression of the gene. This method can also lead to the formation of fusion proteins with unpredictable properties.

The more commonly used method involves the translocation of defined VV promoters to a special plasmid vector (insertion vector) and lends itself to optimisation of expression, the synthesis of authentic or fusion proteins and the development of a general expression vector system.

For the latter procedure, Mackett *et al.*, 1982 have constructed VV recombinants expressing foreign genes in a two-stage process (Fig. 1.2). Firstly, a series of insertion vectors that contains a chimeric gene flanked by VV DNA, was constructed. The chimeric gene consisted of a VV promoter region, (including the transcriptional start site and upstream regulatory sequences), and genetically engineered restriction endonuclease sites for introduction of the foreign DNA. The flanking VV DNA determines the site at which foreign DNA is inserted in the viral genome. To preserve the infectivity of the virus, the foreign gene must be inserted into a region of the genome that is not essential for viral growth. Several non-essential regions have been identified of which the thymidine kinase (TK) gene, is often used.

In the next stage, the chimeric gene is inserted into VV. This is achieved by transfection of wild-type virus infected cells with the insertion vector. Homologous recombination occurs between the VV sequences flanking the chimeric gene and the viral genome, thus producing a recombinant virus. The foreign DNA segments that have been introduced, typically have their natural initiation and termination codons so that authentic gene products will be formed.

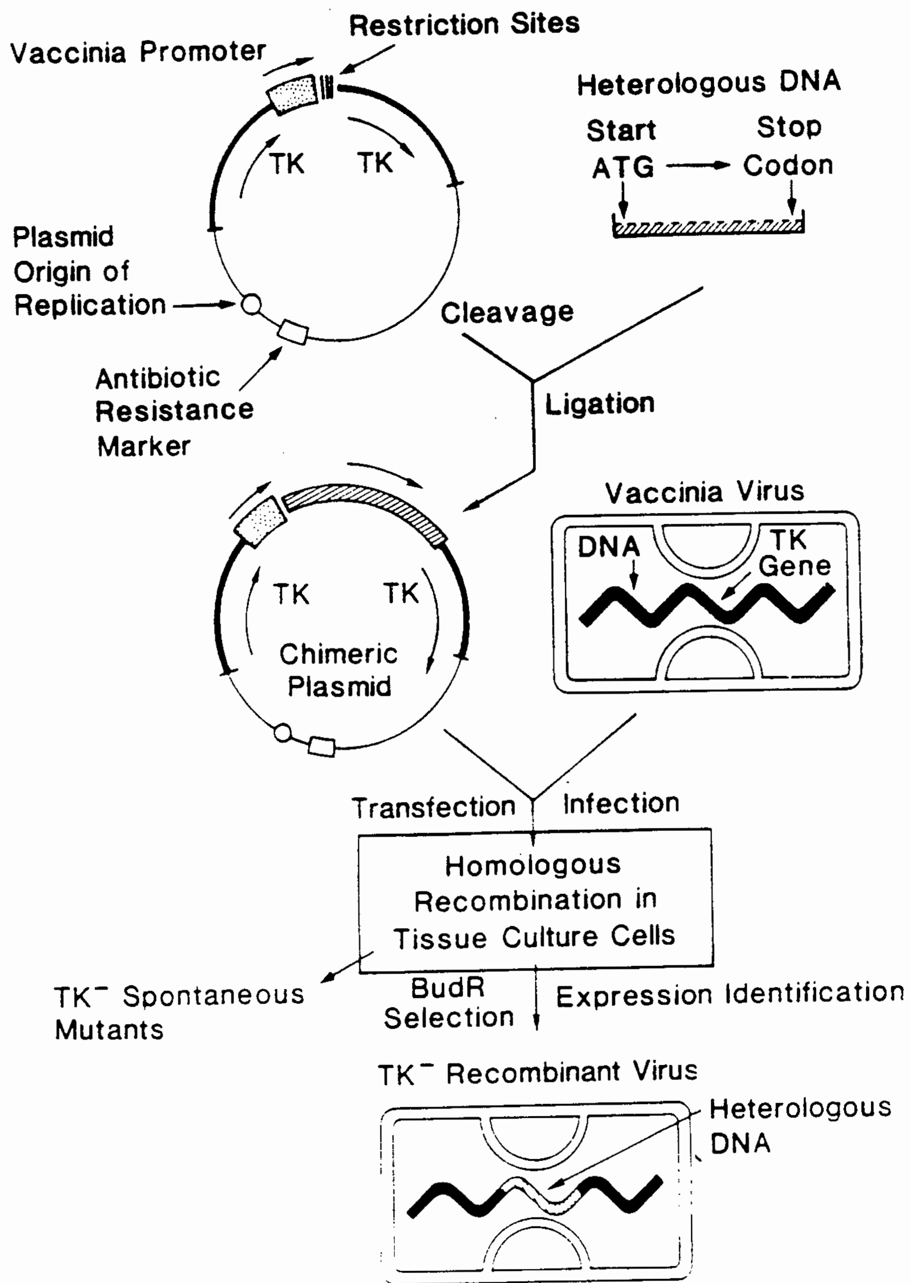


Figure 1.2

Insertion of heterologous DNA coding sequences into VV by insertional inactivation of the virus thymidine kinase (TK) gene. A chimeric plasmid is constructed in such a way that the coding sequences are inserted downstream of a VV transcription site that follows VV promotor sequences; this casette interrupts coding sequences for the VV TK gene. Cells already infected with VV are transfected with the chimeric plasmid before the peak of viral DNA replication. The infection is then allowed to proceed to completion. Three types of progeny virus are produced: wild-type TK^+, spontaneous TK^- mutants and TK^- recombinants. TK^- viruses are isolated by plaque purification using TK^- cells that are maintained in overlay medium containing BUdR. Recombinants are then differentiated from spontaneous mutants by hybridisation and immunological methods using cells infected with virus from individual virus plaques. (Taken from Mackett *et al.*, 1985).

Both the level and regulation of expression of the foreign gene are determined by the promoter used. These promoters can be classified as being early, early/late and late according to the interval post infection, during which a specific gene is expressed. Genes that are controlled by early/late promoters, are expressed throughout an infection and one such promoter, the P7.5 promoter, is commonly used to drive the expression of foreign genes.

1.7.3 Selection of recombinants

With the standard $CaCl_2$-transfection method, only a small percentage (about 0.1%) of virus progeny will be recombinants. A method of selection or screening between wild and recombinant viruses is therefore essential. Various ways of selection have been developed, e.g. plaque hybridisation (Panicali *et al.*, 1983), thymidine kinase (TK)-negative selection (Mackett *et al.*, 1982) and β-galactosidase expression (Chakrabarki *et al.*, 1985). Of these, TK-negative selection has been widely used and is based on the fact that insertion of foreign DNA into the TK-gene, results in loss of TK-activity which can be positively selected for. Disadvantages of this method, however, include requirements for:

(i) special TK-negative VV susceptable cell lines to be used
(ii) the use of a mutagenic selective agent, 5-bromo-deoxi-uridine (BUdR)

In addition, spontaneous TK-mutants arise at a high frequency, necessitating additional steps to distinguish them from recombinants.

Franke *et al.*, 1985, developed a method using the neomycin resistance gene as a dominant selectable marker. A further dominant one-step plaque selection system was developed by Falkner and Moss (1988), based on expression of the *E. coli* xanthine-guanine phosphoribosyl transferase (gpt) gene. Mycophenolic acid (MPA), an inhibitor of purine metabolism, reversibly blocks formation of VV plaques on a variety of cell lines. This inhibition is overcome by co-expression of the foreign gene and gpt-gene and the addition of xanthine and hypoxanthine to the medium. Synthesis of xanthine-guanine phosphoribosyl transferase enables only recombinant viruses to form plaques in the selective medium.

1.8 EXAMPLES OF VECTORED VACCINIA VIRUS EXPERIMENTAL VETERINARY VACCINES

1.8.1 Vaccinia : rabies vaccine

A vaccinia-rabies recombinant (V-RG) in which the surface spike glycoprotein (G) gene of the ERA strain of rabies virus was expressed after insertion into the TK region of Copenhagen VV, was first constructed by Kieny and coworkers (1984). Mice, rabbits (Wiktor *et al.*., 1984), skunks (Tolson *et al.*, 1987), raccoons (Rupprecht *et al.*, 1986) and foxes (Blancou *et al.*, 1986) inoculated intradermally with the recombinant produced neutralising antibodies and were protected against lethal rabies virus challenge. In addition, raccoons and foxes were fully protected via the oral vaccination route.

Safety trials in numerous wild animal species have been carried out: none of these indicated that V-RG was pathogenic (Artois *et al.* 1990) although limited horizontal transmission occurred from vaccinated animals (Rupprecht *et al.* 1988). In 1987 a field trial was carried out in Belgium in a restricted military zone to test V-RG placed in an oral bait, namely chicken heads (Pastoret *et al.*, 1988). No indication of a vaccinia virus infection was found in animals which had taken the baits. Since that trial, the V-RG vaccine has been used in a manufactured bait in more extensive areas of Belgium (Brochier *et al.*, 1991) and in Northern France.

In the USA, where raccoons and skunks are important carriers of the disease, field trials were first carried out on Parramor Island, off the coast of Virginia. Permission has recently been granted to carry out field trials in Pennsylvania. The main rabies threat in South Africa is the domestic dog and similar approaches are currently being investigated for oral vaccination with recombinants in this area.

1.8.2 Vaccinia : Venezuelan equine encephalitis (VEE) vaccine

Kinney and colleagues (1989) produced VV recombinants expressing the 26S polygene of VEE, which was normally cleaved to form all of the virion structural proteins. Intradermally inoculated mice developed VEE virus neutralising antibodies and were protected against an otherwise lethal challenge with virulent virus. The recombinants were as effective as the attenuated-live virus vaccine in inducing cross-protection against challenge with subtypes of VEE virus isolated from outbreaks.

These recombinants are now being developed as candidate vaccines.

1.8.3 Vaccinia : rinderpest vaccine

Rinderpest virus (RPV) is a member of the morbillivirus group and is considered to be one of the most economically important diseases of cattle in Africa, the Middle East and Asia. Because morbilliviruses have a negative sense RNA as genome, VV recombinants that carry a DNA copy (cDNA) of the mRNA for a protein (which plays an important role in protection), the F protein, were constructed (Barett *et al.*, 1989). The F protein was correctly expressed and processed in vitro and recombinants were able to elicit RPV neutralising antibodies in intradermally inoculated rabbits. These rabbits were also protected against lethal challenge with RPV. An experiment in which a number of cattle were inoculated with the RPV-F protein recombinant VV followed soon (Belsham *et al.*, 1989) and in this case, all cattle challenged were also protected. Non-vaccinated control animals developed acute rinderpest and died within 8 days after challenge.

1.9 EXTENSION TO HOST-RANGE RESTRICTED POXVIRUSES

Complications associated with the use of VV (see section 1.6.2) are for many sufficiently severe to argue against the widespread use of VV recombinants as human as well as veterinary vaccines. For veterinary purposes, the risks involve the animal handler, rather than the animal to be vaccinated. In light of this view, an alternative approach which allows the utilization of much experience gained with VV, is being investigated. This is the development of those poxviuses which have host-ranges that are restricted to particular target animal groups and are therefore not capable of replicating and causing disease in humans.

One of these, fowlpox virus (FPV), which is limited to avian species, has been exploited to express a variety of genes. Taylor *et al.* (1988a) were the first to develop a FPV recombinant expressing the haemaglutinin molecule from a highly virulent avian influenza virus. On immunisation of chickens and turkeys, the recombinant offered protection against a lethal challenge with either the homologous or a heterologous influenza virus strain. A strain of the Newcastle disease virus (NDV) has been expressed in the same way (Boursnell *et al.* 1990) and although low levels of antibody was induced, protection in chickens could be demonstrated.

The fact that FPV has been reported to induce a cytotoxic effect not associated with the production of infectious progeny virus in human amnion cells (Burnett *et al.*, 1968), led to experiments in which the rabies virus glycoprotein was expressed under the control of a VV promoter, using FPV as a vector (Taylor *et al.* 1988b), in non-avian species. The level of rabies glycoprotein expression was sufficient to induce a specific immune response in mice, cats and dogs, and the immune response was protective against a live rabies virus challenge.

No evidence could be found that either the parental FPV strain or the recombinant, could induce productive infection in non-avians. This ability provides a built-in safety feature in vaccination procedures, since the potential for transmission to other species or to non-vaccinated individuals would be greatly reduced. The use of FPV as a non-replicating vector may also be indicated in immuno-compromised individuals, who might otherwise be susceptible to generalized infection by a replicating vector (Taylor *et al.*, 1988b). This concept was extended to another avipox virus with the construction of a canarypox-rabies recombinant virus (Taylor *et al.*, 1991), which was significantly better at eliciting neutralising antibodies and was about 100 times more effective in protecting dogs and cats against lethal rabies challenge than the FPV recombinant.

Swinepox virus (SPV), another poxvirus with limited host-range, is also under investigation as a species-specific recombinant vaccine vector. The clinical presentation is that of a mild, self-limiting infection with lesions detected only in the skin and regional lymph nodes. Investigation to the physical, molecular and biological characterization of the virus and development within the host has begun (Massung and Moyer, 1991a and b) and the TK-gene has been isolated (Feller *et al.*, 1991).

1.10 LUMPY SKIN DISEASE VIRUS (LSDV)

1.10.1 Introduction

The promising results in the research of FPV and SPV have prompted interest in the development of Capripoxviruses, of which lumpy skin disease virus (LSDV) is a member, as potential host-range restricted poxvirus vectors. Capripox virus growth is limited to sheep, goats and cattle in those areas of the world where poxvirus diseases of these animals are endemic (Africa, Middle East and Asia).

1.10.2 History

Lumpy skin disease is an acute, subacute or inapparent viral disease of cattle. Other members of the Capripox genus cause sheeppox and goatpox. Lumpy skin disease (LSD) was first described in Northern Rhodesia (Zambia) in 1929 (MacDonald, 1931), after which it spread to Bechuanaland (Botswana) in 1943 (Von Backstrom, 1945) and reached South Africa towards the end of 1944 (Thomas and Marè, 1945), causing a severe outbreak. It was estimated that 75% of the cattle population in South Africa was affected, with up to 75% mortality on some infected premises. Sporadic outbreaks of the disease have recurred since that time.

In 1957, LSD reached epidemic proportions in Kenya (MacOwen, 1959), where the disease was relatively mild with only 10 LSD associated deaths. The LSD outbreak was associated with an outbreak of sheeppox in indigenous fat-tailed sheep. Experiments in which sheep injected with a Kenyan strain of LSDV (Londiani) and cattle injected with a sheeppox strain both developed skin lesions, led to the suggestion that sheep might possibly act as carriers of LSDV (Capstick, 1959). Capstick and Coakley (1961) showed that cattle could be immunised against LSD using a Kenyan strain, Kedong of sheeppox virus. In South Africa, Capripox has never been reported in sheep or goats, although they are kept in close contact with cattle. In other parts of the world, e.g. Bangladesh, India, Yemen and the Middle East, sheeppox and goatpox are the prevalent Capripoxviruses.

Successful vaccination with a live LSD virus is practised in Kenya and South Africa and vaccinated animals have a life-long immunity. A live vaccine based on the Kedong strain of sheeppox (Capstick and Coackley, 1961) has been commercially produced in Kenya for immunisation of sheep, goats and cattle. A local reaction occurs at the site of injection in 10% of cases but produces no ill effects on general health or production. Although no excretion of virus from vaccinated cattle is thought to occur it is generally considered wise to use the vaccine only in sheeppox endemic areas (Davies and Otema, 1981). An attenuated vaccine for use in sheep and goats, based on the sheep isolate 0240, has also been developed (Kitching *et al.*, 1987).

In South Africa, the Neethling strain of LSDV has been attenuated for vaccine purposes by first passaging it in embryonated eggs (20 passages), after which it was adapted to tissue culture. Since the 1960's it has been produced routinely in tissue culture (Weiss, 1968) and used as a vaccine. Approximately 50% of vaccinated cattle show a reaction at the injection site and a temporal fall in milk production may occur. Following vaccination, some cattle have no detectable antibody,

but still become immune. Calves from vaccinated cows have a colostrum derived passive immunity which persists for six months (Weiss, 1968). This may interfere with efficient vaccination of calves less than six months old. No cases of LSD were reported in South Africa during a 15 year period ranging from 1975-1989. However, in 1990 a serious outbreak occured which soon spread throughout the whole of Africa. Lumpy skin disease has not yet been eradicated from any country in which it has appeared.

1.10.3 Epidemiology and transmission

Epidemics of LSD have been associated with the rainy season and locations (along water sources) suggested transmission in cattle by insect bite. The virus has been recovered from the flies *Stomoxys calcitrans* and *Biomyia fasciata* (Kitching and Mellor, 1986). The virus may be transmitted directly or indirectly through water contaminated with infective saliva (Weiss 1968).

1.10.4 Clinical signs

The initial clinical signs of cattle naturally infected with lumpy skin disease virus are lachrymation, salivation, loss of appetite, nasal discharge and a febrile response following an incubation period of 2 to 5 weeks. Eruption of circumscribed, firm, round and raised nodules (0.5 to 5cm in diameter) occurs in the skin that may involve the entire body, within 48 hours of the first rise in temperature (Weiss, 1968). Soft, yellowish-grey nodules may appear in the mucous membranes of the mouth, nose, vulva and prepuce which give rise to erosions and ulcers. In addition, lymphadenitis, especially of the prescapular, precrural and subperotid, is always observed. The nodules usually undergo complete necroses and form ulcers. The overlaying exudate becomes inspisated and the dried scabs which are formed are shed in 3 to 5 weeks. Healing is slow, as the focal inflammatory reaction may persist for several months and then complete scars are left.

Although the mortality rate is low (less than one to ten percent), the disease is of major economic importance through indirect losses resulting from emaciation, temporal or permanent cessation of milk production in lactating cows (Weiss, 1968), infertility in bulls and permanent damage to hides, which affects the quality of the leather (Green, 1959). Recovered animals develop a life-long immunity and are resistant to re-infection. Their sera contain neutralising antibodies which persists for at least 5 years (Weiss, 1968). Passive immunity acquired by calves through colostrum may

persist for 6 months.

1.10.5 Propagation of LSDV

LSDV replicates in a variety of cell cultures, including lamb and calf kidney, adrenal, testes, calf muscle, rabbit fetal kidney and baby hamster kidney cells (Prydie and Coackley, 1959). The cells most frequently used for isolation are primary lamb testis (LT), lamb kidney (LK), calf kidney and calf testicle cells. LSDV also replicates in embryonating chicken eggs without producing remarkable lesions (Van Rooyen *et al.*, 1959).

1.10.6 Antigenic relationship between strains of Capripoxviruses

Although the Capripoxviruses are classified according to whether they cause clinical disease in sheep, goats or cattle, most strains do not have absolute host specificity (Capstick 1959, Kitching and Taylor, 1985). The majority of those examined did however, show a preference for growth in either sheep, goats or cattle. Strains also varied in their pathogenicity in different species and different breeds.

Serological as well as cross-protection studies indicated a close antigenic relationship between isolates from all three host species. Direct and indirect fluorescent antibody and serum neutralisation tests could not distinguish the viruses of LSD and Kenya sheep-and goatpox from each other or from sheeppox and goatpox strains from the Middle East (Davies and Otema, 1981). Results of cross-neutralisation tests (Kitching and Taylor, 1985) showed that immune serum from Yemen, India and Nigeria isolates of sheeppox and goatpox were equally effective in neutralising homologous and heterologous strains. These results provided support to the suggestion that distinction between sheeppox and goatpox solely on clinical grounds can not be justified.

Another confirmation of the close antigenic relationship was demonstrated by cross-protection between isolates of capripoxvirus, provided by passive immunisation or colostral antibody (Kitching, 1986). Sheep were protected against a virulent Nigerian isolate of sheeppox virus using serum against Yemen goatpox and against virulent Yemen goatpox using serum against Oman sheeppox. Lambs born to ewes hyperimmunised with different isolates of capripox were protected against challenge with Yemen goatpox.

Kitching *et al.*, 1986 labelled proteins from various isolates of sheeppox, goatpox, sheep-and goatpox and LSD (Neethling) viruses with [^{35}S] Methionine to show that the major structural polypeptides of those viruses co-migrated on polyacrylamide gels. The agar gel immunodiffusion test with radiolabelled antigen preparations was used to identify a major common precipitating antigen of 67 kDa, but could not distinguish between the different members of the capripoxvirus group.

1.10.7 Genomic organization and relationship between isolates

1.10.7.1 Restriction enzyme analysis

Different isolates of Capripoxvirus have similar linear DNA genomes of between 143 and 147 kb in length as calculated by summation of the lengths of fragments yielded by the Hind III restriction enzyme (Gershon and Black, 1988).

Restriction enzyme analyses have been used to compare the relatedness of genomes of different Capripoxviruses (Black *et al.*, 1986). In the first study, the DNA of 12 isolates was digested with Hind III and the patterns of fragments generated analysed by agarose gel electrophoresis. It was shown that there is a close degree of similarity between restriction patterns of isolates regardless of animal origin. Based on calculations of Schumperli *et al.*, 1977, Black *et al.,* 1986 concluded that the genome homology of the Capripoxviruses must be greater than 80%. Isolates from different animal species did however, have specific differences in their patterns, so that a sheeppox group and a goatpox group could be distinguished.

The extension to maps of other restriction enzymes, notably Ava I and Sal I, demonstrated that the genome of the Kenya cattle isolate KC-1 does not posess a high degree of nucleotide sequence homology with genomes of either Orthopox-, Parapox-, Leporipox- or African swine fever viruses (Gershon and Black, 1987).

The degree of nucleotide sequence divergence between the genomes of typical sheep, goat and cattle isolates was calculated from numbers of conserved and non-conserved Hind III, Pst I, Ava I and Sal I sites (Gershon and Black, 1988). The three groups were calculated to be between 95.8% and 97% homologous at nucleotide sequence level. The comparisons demonstrated that the typical cattle and sheep isolates were more closely related to each other than either was to the typical goat

isolate. This in turn indicated that the sheep and cattle isolates diverged from the goat isolate prior to diverging from one another. The genomes of cattle isolates KC-I and South African cattle (SAC-1) were found to be closely related, but differed at two Hind III sites. Evidence suggested that isolates could arise from recombination between goat-type and cattle-type genomes (Gershon and Black, 1988).

Restriction enzyme maps demonstrated that relatively small deletions are present on genomes when compared to one another (Gershon and Black, 1988) and these deletions are not confined to the terminal or near-terminal regions of the genome. By analogy to the Orthopoxvirus genome, *host-range genes* would be expected to be located near the terminal regions. This data led to their suggestion that the stability of Capripox genomes are related to a more restricted set of genes responsible for host-range (Gershon and Black, 1988).

1.10.7.2 Terminally repeated regions

As is common to other poxviruses the capripox genome processes terminally repeated regions (Gershon and Black, 1987, Gershon and Black, 1988). These regions were found to be 1.13 kb - 6.23 kb in length in the cattle isolate KC-1 (Gershon and Black, 1987). By mapping various r.e. sites present within the terminal 10 kb of the genome of the Indian sheep isolate Ing-1, the terminal repeats were shown to be between 2.25 kb and 3.40 kb in length and inverted with respect to one another (Gershon and Black, 1988).

1.10.7.3 Non-essential regions

For the development of Capripox as a live recombinant vector, non-essential regions into which foreign genes could be inserted, have to be identified. Four such regions from the isolate KS-1, namely the TK-gene, the CT3C region, CT4 region and Q2 region, have already been identified (Bostock, 1990).

The TK-gene (ORF CF8) is contained within the S fragment produce by digestion of the DNA of the KS-1 isolate with Hind III. Two other complete ORFs, namely CF7 and CF8a and two incomplete ORFs, namely CF6 and CF9, have been identified in this fragment (Fig. 1.3).

Sequencing data have shown that the TK-gene and ORFs CF6, CF7 and CF9 are homologous to 4 contiguous ORFs from the central region of VV DNA (Gershon and Black, 1989). They also match

four ORFs found in the FPV genome. The other complete ORF, CF8a, has no homologue in VV or FPV DNA, but is homologous to a region downstream of the TK-gene of Shope Fibroma virus (SFV) DNA. The Orthopox-, Leporipox-, Avipox- and Capripoxviruses thus posess genes in common and there is a resemblance in terms of genetic organisation.

The nucleotide sequence also revealed that the S fragment is more A + T rich than the equivalent regions of VV, SFV and FPV (Gershon and Black, 1989). A high A + T content for the whole genome of the capripox isolate KS-1 is indicated by the relatively high frequency of A + T-rich r.e. sites, such as Dra I (TTTAAA) and Hind III (AAGCTT) and relatively low frequency of G + C-rich sites, such as those for Pst I (CTGCAG) and Sal I (GTCGAC) (Gershon and Black, 1987).

1.10.7.4 LSDV recombinants

Recombinational plasmids (analogous to those described for VV), which each contain one of the above mentioned non-essential capripox region and the VV P7.5 promoter, have been constructed (Bostock, 1990). Experiments are in progress to make Capripoxvirus recombinants carrying and expressing the F protein gene of the rinderpest virus. Construction of a Capripox virus expressing the 67 kDa surface antigen (p67) of *Theileria parva* sporozoites has also been reported (Juarrero *et al.*, 1991). This was achieved by heterologous recombination of the VV TK-gene with the capripox TK gene. The recombinant plasmid vector used contained the gene coding for p67 under regulatory control of the P7.5 promoter of VV. The *E. coli* gpt gene was used as a selective marker upon which recombinants were selected in the presence of mycophenolic acid. Expression of the p67 antigen was confirmed in Western blot analysis. The capacity of the recombinant to immunise against *T. parva* is currently being evaluated.

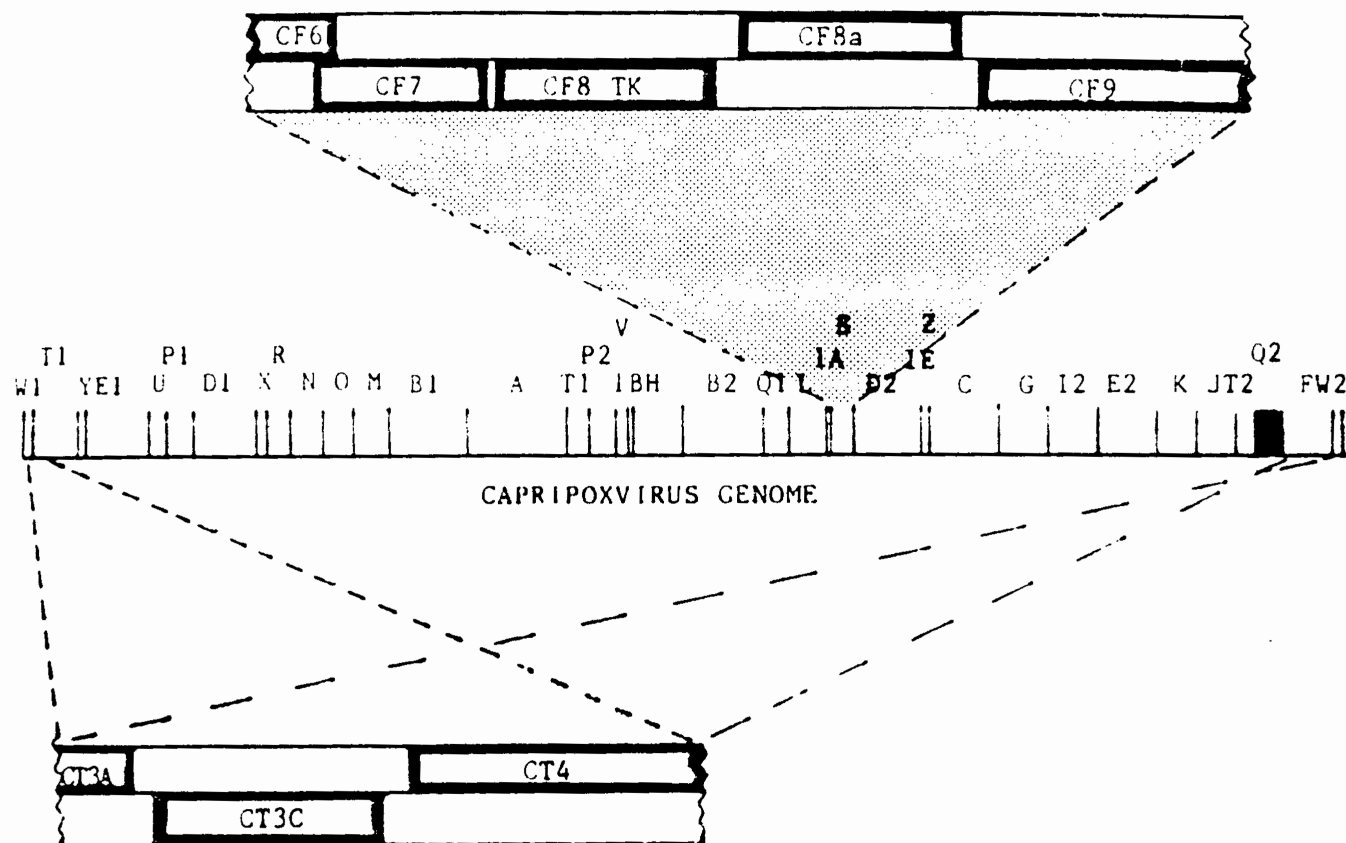


Figure 1.3

The Hind III restriction enzyme map of the KC-1 isolate of capripoxvirus. The individual fragments are assigned letters according to decreasing size. Above the map is shown an expanded region covering the S fragment which contains the non-essential TK gene (CF8 TK) and below is an enlarged map of the terminal regions which contain the CT3C and CT4 non-essential regions. The Q2 non-essential region is black. (Bostock, 1990).

1.11 OTHER VIRUSES CONSIDERED AS LIVE VECTORS

1.11.1 Adenoviruses

The current human adenovirus vaccine protects against acute respiratory disease and has been administred to several million USA soldiers without significant complications. It consists of two non-attenuated strains, types 4 and 7 and is given orally as a gelatin-coated tablet from which the virus is released after passage through the stomach. The virus then replicates in intestinal cells and induces effective immunity without causing the disease.

Recombinant adenovirus vaccines would offer the following advantages: they would have a low cost, would not require purification or be pyrogenic, they could be administred orally and induce immune response at the mucosal surface and they would have a narrow specific host range (WHO meeting, 1989). The small ds DNA genome (36 kb) of the virus however, places limitations on the amount of foreign DNA that may be inserted.

The hepatitis B virus surface antigen (HBsAg) was the first antigen to be inserted into a non-essential region, E3, of the viral genome by homologous recombination (Morin *et al.*, 1987). The recombinants were tested in hamsters where they evoked antibodies to both adenovirus and HBsAg and when inoculated intravenously into chimpanzees, produced partial protection against challenge (Lubeck *et al.*, 1989). Recombinant adenovirus vaccines, containing the structural genes env, gag and the regulatory protein rev, directed against HIV have also been developed (Chanda *et al.*, 1990). The HIV envelope sequences were biologically active, as they bound to the CD4 receptor, but the level of expression was low.

Although the prospect of having vaccines that could be administered orally is exciting, the limited heterologous gene carrying capacity of adenoviruses, their nuclear site of DNA replication, their mRNA splicing capacity, their ability to cause transformation in cell cultures and tumors in animals (Graham, 1984) and their persistence in lymphoid tissues represent unanswered concerns. Furthermore, no data is also available on safety in children or immuno-compromised individuals.

1.11.2 Herpesviruses

In general, herpesviruses evoke very strong cell-mediated immune responses as well as strong and long-lasting humoral responses (Esposito and Murphy, 1989). Additionally, the viruses have rather narrow specific host ranges, which is considered advantageous in any live virus vaccine. Particular herpesviruses are also associated with neurological and neoplastic diseases however, and all herpesviruses are associated with persistent infection and life-long carriage. These characteristics, unless further defined and absolutely controlled, would be considered unacceptable in any live virus vaccine.

Herpes simplex virus (HSV) has been used successfully as a vector for expressing hepatitis B surface antigen (Shih *et al.*, 1984) and for Epstein Barr virus nuclear antigen I (Hummel *et al.*, 1986). Unique prototype herpes simplex virus vectors have been developed (Meigner *et al.* 1988) which consist of HSV type I virus from which 15% of the genome has been deleted and into which genes for HSV type 2 virus immunogenic proteins have been inserted. Because of the extend of the deletions, these viruses have genomic space for the insertion of more than 10 kb of heterologous DNA. Safety and immunogenicity of these recombinants have been examined. These recombinants do not disseminate from the site of inoculation, nor do they cause systemic infection and neurovirulence has been eliminated. Both recombinants have been shown to protect mice, guinea pigs, rabbits and 2 species of primates against challenge with wild- type HSV type 1 and 2 viruses. These viruses could be considered as human vectored vaccines against several diseases.

1.12 LIVE BACTERIAL VECTORS

1.12.1 Introduction

Traditionally, bacterial vaccines have been either inactivated toxins such as tetanus toxoid or whole-cell vaccines such as the pertussis vaccine, which proved to be highly effective. However, for many bacterial pathogens, killed preparations provide only short-term immunity and low efficacy (Dougan *et al.* 1989). Subunit vaccines do not generally stimulate strong cellular immunity, but if living organisms persist in particular tissues of the host, high cellular immunity is achieved. (Collins and Mackaness, 1968).

Pathogenic bacteria can be attenuated by mutations which prevent their virulence, but do not influence their ability to induce a protective immune response. Bacteria can be genetically engineered to express foreign antigens, which opens up the possibility of devising multivalent live bacterial vaccines. Because bacteria can be controlled with antibiotics, adverse effects in immuno-supressed subjects are not such a critical issue as it would be in virus-vectored vaccines (WHO meeting, 1989).

1.12.2 Salmonella

Salmonella is a facultative intracellular pathogen, therefore cell-mediated immunity is more important than humoral immunity in resistance to the disease, salmonellosis (Fields *et al.* 1986, Dougan *et al.* 1987). For this reason, many investigators have considered the possible use of attenuated Salmonella for the expression of foreign antigens.

A galactose epimerase (gal E) mutant of *S. typhi*, designated TY21a, has been constructed by Germanier and Furer (1975) and is used extensively as a vaccine for the prevention of typhoid fever. Extensive evaluation in humans has proven it to be extremely safe (Gilman *et al.*, 1977). In theory, it should be possible to express protective antigens from any pathogen in Salmonella vaccine strains and thus deliver heterologous antigens to the immune system (Dougan *et al.* 1987). Formal *et al.* (1981) were the first to use Salmonella as a multivalent vaccine by introducing *Shigella sonnei* antigens into Ty21a. Ty21a has also been used as a vector for the expression of cholera (Clements and El-Morshidy, 1984) and enterotoxigenic *E.coli* (Stevenson and Manning, 1985, Dougan *et al.* 1986).

The genetic strategy used for Salmonella vectors is expression of the heterologous antigens from genes encoded on recombinant plasmids introduced as extrachromosomal DNA into the vaccine strains. Some plasmid constructs however, are lost once antibiotic selective pressure is relieved *in vivo* (O'Callaghan *et al.* 1988) The cloned sequences may also undergo deletion or other rearrangements. In order to stabilize the expression of antigens *in vivo*, Strugnell and his colleagues 1990, integrated the heterologous gene into the Salmonella chromosome. They constructed a chromosomal integration vector consisting of an antibiotic resistance gene, the aro C gene into which the foreign DNA can be cloned and on either side of it, flanking *S. typhimurium* DNA. The flanking regions are then used to integrate the foreign DNA into the Salmonella chromosome utilising homologous recombination.

Despite its impressive safety record and proven efficacy, the use of Salmonella Ty21a strain suffers from several drawbacks: it grows slowly and transforms and recombines less well than the parent strain. Multiple doses are needed to achieve 60 - 70% protection (Hirschel *et al.*, 1985) Initial trials of the attenuated *S.typhi* constructs, expressing the O antigen of *S. sonnei* (Black *et al.*, 1987) and the O antigen of *Vibrio cholera* were only partially successful.

Ty21a expressing *S. sonnei* antigen was safe and immunogenic when given to North American volunteers. The initial lyophilised vaccine lots conferred significant protection against dysentry. However, subsequent lots failed to protect significantly (Black *et al.*, 1987). Even a manufacturing protocol specifically designed to preserve flagellar structure (which was found to differ in the initial and subsequent lots), did not improve the efficacy of the bivalent vaccine (Herrington *et al.*, 1990).

Although Ty21a expressing *V. cholerae* Inaba O antigen elicited strong anti- *S.typhi* and modest anti-Inaba serological responses, diarrhoea still occurred in most of the vaccinated candidates after challenge with *V.cholerae.* Flynn and colleagues (1990) were the first to demonstrate that cytotoxic T-cells can also be elicited against a specific foreign antigen (of *Plasmodium yoelli*) expressed in Salmonella (Flynn *et al.*, 1990). Although Salmonella has great potential, further research is needed in order to develop it as a bacterial vectored vaccine.

1.12.3 Bacillus Calmette-Guerin

The most widely used vaccine against tuberculosis is the live bacterial vaccine BCG (Bacillus Calmette-Guerin), which is an avirulent derivative of *Mycobacterium bovis*. Distinct advantages of using BCG as a vector for expression of foreign antigens include the facts that the vaccine has superb adjuvant activity, being able to induce both humoral and cell-mediated immunity, and has been administered to billions of people within the first months of life with remarkably few side effects (Lotte *et al.*, 1984). The bacterial genome can also accommodate substantial amounts of additional DNA.

A host-vector system of BCG, which is a fundamental aspect of the development of recombinant live BCG vaccines, has recently received increased attention (Jacobs *et al.*, 1987). Random fragments of a ds DNA bacteriophage from *M. smegmatis* were cloned into a cosmid vector and the recombinants used to transfect *M. smegmatis* to generate viable phage (called phasmids, because they were composites of functional plasmid and phage DNA). The phasmid DNA was used

successfully to infect several strains of BCG.

Matsuo and colleagues (1990) were the first to report the expression and secretion of a foreign human immuno-deficiency virus (HIV-1) antigen from BCG. With the help of *E. coli* -mycobacteria shuttle vectors, they constructed secretion vectors in which an extracellular α-antigen of *Mycobacterium kansassi* was utilized as a carrier. The epitope of HIV-1 antigen was effectively secreted from BCG using this system, and could be used widely to secrete other foreign antigens. It remains to be determined whether expressed antigens will be effective in eliciting substantial immunity and protection. BCG is not a vector to be used in cattle, however, since herds are routinely tested for tuberculine infectivity. Its use in cattle would therefore result in false positive signals upon testing.

CHAPTER TWO

ISOLATION AND CLONING OF LUMPY SKIN DISEASE VIRAL DNA

2.1 INTRODUCTION

Most molecular biological investigations on LSDV have been done on the Kenya cattle isolate KC-1. Restriction enzyme maps based on digestion of the genome with Hind III, Pst I, Ava I and Sal I have been compiled (Gershon and Black, 1987). In contrast, apart from a Hind III r.e. map (Gershon and Black, 1988), nothing concerning the molecular biology of the South African vaccine strain, Neethling, has been reported.

Several facts support the idea that these two strains differ from each other. Firstly, there are differences in the severity of the disease. Whereas the SA strain caused a high mortality (of up to 75%) in the 1944 outbreak (Thomas and Mare, 1945), the KC-1 strain caused only 10 deaths in the Kenya epidemic in 1957 (MacOwen, 1959). Secondly, KC-1 has the ability to spread from cattle to sheep (see section 1.10.2), while Capripox infection has never been reported in South African sheep or goats. Finally, although a comparison of the Hind III profiles of the two strains demonstrated that the genomes were closely related, they differed at two Hind III sites (Gershon and Black, 1988).

In the light of the above, it is important to characterise the genome of the S.A. Neethling vaccine strain before it can be considered for use as an expression vector for foreign genes. As a departure point for molecular investigations, the LSDV genome was cloned. Cloning required purified viral DNA of good quality. Since poxviruses are mostly cell-associated, host cell DNA generally co-purifies with viral DNA. This contaminating host DNA might be ligated into the vector during cloning procedures, necessitating lengthy steps to discriminate between cloned viral and cloned host DNA fragments. It is therefore essential to reduce the amount of contaminating host cell DNA in viral DNA preparations. Various methods for the purification of poxvirus DNA have been described. A comparison of three of these methods showed that LSDV DNA, free from host cell DNA contamination, could be isolated from virions.

Because the size of the LSDV genome (approximately 143 kb), purified DNA cannot be cloned as a single unit, but has to be cleaved into smaller fragments, which are more easily cloned. Restriction enzyme profiles were compiled to select an enyme that fragments the genome into suitable fragments for cloning. A transcription vector was chosen for the cloning experiments as it is anticipated that further studies will include mRNA investigations. The LSDV clones obtained in the cloning experiments were analysed by hybridisation studies to determine the percentage of the LSDV genome obtained as clones.

2.2 MATERIALS

2.2.1 Materials for the isolation of viral DNA

Item	Manufacturer
Trypsin	Difco (USA)
DNase (RNase-free)	Boehringer Mannheim
β-Mercaptoethanol	BDH Chemicals
Proteinase K	Boehringer Mannheim
N-lauryl sarcosinate (NLS)	Sigma (USA)
Low-melting point agarose (Seaplaque)	FMC Bioproducts (US)

2.2.2 Materials for the growth of bacteria

Item	Manufacturer
Tryptone	Difco (USA)
Yeast extract	Biolab Chemicals
Agar	Biolab Chemicals
Ampicillin	Boehringer Mannheim
5-Bromo-4-chlor-3-indolyl-3-D-galactopyranoside(X-gal)	Boehringer Mannheim
Isopropyl-3-D-galacto-pyranoside (IPTG)	Boehringer Mannheim

2.2.3 Plasmid

The Bluescribe plasmid (amp^r) (Stratagene, USA) was used for cloning purposes.

2.2.4 Radioisotopes

Deoxycytidine 5'-[α-^{32}P]-triphosphate triethylammonium salt with a specific activity of 1200 Ci/mMol) and a radioactive concentration of 10 MCi)/ml (370 MBq/ml) was purchased from Amersham International (UK).

2.2.5 Photographic materials

Item	Manufacturer
Ilford PAN F film 35 mm	Ciba Geigy, (Pty) Ltd(Switzerland)
Cronex MRF 31 X-Ray film	DuPont (USA)

2.2.6 Virus isolate

The vaccine strain Neethling, an attenuated strain of Lumpy skin disease virus isolated from South African cattle, was used throughout this investigation. It was kindly supplied by Dr. B.J. Erasmus of the Onderstepoort Veterinary Institute (OVI).

2.2.7 Cell cultures

The Madin and Darby bovine kidney (MDBK) cell line was used for the propagation of virus and was originally obtained from the American Type Tissue Culture Collection (12301 Parklawn Drive, Rockville Drive, Rockville, Maryland USA, 20852). The cells were grown on Roux flasks or roller bottles (RB) in modified Eagles medium (MacPherson and Stoker,1962), supplemented with 5% bovine serum. Virus titrations were performed on primary calf fetal kidney (CFK) cells kindly prepared by the Vaccine Development Section, OVI.

2.2.8 Other materials

Item	Manufacturer
Restriction enzymes	Boehringer Mannheim
Lysozyme	Boehringer Mannheim
T4 DNA ligase	Boehringer Mannheim
Bacteriophage λ DNA	Boehringer Mannheim
Alkaline phosphatase from calf intestine	Boehringer Mannheim
Nick translation kit	Amersham International
Multiprime labelling kit	Amersham International
Sephadex G75	Pharmacia Fine Chemicals
Hybond^{N} nylon membrane	Amersham International
Bovine Serum Albumin (BSA)	Boehringer Mannheim
Fetal calf serum	Highveld Biological (Pty)Ltd.
Ficoll 400	Pharmacia Fine Chemicals
Ultrapure agarose (Seakem)	FMC Broproducts (USA)

2.3 METHODS

2.3.1 Virus propagation

LSDV was propagated in tissue culture according to a procedure developed by Mr. L.M. Pieterse of the Vaccine Development Section, OVI. This procedure was aimed at obtaining higher yields of virus antigen for vaccine production. Because a relatively small percentage of cells are infected with LSDV after initial infection and LSDV seem to have a preference for actively dividing cells (Mr. Pieterse, personal communication), this method entailed two passages of infected cells untill most cells in the monolayer were infected.

MDBK cells were grown as monolayers on roller bottles (RB) untill they were 80% confluent. The medium was removed and cells rinsed with serum-free Eagles medium. Cells were then infected with LSDV stock at 0.1 focus forming units (ffu)/cell, followed by addition of just sufficient medium to cover the cells. Viruses were allowed to adsorb for 60 min, whereupon more medium was added and the cells incubated at 37°C. After the first signs of a cythopathic effect (5 days p.i.) the infected cells were divided 1:3 in Eagles medium containing 5% fetal calf serum (FCS) and incubated at 37°C for a further 5 days. A second passage of the infected cells was then performed. When approximately 90% of cells showed a cythopathic effect, the infected cells were harvested.

2.3.2 Virus titrations

Titrations were performed on 25 cm^2 plastic flasks (Nunc). Serial tenfold dilutions of LSDV viral stock to be titrated, were made in Eagles medium. Each dilution of virus was inoculated on a confluent monolayer of CFK cells that was rinsed with serum-free medium. Viruses were allowed to adsorb for 60 min, more serum-free medium was added and flasks were incubated at 37°C. After 7 days, the number of ffu in each dilution was counted. An estimation of the virus concentration calculated as ffu/ml could then be made.

2.3.3 Purification of LSDV

LSDV was purified according to a procedure described for vaccinia virus (Mackett *et al.*, 1985). The purification was performed at 4°C.

LSDV-infected cells were collected from 20 roller bottles by either glass beads or trypsination. The cells were concentrated from the medium by low speed centrifugation (1000xg) for 30 min and resuspended in 10mM Tris-HCl pH 8 at a concentration of $1x10^8$ cells/3 ml. After a 10 min incubation period on ice, the cells were dounced 20 times and the cellular debris removed by centrifugation at 750xg for 7 min. The supernatant was collected, 1/100 volume of trypsin (25mg/ml) was added and the suspension incubated at 37°C for 30 min. The virus was pelleted by centrifugation at 25 000xg for 90 min through half a volume cushion of 36% sucrose in 10mM Tris-HCl pH 8. The pellet was resuspended in a small volume of 10mM Tris-HCl pH 8 and sonicated for 60 sec. $MgCl_2$ was added to a final concentration of 10mM and 1μl/ml DNase (1mg/ml) was added. The viral suspension was then incubated at 37°C for 30 min. This was loaded onto 15-40% sucrose gradients and centrifuged at 18750xg for 45 min in a Beckman SW41 rotor. The viral band was collected, an equal volume of 10mM Tris-HCl pH 9 added and the virus concentrated by centrifugation at 20 000 rpm in a Beckman SW27 rotor for 15 min. The purified virus was finally resuspended in a small volume of 10mM Tris-HCl pH 9 and stored at 4°C.

2.3.4 Purification of LSDV DNA

2.3.4.1 Purification from a crude preparation of virus

Viral DNA was purified from a crude preparation of virus after cultivation in cell culture using a method described for Orthopox viruses (Esposito *et al.*, 1981), as follows:

Monolayer MDBK cells were infected at a multiplicity of infection (m.o.i.) of 0.1 ffu/cell and passaged twice as described (section 2.3.1). The cells of 10 RB were collected by centrifugation at 100xg for 30 min and washed in an isotonic buffer (10mM Tris-HCl pH 8, 150mM NaCl, 5mM EDTA). The cell pellet was suspended in 36 ml of hypotonic buffer (10mM Tris-HCl pH 8, 10mM KCl, 5mM EDTA) and placed at 0°C for 10 min. βME (0.1 ml) and 10% (v/v) Triton X-100 (4ml) was added and the incubation continued at 0°C for another 10 min with occasional gentle mixing. This treatment solubilised the plasma membranes and released viral cores, the cell nuclei and cytoplasmic

organelles. Cell nuclei were removed by centrifugation at 2000 rpm for 7 min at 4°C and the supernatant fluid was centrifuged at 13 000 rpm for 60 min at 4°C in a Beckman SW35 rotor to pellet the viral cores. Cores were resuspended in 3.2 ml of ice-cold buffer (10mM Tris-HCl pH 8, 1mM EDTA, mixed with 60μl of βME, 200μl of proteinase K (500 μg) and 0.8 ml of 20% (w/v) N-lauryl sarcosinate(NLS)). The mixture was incubated at 4°C for 30 min. The NLS lysed the cores and released the DNA. Sucrose, 7.6 ml of a 54% (w/v) solution in water was added and the lysate incubated at 55°C for 2 hr to digest proteins. Non-specific binding of residual material to the DNA was inhibited by adding 1.6 ml of 5M NaCl to the digested DNA. DNA was purified from the core digest by three phenol:chloroform:iso- amylalcohol (50:48:2) extractions. The final aqueous phase was removed and extracted twice with an equal volume of chloroform: isoamylalcohol (96:4). DNA was precipitated by adding 2.5 volumes of 96% EtOH, collected by centrifugation at 4 000 rpm for 30 min at 4°C, rinsed in 70% EtOH, air-dried for 5 min and resuspended in 1xTE (1mM EDTA, 10mM Tris-HCl PpH 7.5) buffer. DNA was stored at 4°C.

2.3.4.2 Purification by pulsed field gel electrophoresis

Pulsed field gel electrophoresis (PFGE) facilities allows the seperation of viral DNA from host cell DNA and RNA on agarose gels. Viral DNA may then be recovered from the agarose. The protocol followed was described for the extraction of swinepox virus (SPV) from porcine kidney cells (Massung and Moyer, 1991a).

MDBK cells were infected at a m.o.i. of 0.1 ffu/cell and harvested after two passages of the infected cells as described (section 2.3.1). The cell concentration/ml medium was then determined by a haemacytometer (Neubauer).

Cells were collected by centrifugation at 100xg for 15 min and resuspended in 0.5 ml phosphate-buffered saline (10mM sodium phosphate 150mM NaCl pH 7.2) that contained 40mM EDTA. This was incubated at 37°C for 5 min. One-half ml of 1.5% low-melt agarose containing 120mM EDTA and prewarmed to 42°C was added to the cells and gently mixed until a uniform suspension was achieved. The suspension was transferred to an agarose plug mold (supplied with the Rotavor system) and the agarose allowed to solidify for 15 min. After solidification, the plugs were removed from the mold and incubated for 12-16 hr at 50°C in plug lysis buffer (1% NLS, 100μg/ml proteinase K, 10mM Tris-HCl pH 7.5, 200mM EDTA). The lysis buffer was then removed and replaced with sterile 0.5X TBE electrophoresis running buffer (1X TBE is 89mM Tris base, 89mM Boric acid, 1mM

EDTA) and equilibrated at 4°C for 6 hr with three changes of 0.5X TBE buffer. The viral DNA was separated from the cellular RNA and DNA contained within the agarose plug by PFGE using the Rotavor[R] Type V (Biometra) system. The following conditions were used:

Interval	80-20	log (s)
Angle	115-95	log (°)
Voltage	220	V
Rotor speed	7	
Temperature	13	°C
Agarose	1	% low melting point
Buffer	25	mM TBE

After analysis of the trial gel, a preparative gel was made with the appropriate amount of cells. The isolated band of viral DNA was visualised by staining with EtBr, excised from the gel and electroeluted from the agarose by electrophoresis for 2 hr at 100 V in dialysis tubing followed by a 30 s reverse pulse. The electroeluted viral DNA was ethanol precipitated, resuspended in 1X TE and stored at 4°C.

2.3.4.3 Extraction of DNA from purified virions

Purified virus (2.3.3) was diluted with an equal volume of 1% SDS, 20mM ß-ME, 20mM EDTA solution. Proteinase K was added to a final concentration of 100μg/ml and the mixture incubated at 37°C for at least 30 min. DNA was purified by extracting it twice with phenol saturated with 100mM Tris-HCl buffer pH 8, followed by two chloroform extractions. This was done by adding an equal volume of phenol, low speed rocking for 5 min and separating the phenol and aqueous phases by centrifugation at 6 000 rpm for 5 min at 20°C. The final aqueous phase was removed and extracted with chloroform in the same way. A tenth volume of 3M NaAc and 2.5 volumes of 96% EtOH were added to the final supernatant, whereupon viral DNA immediately precipitated. The DNA was collected by centrifugation at 4 000 rpm for 30 min, rinsed with 70% EtOH, lyophilised, resuspended in 1X TE buffer and stored at 4°C.

2.3.5 Restriction enzyme digestion of DNA

Restriction endonucleases (r.e.) are enzymes that cleave double stranded (ds) DNA in a sequence-dependant manner at specific sites within or adjacent to the sequences. The efficiency of the cleavage reaction is , in part, dependent upon the purity of the DNA. Digestions were carried out in the recommended high (100mM NaCl), medium (50mM NaCl) or low (10mM NaCl) salt buffer supplied with the enzymes and the reaction mixtures contained 4 units of enzyme per μg of viral DNA. Incubation was for 120-180 min at the recommended temperature for the enzyme concerned. The digestion products were analysed in agarose gels.

In cases where r.e. digestions were performed on DNA imbedded in agarose, bovine serum albumin to a final concentration of 0.1mg/ml and spermidine to a final concentration of 0.5mM, was added to the reaction volume (200μl). Also, more enzyme (8-20 units per μg of DNA) was used. Incubation was performed at the recommended temperatures for at least 4 hr.

2.3.6 Agarose gel electrophoresis

Restriction enzyme digested DNA was analysed by electrophoresis in horisontal 0.8% agarose slab gels using custom-built electrophoresis equipment. The electrophoresis buffer contained 0.5X TBE (1X TBE is 89mM Tris base, 89mM boric acid,1mM EDTA) and gels were made according to the method described by Sambrook *et al.*, 1989. DNA samples were mixed with 6X loading buffer (0.25% bromo-phenol blue, 40% sucrose in water) prior to loading on the gel. Electrophoresis was performed at 20mA for 16 hr, after which the gels were stained with EtBr (0.5μg/ml) and visualised by UV fluorescence. The gels were destained in buffer for 60 min and photographed using Ilford PAN F film and a red and orange filter.

2.3.7 Estimation of fragment sizes

The sizes of DNA fragments were estimated by comparing the migration distance in an agarose gel of the unknown fragments with that of molecular size markers of a known size, in the same gel during the same electrophoretic run.

The molecular markers used, were λ cL857 Sam7 (Boehringer Mannheim) digested with Hind III

and pAT153 digested with Hinf I. With the sizes of the fragments known, the distance they had moved in a gel was plotted (x-axis) against their molecular size in base-pairs (y-axis) on logarithmic paper to form a logarithmic curve. The distance the fragments of unknown size had migrated in the same gel, was measured and the sizes thus determined from the curve. Using a 0.8 % agarose gel, it was impossible to accurately size fragments larger than 20 kb.

2.3.8 Cloning of LSDV DNA fragments

2.3.8.1 Purification of fragments by electroelution

After electrophoretic seperation of r.e. digested DNA (sections 2.3.5 and 2.3.6), fragments of interest were selected and excised from the gel. Each gel strip was put into dialisis tubing, to which 3 ml of 0.5X TAE buffer (40mM Tris-HCl pH 7.9, 5mM ammonium acetate and 1mM EDTA) was added and the ends of the tubes closed off by a plastic clip. The tubes were placed diagonally in an electrophoresis tank, containing sufficient 0.5X TAE buffer to just cover the dialysis tubes. An electric current (100 V) was applied for 2 hr, followed by a reverse pulse for 30 s, to dislodge the DNA adhering to the sides of the dialysis tubing. Each dialysis tube was carefully opened and the buffer removed. The tubes were rinsed once with buffer, which was combined with the buffer previously removed. DNA was precipitated at -70°C for 2 hr by adding 1/10 volume of 3M NaAc and 2.5 volumes of 96% EtOH and collected by centrifugation at 6 000 rpm for 30 min at 4°C. The DNA was washed with 70% EtOH, lyophilised and resuspended in a small volume of 1X TE buffer.

2.3.8.2 Size fractionation using sucrose gradients

In this method, seperation of r.e. digested DNA is accomplished by zonal centrifugation using high-salt sucrose gradients (Ausubel *et al.*, 1989). DNA fragments migrate through linear sucrose gradients at a rate that is dependant on their size. By collecting relevant fractions of a gradient, fragments of a required size range can be selected.

Linear 10-40% sucrose gradients were prepared by hand in SW41 centrifugation tubes. Restriction enyzme digested DNA was heated to 65°C for 5 min to dissosiate any DNA aggregates prior to layering it on top of the gradients. A maximum of 0.2 mg of viral DNA was used per tube. The gradients were centrifuged in a Beckman SW41 rotor at 30 000 rpm for 16 hr at 20°C and then

fractionated in Eppendorf tubes by removing 500μl aliquots from the top. The size of the collected DNA fractions was determined by subjecting a 30μl sample of each fraction to agarose gel electrophoresis using a 0.9% agarose gel.

Each fraction was then divided into 3 Eppendorf tubes (170μl in each), 330μl of water and 1ml of 100% EtOH was added and the tubes placed at -20°C overnight. The DNA precipitate was collected by centrifugation at 6 000 rpm for 30 min at 4°C. Fractions that contained the DNA fragments of interest were pooled and resuspended in 1X TE buffer. This was used directly for ligations into plasmid vectors.

2.3.8.3 Ligation of DNA fragments

Vector DNA (pBS) was linearised by digestion with the appropriate r.e. Linearised vector (5μg) was incubated in the presence of 20mM Tris-HCl pH 8.0, 1mM $MgCl_2$, 1mM $ZnCl_2$ and 1 unit of calf intestinal alkaline phosphatase at 37°C for 30 min, to remove its 5'-terminal phosphatase residues, in order to reduce the possibility of recircularisation of the vector. The phosphatase was removed and vector DNA recovered by a phenol:chloroform:isoamylalcohol (25:24:1) extraction and EtOH precipitation, respectively. LSDV DNA fragments were used at a concentration of 0.5μg per ligation reaction. Ligations were carried out overnight at 15°C in a final volume of 20μl with 1 unit of T4 DNA ligase in a buffer containing 50mM Tris-HCl pH 7.6, 10mM $MgCl_2$, 5% (w/v) PEG 8 000, 1mM ATP and 1mM DTT.

2.3.8.4 Preparation of competent *Eschericia coli* cells

The use of $CaCl_2$ to enhance the uptake of foreign DNA by bacterial cells was first demonstrated by Mandel and Higa (1970). It is based on the fact that bacterial cells are made more susceptible to the uptake of foreign DNA by treatment of the cells with $CaCl_2$ at 0°C. If plasmid DNA that expresses a drug-resistance marker is taken up, the marker allows the transformed bacterial cells to survive in the presence of an antibiotic.

A 3 ml overnight culture of *E. coli* JM105 cells was used to inoculate 400 ml sterile LB medium. The culture was grown to log phase (OD_{590}=0.375) at 37°C with shaking and then placed on ice for 10 min. The cells were harvested by centrifugation at 4 000xg for 5 min at 4°C. The cell pellet was

resuspended in 200 ml ice-cold 0.1M $CaCl_2$, incubated on ice for 45 min and centrifugated again at 4000xg for 5 min at 4°C. The cells were then carefully resuspended in 4 ml ice-cold 0.1M $CaCl_2$ containing 15% sterile glycerol and either used after 1 hr at 4°C, or aliquotted into 500μl fractions and stored at -70°C.

2.3.8.5 Transformation procedure

The ligation reaction mixture (20μl) was added to 150μl of competent *E. coli* cells and placed on ice for 30 min. Uptake of DNA was induced by incubating the cells at 42°C for 2 min followed by a 3 min cooling down on ice. LB medium (0.8 ml, preheated to 37°C) was added and the cells were incubated at 37°C for 1 hr to allow for the expression of the antibiotic resistance gene encoded by the vector. The cells were then plated onto LB media containing 1.5% agar, ampicillin (100μg/ml), X-gal and IPTG using a spreading technique described by Sambrook *et al.*, 1989 and incubated at 37°C overnight. X-gal is a substrate for, and IPTG is an inducer of ₀-galactosidase activity, which is encoded by the *lacZ* gene of pBS. When grown on media containing the substrate and inducer, *E. coli* JM105(pBS) is blue in colour. If the *lacZ* gene is interrupted by the insertion of DNA in to the MCS of pBS (Fig 2.6), the bacterial colonies are white.

2.3.9 Purification of plasmid DNA

The alkaline lysis method of plasmid DNA extraction described by Birnboim and Doly (1979) with the modifications of Ish-Horowicz and Burke (1981), was used to isolate plasmid DNA from bacterial cells. This method depends upon the fact that a narrow pH range (about 12-12.5) exists within which linear DNA, but not covalently closed circular (ccc) DNA, is denatured. Plasmid DNA was purified on either a large (from a 400ml overnight culture) or a small (from a 10ml overnight culture) scale. Volumes for the small-scale purification are indicated in brackets:

Sterile Luria broth (LB) medium (1% bacto-tryptone, 0.5% bacto-yeast and 1% NaCl adjusted to pH 7.5) containing the appropriate antibiotic (100μl/ml Amp), was inoculated from a bacterial stock culture and incubated at 37°C overnight with shaking. The bacterial cells were harvested by centrifugation at 5 000xg for 10 min at 4°C and the cell walls were weakened by resuspending in 10 ml (1ml) of a solution containing 50mM glucose, 10mM EDTA, 25mM Tris-HCl pH 8 and 5 mg/ml lysozyme and incubating on ice for 5 min. Complete lysis of the cells was achieved by adding 20

ml (2ml) 0.2 M NaOH/1% SDS and incubating on ice for a further 5 min. Protein, high molecular mass RNA and chromosomal DNA were precipitated by the addition of 10 ml (1 ml) 3M potassium-acetate pH 4.8. After 10 min on ice, the cellular DNA and bacterial debris were pelleted by centrifugation at 5 000xg for 15 min at 4°C. The plasmid DNA was precipitated by the addition of isopropanol (1/2 vol) to the supernatant and collected by centrifugation as described above. The DNA pellet was resuspended in 1X TE buffer (1mM EDTA, 10mM Tris-HCl pH 7.5) and contaminating low molecular mass RNA was removed by precipition with half a volume of 7.5M ammonium-acetate. The purified plasmid DNA was finally recovered by precipitation with 2 volumes of 96% EtOH. After washing with 70% EtOH, the DNA pellet was lyophilised, resuspended in 1 ml (50μl) 1X TE buffer and stored at 4°C.

2.3.10 CsCl density gradient centrifugation

In order to obtain a preparation completely free of contaminating RNA and protein, the plasmid DNA was in some instances purified further by centrifugation to equilibrium in CsCl-ethidium bromide (EtBr) density gradients. Because EtBr unwinds DNA when it intercalates, less EtBr can be bound by a ccc plasmid than can be bound by a nicked plasmid or chromosomal DNA. Since binding of EtBr to DNA lowers its density, the plasmid band appears at higher density (lower down in the tube) in equilibrium density gradients.

The plasmid DNA (1ml) was mixed with 14 ml of a 1g/ml solution of CsCl in water and EtBr was added to a final concentration of 600μg/ml. The gradient was centrifuged to equilibrium at 42 000 rpm for 16 hr at 20°C in a Beckman Vac50 rotor. Two bands of DNA were visible in UV light. The upper band consists of linear bacterial DNA and nicked circular plasmid DNA, while the lower band consists of ccc DNA. The lower band was collected with a syringe by inserting a #21 hypodermic needle into the side of the centrifuge tube. The fluid was then transferred to a Corex tube. EtBr was removed by extracting three times with an equal volume of water-saturated butanol and the DNA was recovered by ethanol precipitation. The lyophilised plasmid DNA was resuspended in 0.8 ml of 1X TE buffer and stored at 4°C.

2.3.11 Characterisation of LSDV clones

2.3.11.1 Dot-spot hybridisation

A Biorad blotting apparatus was used to immobilise DNA onto Hybond^N nylon membranes. The dsDNA was denatured before blotting by boiling at 100°C for 10 min and subsequent snap-cooling on ice for 3 min. Blotting was achieved by vacuum action through the blot apparatus. The membrane was removed and DNA again denatured by floating the membrane (DNA side up) on filter paper saturated with 0.5M NaOH, 1.5M NaCl for 5 min, followed by neutralisation on filter paper saturated with 1.5M NaCl, 0.5M Tris-HCl pH 8, 1 mM EDTA for 5 min. The membrane was allowed to air-dry and DNA then fixed by UV-light exposure (312 nm) for 3 min on each side. It was sealed in a plastic bag and prehybrised in a solution (50% formamide, 5X Denhardts, 20X SSC 0.05M Na-P buffer pH 6.8 and 0.5 mg/ml salmon sperm DNA) at 42°C for at least 4 hr prior to hybridisation. If not used immediately, blots were stored in prehybridisation solution at 4°C.

2.3.11.2 Southern blotting

Electrophoretic separated r.e. digested DNA was transferred to a membrane to be used in hybridisation reactions by a procedure developed by Southern (1975).

After digestion of LSDV DNA with the appropriate r.e. (section 2.3.5), the fragments were separated electrophoretically in 0.8% agarose gels (section 2.3.6). The DNA fragments were denatured by gently rocking the gel in a denaturing solution (1.5M NaCl, 0.5M NaOH) for 15-30 min. This was repeated by changing the denaturing solution twice. The gel was then neutralised by soaking it twice in a 1.5M NaCl, 0.5M Tris-HCl pH 7.5, 1mM EDTA solution for 30 min each. The single stranded DNA was then ready to be transferred to a Hybond^N nylon membrane. The transfer was effected by a vacuum using 2X SSC as blotting buffer. The vacuum was applied for 45 min after which the membrane was removed and air-dried. DNA was fixed on the membrane by UV-light exposure (312 nm) for 3 min on each side. The membranes were further treated as described in section 2.3.11.1

2.3.12 Labelling of DNA to be used as probes

2.3.12.1 Nick-translation

Relevant DNA was labelled with ^{32}P in a nick-translation reaction using the enzyme *E. coli* DNA polymerase I. This enzyme adds nucleotides to the 3'OH-terminus that is created when one strand

of a dsDNA molecule is nicked. In addition, the enzyme removes nucleotides from the 5'-side of the nick by its 5'- to 3'- exonuclease activity. The elimination of nucleotides from the 5'-side together with the sequential addition of nucleotides to the 3'-side, results in movement of the nick (nick translation) along the DNA. By replacing the pre-existing nucleotides with highly radioactive nucleotides, it is possible to prepare ^{32}P-labelled DNA (Sambrook *et al.*, 1989).

The commercial kits nick translation mixture comprised 0.05M Tris-HCl pH 7.4, 5mM $MgCl_2$, 0.1mM dTTP, 0.1mM dGTP, 0.1mM dATP, 10μCi ^{32}P dCTP (10μCi/μl with a specific activity of 800 Ci/mMol), 7.7 units of DNA polymeraseI and 2μl of the recombinant plasmid or insert. The reaction was carried out for 60 min at 15°C after which the reaction was terminated by placing the mixture on ice. The labelled DNA was separated from free nucleotides using a Sephadex G75 column.

The Sephadex G75 column was packed in a Pasteur pipette and equilibrated with STE-1 buffer containing 0.1% SDS. The labelled probe was loaded onto the column and eluted using the same buffer. A first fraction of 0.5ml was collected and 0.1 ml samples thereafter. All samples were counted for the presence of ^{32}P-cpm. The relevant fractions were pooled and denatured by heating at 95°C for 5 min, followed by snap-cooling, just prior to hybridisation.

2.3.12.2 Random priming

The principal of this labelling method lies in the use of random sequence hexanucleotides to prime DNA synthesis on denatured template DNA at numerous sites along its length (Feinberg and Vogelstein, 1983). The Klenow fragment of DNA polymerase I, which lacks the 5'-3' exonuclease activity of the intact enzyme, is used in this reaction. It ensures that labelled nucleotides that are incorporated by the polymerase are not subsequently removed as monophosphates. The amount of newly synthesised DNA often exceeds the amount of input DNA. It is therefore likely that strand displacement can occur, so that the same region of a given single strand of input DNA may be copied more than once, primed from different hexanucleotides.

Because a very high specific activity DNA probe is obtainable with relatively small quantities of added labelled nucleotides, this method was chosen for labelling of genomic probes and large recombinant plasmid probes, using a multiprime DNA labelling kit (Amersham). Very small amounts of input DNA (30-100ng), diluted in 10mM Tris-HCl pH 8.0, 1mM EDTA to a volume of 21μl, was denatured by heating at 95°C for 5 min and chilling on ice for 3 min. Unlabeled dATP, dGTP and

dTTP (4μl of each), 5μl multiprime DNA labelling buffer, 2μl Klenow DNA polymerase, 5μl [^{32}P]-dCTP and 5μl of random sequence hexamers was added to the single stranded DNA and the reaction was allowed to proceed at room temperature for a minimum for 6 hr, up to 24 hr. The labelled DNA was separated from free nucleotides using a Sephadex G75 column as described (section 2.3.12.1).

2.3.13 Hybridisation of probes with immobilised DNA

Hybridisation was carried out after membranes were pre-hybridised in a solution (50% formamide, 1X Denhardts solution, 4X SSC, 0.02M Na-P buffer pH 6.8, 0.05mg/ml denatured herring sperm DNA) for at least 4 hr at 42°C. The solution was then replaced with hybridisation solution with the same composition, except that less sperm DNA (0.01 mg/ml) was added. Before the labelled probe was added to the hybridasation soluton, it was denatured by heating at 95°C for 5 min and snap-cooled on ice. Hybridisation was allowed to proceed for at least 16 hr at 42°C after which the membrane was removed and washed at the required stringency choosing an appropriate temperature and buffer. If high stringency washes were required, 3 washes in 0.1X SSC buffer, 0.1% SDS were performed with shaking at 65°C for 15-30 min each. Lower stringency washes were performed at 42°C with 3 changes of 1X SSC buffer, 0.1% SDS after 15 min each.

The membrane was then removed from the washing solution and loaded in an X-ray casette. An X-ray film (Cronex MRF31) and a [^{32}P]-intensifying screen were placed over the membrane and the casette kept at -70°C for a suitable time. Upon development of the X-ray film, the results could be observed on the autoradiogram.

2.4 RESULTS

2.4.1 Growth of Lumpy skin disease virus in tissue culture and virus titrations

LSDV can be propagated in a variety of cell cultures (Weiss, 1968) of which primary lines are mostly used. For this study, the Madrin Darby bovine kidney (MDBK) cell line (Fig 2.1A) was used. This is an established cell line which can be propagated indefinitely. Cytopathic changes in LSDV-infected MDBK cells were first noted 4 to 5 days p.i., when foci became visible (Fig. 2.1B). The infection had little effect on the surrounding monolayer, indicating that the virus remained localised and did not spread throughout the monolayer. The highest titres on MDBK cells were obtained after two passages of virus infected cells (described in 2.3.1) and ranged between 1×10^5 and 5×10^5 ffu/ml.

2.4.2 Purification of viral DNA

Several methods for poxvirus DNA purification have been described in the literature. This section describes the results obtained for LSDV DNA purification using three different methods.

2.4.2.1 Purification from a crude preparation of virus

Esposito *et al.*, 1981 developed a method for purification of Orthopox virus strains that grow poorly and thus present difficuties in obtaining sufficient amounts of viral DNA. This method was investigated, since LSDV grows relatively slowly and does not reach high titres in cell culture. MDBK cells were infected with 0.1 ffu/cell, harvested after two passages and purified (section 2.3.4.1). As much as 5mg LSDV DNA/20 RB of infected cells was obtained in this way, but viral DNA was excessively contaminated with genomic bovine DNA. This was seen as a smear accompanying the viral DNA band when undigested DNA was electrophoretically analysed (Fig. 2.2 lane b) on 0.8% agarose gels. The contamination was also later confirmed by hybridisation studies (see section 2.4.5). A further indication of the poor quality of the DNA, was the restriction enzyme profiles it produced. The DNA was digested with restriction enzymes (Fig. 2.2) Bam HI (lane c), Pst I (lane d) and Sal I (lane f). In no case, however, were these digestions complete, resulting in poor r.e. profiles. No digestion of viral DNA occured with Hind III (lane e) or Sma I (lane g), although control digestions proved that these enzymes were indeed functional (data on control digestions not shown).

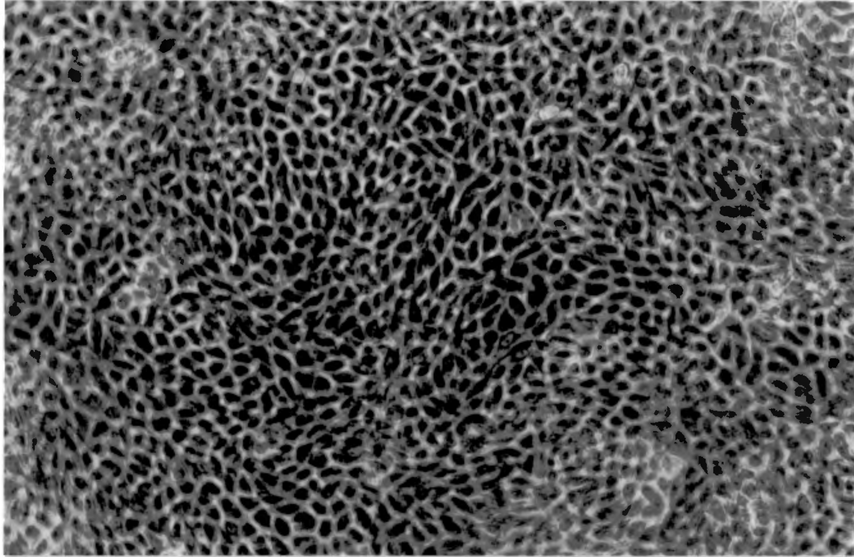


Figure 2.1 A An uninfected monolayer of Madrin Darby bovine kidney (MDBK) cells.

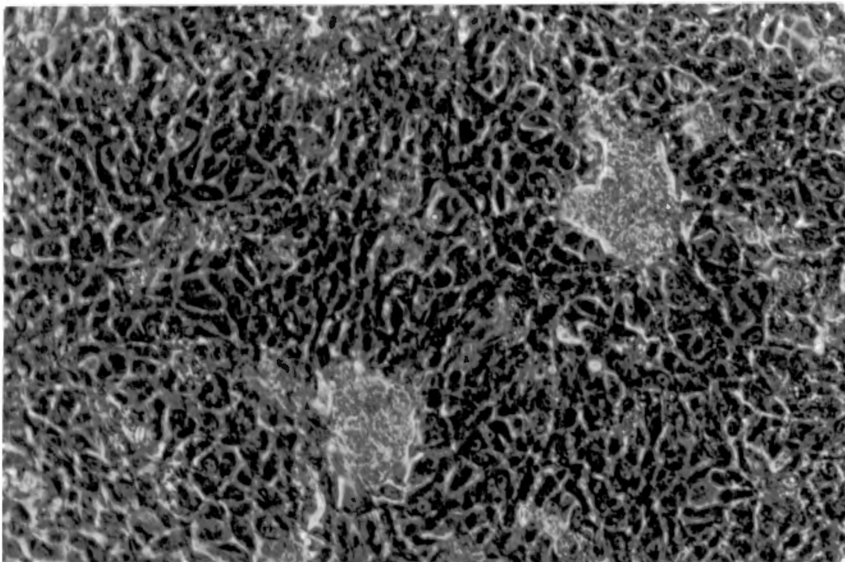


Figure 2.1 B Foci produced by a LSDV infection on MDBK cells, 5 days post infection.

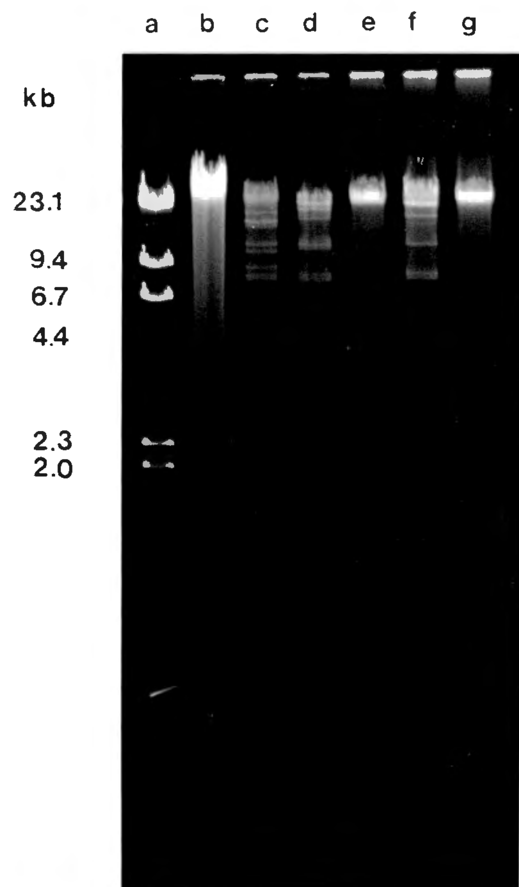


Figure 2.2 Agarose gel (0.8%) electrophoretic analysis of Lumpy skin disease viral DNA extracted from a crude viral preparation. DNA was digested with the restriction enzymes Bam HI (c), Pst I (d), Hind III (e), Sal I (f) and Sma I (g). Undigested DNA is shown in lane b and Hind III digested λ DNA (lane a) was included as size marker.

It should be noted that Esposito's method was not developed specifically for the isolation of DN from MDBK cells. Still, the method was applied to viruses cultivated in a variety of cells, including primary chick embryo fibroblast and rabbit kidney cells. The formulation of the buffers used for lysing the cells and cores might, however, not be applicable to MDBK cells. Nevertheless, the high DNA yields that were obtained, justifies further investigation to optimise conditions to be used in this specific case.

2.4.2.2 Pulsed field gel electrophoresis

In an effort to obtain purified LSDV DNA preparations free of contaminating bovine DNA, the use of pulsed field gel electrophoresis as described for purification of SPV DNA (Massung and Moyer, 1991a) was investigated. Under pulsed field conditions in an agarose matrix, very large genomic DNA is separated from the smaller viral DNA. The viral DNA band can then be excised in a gel slice and subsequently purified from the agarose.

MDBK cells were infected with LSDV as described (section 2.3.1), harvested and imbedded in agarose plugs. The infected cells were lysed in the plugs and subjected to electrophoresis under pulsed field conditions (section 2.3.4.2). In order to standarise the conditions for a preparative gel, various amounts of infected cells were subjected to electrophoresis. (Fig. 2.3 A). It was judged that 1.5×10^7 infected cells (lane f) was the appropriate amount to be used. This resulted in a sharp viral DNA band of between 100 kb and 150 kb in length, without overloading (lane e) or underloading (lane g) the gel slot. A preparative gel in which 1.5×10^7 cells/ slot was used, is depicted in Fig. 2.3B.

Using this protocol, viral DNA could be separated completely from contaminating bovine DNA. It could, however, not be recovered satisfactorily from the agarose gel. Due to the large size (approximately 145 kb) of the viral genome, electroelution was the only realistic option to consider. Although Massung and Moyer, 1991a recovered 50μg of SPV DNA/10^7 cells using electroelution and their protocol was followed, only minute amounts of LSDV DNA was recovered in this way. Even doubling the elution period did not improve the yield.

The ability to digest the excised viral DNA band directly in the agarose slice (section 2.3.5) was also investigated, as this would eliminate the need for elution. Digestions with the r.e. Pst I and Bam HI were performed. Electrophoretic analysis in a 0.8% agarose gel (Fig. 2.3C) showed that no digestion had occured with either Pst I (lane c) or Bam HI (lane d).

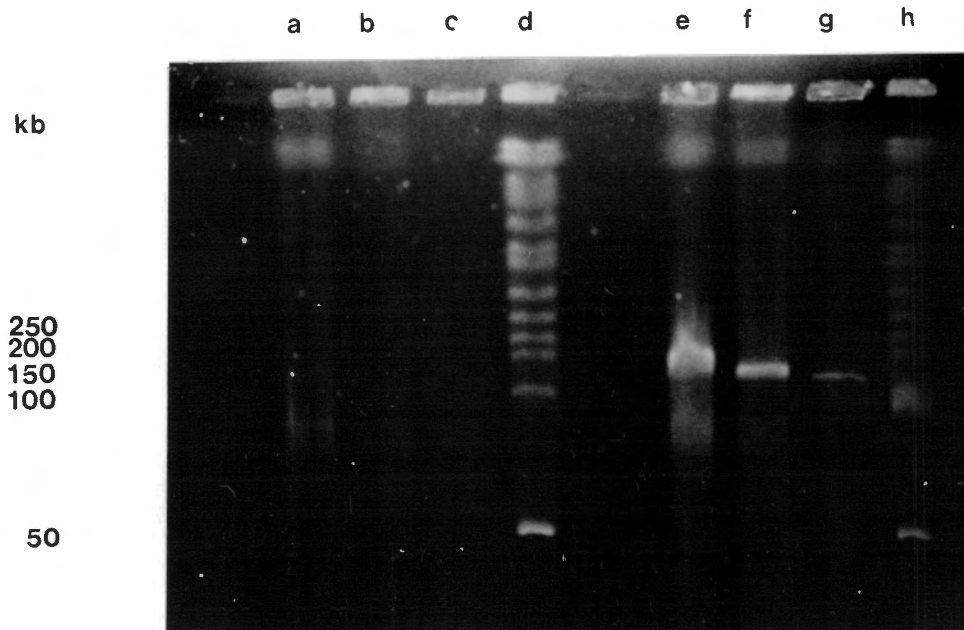


Figure 2.3 A Standarisation of conditions for pulse field gel electrophoresis. Different amounts of cells, namely $3x10^7$ (a & e), $1.5x10^7$ (b & f), $5x10^6$ (c & g) were imbedded in agarose plugs and analysed on a 1% agarose gel under the following pulsed field conditions: Interval 80-20 log (s), Angle 115-95 log (°), Voltage 220 V, Rotor speed 7, Buffer 25mM TBE, Temperature 13°C. Uninfected MDBK cells (a-c) and LSDV-infected cells (e-g) were included. A λ ladder was used as size marker (d) and (h).

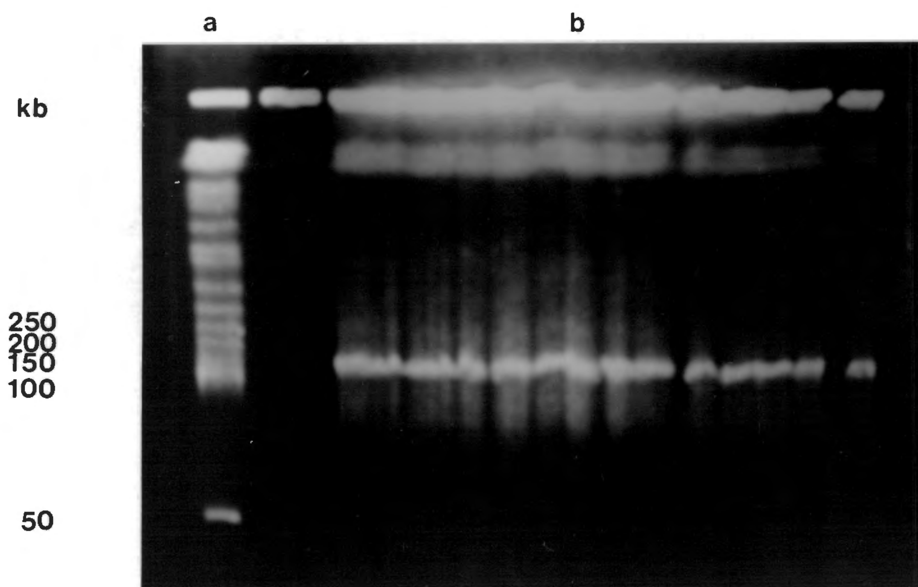


Figure 2.3 B Preparative 1% agarose gel run under pulsed field conditions. (a) Lambda ladder molecular weight marker (b) DNA from LSDV-infected cells, $1.5x10^7$ cells/slot.

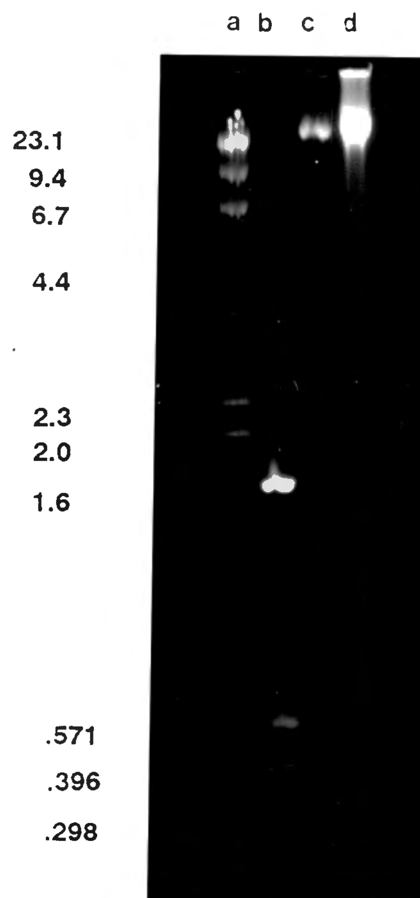


Figure 2.3 C Agarose gel (0.8%) electrophoretic analysis of LSDV DNA excised from a pulsed field gel and digested directly in the excised slice by Pst I (c) or Bam HI (d). Hind III digested λ DNA (a) and Hinf I digested pAT153 (b) were used as size markers.

Since adequate amounts of LSDV DNA separated by PFGE could not be eluted from the agarose gel, and nor was it possible to restrict the DNA in the agarose, this purification method was not of practical use. Consequently, a third method of viral DNA purification, i.e. by extracting DNA from purified virions, was investigated.

2.4.2.3 <u>Purification from virions</u>

Monolayers of MDBK cells (20 Rb) were infected and harvested as described (2.3.1). LSDV virions were purified from these cells by lysing the cells, removing the cellular debris, concentrating virus from the supernatant and subjecting it to rate sonal centrifugation through sucrose gradients (2.3.3). The viral band was collected from the gradients and DNA extacted from the virions by phenol-chloroform extractions (2.3.4.3).

Although this protocol was time consuming and yielded less LSDV DNA (1mg/20 Rb) than the protocol whereby DNA was extracted directly from infected cells (5mg/20 Rb), the quality obtained was good. The DNA could be digested with all enzymes used (Fig. 2.4 A and B), resulting in well defined r.e. profiles. Undigested DNA showed no smear associated with the viral DNA band (Fig. 2.4 B lane c). This was taken to indicate the virtual absence of contaminating bovine DNA. This DNA was used to compile restriction enzyme profiles, for estimatation of the sizes of r.e. fragments yielded (section 2.4.3).

2.4.3 <u>Restriction enzyme analysis of LSDV DNA.</u>

The large size of the LSDV genome (approximately 145 kb) necessitated its fragmentation to facilitate cloning and easy manipulation. Various restriction enzymes were used to obtain r.e. profiles, from which decisions could be made as to which enzyme(s) would be appropriate for cloning.

The aim was to clone as large a portion of the genome as possible, without producing an unmanagable number of clones. To accomplish this, an attempt was made to clone large fragments, preferably of an appropriate size for either plasmid (0-10 kb) or phage lambda vectors (8-20 kb), since these systems were available in our laboratory. In the previous section (2.4.2.3), various r.e. digestions were performed on purified LSDV DNA (Fig. 2.4 A and B). Digestions of the

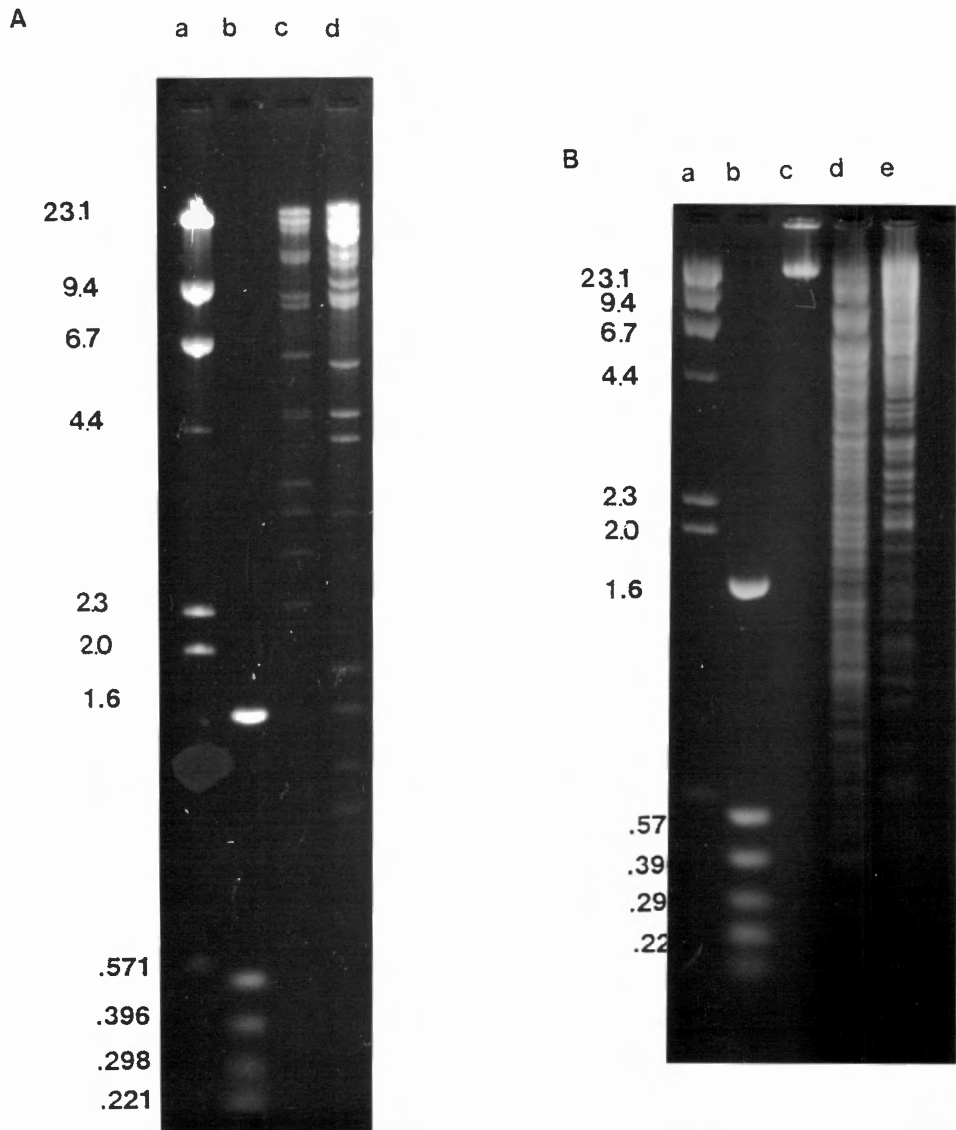


Figure 2.4 Agarose gel electrophoretic analysis of LSDV DNA extracted from purified virions and digested with various restriction enzymes.

2.4 A LSDV DNA digested with Pst I (c) and Bam HI (d). Molecular weight markers pAT153/Hinf I (b) and λ/Hind III (a) were included as size markers.

2.4 B LSDV DNA digested with Hind III (d) and Eco RI (e). Undigested DNA is shown (c), while pAT153/Hinf I (b) and λ/Hind III (a) were included as size markers.

genome with Eco RI (Fig 2.4 B lane e) and Hind III (Fig 2.4 B lane d) produced many fragments ranging in size from between 0.5 kb and 5 kb. On the other hand, Sal I (Fig. 2.2 lane f) yielded onlya few fragments, most of which were larger than 10kb and thus not suitable for these cloning systems. LSDV DNA digested with Pst I yielded 9 fragments (Fig. 2.4 A lane c) while Bam HI (Fig. 2.4 A lane d) produced 11 fragments of between 1 kb and 10 kb. Both enzymes, however, also yielded a few larger fragments. Much of the genomic DNA could, however, potentially be obtained in only a few plasmid or phage clones. It was thus decided to clone segments generated by Pst I and Bam HI.

The length of each of the fragments generated by LSDV DNA digestion with Pst I and Bam HI was estimated from a logarithmic curve (Fig. 2.5), based upon molecular weight size markers. The fragments (named in alphabetical order from the largest to the smallest) and their estimated lengths, are summarised in Table 2.1. A published result of a LSDV strain isolated in Kenya (KC-1) after digestion with Pst I (Gershon and Black, 1987) is also included. No results of LSDV strains digested with Bam HI have as yet been published.

One obvious difference between the profiles of the LSDV vaccine Neethling strain and that of the Kenyan KC-1 strain was established, namely that Neethling produced 14 Pst I fragments, while KC-1 produced only 12. There were, nevertheless, apparent similarities since for most of the restriction fragments reported for KC-1, comparable size fragments were obtained with the Neethling DNA (Table 2.1, fragments A-H, K, L and N). There were, however, some bands for which corresponding fragments could not be identified (Table 2.1, fragments I,J and M of the Neethling strain).

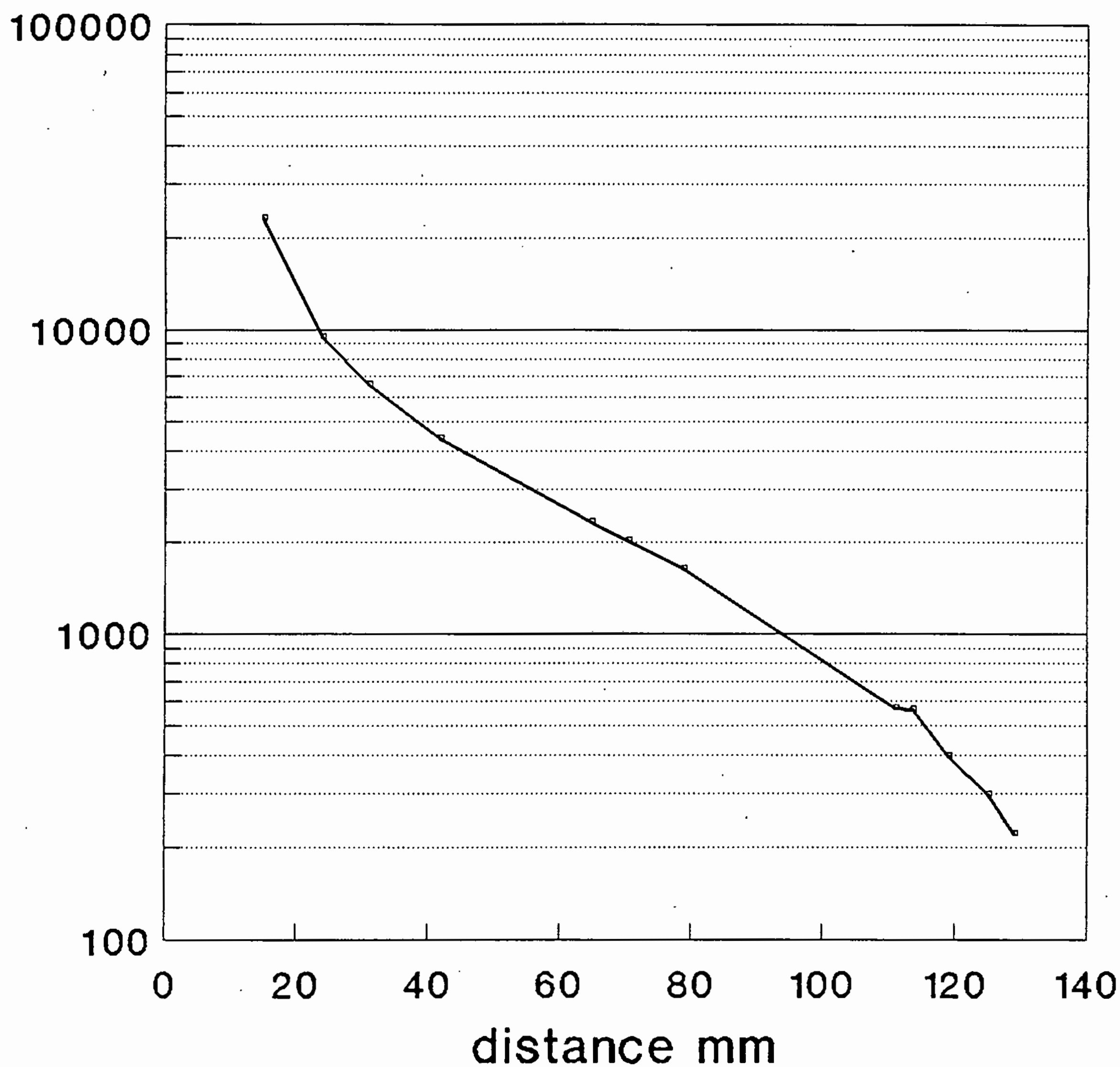


Figure 2.5 Logarithmic curve for estimation of fragment sizes. The distance a fragment migrated (x-axis) was measured in mm from the inner edge of the loading well. The molecular size (in bp) was plotted on the y-axis on a logarithmic scale. Molecular size markers λ DNA/Hind III and pAT153/Hinf I were used to draw the curve.

Table 2.1 Fragment sizes of LSDV (Neethling vaccine strain) DNA generated after restriction enzyme digestion with Pst I and Bam HI respectively.

FRAGMENT	MOLECULAR SIZE (kb)		
	PstI	PstI*	BamHI
A	33	39.1	35
B	23	24.2	21
C	19	18.9	15
D	12.7	13.1	12.95
E	12.2	11.3	11.5
F	8.9	8.0	10
G	8.2	7.8	8.6
H	6.4	7.0	8.2
I	4.7	5.7	6.0
J	4.6	3.5	4.7
K	3.5	3.1	4.2
L	3.15	2.2	2.8
M	2.72		1.83
N	2.3		1.62
O			1.33
P			1.12
TOTAL LENGTH	143.87	141.11	145.85

Fragments yielded by Pst I digestion of DNA of the Kenia isolate (KC- 1) of LSDV (Gershon and Black, 1987) are included (*) for comparison.

2.4.4 Cloning of the LSDV genome

Identification of non-essential genes and promoter areas for the development of LSDV as a vector depends on the availablility of LSDV clones. Two vector systems, namely a phage lambda system, which can accomodate inserts of 8 kb to 20 kb in length and bacterial plasmid vectors, capable of accomodating inserts ranging from very small (a few hundred base pairs) to about 10 kb, were considered. The lambda system, pEMBL12, was investigated but rejected in favour of a plasmid vector after a few unsuccesful cloning attempts. These results are therefore not presented.

Instead, the bacterial plasmid vector pBluescribe (Fig. 2.6) was chosen. Firstly, it contains the lacZ gene which offers a blue-white selection system, allowing easy identification of recombinants. Secondly, it also has a multiple cloning site (MCS) which offers a variety of unique restriction enzyme sites. Furthermore, pBluescribe contains the T3 and T7 RNA polymerase initiation regions which are potentially useful to allow transcripts to made of the cloned fragment. Lastly, pBS contains the sites for the M13 forward and reverse primers, facilitating the direct sequencing of inserts.

2.4.4.1 Selection of a specific size range of LSDV fragments to be cloned

During a cloning procedure, bacterial plasmids usually tend to incorporate fragments of a relatively small size (<4kb) in preference to those of larger size. To circumvent this tendency which would result in only small fragments being cloned, two methods of selecting fragments of a specific size range for cloning were investigated, namely electroelution and sucrose gradient fractionation.

LSDV DNA was digested with Pst I and Bam HI respectively and a sample subjected to agarose gel electrophoresis to determine whether the digestion was complete. For electroelution, the remainder of the digested DNA was then electrophoresed and fragments of different size ranges excised from the gel. The DNA was purified by electroeluting each gel slice separately. Although small fragments could be recovered satisfactorily by this method, the yield of larger fragments was poor. Since the aim was to specifically enrich for the large fragments, this method was rejected.

For the second protocol, r.e. digested LSDV DNA was separated on a sucrose gradient at a high speed of centrifugation. Fractions of the gradient were collected and a sample of each electrophoresed, as this gave an indication as to which fractions were to be used for cloning.

In the case of Pst I digested LSDV DNA, fractions 7-14 and 15-25 (Fig. 2.7 A) were pooled respectively. Fractions 1-4 and 5-25 (Fig. 2.7 B) were pooled similarly after a Bam HI digestion. All pools were subsequently used for cloning into the plasmid vector pBluescribe.

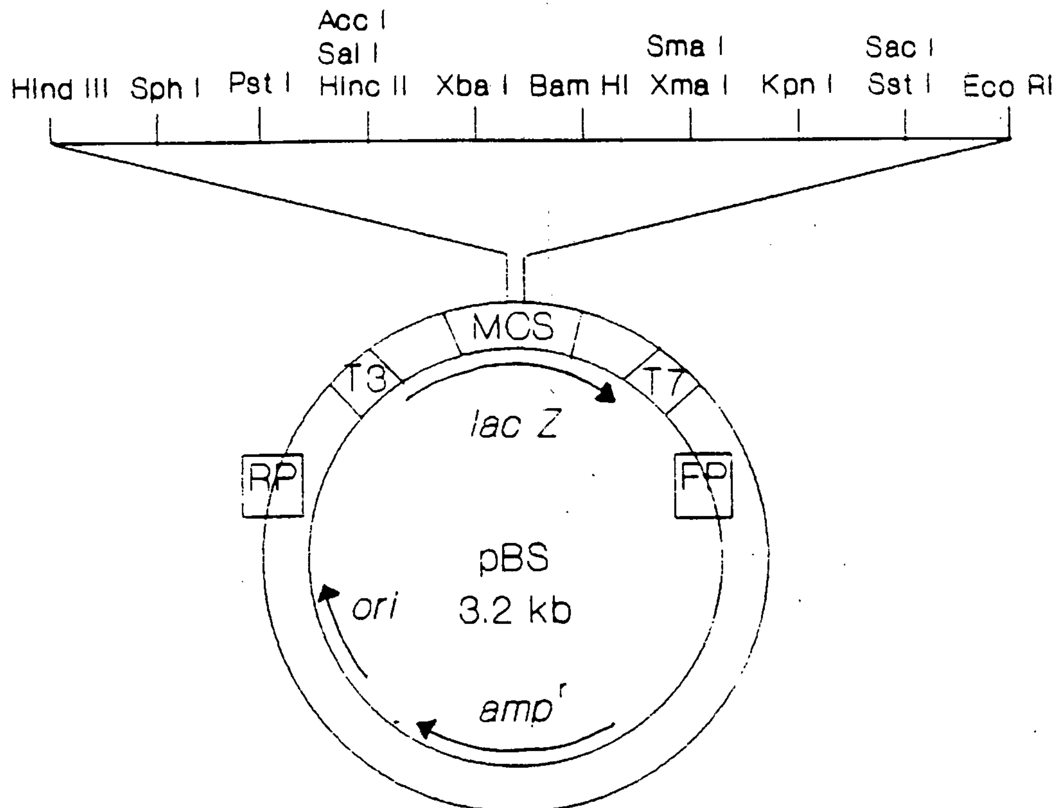


Figure 2.6 The design of the pBluescribe (pBS) transcription vector. This plasmid confers resistance to ampicillin and contains the M13 multiple cloning site (MCS). The LacZ gene offers a blue-white selection system of recombinants. The positions of the T3 and T7 phage promoters as well as of the M13 forward and reverse primer sequences are indicated.

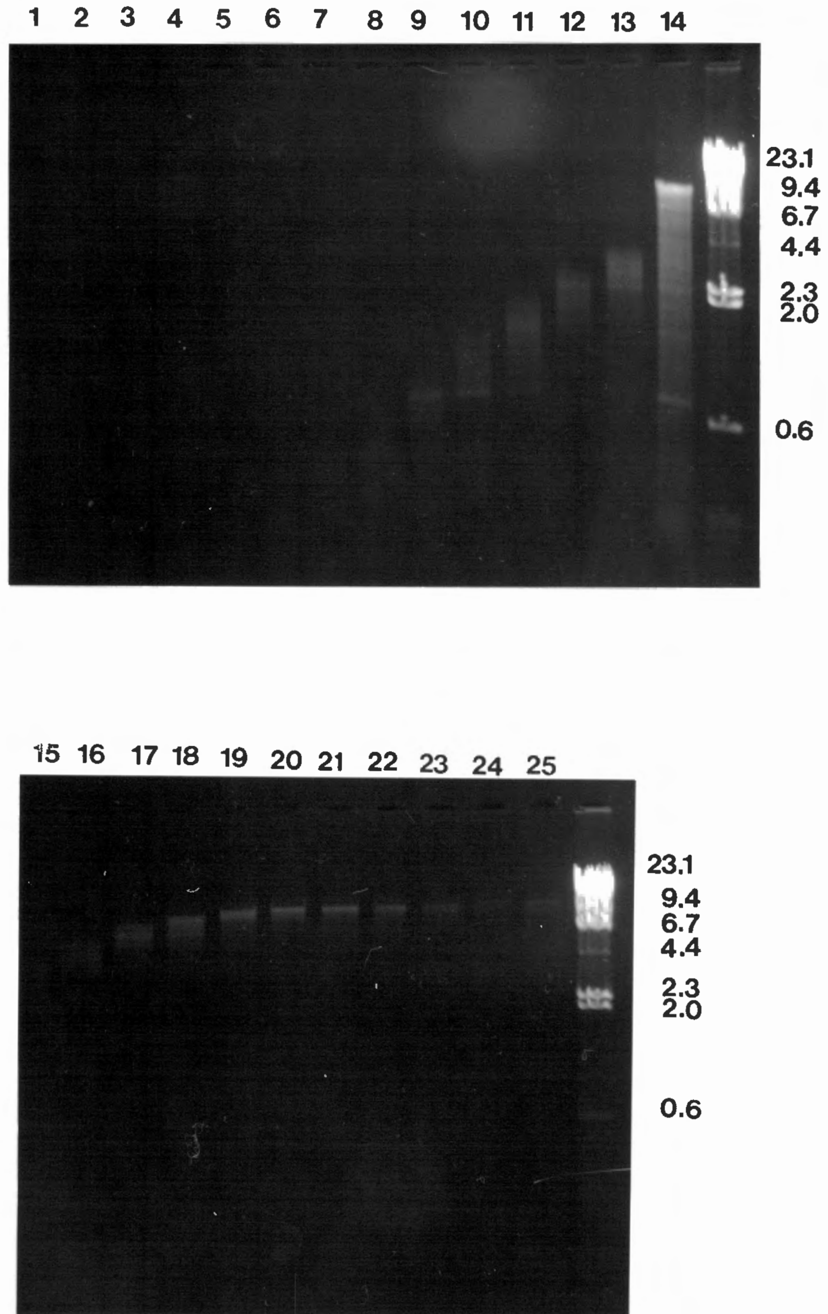


Figure 2.7 A Agarose gel electrophoretic analysis of fractions collected after sucrose gradient fractionation of Pst I digested LSDV DNA. Gradients were centrifuged at 30 000 rpm for 16 hr at 20°C. Fractions 7-14 and 15-25 were pooled respectively for cloning.

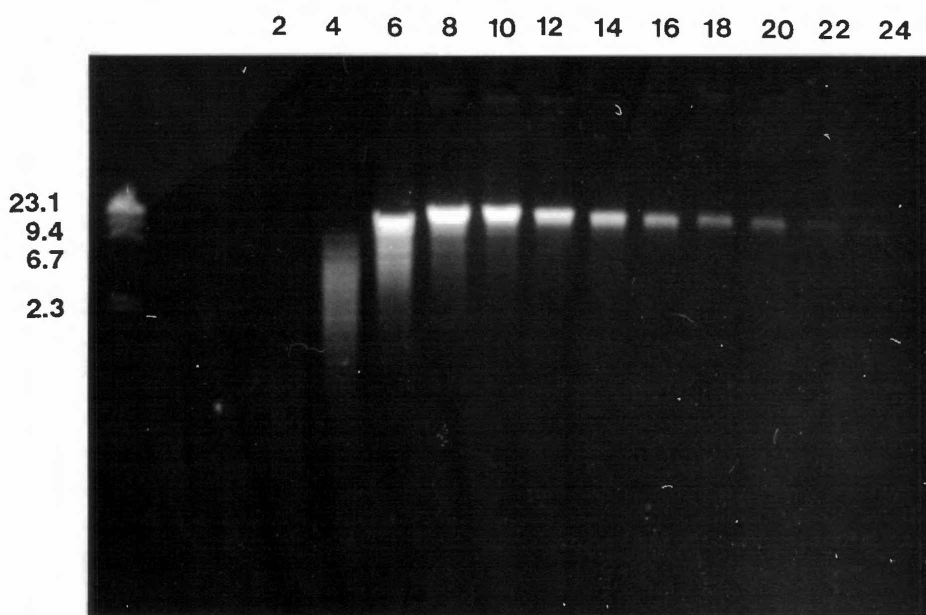


Figure 2.7 B Agarose gel electrophoretic analysis of fractions collected after sucrose gradient fractionation of Bam HI digested LSDV DNA. The gradient was centrifuged at 30 000 rpm for 16 hr at 20°C. Fractions 1-4 and 5-24 were pooled respectively for cloning.

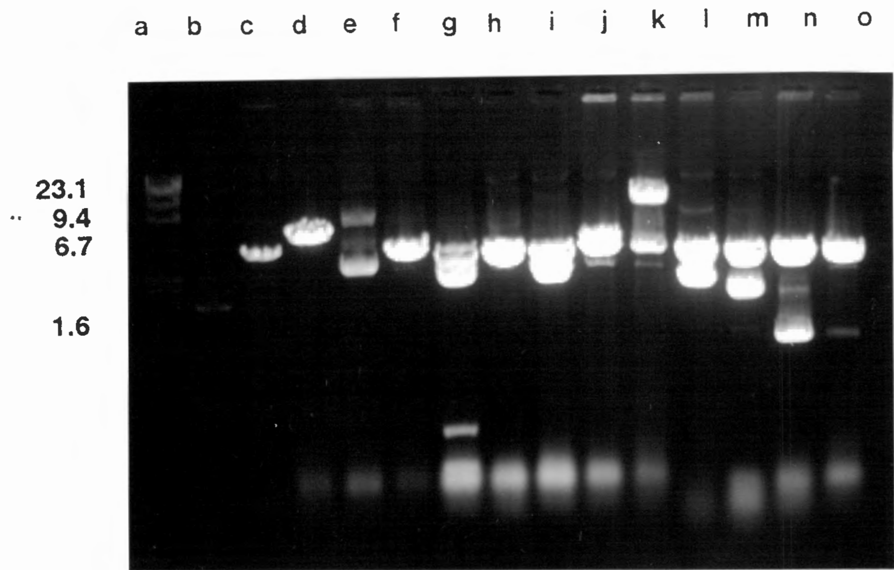
2.4.4.2 Selection of recombinant clones

White colonies, indicative of recombinants, were obtained after cloning Pst I and Bam HI LSDV fragments into pBS by plating transformed *E. coli* cultures on Amp/X-gal/IPTG agar plates. Pooled fragments that were obtained by sucrose gradients (previous section 2.4.4.1), were used. Cloning of the smaller range of fragments produced approximately 40% white colonies, while cloning of the larger range produced approximately 15% . Forty Pst I and sixty Bam HI recombinants were picked and propagated in an overnight culture. A small scale plasmid extraction was performed on each culture and the plasmids digested with either Pst I or Bam HI, depending on the recombinant. These were analysed on 1% agarose gels to confirm the presence of inserts. Examples of some of these gels are shown (Fig. 2.8 A and B).

2.4.5 Screening for LSDV-specific clones

Prior to obtaining LSDV DNA preparations of good quality (purified from virions 2.4.2.3), cloning was performed with viral DNA purified directly from cell cultures (section 2.4.2.1). Since this DNA was contaminated with bovine genomic DNA, it was anticipated that a percentage of these recombinants would contain bovine and not LSDV inserts. Screening for LSDV-specific clones was performed by subtractive dot-spot hybridisation (section 2.3.11.1). Extracted plasmid DNA from each recombinant was immobilised onto nylon membrane, denatured and hybridised to a bovine genomic DNA probe, extracted directly from MDBK cells (section 2.3.4.1) and labelled by random priming (section 2.3.13.2). As controls, genomic LSDV DNA (Fig. 2.9 A, lane a8) purified by Esposito's method, genomic bovine DNA (Fig. 2.9 A, lane b8) and pBluescribe vector DNA (Fig. 2.9 A, lane c8) were also spotted. High stringency washes (> 90% homology) of the membrane ensured that positive hybridisation signals (light spots, Fig. 2.9 A) were those representing cloned bovine DNA. Such recombinants were discarded, whereas those which did not hybridise to the bovine probe, were assumed to be LSDV-specific. This result also confirmed that the LSDV DNA purified directly from cell culture was indeed contaminated, as it reacted positively to the bovine probe (Fig. 2.9 A, lane a8). As was expected, the strongest hybridisation signal was obtained from the positive control, i.e. the immobilised bovine genomic DNA (Fig. 2.9 A, lane b8).

To confirm the identity of those recombinants which did not react to the bovine probe, an identical blot was hybridised to a labelled genomic LSDV DNA probe (Fig. 2.9 B). This, however, was unsuccessful as only the controls, namely purified genomic LSDV (Fig. 2.9 B lane a8) and



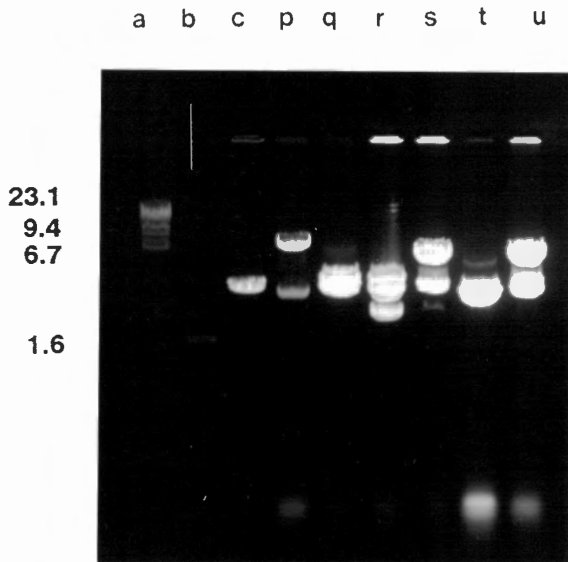
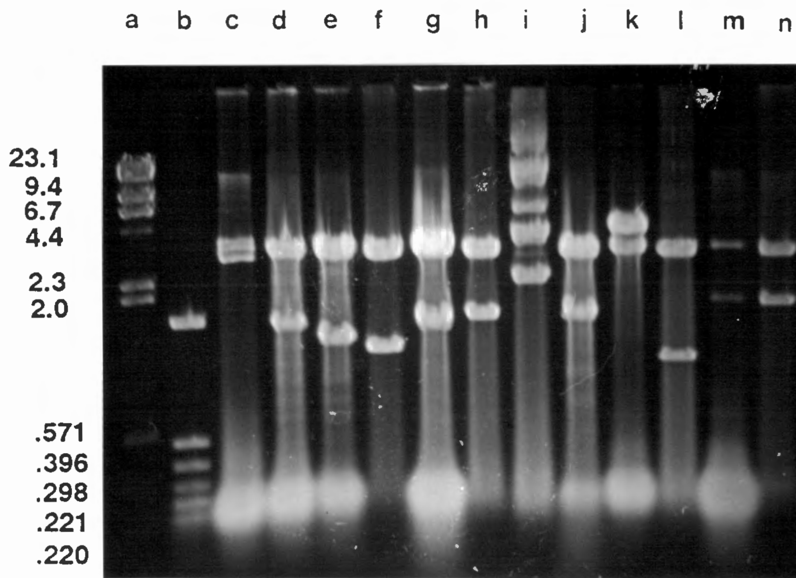


Figure 2.8 A Agarose gel electrophoretic analysis of recombinants obtained after cloning Pst I digested LSDV fragments into pBS. White colonies were selected, plasmids extracted and DNA digested with Pst I (d-u), to determine which contained an insert. Linearised pBS (c) was included as a positive control, while Hind III-digested λDNA (a) and Hinf I-digested pAT153 (b) were used as molecular size markers.



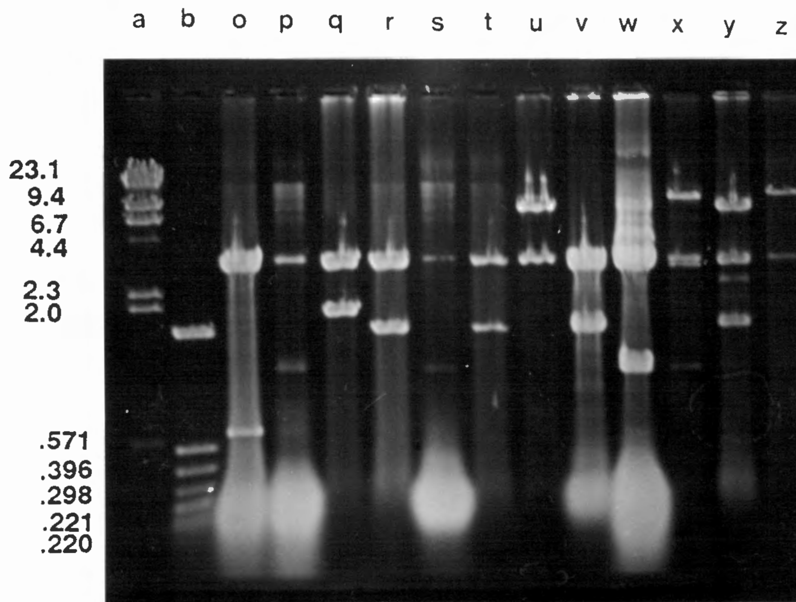
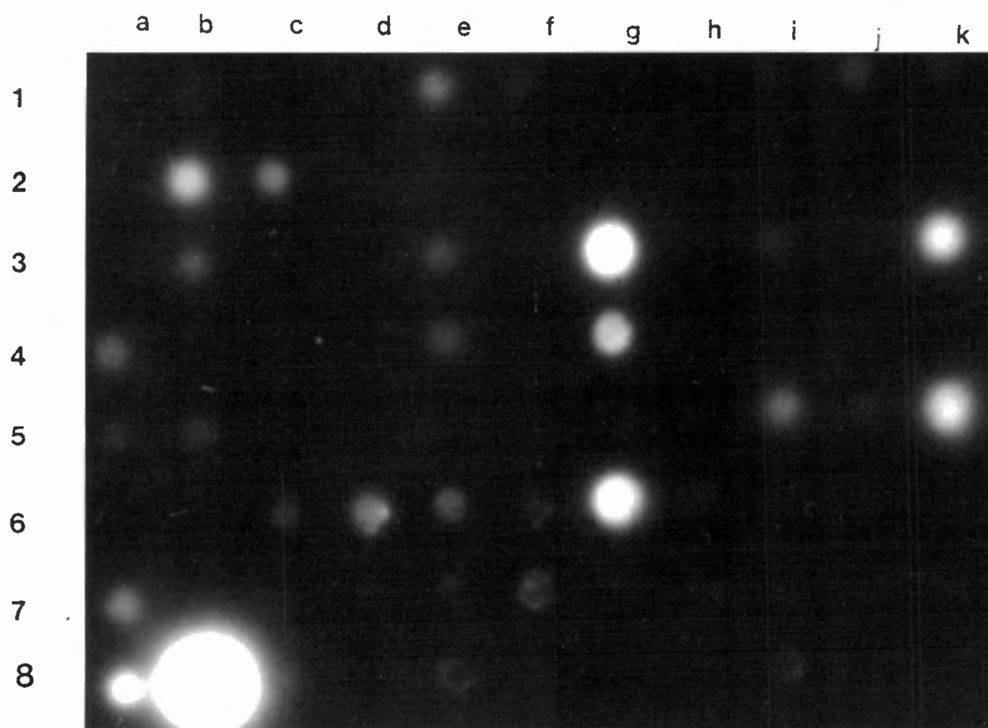


Figure 2.8 B Agarose gel electrophoretic analysis of recombinants obtained after cloning Bam HI digested LSDV fragments into pBS. White colonies were selected and extracted plasmids digested with Bam HI to determine which contained an insert (c-z). Molecular size markers λ/Hind III (a) and Hinf I/pAT153 (b) were used.



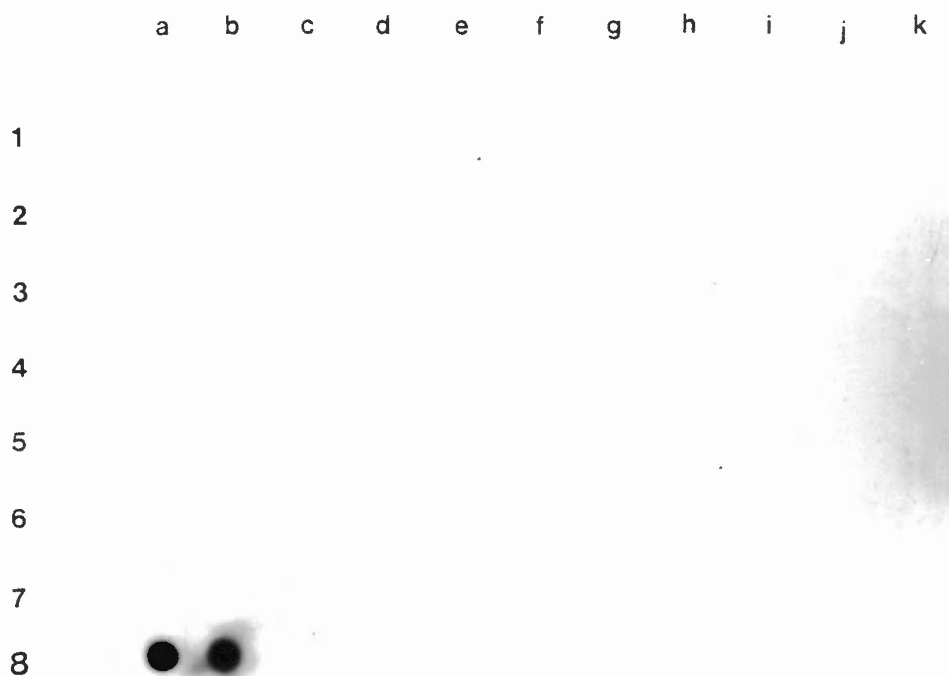


Figure 2.9 A and B Dot-spot hybridisation of recombinant pBS clones (rows 1-7) to a (A) bovine genomic and (B) LSDV genomic probe. Identical membranes were used. Purified LSDV (lane a8), bovine (lane b8) and pBS (lane c8) DNA were spotted onto the membranes as controls.

bovine (Fig. 2.9 B lane b8) DNA hybridised to the probe. The immobilised bovine DNA cross-hybridised to the LSDV probe, since the viral DNA used to prepare the probe was slightly contaminated with bovine DNA.

The absence of any hybridisation signals from the recombinants, may be explained by taking copy numbers into account. Since the entire genome of LSDV was used as a probe, only a limited number of any specific segment would be present and labelled. As such, even if hybridisation did occur, it could not result in a positive signal. This problem was solved by reversing the approach and using DNA from a specific recombinant as a probe, thereby labelling only the insert of interest. Each recombinant was radioactively labelled by nick translation (section 2.3.13.1) and hybridised to immobilised LSDV- and bovine genomic DNA respectively (Fig. 2.11 B).

2.4.6 Southern blotting of LSDV recombinants

To identify the cloned LSDV fragments (cloned as either Pst I or Bam HI fragments), Southern blotting was performed (section 2.3.11.2). Preparative agarose gels of either Pst I (Fig. 2.10 A) or Bam HI (Fig. 2.10 B) digested LSDV DNA were made and the DNA blotted onto a nylon membrane. A membrane was cut into strips that were then hybridised to each prospective LSDV recombinant, identified in the previous section (2.4.5).

The specific Pst I or Bam HI fragment a recombinant contained, in the case of those accommodating small LSDV fragments, was easily identified from autoradiographs in this way (Fig. 2.11 A and 2.12. This included those containing the Bam HI fragments (Fig 2.12) M (pKV28), N (pKV13, pKV17, pKV36, pKV39, pKV44), O (pKV4) and P (pKV20, pKV33, pKV46) and also recombinants containing the Pst I fragments (Fig 2.11 A) K (pKV9) and N (pKV6). Results concerning these recombinants are summarised in table 2.2 (page 72).

In the case of recombinants containing large LSDV fragments, the specific Pst I or Bam HI fragment could, however, not be identified by Southern blotting, since the large fragments were not sufficiently separated on preparative agarose gels and had a poor resolution (Fig. 2.10 A and B). As such, those containing identical sized fragments could also not readily be identified. The identification of the specific large fragment a recombinant contained, was instead performed by double digestion of the DNA.

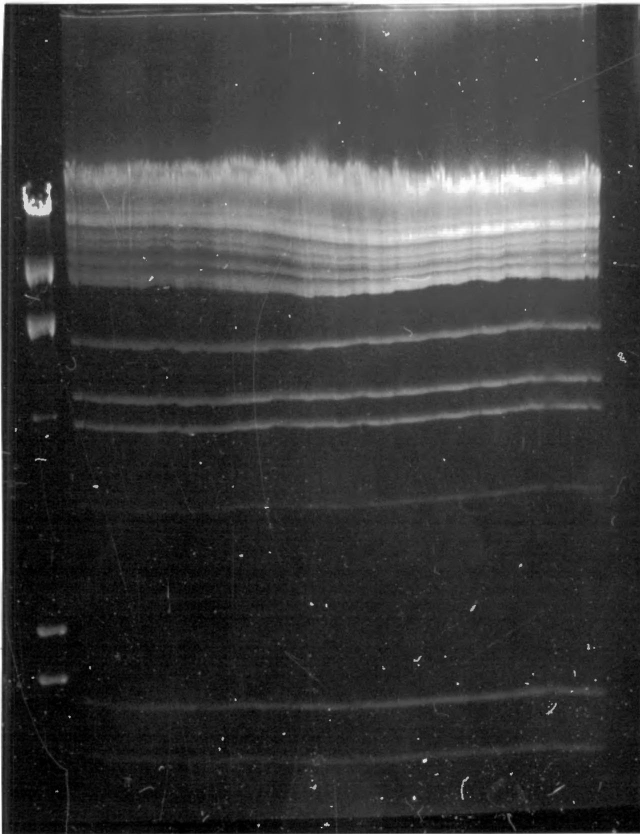


Figure 2.10 A
A preparative agarose gel of Pst I digested LSDV DNA.

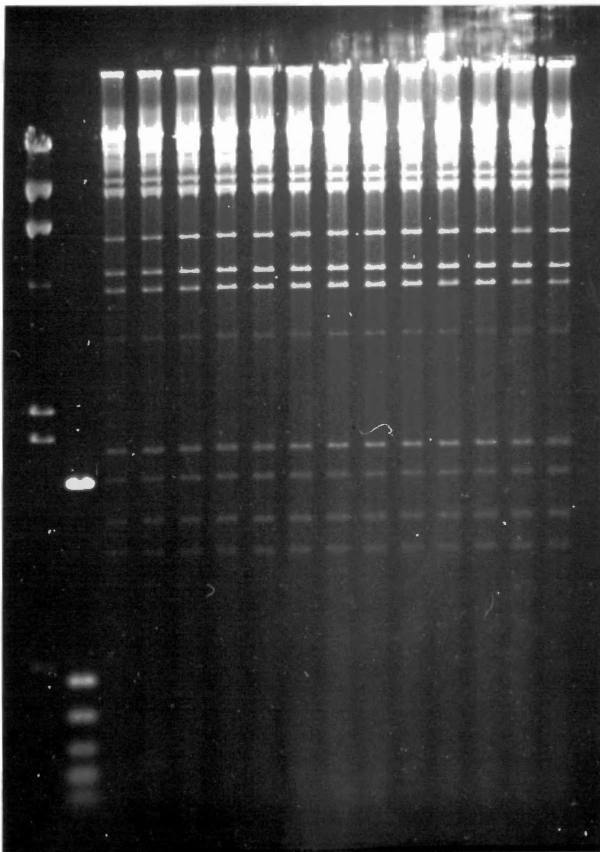


Figure 2.10 B
A preparative agarose gel of Bam HI digested LSDV DNA.

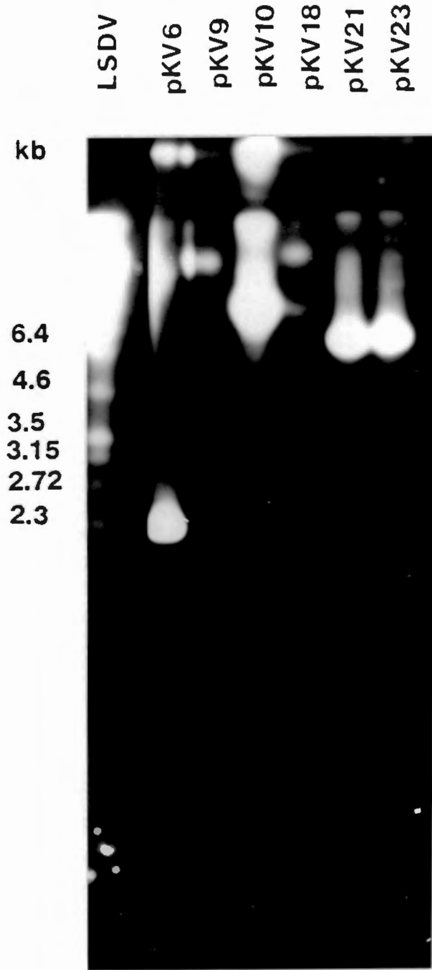


Figure 2.11 A
Autoradiogram of Southern blotting performed on Pst I digested LSDV. Pst I recombinants as indicated above the autoradiograph, were used as probes. A genomic LSDV probe was included as a control.

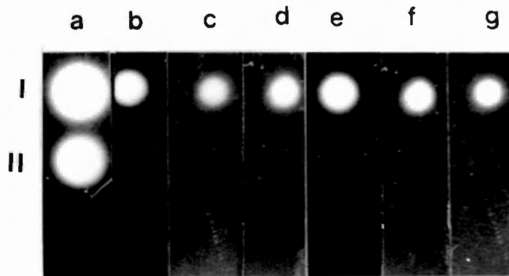


Figure 2.11 B
Dot spot hybridisation of the pKV6 (b), pKV9 (c), pKV10 (d), pKV18(e), pKV21 (f) and pKV23 (g) probes to genomic LSDV (I) and bovine (II) DNA. A genomic LSDV probe (a) was included as a control.

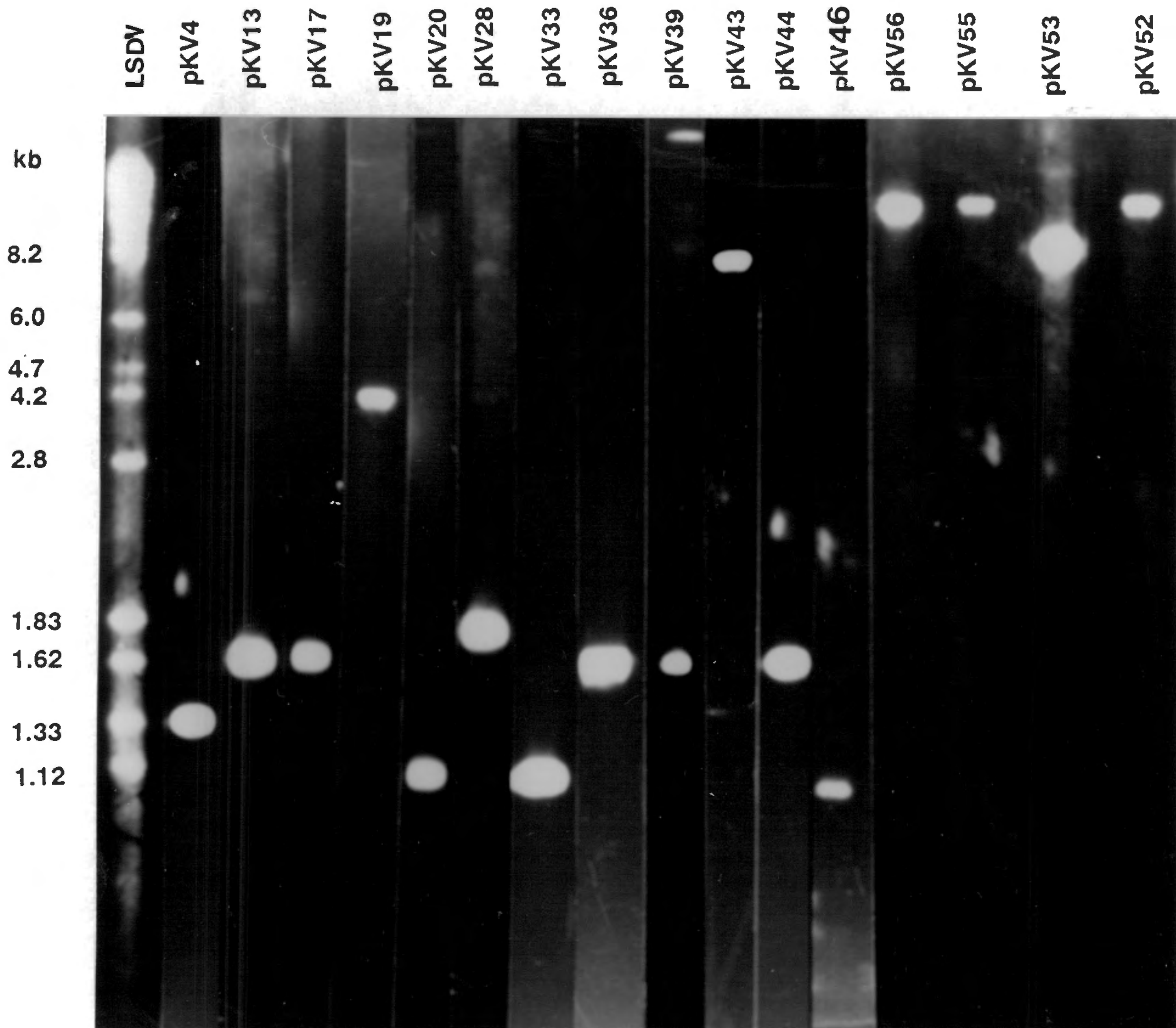


Figure 2.12 Autoradiograph of Southern blotting performed on Bam HI digested LSDV. DNA of Bam HI recombinants as indicated above the autoradiograph, were used as probes. A genomic LSDV probe was included as control. Dot spot hybridisation of these clones to genomic LSDV and bovine DNA (as in Fig. 2.10 B) was also performed to confirm that they were indeed LSDV-specific, but results are not presented.

2.4.7 Double digestion

In cases where the identity of the large sized cloned LSDV DNA fragments could not be identified by Southern blotting (Fig. 2.11 A pKV10, pKV18, pKV21, pKV23 and Fig. 2.12 pKV43, pKV52, pKV53, pKV55 and pKV56), double digestion was performed on DNA of those recombinants. The DNA was digested simultaneously with the r.e. used to clone the fragment (which yielded the intact vector pBS and the insert) and with another r.e. present in the multiple cloning site of the vector (which cleaved the insert into smaller pieces, but not the vector). In this way the length of the insert could be determined accurately, allowing identification of the specific fragment. Furthermore, recombinants containing the same fragment, had the same r.e. profile after this double digestion procedure and could thus be identified.

Pst I recombinants pKV10, pKV18, pKV21 and pKV23 were digested with either Pst I + Eco RI or Pst I + Cla I. Digestions with Pst I, which yielded the intact insert, were also performed. Electrophoretic analysis (Fig. 2.13 A) indicated that pKV21 and pKV23 (lanes i and j; m and n) contained the same insert, since they had the same r.e. profiles after the double digestions, i.e. neither had Eco RI or Clä I sites in their inserts. For furher conformation these two recombinants were also digested with Pst I + Hind III (results not shown) and again, both had no internal Hind III site. It would therefore seem that pKV21 and pKV23 contain the same insert. It might, however, be a coincidence that neither has internal recognition sites for these restriction enzymes and that they actually do contain different inserts. Definitive proof can be obtained by cross-hybridising the pKV21 and pKV23. pKV10 and pKV18 showed different r.e. profiles after double digestions with the same combination of restriction enzymes (Fig. 2.13 A lanes g and h ; k and l) and therefore contain different inserts.

Bam HI recombinants pKV43, pKV52, pKV53, pKV55 and pKV56 were analysed by digesting them with Bam HI (which yields the intact insert), Bam HI + Hind III and Bam HI + Eco RI (Fig. 2.13 B). The results of the Bam HI digestion indicated that more than one insert was apparently cloned into pKV52 and pKV53 (lanes b and c), which might be attributed to either incompletely digested LSDV fragments or incorporation of more than one fragment.

Double r.e. digestions of pKV55 and pKV56 (Fig. 2.13 B lanes l and m ; q and r) resulted in identical r.e. profiles, verifying that the same LSDV fragment had been cloned in both recombinants. Recombinants pKV43 and pKV53 (Fig. 2.13 B lanes i and k ; n and p) also had similar, but not identical profiles. Two additional inserts (sizes 1.7 and 2.4) were present in pKV53 (Fig. 2.13 B lane

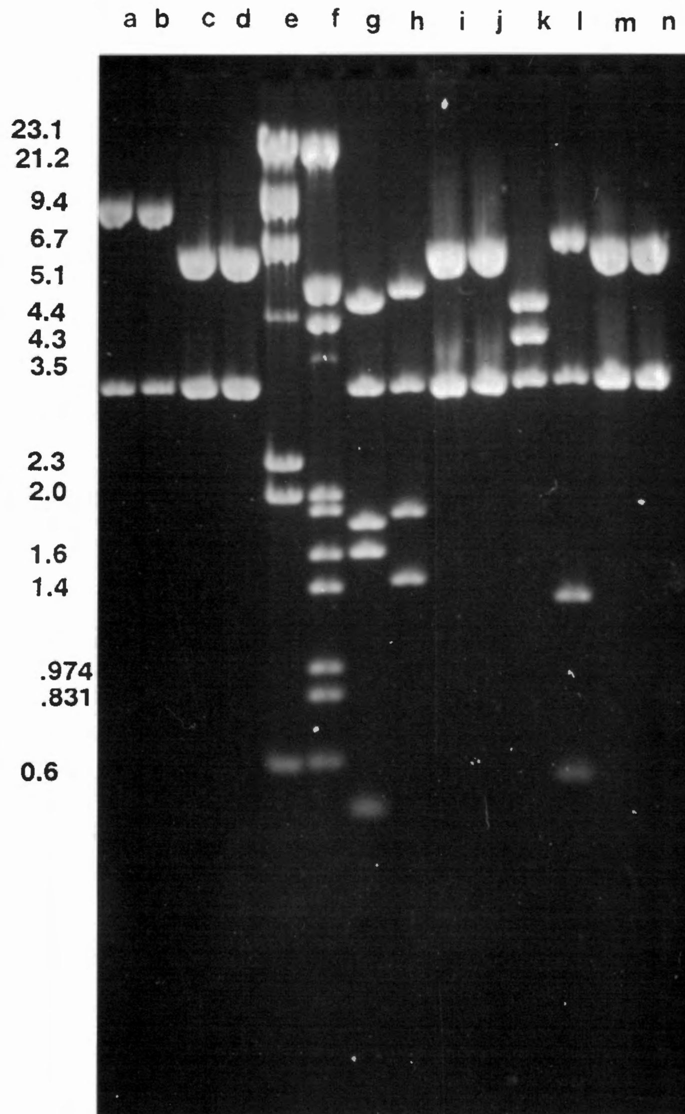


Figure 2.13 A Electrophoretic analysis of LSDV/Pst I recombinants after double digestions.
(a) pKV10/Pst I (b) pKV18/Pst I (c)pKV21/Pst I (d)pKV23/Pst I
(e) λ/Hind III (size marker) (f) λ/Hind III+ Eco RI (size marker)
(g) pKV10/Pst I + Eco RI (h) pKV18/Pst I + Eco RI (i) pKV21/Pst I + Eco RI
(j) pKV23/Pst I + Eco RI (k) pKV10/Pst I + Cla I (l) pKV18/Pst I + Cla I
(m) pKV21/Pst I + Cla I (n) pKV23/Pst I + Cla I

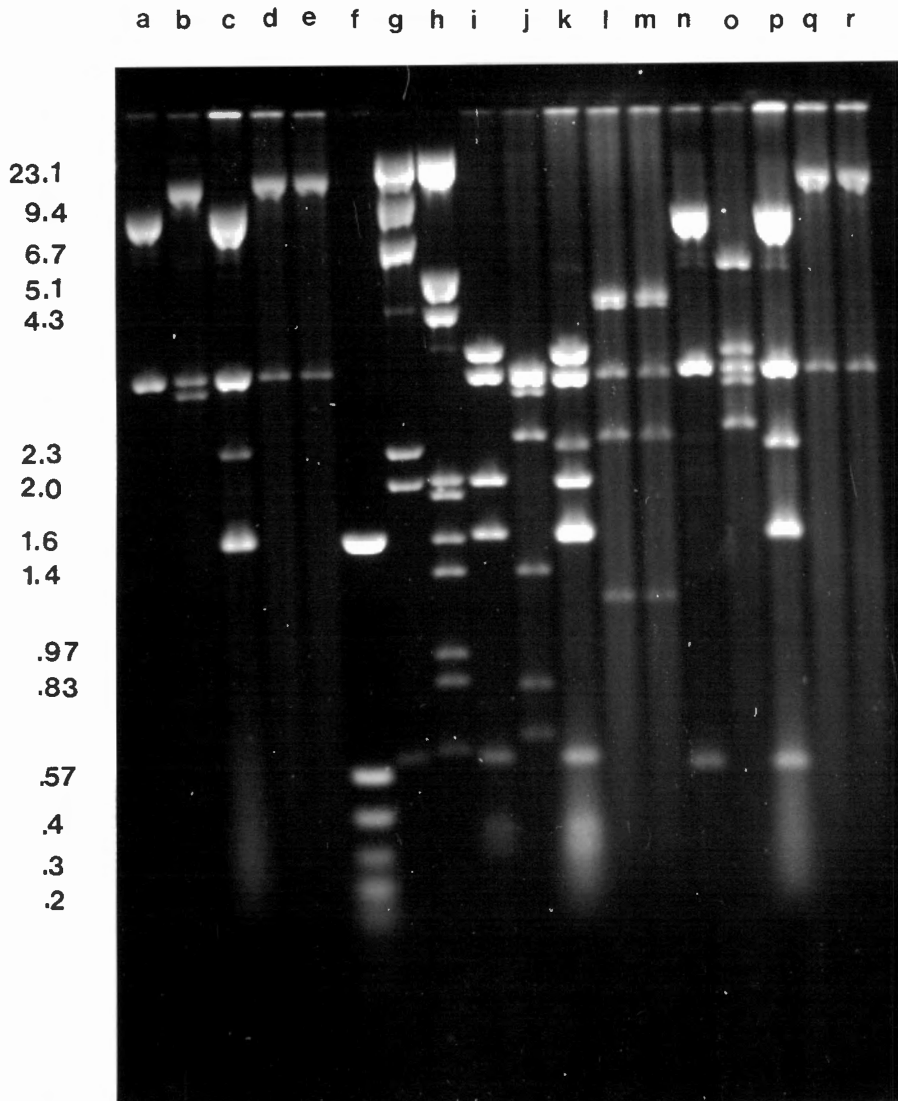


Figure 2.13 B Electrophoretic analysis of LSDV/Bam HI recombinants after double digestions.
(a) pKV43/Bam HI (b) pKV52/Bam HI (c) pKV53/Bam HI (d) pKV55/Bam HI
(e) pKV56/Bam HI (f) pAT153/Hinf I (size marker) (g) λ/Hind III (size marker)
(h) λ/Hind III/Eco RI (size marker) (i) pKV43/Bam HI + Hind III
(j) pKV52/Bam HI + Hind III (k) pKV53/Bam HI + Hind III (l) pKV55/Bam HI + Hind III
(m) pKV56/Bam HI + Hind III (n) pKV43/Bam HI + Eco RI (o) pKV52/Bam HI + Eco RI
(p) pKV53/Bam HI + Eco RI (q) pKV55/Bam HI + Eco RI (r) pKV56/Bam HI + Eco RI

c, k and p). In the Bam HI + Hind III digestion of pKV53 (lane k) two 1.7 kb fragments were present, evident from the double intensity of the band. On the Southern blot, however, both pKV43 and pKV53 hybridised at the same position (Fig. 2.12), i.e. to a fragment of approximately 8 kb in length. Surprisingly, pKV53 did not hybridise to any other LSDV fragment,
which would be expected, considering the fact that it apparently contained 3 Bam HI LSDV fragments. The extra fragments were not of bovine origin, since pKV53 did not hybridise to genomic bovine DNA in dot-spot hybridisation (result not shown). Apart from speculating that these extra fragments may be due to star activity of Bam HI, no concrete explanation can be given. They were not further investigated and omitted from calculations.

The total length of both inserts of pKV52 as calculated from the double digestions (Fig. 2.13 B lanes j and o) was found to be 14.3 kb. Since the smaller insert was 2.8 kb (from Fig. 2.13 B lane b), the larger fragment would be 11.5 kb in length, i.e. the E fragment of Bam HI digested LSDV DNA. On the Southern blot (Fig. 2.12) pKV52 hybridised only to a fragment of approximately 14 kb. This could be explained if the LSDV DNA used for the preparative gel was not digested to completion and the pKV52 probe all hybridised to the incompletely digested fragment. It therefore seems as if pKV52 contains the E and a fragment of 2.8 kb, corresponding to the L fargments of Bam HI digested LSDV DNA.

A summary of the results of the double digestions, where sizes of all inserts were calculated from logarithmic curves (section 2.3.7) are given in table 2.2 *). In this table the combined results of the Southern blotting and double digestions as to which Pst I and Bam HI fragments were finally cloned, are presented.

Table 2.2 A summary of the specific LSDV Pst I (A) and Bam HI (B) fragments that had been cloned based on analysis of recombinants by Southern blotting and double digestions (*).

Recombinant	Pst I fragment	Size in kb
pKV6	N	2.3
pKV9	K	4
* pKV10	F	8.9
* pKV18	G	8.2
* pKV21, pKV23	H	6.4
Total length cloned as Pst I fragments :		29.8

Recombinant	Bam HI fragment	Size in kb
pKV20, pKV33, pKV46	P	1.12
pKV4	O	1.33
pKV13, pKV17, pKV36, pKV39, pKV44	N	1.62
pKV28	M	1.83
* pKV43	H	8.3
* pKV53	H + unknown	8.3
* pKV52	E + L	11.5 + 2.8
* pKV55, pKV56	D	12.95
Total length cloned as Bam HI fragments :		41.45

Total length of the LSDV genome cloned (Pst I	+	Bam HI fragments) :		
29.8 kb	+	41.45 kb	=	71.25 kb

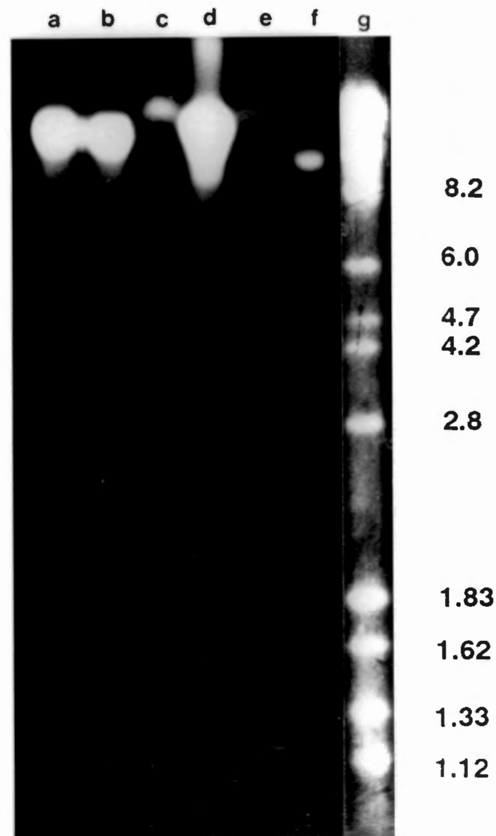


Figure 2.14 Autoradiograph of Southern blotting performed by hybridising the different Pst I recombinants to Bam HI digested LSDV DNA. pKV6 (a), pKV9 (b), pKV10 (c), pKV18 (d), pKV21 (e) and pKV23 (f) were used as probes. A genomic LSDV probe hybridised to Bam HI digested genomic LSDV DNA was included as a control (g).

To calculate the LSDV genome portion which was finally cloned, the possibility that the Pst I recombinants contained the same or overlapping regions as the Bam HI recombinants, were considered. For verification, the Pst I recombinants were radioactively labelled and hybridised to electrophoretically separated Bam HI digested LSDV DNA, blotted onto a nylon membrane (section 2.3.11.2). The reasoning being that, because it was now known which LSDV/Bam HI fragments had been cloned (Table 2.3), LSDV/Pst I recombinants that hybridised to those specific Bam HI fragments shared mutual sequences with it, signifying common inserts..

Results (Fig. 2.14) demonstrated that all of the Pst I recombinants hybridised to very large Bam HI fragments, i.e. those larger than was cloned. It was therefore concluded that none of the recombinants shared common inserts. The total length of the LSDV genome cloned was subsequently calculated by adding the cloned Pst I (29.8 kb, table 2.3) and Bam HI (41.45 kb, table 2.3) fragments, amounting to 71.25 kb. This represents nearly 50 % of the 143 kb LSDV genome.

2.5 DISCUSSION

In this study, approximately 50% of the LSDV genome (Neethling vaccine strain) was cloned into the plasmid vector, pBluescribe. To accomplish this, purified DNA of good quality had to be acquired. Three methods of purification were investigated: DNA was purified from a crude viral preparation (Esposito *et al.*, 1981) and although relatively large quantities (5mg LSDV DNA/20 RB) could be obtained in this way, the LSDV DNA was excessively contaminated with bovine DNA. Since such contamination complicates cloning procedures, this was not considered to be an appropriate method. Secondly, LSDV DNA was separated from bovine DNA by pulsed field agarose gel electrophoresis and extracted from the gel by electroelution. The problem of contaminating DNA was overcome, but inadequate amounts of LSDV DNA for cloning were recovered. The best quality of reasonable amounts (1mg/20 RB) of LSDV DNA was finally obtained by extracting it from purified virions. Although DNA yields were lower than was obtained by direct extraction, almost no contaminating bovine DNA was present.

Using the DNA isolated from purified virus, various restriction enzyme profiles were compiled. Pst I and Bam HI digestions yielded several fragments of 3 kb to 10 kb in size and were thus used for cloning. The LSDV genome length was estimated from these restriction enzyme profiles and found to be approximately 145 kb, a size similar to that of the Kenya isolate, KC1. (Gershon and Black, 1987). A limitation was that lengths of the large fragments (> 20 kb) could not be determined accurately due to the poor resolution in the agarose gel system used. This estimate is therefore likely to be subject to an error rate, which might be as high as 15% . The use of pulsed field gel electrophoresis could conceivably improve the resolution of the large fragments and allow more accurate sizing of the genome. Since accurate sizing was not one of the aims of this investigation, PFGE was not performed for this purpose.

DNA isolated from the LSDV Neethling and KC-1 (Gershon and Black, 1987) isolates yielded different Pst1 restriction enzyme digestion profiles. (see table 2.1, page 54). The Neethling strain produced 14 Pst I fragments, in contrast to the 12 of KC-1. There were, nevertheless, apparent similarities since for most of the restriction fragments reported for KC-1, comparable size fragments were obtained with the Neethling DNA (table 2.1, fragments A-H, K, L and N). There were, however, some bands for which corresponding fragments were not identified. It should be noted that a limitation of such comparisons is that it is impossible to determine the origins of such discrepancies. Despite this, the differences in restriction enzyme profiles are an indication that the Neethling and KC-1 isolates represent two distinct strains.

The major aim of this section of the work was the molecular cloning of the genome. Since restriction enzyme digestions yielded a wide range of fragment sizes, it was necessary to enrich for the larger fragments by means of sucrose density gradient centrifugation to obviate producing an unmanageable number of clones and duplication of small fragments. DNA fragments of 8.2 kb (G fragment of Pst I/LSDV), 8.9 kb (F fragment of Pst I/LSDV), 8.3 kb (H fragment of Bam HI/ LSDV), 11.5 kb (E fragment of Bam HI/LSDV) and 12.95 kb (D fragment of Bam HI/LSDV) were successfully cloned into the Bluescribe plasmid. Together with a number of smaller segments, this represents approximately 50% of the LSDV genome. These relatively large inserts are not likely to be deleted from this plasmid according to the product information sheet of the supplier.

The availabilty of these clones now opens the door for further molecular characterisation of the LSDV (Neethling) genome. One of the projected aims is to identify strong promoter sequences present in the cloned fragments. A strong promoter is required to drive the expression of foreign genes if LSDV is to be used as an recombinant vaccine vector. The amount of transcribed mRNA is an indication of the strength of a promoter and has been used for promoter identification (Kumar and Boyle, 1990) by means of Northern blot hybridisation. Messenger RNA will be extracted from LSDV infected cells and hybridised to each recombinant obtained in this study. This will allow identification of specific recombinants which contain genes that are strongly expressed. Such genes may probably be under the control of strong promoters. The DNA from such recombinants may then be examined further to accurately locate the structural gene and its accompanying promoter. This should facilitate further detailed characterisation of the promoter.

CHAPTER THREE

TAILORING AND SEQUENCING OF BTV4 VP5

3.1 INTRODUCTION

Even though the development of LSDV as a live recombinant vector is still in its initial stages, a trial gene to be cloned and expressed in this system was prepared and sequenced. The gene decided upon was a gene of South African bluetongue virus serotype 4, coding for viral protein 5 (SA-BTV4 VP5), a component of the outer capsid of the virus. This was partly done in order to gain experience in the methodology involved, but would also serve other purposes as this gene is to be cloned into the vaccinia virus system by other researchers in our laboratory.

At least 24 BTV serotypes have been identified (Gorman *et al.*, 1983; Knudson and Shope, 1985) and VP2 has been shown to be the principal serotype-determining antigen (Huismans and Erasmus,1981; Kahlon *et al.*, 1983, Mertens *et al.*, 1989). It elicits neutralising antibodies in sheep, capable of protecting against virulent challenge (Huismans *et al.*, 1987). Wade-Evans *et al.* (1988) have shown that VP5 may play a role in the determination of serotype and there is evidence that immunisation with soluble VP2 and VP5 can induce higher titres of neutralising antibodies than immunisation with VP2 alone (Huismans *et al.*, 1987; Roy *et al.*, 1990). The actual function of VP5 is however not yet known. Expression in a recombinant expression system, such as VV or LSDV might facilitate knowledge in this regard.

Huismans and Cloete (1987) have cloned the cDNA form of BTV4 VP5 into the bacterial plasmid vector pBR322 and designated the recombinant p73, using a GC-homopolymeric tailing strategy. This strategy involved the addition of homopolymer tails in two different stages. In the first stage poly(A) tails were synthesised at the 3'-termini of the dsRNA segment using *E. coli* polyA polymerase, to provide oligo-dT priming sites for reverse transcription. Secondly, oligo-dC tails were added to the 3'-termini of the cDNAs. The cDNA was then annealed to Pst I-digested oligo-dG tailed pBR322 vector, to produce the recombinant. It is known, however that these homopolymeric sequences which are present at the termini of the gene, inhibit expression of the cloned gene (eg. Steuber *et al.* 1984; Galili *et al.* 1986). It is therefore necessary to remove these sequences prior to cloning into an expression system. This chapter describes the strategy followed to tailor the gene by means of the polymerase chain reaction (PCR) and the sequencing of the resulting BTV4 VP5 PCR tailored gene.

3.2 MATERIALS

3.2.1 Primers

The following primers were synthesised and supplied by the Biochemistry department, UCT under contract to Beckman Instruments.

OP-12 PRIMER

Sequence	:	5'-	ATCC	GGATCC	GTAAGTGTAAGCTTC -3'
				BamHI	3'-terminal of (-) strand

Composition : OP-12 is a 25-mer consisting of 15 deoxynucleotides complementary to the 3'-terminal end of the BTV4 VP5 non-coding strand and preceded by a 5'-non-complementary decanucleotide sequence which specifies a Bam HI site (underlined). The 4-nucleotide 5'-extension sequence was included to stabilise the r.e. site.

OP-11 PRIMER

Sequence	:	5'-	ATCC	GGATCCG	TTAAAAGTATTCTC -3'
				BamHI	3'-terminal of (+) strand

Composition : OP-11 is a 25-mer consisting of 15 deoxynucleotides complementary to the 3'-terminal end of the BTV4 VP5 coding strand and preceded by a 5'-non-complementary decanucleotide sequence which specifies a Bam HI site (underlined). The 4-nucleotide 5'-extension sequence was included to stabilise the r.e. site.

3.2.2 Plasmids

Recombinant plasmid p73 (amp^r, tet^r) which contains the VP5-gene of bluetongue virus serotype 4 (Huismans and Cloete, 1987) was kindly provided by Prof. H. Huismans, Department of Genetics, University of Pretoria, Pretoria. Plasmid pUC13 was purchased from Clontech Laboratories, Inc. (USA).

3.2.3 Radio-isotopes

Deoxyadenosine 5'-α-[^{35}S]-thiotriphosphate triethylammonium salt with a specific activity of 1000 Ci/mMol and a radioactive concentration of 10 mCi/ml (370 MBq/ml) was purchased from Amersham International (UK).

3.2.4 Reagents used for sequencing

Item	Manufacturer
TaqTrack™ sequencing kit	Promega (USA)
RNaseA	Boehringer Mannheim
PEG 6000	Sigma (USA)
M13 forward and reverse primers	

3.2.5 Photographic material

Item	Manufacturer
Ilford PAN F film 35mm	Ciba Geigy, (Pty)Ltd.(Switzerland)
Cronex MRF 31 X-Ray film	Dupont (USA)

3.2.6 Other materials

Item	Manufacturer
Geneclean(TM) kit	Bio 101 Incorporated (USA)

3.3 METHODS

3.3.1 Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) procedure was originally described (Saiki *et al.*,1985) as a means of amplifying a specific region of which the flanking sequences are known. It also provides a method whereby the ends of cloned genes may be tailored (Dennis, 1990). The reaction consists of successive cycles of denaturation and extension of sequence-specific primers by a heat resistant DNA polymerase of *Thermus aquaticus* (Taq DNA Polymerase).

The DNA template (100-500ng of dsDNA) was diluted in the presence of an excess (2.5μg each) of the oligonucleotide primers OP-11 and OP-12 to a volume of 38μl in water. To this was added 10μl 1mg/ml BSA; 8μl each of 10mM dATP, dCTP, dGTP and dTTP and 20μl 5X Taq polymerase buffer (0.335M Tris-HCl pH 8.8, 33.5mM $MgCl_2$, 83mM $(NH_4)_2SO_4$, 50mM β-ME) which brought the reaction mix to a final volume of 100μl. To avoid evaporation, liquid paraffin (100μl) was placed carefully over the reaction mix and the DNA was denatured at 94°C for 5 min. The primers were then allowed to anneal by incubating the mix at 37°C for 2 min, followed by the addition of 1μl (2.5 units) of Taq polymerase through the paraffin layer. The extension reaction was carried out by incubating at 72°C for 6 min. Twenty cycles of PCR (denaturation 1 min at 94°C, annealing 2 min at 37°C, extension 6 min 72°C) were performed manually. A sample of the reaction mix was analysed in a 1% agarose gel.

3.3.2 Purification of DNA from agarose by the Geneclean™ method.

DNA was purified according to the manufacturer's instructions as outlined below. After electrophoretic separation of the DNA, the VP5-gene band was cut out of an EtBr-stained gel and mixed with the saturated NaI solution. The gel was dissolved at 55°C for 5 min followed by the addition of 1μl glassmilk™ per 0.5μg DNA. Glassmilk™ is a silica matrix in water which binds rapidly to DNA, but not RNA at ambient temperature. After 5 min, the silica matrix with bound DNA was pelleted by centrifugation in an IEC Centra-M benchtop microcentrifuge and washed three times with ice-cold NEW™ (NaCl, Ethanol, Water) -wash. The DNA was finally eluted from the silica by resuspending in 15μl water and incubating at 55°C for 2 min. The elution step was repeated twice more to yield a final volume of 45μl purified DNA. 10μl of this DNA was used for the ligation step.

3.3.3 Ligation procedure

Vector DNA was linearised by digestion with the BamHI restriction enzyme. The vector was dephosphorilated by adding alkaline phosphatase enzyme and incubating at 37°C for 30 min. The enzyme was then removed by extraction with an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) and the vector DNA recovered by ethanol precipitation. Ligations were carried out overnight in a final volume of 20μl with 1 unit of T4 DNA ligase at 15°C in a buffer containing 50mM Tris-HCl pH 7.6, 10mM $MgCl_2$, 5% (w/v) PEG 8000, 1mM ATP and 1mM DTT.

3.3.4 Transformation procedure

Transformation was performed as described in Chapter two, section 2.3.8.5.

3.3.5 Sequencing

Sequencing was performed on plasmid DNA using the commercially available TaqTrack^{TM} system, which is an enzymatic dideoxynucleotide method (Sanger *et al.*,1987) with a labelling method developed by Tabor and Richardson (1987). In this method, the sequencing reaction is separated into a labelling step and an extension/termination step. In the first step, the primer is extended a short distance using limiting concentrations of the dNTPs and a single radiolabeled dNTP. In the second step, the extended primers are further extended in the precence of both dd- and dNTPs. Sequencing is performed with the Taq DNA polymerase enzyme, which is a thermostable enzyme purified from *Thermus aquaticus* and replicates DNA at 74°C.

3.3.5.1 RNase-PEG precipitation of plasmid DNA

All reactions were carried out in 1.5 ml Eppendorf tubes. CsCl-purified plasmid DNA (30μg in 150μl water) was incubated with 3μl 10mg/ml RNAse A at 37°C for 30 min. DNA was precipitated with the addition of 90μl PEG solution (20% PEG 6000, 2.5M NaCl) followed by incubation on ice for 15 min. The precipitate was collected by centrifugation for 15 min in a Sigma MK2 Eppendorf benchtop centrifuge, rinsed with 70% EtOH, lyophilised and resuspended in deionised distilled water.

3.3.5.2 Denaturation of template DNA

For a single sequencing reaction, 4μg DNA was denatured in 0.2M NaOH, 2mM EDTA for

5 min at room temperature. The mixture was neutralised by the addition of 0.4 volumes of 5M ammonium-acetate and the DNA precipitated with 3 volumes of 96% EtOH. After 10 min at -70°C, the DNA was collected by centrifugation for 10 min in a Sigma MK2 benchtop centrifuge, rinsed with 70% EtOH and lyophilised.

3.3.5.3 Annealing template and primer

The denatured DNA was redissolved in 16µl of deionised distilled water and 5µl Taq DNA polymerase 5X buffer (250mM Tris-HCl pH 9, 50mM $MgCl_2$), 2µl of the extension/labelling mix (7.5µM each of dGTP, dTTP and dCTP) and 2µl (2ng) of the appropriate sequencing primer added. The mixture was incubated at 37°C for 10 min.

3.3.5.4 Extension/labelling reaction

To the 25µl of annealed primer-template mixture, the following were added: 2µl of α-^{35}S-dATP (1000Ci/mMol) and 1.6µl of sequencing grade Taq DNA polymerasel (2.5µg/µl). The reaction was incubated at 37°C for 5 min. This reaction is carried out at 37°C rather than 70°C to slow down the incorporation rate of the enzyme and thereby limit the number of bases incorporated in this step. This allows the sequence to be read close to the primer.

3.3.5.5 Termination reaction

Four tubes were labelled A, C, G, and T and 1µl of the appropriate d/ddNTP mix, with the following composition, was placed in its corresponding tube:

Component	**G nucleotide mix**	**A nucleotide mix**	**T nucleotide mix**	**C nucleotide mix**
ddGTP	50µM	-	-	-
ddATP	-	350µM	-	-
ddTTP	-	-	600µM	-
ddCTP	-	-	-	160µM
dGTP	25µM	250µM	250µM	250µM
dATP	250µM	25µM	250µM	250µM
dTTP	250µM	250µM	25µM	250µM
dCTP	250µM	250µM	250µM	25µM

When the extension/labelling reaction was complete, 6μl of this mix was aliquotted to each of the four tubes, mixed and incubated at 70°C for 5 min. The reaction were stopped by the addition of 4μl stop solution (95% formamide, 10mM NaOH and 0.05% each of Bromophenol Blue and Xylene Cyanol FF). The tubes were heated to 90°C for 5 min immediately before loading 3 μl samples onto a gel.

3.3.5.6 Polyacrylamide gel electrophoresis

The sequencing reactions were analysed by polyacrylamide gel electrophoresis in gels containing 6% acrylamide (from 38% acrylamide, 2% bisacrylamide stock solution), 8M urea and 1X TBE (90mM Tris-HCl pH 8.3, 90mM boric acid, 2.5mM EDTA). Electrophoresis was carried out in 0.5X TBE on a BRL model S2 sequencing apparatus using an LKB 2197 power supply. Gels were run at 2000 V, equivalent to 70 Watts and 40 mA. After electrophoresis, the gel was dried and exposed to X-Ray film for 16 hr at room temperature.

3.4 RESULTS

3.4.1 Tailoring of the cloned BTV4 VP5-gene segment using PCR

The tailoring of cloned cDNA fragments by means of the polymerase chain reaction (PCR) was originally described by Dennis (1990). The method involves the use of oligonucleotide primers complementary to the cDNA sequences immediately adjacent to the homopolymer sequences, to produce amplification products. An outline of the procedure is given in Fig. 3.1. Denatured dsDNA recombinant p73 was used as template for the PCR procedure to generate large amounts of the VP5 DNA it contained. Because only a small amount of starting material was used, the majority of the products contained the termini which were specified by the primers. Primers were constructed in such a way that they contained additional 5'-terminal non-complementary (to template) nucleotides, specifying a r.e. site. The cDNA was thus tailored to contain a Bam HI r.e. site at each termini. This greatly facilitated the eventual cloning of the cDNA fragment and had the added advantage that only molecules containing the r.e. site at both termini, were efficiently ligated to form functional recombinant plasmids.

The PCR product was analysed by agarose gel electrophoresis (Fig 3.2). Amplification yielded a major discrete DNA band of approximately 1.6 kb in length (lane c) when compared with the size marker (lane 2), which was in agreement with the length of the originally cloned fragment. The DNA fragment was purified from excess primers and unincorporated dNTP's, by excising the band from the gel and purifying it by the Geneclean™ procedure. After restriction with Bam HI, the tailored and amplified DNA fragment was ligated into the Bam HI site of pUC13 (Yanisch-Perron, 1985). The ligation mixture was used to transform competent E. coli JM105 (Yanisch-Perron, 1985) cells to ampicillin resistance. On Amp/X-gal/IPTG plates, approximately 33% of the colonies were white, indicating the presence of recombinant plasmids. Five of these colonies were picked, the plasmids extracted, digested with Bam HI and subjected to agarose gel electrophoresis (Fig. 3.3). Two did not contain a recombinant plasmid (lanes d and e) and were ignored. In the other cases, plasmids had incorporated a Bam HI fragment of the apparent correct size when compared with linearised pUC13 vector DNA (lane a). One recombinant (lane b) was selected for sequencing analysis and designated pWF1.

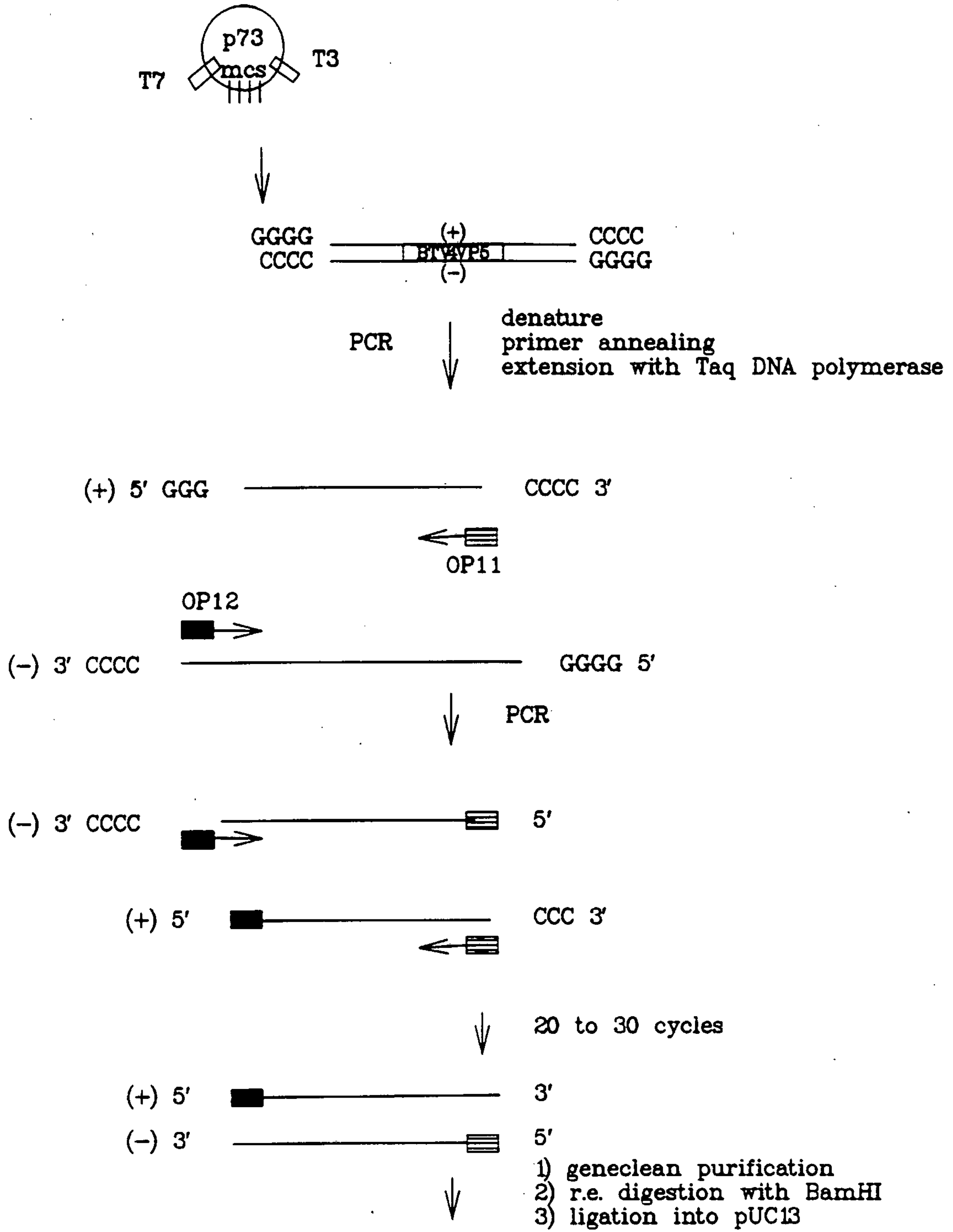


Figure 3.1

Strategy for the removal of GC-homopolymer tails using the polymerase chain reaction.

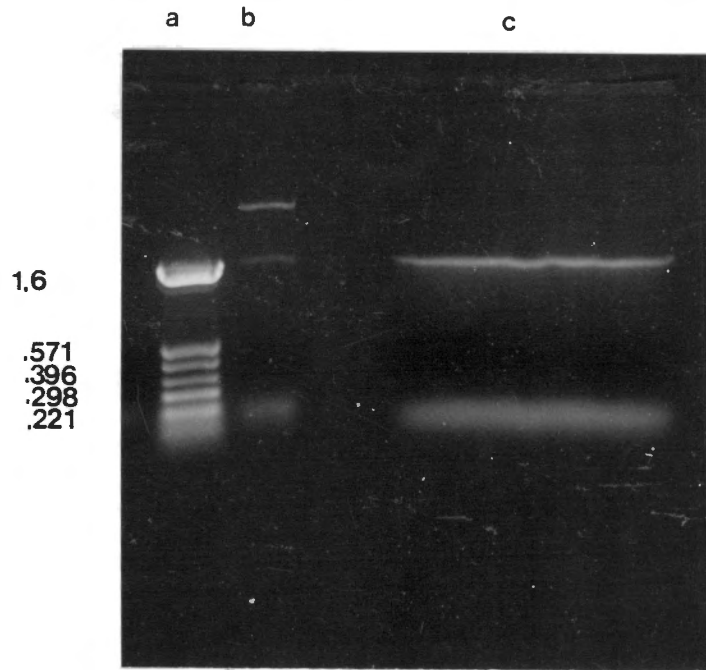


Figure 3.2 Agarose gel analysis of the PCR product after removal of the GC-tails (c). Pst I digested p73 (b), which yielded the vector and the BTV4 VP5 gene insert, was included as a control and pAT153/Hinf I was used as a size marker.

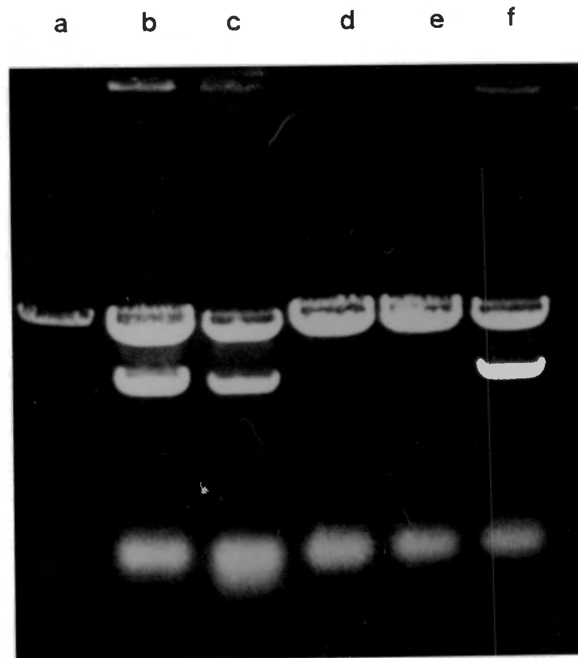


Figure 3.3 Agarose gel electrophoretic analysis of BamHI digested recombinant plasmids after ligation with the tailored VP5-gene (lanes b-f). Linearised pUC13 vector DNA (lane a) was included as a control.

3.4.2 Sequencing strategy

It has been reported that Taq polymerase may have an overall error frequency of 0.25% in typical amplification reaction (Saiki *et al.*, 1988). Sequencing was thus undertaken not only to extend current knowledge of sequences of the VP5-genes of different serotypes of BTV (BTV4 VP5 sequence is not yet known), but also to confirm that the open reading frame (ORF) had not been disturbed by the PCR reaction. An ORF is essential for further expression studies. Sequencing would also confirm that homopolymer tails had indeed been removed.

The pUC13 vector (Yanish-Perron, 1985) contains the M13 multiple cloning site (MCS) which facilitates the use of the M13 forward and reverse primers for sequencing purposes. Because the gene to be sequenced is approximately 1.6 kb in length, sequencing could not be performed from these primers alone (approximately 400bp can be sequenced from a single primer). The MCS offers a series of r.e. sites which are situated close to each other to form a so called cloning "casette" which is very convenient for the purpose of subcloning. By subcloning two regions of the VP5-gene and with the using custom made primers that were synthesised as the sequencing progressed, the complete nucleotide sequence was determined.

3.4.3 Preparation of subclones

Sequencing of approximately 350 nucleotides (nt) of each terminus of the cloned VP5-gene in pWF1 with the M13 forward and reverse primers, verified that tailoring had been sucessful. Homopolymer tails were removed and replaced by the Bam HI r.e. site. Restriction enzyme sites in these terminal regions that could be used for further subcloning, were identified using the Microgenie computer sequencing programme. The first subclone, pWF2, was constructed by excising a Hind III fragment of 180 bp in length from the 3'-terminus of the cloned gene and religating the plasmid. This made sequencing of nucleotides 1100-1400 possible. The other subclone, pWF3, was constructed by excising a Pst I/Sph I fragment from pWF1, purifying it using the Geneclean™ procedure and ligating it into the mulitiple cloning site of pUC18 (yanisch-Perron, 1985) by forced cloning. In this way, 300 bp of the 5'-terminus of the cloned VP5-gene was deleted and sequencing of nucleotides 300 to 570 was accomplished. Sequencing of the remaining nucleotides was accomplished by using primers OP39 (for sequencing of nts 570-850) and OP31 (for sequencing of nts 850-1100). A diagram illustrating the constructed subclones and primers is given (Fig. 3.4), showing the manner in which the cloned VP5-gene was spanned for determination

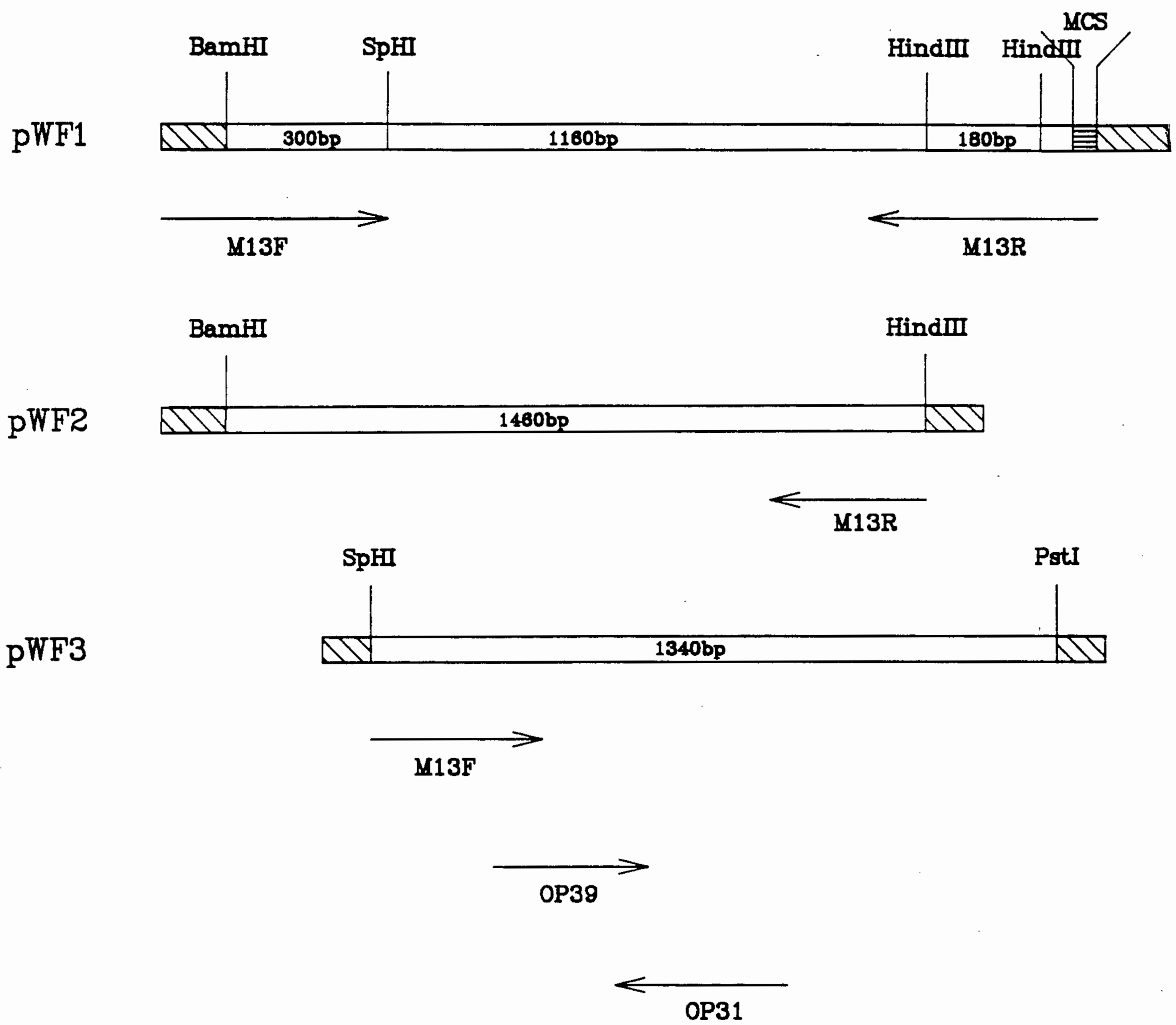


Figure 3.4 Sublones constructed and primers used for the purpose of sequencing the cloned BTV4 VP5-gene.

of its nucleotide sequence. Sequencing was performed in only one direction, i.e. on either the (+) or the (-) strand of the cloned DNA, depending on the primer used.

3.4.4 The complete nucleotide sequence of the VP5-gene of SA-BTV4

The complete nucleotide sequence of the BTV4 VP5-gene in its coding (mRNA) sense is presented in Fig. 3.5, with the predicted amino acid sequence indicated below it. The VP5-gene segment is 1638 base pairs in length. The coding strand has a calculated base composition of 23.2% U, 32.0% A, 17.6% C, 27.2% G with a AT content of 55.2%. The gene contains a single long open reading frame stretching from nts 30 to 1607. The first 28 nts at the 5'-terminus and 31 nts at the 3'-terminus represents non-coding sequences. The 5'- and 3'- termini contain the characteristic consensus sequences of orbivirus RNA segments, namely 5'-GTTAAA... and ...ACTTAC-3' (underlined in Fig. 3.5). The open reading frame codes for a 526 amino acid protein. The protein of BTV4 has a M_r of 59 274 Da, similar to that of VP5 of BTV10, which is 59 163 Da (Purdy *et al.*, 1986). It contains 3 cystein residues at positions 152, 315 and 383. The amino acid (aa) composition of VP5 (summarised in table 3.1), shows that the most abundant aa is glutamic acid while few tryptophan residues are present. The protein is also rich in certain non-polar aa such as alanine and isoleucine. It contains 15.4% acidic and 13.1% basic amino acids.

***

10 20 30 40 50 60 70 80 90 100 110

5'-GTTAAAAGTATTCTCCTACTCGCAGAAGATGGGGAAGATAATTAAATCGCTAAGTAGATTTGGAAAGAAAGTTGGAAACGCATTGTCGTCAAACACAGCGAAGAAAATTT

MetGlyLysIleIleLysSerLeuSerArgPheGlyLysLysValGlyAsnAlaLeuSerSerAsnThrAlaLysLysIleT

120 130 140 150 160 170 180 190 200 210 220

ATTCAACTATTGGGAAAGCAGCGGAACGTTTTGCTGAAACGGAGATCGGTGCGGCGACAATAGATGGGTTGGTGCAGGGTAGCGTTCATTCCATAATCACAGGTGAATCG

yrSerThrIleGlyLysAlaAlaGluArgPheAlaGluThrGluIleGlyAlaAlaThrIleAspGlyLeuValGlnGlySerValHisSerIleIleThrGlyGluSer

230 240 250 260 270 280 290 300 310 320 330

TATGGAGAGTCAGTTAAACAGGCAGTTCTTCTCAACGTGTTAGGTACAGGTGAAGAGTTACCAGATCCACTGAGCCCTGGCGACCGTGGCATGCAAACGAAAATAAAGGA

TyrGlyGluSerValLysGlnAlaValLeuLeuAsnValLeuGlyThrGlyGluGluLeuProAspProLeuSerProGlyAspArgGlyMetGlnThrLysIleLysGl

340 350 360 370 380 390 400 410 420 430 440

GTTGGAAGATGAGCAGCGGAATGAGCTTGTCCGATTGAAATACAATAAAGAGATAACAAAAGAGTTTGGGAAGGAGCTAGAAGAAGTGTACGATTTCATGAATGGCGAGG

uLeuGluAspGluGlnArgAsnGluLeuValArgLeuLysTyrAsnLysGluIleThrLysGluPheGlyLysGluLeuGluGluValTyrAspPheMetAsnGlyGluA

450 460 470 480 490 500 510 520 530 540 550

CGAAAGAGGAGGAAGTGGTTCAGGAACAATACTCAATGCTATGCAAAGCGGTAGATTCATATGAGAAAATATTAAAAGCAGAGGACTCGAAAATGGGAATATTAGCACGC

laLysGluGluGluValValGlnGluGlnTyrSerMetLeuCysLysAlaValAspSerTyrGluLysIleLeuLysAlaGluAspSerLysMetGlyIleLeuAlaArg

560 570 580 590 600 610 620 630 640 650 660

GCATTGCAGCGGGAGGCTTCAGAGAGAAGTCAAGATGAAATCAAAATGGTGAAGGAGTACAGACAGAAGATTGATGCGCTTAAAAATGCGATCGAGATTGAACGAGACGG

AlaLeuGlnArgGluAlaSerGluArgSerGlnAspGluIleLysMetValLysGluTyrArgGlnLysIleAspAlaLeuLysAsnAlaIleGluIleGluArgAspGl

670 680 690 700 710 720 730 740 750 760 770

AATGCAGGAGGAGGCGATCCAGGAGATTGCCGGAATGACCGCTGACGTCTTAGAGGCGGCATCGGAGGAGGTGCCATTAATCGGCGCAGGTATGGCCACAGCTGTAGCGA

yMetGlnGluGluAlaIleGlnGluIleAlaGlyMetThrAlaAspValLeuGluAlaAlaSerGluGluValProLeuIleGlyAlaGlyMetAlaThrAlaValAlaT

780 790 800 810 820 830 840 850 860 870 880

CCGGTAGGGCGATAGAAGGCGCATATAAATTGAAGAAGGTCATAAACGCGTTAAGTGGGATTGATTTATCGCATATGAGGAGTCCGAAGATTGAACCCACTATCATCGCC

hrGlyArgAlaIleGluGlyAlaTyrLysLeuLysLysValIleAsnAlaLeuSerGlyIleAspLeuSerHisMetArgSerProLysIleGluProThrIleIleAla

890 900 910 920 930 940 950 960 970 980 990

ACGACATTGGAGCATCGATTTAAAGAGATACCGGACGAGCAGCTGGCGGTAAGTGTTTTGAATAAAAAGACAGCGGTAACTGATAACTGCAATGAGATCGCGCATATCAA

ThrThrLeuGluHisArgPheLysGluIleProAspGluGlnLeuAlaValSerValLeuAsnLysLysThrAlaValThrAspAsnCysAsnGluIleAlaHisIleLy

1000 1010 1020 1030 1040 1050 1060 1070 1080 1090 1100

ACAAGAGATATTACCAAAGTTTAAACGGATTATGGATGAGGAGAAGGAGATTGAAGGAATAGAGGATAAAGTGATTCATCCGCGTGTGATGATGAGGTTCAAAATTCCTA

sGlnGluIleLeuProLysPheLysArgIleMetAspGluGluLysGluIleGluGlyIleGluAspLysValIleHisProArgValMetMetArgPheLysIleProA

1110 1120 1130 1140 1150 1160 1170 1180 1190 1200 1210

GAACGCAGCAACCGCAAATCCACATTTATGCAGCCCCCTGGGATTCTGATGATGTGTTTTTCTTTCATTGCGTTTCACACCATCATCGGAACGAATCTTTTTTCCTGGGA

rgThrGlnGlnProGlnIleHisIleTyrAlaAlaProTrpAspSerAspAspValPhePhePheHisCysValSerHisHisHisArgAsnGluSerPhePheLeuGly

1220 1230 1240 1250 1260 1270 1280 1290 1300 1310 1320

TTTGATCTAGGAATCGATGTCGTTCACTTTGAAGATTTAACCAGCCATTGGCACGCATTGGGACTAGCGCAAGAGGCGAGCGGGCGTACGTTAACGGAGGCGTATCGTGA

PheAspLeuGlyIleAspValValHisPheGluAspLeuThrSerHisTrpHisAlaLeuGlyLeuAlaGlnGluAlaSerGlyArgThrLeuThrGluAlaTyrArgGl

1330 1340 1350 1360 1370 1380 1390 1400 1410 1420 1430

GTTTCTCAATTTATCAATTTCAAGCACATATAGCAGCGCTATACATGCGAGGCGTATGATTAGGTCACGAGCAGTACACCCAATTTTTCTAGGATCGATGCACTACGATA

uPheLeuAsnLeuSerIleSerSerThrTyrSerSerAlaIleHisAlaArgArgMetIleArgSerArgAlaValHisProIlePheLeuGlySerMetHisTyrAspI

1440 1450 1460 1470 1480 1490 1500 1510 1520 1530 1540

TTACGTATGAAGCTTTGAAAAACAACGCGCAGAGAATAGTCTATGATGAGGAACTGCAAATGCATATTCTGAGGGGACCTTTGCACTTTCAGCGTCGAGCCACTCTGGGG

leThrTyrGluAlaLeuLysAsnAsnAlaGlnArgIleValTyrAspGluGluLeuGlnMetHisIleLeuArgGlyProLeuHisPheGlnArgArgAlaThrLeuGly

***

1550 1560 1570 1580 1590 1600 1610 1620 1630

GCGTTGAAATTTGGAGTTAAAATATTAGGCGATAAAATTGATGTTCCCCTCTTCTTACGAAATGCATGAACGCAGCGAGGGAGAAGCTTACACTTAC-3'

AlaLeuLysPheGlyValLysIleLeuGlyAspLysIleAspValProLeuPheLeuArgAsnAla

Table 3.1 The amino acid composition of the SA BTV4 VP5 protein.

AMINO ACID	NO OF RESIDUES	% OF SEQUENCE
Alanine	46	8.7
Arginine	29	5.5
Asparagine	16	3.0
Aspartic acid	26	4.9
Cysteine	3	0.6
Glutamine	20	3.8
Glutamic acid	55	10.5
Glycine	33	6.3
Histidine	18	3.4
Isoleucine	46	8.7
Leucine	44	8.4
Lysine	40	7.6
Methionine	16	3.0
Phenylalanine	19	3.6
Proline	15	2.9
Serine	32	6.1
Threonine	23	4.4
Tryptophane	2	0.4
Tyrosine	14	2.7
Valine	29	5.5
Acidic (Asp+Glu)	81	15.4
Basic (Arg+ Lys)	69	13.1
Aromatic (Phe+Trp+Tyr)	35	6.7
Hydrophobic (Aromatic + Ile + Leu + Met + Val)	170	32.3

3.5 DISCUSSION

A cloned DNA copy of dsRNA gene VP5 (SA-BTV serotype 4) was successfully tailored by utilising the Polymerase Chain Reaction in a method described by Dennis (1990). This involved removal of the G/C homopolymeric flanking sequences from the gene, a consequence of the original cloning strategy. Furthermore a BamHI site was incorporated immediately adjacent to both the 5'- and 3'-terminal VP5-gene ends, which made cloning of the tailored product into the plasmid vector pUC13 possible.

The complete nucleotide sequence of this tailored VP5-gene was then determined, although not fully in both directions, i.e. on both cloned strands. From the sequence data, it is apparent that a full-length copy of the gene had been retained and cloned after tailoring, since the conserved sequences at the 5'- and 3'- termini were still present (Mertens and Sanger, 1985). It was also clear that the polymerase chain reaction had not resulted in mutations which disturbed the ORF, as it was of the same length as that previously reported for VP5 ORF's (e.g. Purdy *et al.*, 1896, Wade-Evans *et al.*, 1988). The gene is 1638 bp in length and encodes a protein of 526 amino acids.

The full length VP5-gene of the United States isolates BTV10 (Purdy *et al.*,1986), BTV2 (Hirasawa and Roy, 1990) and BTV13 (Oldfield et al., 1991), the South African isolate BTV1 (Wade-Evans *et al.*, 1988), the Australian isolate BTV1 (Gould and Pritchard, 1988) and a related Orbivirus, namely EHDV-1 (Itawa *et al.*, 1991) has been cloned and sequenced. A comparison of the nucleotide sequences (Fig. 3.6) as well as the predicted amino acid sequences (Fig 3.7) of all these serotypes, including that of BTV4 VP5, is presented.

The VP5-gene of SA-BTV4 is the same length than that of US-BTV10 (1638 bp), which is three nucleotides longer than that of US-BTV2 and both isolates of BTV1 (from South Africa and Australia), with a length of 1635 bp. It is one nucleotide longer than that of US-BTV13 (1637 bp) and five nucleotides shorter than the VP5-gene of EHDV-1 (1340 bp).

The alignment of the predicted VP5 amino acid sequence (Fig 3.7) demonstrates the close similarity of all these proteins. In the case of the bluetongue serotypes, no gap is required for maximum homologous allignment, while allignment of the EHDV-1 protein requires only three gaps. The VP5 proteins of all of the BTV serotypes determined thus far are 526 aa in length, in contrast to the VP5 protein of EHDV-1, which contains 527 aa. All of the VP5 proteins contain a conserved cysteine residue at position 383. Itawa *et al.*, 1991 proposed that this may indicate the importance

```
                                                         ●●●
BTV4 VP5        GTTAAAA-GTATTCTCCTACTCGCAGAAGATGGGAAGATAATTAAATCG   50
BTV10 VP5       GTTAAAAAGTGTTCTCCTACTCGCAGAAGATGGGAAGATAATCAAATCG
BTV1(SA)        GTTAAAAAGTGCCCCCTTA---GC-GAAGATGGGTAAAGTCATACGGTCC
BTV1(Aus)       GTTAAAAAGTGT-CCCTTA---GC-GAAGATGGGTAAAGTCATACGATCT
BTV13 VP5       GTTAAAAAGT-TCCTCAAGATCGCGGAAGATGGGGAAGATCATAAAATCA
BTV2 VP5        GTTAAAAAGTGTACCCTTA---GC-GAAGATGGGTAAAGTCATACGATCT
EHDV VP5        GTTAAAAAGTGGTGCGTAGCTTGCATAA--TGGGCAAGTTCATTAAACAA
                ******* **            **  **  **** **  *  **

                                                                   100
BTV4 VP5        CTAAGTAGATTTGGAAAGAAAGTTGGAAACGCATTGTCGTCAAACACAGC
BTV10 VP5       CTTAGTCGTTTTGGAAAAAAGGTCGGGAATGCTTTGACATCGAATACAGC
BTV1(SA)        TTAAACCGATTTGGCAAAAAGGTAGGCAACGCGTTAACTTCTAATACCGC
BTV1(Aus)       CTAAGCCGATTCGGAAAAAAGGTAGGTAACGCATTGACATCAAATACGGC
BTV13 VP5       CTTAGCCGTTTTGGCAAAAAAGTAGGTAATGCGTTAACCTCGAATACAGC
BTV2 VP5        CTGAGTAGATTCGGGAAGAAAGTAGGTAGCGCACTCACATCAAATGCGGC
EHDV VP5        CTGAGTAAGTTTGGTAAAAAGGTGGGGGAGCGCTAACGTCAAATACCGC
                 * *     ** ** ** ** ** **    **  *  * ** **  * **

                                                                   150
BTV4 VP5        GAAGAAAATTTATTCAACTATTGGGAAAGCAGCGGAACGTTTTGCTGAAA
BTV10 VP5       GAAAAAGATTTACTCAACGATTGGAAAAGCAGCGGAACGTTTTGCAGAAA
BTV1(SA)        AAAAAAGATCTATAGTACAATTGGAAAAGCGGCGGACGAATTTCTTGAGA
BTV1(Aus)       GAAGAAAATATATAGTACGATCGGGAAGGCGGCCGAAAGATTTGCGGAGA
BTV13 VP5       TAAGAAGATTTATAATACAATCGGGAAGGCAGCGGAGCGTTTTGCTGAAA
BTV2 VP5        TAAAAAGATTTATAGTACAATAGGCAAAGCAGCTGAAAGATTCGCGGAAA
EHDV VP5        AAAAAAGATTTATAAGACAATCGGGGATACGGCAGTACGATTCGCGGAGA
                 ** ** ** **    ** ** **  *  * ** *     **    **  *

                                                                   200
BTV4 VP5        CGGAGATCGGTGCGGCGACAATAGATGGGTTGGTGCAGGGTAGCGTTCAT
BTV10 VP5       GTGAAATAGGTGCGGCAACGATTGATGGGTTAGTACAAGGAAGCGTTCAC
BTV1(SA)        GTGAAATAGGTTCAGCGGCGATCGATGGATTAGTACAGGGAAGCGTACAT
BTV1(Aus)       GCGAAATTGGATCGGCAGCAATTGATGGGCTTGTACAAGGAAGTGTACAC
BTV13 VP5       GTGAGATTGGTTCGGCCGCGATTGATGGACTGGTTCAAGGAAGCGTTCAT
BTV2 VP5        GTGAAATTGGTTCAGCGGCGACTGATGGCTTAGTGCAGGGTAGCGTACAT
EHDV VP5        GCGATATCGGCTCGGCAGCAATTGATGGACTAATCCAGGGCAGTGTTGAA
                  ** ** **  * **  * *  *****  *  * ** ** ** **  *

                                                                   250
BTV4 VP5        TCCATAATCACAGGTGAATCGTATGGAGAGTCAGTTAAACAGGCAGTTCT
BTV10 VP5       TCAATTATAACAGGAGAATCGTATGGGGAATCGGTGAAACAGGCGGTCCT
BTV1(SA)        TCAATCATAACGGGCGAATCTTACGGCGAATCTGTGAAACAAGCTGTGTT
BTV1(Aus)       TCGATATTAACAGGTGAATCATATGGCGAATACGTTAAACAGGCAGTCTT
BTV13 VP5       TCGATTATCACGGGGGAATCCTACGGTGAATCAGTTAAGCAAGCGGTGCT
BTV2 VP5        TCAATATTGACAGGCGAGTCTTACGGCGAATCGGTTAAACAGGCAGTCCT
EHDV VP5        TCGGTATTAACGGGTGAATCGTATGGTGAAACCGTAAAGAGAGCAGTCCT
                **   * * ** ** ** ** ** ** **    ** **    ** **  *

                                                                   300
BTV4 VP5        TCTCAACGTGTTAGGTACAGGTGAAGAGTTACCAGATCCACTGAGCCCTG
BTV10 VP5       ATTGAATGTGTTGGGTACAGGCGAAGAATTGCCTGATCCCCTCAGCCCTG
BTV1(SA)        GTTAAATGTGTTGGGGAGTGGTGAGGAAATTCCTGATCCGCTAAGTCCAG
BTV1(Aus)       ACTGAATGTTTTAGGTAGTGGTGAAGAAATTCCAGATCCGTTAAGCCAAG
BTV13 VP5       ATTGAATGTGCTCGGCGCAGGAGATGAGATCCCGGACCCACTTAGTCCTG
BTV2 VP5        ACTGAATGTTCTAGGTAGTGGTGAGGAGATTCCGGACCCGTTGAGCCCGG
EHDV VP5        ATTGAATGTGTTAGGGGCGGGAGATGAAATCCCAGACCCATTAAGCCCCG
                  * ** **  * **    ** ** **  * ** ** **  * **  *  *

                                                                   350
BTV4 VP5        GCGACCGTGGCATGCAAACGAAAATAAAGGAGTTGGAAGATGAGCAGCGG
BTV10 VP5       GTGAGCGCGGCATGCAGACGAAGATAAAGGAATTGGAAGATGAGCAACGT
BTV1(SA)        GAGAGCGAGGGATACAAGCTAAGTTGAAAGAGTTAGAGGACGAGCAACGC
BTV1(Aus)       GGGAAACGGAAATCCAGGCCAAATTAAGGGAGCTAGAAGATGAACAAAGG
BTV13 VP5       GAGAGCGTGGCATCCAGACAAAAATCAAGGAGATCGAGGAAGAGCAGAGG
BTV2 VP5        GTGAGCAAGGTATGCAAACTAAGTTAAAAGAGTTAGAAGAGGAACAAAGG
EHDV VP5        GGGAACAAGGGATACAGAGAAAAATTAAGGAATTAGAAGATGAAATGAAA
                * **    *  ** **    **  * *  **  * ** ** **
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                                                                   400
BTV4 VP5        AATGAGCTTGTCCGATTGAAATACAATAAAGAGATAACAAAAGAGTTTGG
BTV10 VP5       AACGAACTTGTTAGGCTTAAATATAATAAAGAGATCACCGAGAAATTCGG
BTV1(SA)        AATGAATTAGTTCGCTTAAAATATAATGATAAGATTAAGGAAAAATTTGA
BTV1(Aus)       AATGAGCTTGTACGATTAAAATACAATGATAAGATAAAAGAAAAAATTTGG
BTV13 VP5       AATGAGTTAGTAAGAATAAAATACGGAAAAGAGATAAGAGAGAAATTTGG
BTV2 VP5        AATGAGCTAGTGCGATTGAAGTATAACGATAAAATTAAAGAAAAAATTTGG
EHDV VP5        GGTGAAGTGGTGCGGACAAAGCATAACGAACAGATTATACGCCGGTTTGG
                   **  * **  *    **  *     *  * ** *        ** *

                                                                   450
BTV4 VP5        GAAGGAGCTAGAAGAAGTGTACGATTTCATGAATGGCGAGGCGAAAGAGG
BTV10 VP5       TAAAGAGCTCGGAGAAGTTTATGACTTCATGAATGGGGAGGCAAAAGAAG
BTV1(SA)        AAAGGAGCTTGAGGAGGTTTACAATTTTTATAATGGAGAGGCGAATGCTG
BTV1(Aus)       TGAAGAACTCGAGGAGGTATATGAATTTATGAATGGCGCAGCCAAAGCAG
BTV13 VP5       TGAACAATTGGAGGAAATATACCAATTCATGAACGGCGAAGTTAAGGGCG
BTV2 VP5        AGAAGAGCTGGAGCAGGTGTATGAGTTTATGAACGGTGCGGCGAAGGAAG
EHDV VP5        TGCGGATTTAGATGAAGTGTATAAATTCGCAGTCAGTGAGTATAAGGAGG
                     *  * *   *  * **  * **        * *     ** *  *

                                                                   500
BTV4 VP5        AGGAAGTGGTTCAGGAACAATACTCAATGCTATGCAAAGCGGTAGATTCA
BTV10 VP5       TGGAGGCGGTTGAAGAACAGTATACTATGCTATGTAAGGCTGTTGATTCG
BTV1(SA)        AGATTGAAGATGAGAAGCAGTTTGATATACTGAACAAGGCGGTGACCTCG
BTV1(Aus)       AGGTGGAGGACGAAAAGCAATTTGACATACTCAATAAAGCGGTTACCTCA
BTV13 VP5       AAGAAGAACAAGAGGAACAATATAAAGTTTTATGCAAGGCTGTTGATTCA
BTV2 VP5        AAGTCGGAGCTGAGAAGCAGTTTGATATCTTAAGTAAGGCAGTTAATTCA
EHDV VP5        GAGTTGAAGAAAAGGATCAGTTCGAAATATTAAAGAAAGCGTTAACATCT
                     *      *  * ** *      *  *    ** **  *    **

                                                                   550
BTV4 VP5        TATGAGAAAATATTAAAAGCAGAGGACTCGAAAATGGGAATATTAGCACG
BTV10 VP5       TATGAGAAAATCCTGAAGGAGGAAGATTCGAAGATGGCTATTTTAGCGCG
BTV1(SA)        TATAACAAAATTCTTACGGAGGAAGATCTACAGAATCGCCGGTTAGCTAC
BTV1(Aus)       TACAATAAGATTTTAACAGAAGAAGATTTGCAGATGAGGCGATTAGCTAA
BTV13 VP5       TATGAAAACCTACTCGTCACAGAGAATGAGCAAATGCGTACGTTGGCGCG
BTV2 VP5        TACAATAAGATATTAACCGAAGAAGACCAACAAATGCGTCGACTTGCGAT
EHDV VP5        TATGGTGAACTGACGAAAGCGGAGTTTGGTGAATTGAAACGACTGGAAAA
                **     *  *          **        *           * *

                                                                   600
BTV4 VP5        CGCATTGCAGCGGGAGGCTTCAGAGAGAAGTCAAGATGAAATCAAAATGG
BTV10 VP5       TGCGTTACAACGAGAGGCAGCGGAAAGAAGTGAAGATGAAATCAAAATGG
BTV1(SA)        GCGCCTGCAGAAAGAGATCGGAGAAAGAACACATGCGGAGACGGTCATGG
BTV1(Aus)       TGCACTGCAGAAGGAAATTGGCGAGAGGACGCATGCGGAAACAGTTATGG
BTV13 VP5       CGCGTTACAGCGAGAAGCGACTGAACGAACAGAAAACGAATCGAAGATGG
BTV2 VP5        TGCTCTACAGAAAGAGATTGGTGAGAGGACACATGTAGAAACAGCTATGG
EHDV VP5        AGCATTACAGAAGGAGTCGTCAGAAAGATCAAAAGACGAAAGCATGATGG
                     * **    **       **  *     *    **       ****

                                                                   650
BTV4 VP5        TGAAGGAGTACAGACAGAAGATTGATGCGCTTAAAAATGCGATCGAGATT
BTV10 VP5       TAAAAGAGTATAGACAGAAGATTGATGCGCTTAAAAGTGCGATAGAGATT
BTV1(SA)        TGAAAGAATACCGCGATAAAATTGACGCTTTAAAAAATGCGATTGAGGTA
BTV1(Aus)       TGAAGGAGTATCGTAATAAGATTGATGCACTAAAGAATGCAATCGAGATT
BTV13 VP5       TTAAAGAATATAGGCAGAAAATAGATGCCTTAAAGGTTGCAATAGAGGTA
BTV2 VP5        TAAAAGAATATCGGAATAAGATCGACGCTCTTAAAAGTGCAATAGAGATT
EHDV VP5        TCAAAGAATATCGGCAGAAGATTGAAGCATTGAAGGATGCCATTGAGGTT
                * ** ** **  *  * ** ** ** **  * **   *** ** *** *

                                                                   700
BTV4 VP5        GAACGAGACGGAATGCAGGAGGAGGCGATCCAGGAGATTGCCGGAATGAC
BTV10 VP5       GAGCGTGATGGAATGCAGGAAGAGGCTATACAAGAGATCGCTGGAAGGAC
BTV1(SA)        GAAAGAGATGGCATGCAAGAGGAGGCGATACAGGAGATCGCGGGGATGAC
BTV1(Aus)       GAAAGGGATGGCATGCAGGAAGAAGCCATACAAGAAATAGCCGGGATGAC
BTV13 VP5       GAAAGAGATGGTATGCAAGAGGAAGCAATCCAAGAGATCGCGGGTATGAC
BTV2 VP5        GAAAGAGACGGCATGCAGGAAGAGGCGATACAAGAAATAGCGGGGATGAC
EHDV VP5        GAATCGACAGGCATACAGGAGGAAGCGATACAGGAAATAGCAGGGATGAG
                **       ** ** ** ** ** ** ** ** ** ** ** ** * **
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                                                                    750
BTV4 VP5        CGCTGACGTCTTAGAGGCGGCATCGGAGGAGGTGCCATTAATCGGCGCAG
BTV10 VP5       AGCAGACGTGCTAGAGGCGGCATCAGAAGAATTGCCTTTGATAGGTGCCG
BTV1(SA)        CGCAGATGTGTTAGAGGCGGCATCGGAGGAGGTTCCGCTGATTGGTGCGG
BTV1(Aus)       GCGCGGTGTTTTAGAAGCAGCATCGGAAGAAGTCCCCCTGATTGGAGCGG
BTV13 VP5       GGCGGATGTTCTGGAGGCGGCTTCGGAAGAAGTTCCTCTAGTAGGATCGG
BTV2 VP5        TGCTGATGTGTTAGAGGCAGCATCTGAGGAGGTGCCCCTAATCGGAGCGG
EHDV VP5        CGCTGATATTTTGGAGAGTGCAGCGGAGGAAGTGCCCTTGTTTGGAGGGG
                    *   *  * **    **  * ** **  * **  *  * **    *

                                                                    800
BTV4 VP5        GTATGGCCACAGCTGTAGCGACCGGTAGGGCGATAGAAGGCGCATATAAA
BTV10 VP5       GTATGGCAACGGCTGTCGCAACGGACGAGCAATAGAGGGGGCGTATAAA
BTV1(SA)        GGATGGCCACAGCTGTGGCGACAGGGAGGGCTATTGAGGGAGCGTACAAA
BTV1(Aus)       GCATGGCTACCGCGGTTGCAACAGGAAGGGCGATTGAGGGAGCTTATAAG
BTV13 VP5       GAATGGCAACGGCTATTGCAACAGGTAGAGCGATTGAAGGTGCGTACAAA
BTV2 VP5        GTATGGCGACAGCCGTCGCAACGGGAAGAGCGATTGAGGGGGCGTATAAG
EHDV VP5        GGGTGGCAACGAGCATAGCGACCGCGCGTGCAATAGAAGGAGGGTACAAA
                *  **** **     * ** ** *   * ** ** ** ** *  ** **

                                                                    850
BTV4 VP5        TTGAAGAAGGTCATAAACGCGTTAAGTGGGATTGATTTATCGCATAT-GA
BTV10 VP5       TTAAAGAAGGTCATCAATGCACTCAGCGGAATTGACTTATCACATAT-GC
BTV1(SA)        CTCAAAAAAGTGATCAATGCTTTAAGCGGGATCGATTTAACGCATTTTAC
BTV1(Aus)       CTCAAAAAAGTTATTAACGCTTTGAGCGGAATTGATTTAACGCATTT-GC
BTV13 VP5       CTTAAGAAAGTGATAAACGCTTTAAGCGGCATTGATTTATCCCATTT-GC
BTV2 VP5        TTGAAGAAAGTAATTAATGCATTAAGCGGGATAGATCTAACACATTT-GC
EHDV VP5        CTAAAAAAAGTTATTAACGCATTAAGTGGTATAGATCTATCACAC-TTGC
                 * ** ** ** ** ** **  * ** ** ** **  ** * **  *

                                                                    900
BTV4 VP5        GGAGTCCGAAGATTGAACCCACTATCATCGCCACGACATTGGA-GCATCG
BTV10 VP5       GAAGCCCCAAGATCGAACCTACTATTATCGCCACAACTCTTGA-GCATAG
BTV1(SA)        GCACTACGAAAATCGA-CCTAGTGTTGTTTCAACTATTCTTGA-GTACCG
BTV1(Aus)       GCACGCCTAAAATAGAGCCAAGTGTAGTTTCTACTATCTTAGA-GTATCG
BTV13 VP5       GCACTCCGAAAATTGAGCCAACTATGGTTGCGACAACGTTAGA-GCATAG
BTV2 VP5        GTACGCCGAAAATTGAACCGAGTGTAGTTTCTACGATTTTGGA-GTATCG
EHDV VP5        GTACGCCGAAAATACAACCGAAAACATTAGAAGCAATATTAGAAGCACCG
                * *   * ** **  * ** *      *     * *   * ** * *  *

                                                                    950
BTV4 VP5        ATTTAAA--GAGATACCGGACGAGCAGCTGGCGGTAAGTGTTTTGAATAA
BTV10 VP5       GTTTAAA--GATATACCAGATGAACAATTGGCTATTAGCGTATTAAATAA
BTV1(SA)        CGCAAAG--GAGATTCCTGATAACGCTCTGGCTGTTAGTGTTCTGATCAA
BTV1(Aus)       CACTAGA--GCTATTCCTGATAGCGCGTTAGCAGTAAGTGTCCTGTCGAA
BTV13 VP5       ATTCAAG--GAGATACCGGATAAGGAATTGGCTGTAAGCGTTTTGGCGAA
BTV2 VP5        TGCAAAG--GACATTCCAGACAATGCACTCGCAGTCAGCGTGTTGTCGAA
EHDV VP5        ACTAAAGAAGAGATTAAAGATTTATCTTTAGTAGAAGGAATACAAATGAA
                    *    *  **    **        * *      *  *       **

                                                                    1000
BTV4 VP5        AAAGACAGCGGTAACTGATAACTGCAATGAGATCGCGCATATCAAACAAG
BTV10 VP5       AAAGACGGCTGTGGCTGATAATTGCAACGAGATTGCTCATATTAAACAAG
BTV1(SA)        AGAACGTGCGATTCAAGAAAACCACAAAGAACTGATGCATATTAAGAATG
BTV1(Aus)       GAATCGTGCAATCCAGGAAAATCATAAAGAGCTAATACACATAAAAGATG
BTV13 VP5       AAACGATGCGATAGTAGCGAATACTAAAGAGATTAAGCACATAAAAGAGG
BTV2 VP5        GAATCGTGCAATTCAAGAAAACCATAAGGAGTTAATGCATATTAAAGATG
EHDV VP5        GCTACAGAATCTAGAGGAGAATCGTAATGAGGTCCTACACATACAGGAAG
                           *    *  **    ** **  *    ** **  *  * *

                                                                    1050
BTV4 VP5        AGATATTACCAAAGTT---TAAACGGATTATGGATGAGGAGAAGGAGATT
BTV10 VP5       AGATACTACCAAAGTT---TAAACAGATAATGAATGAAGAGAAGGAGATT
BTV1(SA)        AGATATTACCGAGATT---TAAGAAAGCGATGGATGAAGAAAAGGAAATA
BTV1(Aus)       AGATATTACCTCGTTT---TAAAAAAGCAATGGATGAAGAGAAGGAGATA
BTV13 VP5       AGATTCTACCAAAATT---TAAGAAGATCATGGAAGAAGAGAAGGAGTTA
BTV2 VP5        AAATTTTACCGCGTTT---TAAGAAGGCAATGGACGAGGAGAAAGAGATA
EHDV VP5        AGATACTACCAAAATTACGTGAGGCGATGATAGAAGATCATAAAGAGATA
                * **  ****    **   * *       **  * **  * ** **  *
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                                                                   1100
BTV4 VP5        GAA-GGAATAGAGGATAAAGTGATTCATCCGCGTGTGATGATGAGGTTCA
BTV10 VP5       GAG-GGGATAGAGGATAAAGTCATACACCCGAGAGTAATGATGAGGTTTA
BTV1(SA)        ATGCGGGATAGAAGACAAA-TGATCCACCCGAAGGTCATGATGAAGTTTA
BTV1(Aus)       -TGCGGGATAGAAGATAAAACCATACATCCGAAGGTTATGATGAGATTCA
BTV13 VP5       GAA-GGTATAGACGATAAAAAGATACATCCGAGAGTGATGATGAGATTTA
BTV2 VP5        -TGTGGAATTGAGGATAAAACAATACATCCAAAAGTGATGATGAAATTTA
EHDV VP5        G-GTGACGAACGTGACAAGCGTATTCTTCCAAAAACGGCGATGAGATTTA
                    *        ** **    ** *  **         *****  ** *

                                                                   1150
BTV4 VP5        AAATTCCTAGAACGCAGCAACCGCAAATCCACATTTATGCAGCCCCCTGG
BTV10 VP5       AGATCCCACGTACGCAGCAGCCCCAAATTCATATCTATGCAGCCCCATGG
BTV1(SA)        AGATTCCAAGAGCTCAACAGCCGCAGATTCATGTATACAGTGCCCCATGG
BTV1(Aus)       AGATCCCAAGAGCTCAACAGCCTCAAATCCACGTGTATAGCGCTCCATGG
BTV13 VP5       AGGTGCCTAGATCACAACAGCCGCAAATTCACATATACAGTGCTCCGTGG
BTV2 VP5        AGATCCCGAGGTCACAGCAACCGCAAATTCATATTTATACGGCGCCATGG
EHDV VP5        AAGTACCAACTACGCAGCAGCCGGTAATACAAATATACTCAGCTCCCTGG
                *  * **     * ** ** **    ** **  * **    ** ** ***

                                                                   1200
BTV4 VP5        GATTCTGATGATGTGTTTTTCTTTCATTGCGTTTCACACCATCATCGGAA
BTV10 VP5       GATTCGGACGATGTCTTCTTCTTTCACTGTGTGTCTTACCATCATCGGAA
BTV1(SA)        GATTCTGATGATGTGTTTTTCTTTCATTGCATCTCACACCATCATGCAAA
BTV1(Aus)       GACTCAGATGACGTATTCTTCTTTCATTGTATATCGCACCATCACGCCAA
BTV13 VP5       GATTCAGACGATGTATTTTTCTTTCACTGTGTGTCGCACTTCCACGCGAA
BTV2 VP5        GATTCTGATGATGTATTCTTCTTCCATTGTATTTCACATCATCATGCGAA
EHDV VP5        GATTCGGACGATGTGTTTATGTTCCATTGTATATCACATCACCATTTAAA
                ** ** ** ** ** **  * ** ** **  * **  *    **    **

                                                                   1250
BTV4 VP5        CGAATCTTTTTTCCTGGGATTTGATCTAGGAATCGATGTCGTTCACTTTG
BTV10 VP5       TGAATCATTCTTTCTAGGCTTTGATTTAGGAATTGATGTAGTTCATTTTG
BTV1(SA)        TGACTCGTTCTTCTTAGGTTTCGATTTGAGCATTGACTTAGTTCATTATG
BTV1(Aus)       CGAATCATTTTTCCTTGGTTTTGATTTAAGCATCGACCTAGTTCATTACG
BTV13 VP5       TGAATCATTCTTTTTAGGCTTCGATTTGGGAATTGACGTTGTTCATTTCG
BTV2 VP5        TGAATCATTTTTTATAGGATTCGATCTGAGTATCGACTTAGTTCACTATG
EHDV VP5        CGAATCCTCCTTTTTAGGGTTTGATCTTGAATTAGAATATGTGCATTATG
                 ** ** *  **  * ** ** *** *     * **    ** ** *  *

                                                                   1300
BTV4 VP5        AAGATTTAACCAGCCATTGGCACGCATTGGGACTAGCGCAAGAGGCGAGC
BTV10 VP5       AAGATTTAACCAGTCACTGGCACGCGTTAGGGATGGCACAAGAGGCCAGT
BTV1(SA)        AAGATCTCACCGCCCATGCTCATGCCATTGGAGCAGCTCAAACGGCAGCG
BTV1(Aus)       AGGACCTCACAGCACATTGGCACGCGTTAGGTGCGGCACAAATGGCGATG
BTV13 VP5       AGGACCTCGCTGCGCACTGGCATGCGTTAGGCGCAGCTCAAGAAGCAAAG
BTV2 VP5        AGGATTTAACGGCTCATTGGCATGCATTGGGAGCGGCTCAGATGGCTGTA
EHDV VP5        AGGATTTGACTCGCCATTGGCATGCACTGGGGGCAGCACAAGAAGTCACC
                * **  *  *    **    ** **  * **    ** **    *

                                                                   1350
BTV4 VP5        GGGCGTACGTTAACGGAGGCGTATCGTGAGTTTCTCAATTTATCAATTTC
BTV10 VP5       GGACGGACTTTAACGGAGGCTTATAGAGAGTTCCTCAATCTTTCGATTTC
BTV1(SA)        GGACGTACGCTAACGGAAGCATATAGAGAGTTTCTAAATTTGGCTATTTC
BTV1(Aus)       GGACGTACGCTATCCGAGGCATATAAGGAGTTTTTAAATATGGCGATATC
BTV13 VP5       GGACGTACACTGAATGAGGCGTACAGAGAATTTCTTAATTTGTCTATTGG
BTV2 VP5        GGACGTACGTTAACCGAAGCGTATAAAGAATTCTTAAAACTGGCTATTTC
EHDV VP5        GGCCGCAGTTTGAAGGAGGCATATAGCGAATTTTTCCAGTTGGCAGCA-C
                ** ** *   *    ** ** **    ** **  *  *  *  *

                                                                   1400
BTV4 VP5        AAGCACATATAGCAGCGC-TATACATGCGAGGCGTATGATTAGGTCACGA
BTV10 VP5       GAGCACGTTCAGTAGCGC-AATACATGCGAGGCGTATGATAAGATCACGC
BTV1(SA)        AAAGGCATTTGGCA-CGCAAATGCACACGAGAAGATTGGTTAGGTCGAAA
BTV1(Aus)       AAACGCTTATGGGA-CGCAAATGCATACGAGGAGGTTGGTGAGATCAAAG
BTV13 VP5       AAGCGCGTTCA-CATCGCCAATGCATGCGCGGCGTATGATTAGATCAAAA
BTV2 VP5        TAGTACATATGGAA-CACAAATGCATACTCGAAGGTTGATTAGGTCGAAA
EHDV VP5        AGATTGATGGAGCTGGGGCGATACATCAGAAGAGATTGATTCGATCAAAG
                       *            ** **        *  ** *  * **
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                                                                    1450
BTV4 VP5        GCAGTACACCCAATTTTTCTAGGATCGATGCACTACGATATTACGTATGA
BTV10 VP5       GCTGTACACCCTATCTTTTTAGGTTCAATGCATTACGATATAACTTATGA
BTV1(SA)        ACGGTACATCCGATTTACTTAGGTTCCTTGCACTACGATATTTCCTTTTC
BTV1(Aus)       ATGGTGCACCCAATTTACTTGGGCTCTTTACATTATGATATTTCTTTTCT
BTV13 VP5       ACTGTGCACCCCATTTATTTAGGCTCGATGCATTACGATATTACTTATGA
BTV2 VP5        ACGGTGCATCCGATTTATTTGGGATCTCTCCACTATGACATCTCTTTCGC
EHDV VP5        AGTTATCATCCAATTTATTTAGGAGCGATGCACTATGATATCGCGTACCG
                      ** ** ** *   * **  *  * ** ** ** **  * *

                                                                    1500
BTV4 VP5        AGCTTTGAAAAACAACGC-GCAGAGAATAGTCTATGATGAGGAACTGCAA
BTV10 VP5       AGCTCTGAAGAATAATGC-GCAGCGGATAGTTTACGATGATGAATTGCAA
BTV1(SA)        GGATCTGCGTGGAAACGCT-CAGAGAATTGTTTATGACGATGAACTGCAG
BTV1(Aus)       AGATTTACGTGGGAACGCG-CAAAGAATAGTATATGATGATGAGTTACAG
BTV13 VP5       AACGTTAAAGACGAACGCCACAGAGGCTCGA-TATGATGAAGATTTACAA
BTV2 VP5        TGATCTACGAGGGAACGCT-CAACGTATCGTATACGATGACGAATTACAG
EHDV VP5        CGAGTTAAAGAATAATGC-GTTGAAGATCGTAAATGATTCAGAAGTCCAG
                     *       ** **         * *   * **    **  * **

                                                                    1550
BTV4 VP5        ATGCATATTCTGAGGGGACCTTTGCACTTTCAGCGTCGAGCCACTCTGGG
BTV10 VP5       ATGCATATACTTAGAGGACCGTTACACTTCCAGCGCCGAGCAATATTAGG
BTV1(SA)        ATGCACATACTTCGTGGGCCGATACATTTTCAGAGACGGGCAATACTAGG
BTV1(Aus)       ATGCATATACTGCGTGGGCCCATACATTTTCAAAGACGGGCGATCCTGGG
BTV13 VP5       ATGCATATACTGAGGGGGCCCTTACACTTTCAGCGACGCGCTATATTAGG
BTV2 VP5        ATGCACATTTTGCGCGGTCCGCTACATTTCCAGAGACGAGCTATATTAGG
EHDV VP5        AAGCATTTGCTACGAGGGCCAAAGCACTTTCAACGAAGAGCAATATTAAG
                * ***  *  *  * ** **    ** ** **  *  * ** *   *  *

                                                                    1600
BTV4 VP5        GGCGTTGAAATTTGGAGTTAAAATATTAGGCGATAAAATTGATGTTCCCC
BTV10 VP5       TGCGTTAAAATTCGGAGTTAAGATAATAGGCGATAAGATAGATGTTCCCC
BTV1(SA)        GGCTTTGAAATTTGGATGTAAGGTTTTGGGGGACCGTTTAGACGTACCAC
BTV1(Aus)       AGCGTTAAAGTTTGGCTGTAAGGTTTTAGGAGATCGTCTGGACGTACCAC
BTV13 VP5       TGCGTTGAAGTTTGGTGTAAAAGTGTTAGGTGATAAAGTTGATGTTCCGC
BTV2 VP5        CGCGTTAAAGTTTGGGTGCAAGATACTTGGAGATCGGTTGGACGTGCCCC
EHDV VP5        CGCATTGAAAGATGGAGTCAAACTGCTTGGGGGT---GTTGATCTTGCCG
                 ** ** **    **    **  *  * ** *      * **  *  *

                                 ●●●
BTV4 VP5        TCTTCTTACGAAATGCATGAACGCAGCGA-GGGAGAAGCTTACACTTAC
BTV10 VP5       TCTTCTTACGAAATGCTTGAACGCAGCGG-GGGAGGACCTTCCACTTAC
BTV1(SA)        TCTTCTTACGAAATGCTTGAACGCAGCGACGGGAAGCACTTACACTTAC
BTV1(Aus)       TCTTCTTACGAAATGCTTGAACGCAGCGACGGGAAGCACTTCCACTTAC
BTV13 VP5       TCTTCCTAAAAAACGCGTGACAGCGGTGA-TCCCGGGGCTTACACTTAC
BTV2 VP5        TCTTCTTACGAAATCCTTGAACGCAGCGACGGGCAGCACTTACACTTAC
EHDV VP5        AATTTATGCGATACGCGTGAAGGCGGAGCGTGCATACACTTACACTTAC
                  **  *   * *  * ***  ** * *          *** *******
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```
                                                                        50
BTV4         MGKIIKSLSRFGKKVGNALSSNTAKKIYSTIGKAAERFAETEIGAATIDG
BTV10 VP5    MGKIIKSLSRFGKKVGNALTSNTAKKIYSTIGKAAERFAESEIGAATIDG
BTV1(SA)     MGKVIRSLNRFGKKVGNALTSNTAKKIYSTIGKAADEFLESEIGSAAIDG
BTV1(AUS)    MGKVIRSLSRFGKKVGNALTSNTAKKIYSTIGKAAERFAESEIGSAAIDG
BTV13 VP5    MGKIIKSLSRFGKKVGNALTSNTAKKIYNTIGKAAERFAESEIGSAAIDG
BTV2 VP5     MGKVIRSLSRFGKKVGSALTSNAAKKIYSTIGKAAERFAESEIGSAATDG
EHDV VP5     MGKFIKQLSKFGKKVGGALTSNTAKKIYKTIGDTAVRFAESDIGSAAIDG
             *** *. *..****** **.**.***** *** .*  * *..**.*. **

                                                                        100
BTV4         LVQGSVHSIITGESYGESVKQAVLLNVLGTGEELPDPLSPGDRGMQTKIK
BTV10 VP5    LVQGSVHSIITGESYGESVKQAVLLNVLGTGEELPDPLSPGERGMQTKIK
BTV1(SA)     LVQGSVHSIITGESYGESVKQAVLLNVLGSGEEIPDPLSPGERGIQAKLK
BTV1(AUS)    LVQGSVHSILTGESYGEYVKQAVLLNVLGSGEEIPDPLSQGETEIQAKLR
BTV13 VP5    LVQGSVHSIITGESYGESVKQAVLLNVLGAGDEIPDPLSPGERGIQTKIK
BTV2 VP5     LVQGSVHSILTGESYGESVKQAVLLNVLGSGEEIPDPLSPGEQGMQTKLK
EHDV VP5     LIQGSVESVLTGESYGETVKRAVLLNVLGAGDEIPDPLSPGEQGIQRKIK
             *.****.*..******* **.********.*.*.***** *.  .* *..

                                                                        150
BTV4         ELEDEQRNELVRLKYNKEITKEFGKELEEVYDFMNGEAKEEEVVQEQYSM
BTV10 VP5    ELEDEQRNELVRLKYNKEITEKFGKELGEVYDFMNGEAKEVEAVEEQYTM
BTV1(SA)     ELEDEQRNELVRLKYNDKIKEKFEKELEEVYNFYNGEANAEIEDEKQFDI
BTV1(AUS)    ELEDEQRNELVRLKYNDKIKEKFGEELEEVYEFMNGAAKAEVEDEKQFDI
BTV13 VP5    EIEEEQRNELVRIKYGKEIREKFGEQLEEIYQFMNGEVKGEEEQEEQYKV
BTV2 VP5     ELEEEQRNELVRLKYNDKIKEKFGEELEQVYEFMNGAAKEEVGAEKQFDI
EHDV VP5     ELEDEMKGEVVRTKHNEQIIRRFGADLDEVYKFAVSEYKEGVEEKDQFEI
             *.*.* . *.** *    *   *  .* ..* *  .  .       *. .

                                                                        200
BTV4         LCKAVDSYEKILKAEDSKMGILARALQREASERSQDEIKMVKEYRQKIDA
BTV10 VP5    LCKAVDSYEKILKEEDSKMAILARALQREAAERSEDEIKMVKEYRQKIDA
BTV1(SA)     LNKAVTSYNKILTEEDLQNRRLATRLQKEIGERTHAETVMVKEYRDKIDA
BTV1(AUS)    LNKAVTSYNKILTEEDLQMRRLANALQKEIGERTHAETVMVKEYRNKIDA
BTV13 VP5    LCKAVDSYENLLVTENEQMRTLARALQREATERTENESKMVKEYRQKIDA
BTV2 VP5     LSKAVNSYNKILTEEDQQMRRLAIALQKEIGERTHVETAMVKEYRNKIDA
EHDV VP5     LKKALTSYGELTKAEFGELKRLEKALQKESSERSKDESMMVKEYRQKIEA
             * **. **  .   *    *  **.* **.  *  ******.**.*

                                                                        250
BTV4         LKNAIEVERDGMQEEAIQEIAGMTADVLEAASEEVPLIGAGMATAVATGR
BTV10 VP5    LKSAIEVERDGMQEEAIQEIAGMTADVLEAASEEVPLIGAGMATAVATGR
BTV1(SA)     LKNAIEVERDGMQEEAIQEIAGMTADVLEAASEEVPLIGAGMATAVATGR
BTV1(AUS)    LKNAIEIERDGMQEEAIQEIAGMTRGVLEAASEEVPLIGAGMATAVATGR
BTV13 VP5    LKVAIEVERDGMQEEAIQEIAGMTADVLEAASEEVPLVGSGMATAIATGR
BTV2 VP5     LKSAIEIERDGMQEEAIQEIAGMTADVLEAASEEVPLIGAGMATAVATGR
EHDV VP5     LKDAIEVESTGIQEEAIQEIAGMSADILESAAEEVPLFGGGVATSIATAR
             ** ***.*  *.**********. ..**.*.***** *.*.**..**.*

                                                                        300
BTV4         AIEGAYKLKKVINALSGIDLSHMRSPKIEPTIIATTLEHRFKE-IPDEQL
BTV10 VP5    AIEGAYKLKKVINALSGIDLSHMRSPKIEPTIIATTLEHRFKD-IPDEQL
BTV1(SA)     AIEGAYKLKKVINALSGIDLTHFTHYENRPSVVSTILEYRAKE-IPDNAL
BTV1(AUS)    AIEGAYKLKKVINALSGIDLTHLRTPKIEPSVVSTILEYRTRA-IPDSAL
BTV13 VP5    AIEGAYKLKKVINALSGIDLSHLRTPKIEPTMVATTLEHRFKE-IPDKEL
BTV2 VP5     AIEGAYKLKKVINALSGIDLTHLRTPKIEPSVVSTILEYRAKD-IPDNAL
EHDV VP5     AIEGGYKLKKVINALSGIDLSHLRTPKIQPKTLEAILEAPTKEEIKDLSL
             ****.***************.*       *  .  **   .  * *  *

                                                                        350
BTV4         AVSVLNKKTAVTDNCNEIAHIKQEILPKFKRIM-DEEKEIEGIEDKVIHP
BTV10 VP5    AISVLNKKTAVADNCNEIAHIKQEILPKFKQIM-NEEKEIEGIEDKVIHP
BTV1(SA)     AVSVLIKERAIQENHKELMHIKNEILPRFKKAM-DEEKEIMRDRRQMIHP
BTV1(AUS)    AVSVLSKNRAIQENHKELIHIKDEILPRFKKAM-DEEKEICGIEDKTIHP
BTV13 VP5    AVSVLAKNDAIVANTKEIKHIKEEILPKFKKIM-EEEKELEGIDDKKIHP
BTV2 VP5     AVSVLSKNRAIQENHKELMHIKDEILPRFKKAM-DEEKEICGIEDKTIHP
EHDV VP5     VEGIQMKLQNLEENRNEVLHIQEEILPKLREAMIEDHKEIGDERDKRILP
               ..   *   .   * .*. **..****...  *  ...**.    . * *
```

```
                                                                   400
BTV4          RVMMRFKIPRTQQPQIHIYAAPWDSDDVFFFHCVSHHHRNESFFLGFDLG
BTV10 VP5     RVMMRFKIPRTQQPQIHIYAAPWDSDDVFFFHCVSYHHRNESFFLGFDLG
BTV1(SA)      KVMMKFKIPRAQQPQIHVYSAPWDSDDVFFFHCISHHHANDSFFLGFDLS
BTV1(AUS)     KVMMRFKIPRAQQPQIHVYSAPWDSDDVFFFHCISHHHANESFFLGFDLS
BTV13 VP5     RVMMRFKVPRSQQPQIHIYSAPWDSDDVFFFHCVSHFHANESFFLGFDLG
BTV2 VP5      KVMMKFKIPRSQQPQIHIYTAPWDSDDVFFFHCISHHHANESFFIGFDLS
EHDV VP5      KTAMRFKVPTTQQPVIQIYSAPWDSDDVFMFHCISHHHLNESSFLGFDLE
              .  *.**.* .*** *..*.********* ***.*  * *.* *.****

                                                                   450
BTV4          IDVVHFEDLTSHWHALGLAQEASGRTLTEAYREFLNLSISSTYSSAIHAR
BTV10 VP5     IDVVHFEDLTSHWHALGMAQEASGRTLTEAYREFLNLSISSTFSSAIHAR
BTV1(SA)      IDLVHYEDLTAHAHAIGAAQTAAGRTLTEAYREFLNLAISKAFGTQMHTR
BTV1(AUS)     IDLVHYEDLTAHWHALGAAQMAMGRTLSEAYKEFLNMAISNAYGTQMHTR
BTV13 VP5     IDVVHFEDLAAHWHALGAAQEAKGRTLNEAYREFLNLSIGSAFTSPMHAR
BTV2 VP5      IDLVHYEDLTAHWHALGAAQMAVGRTLTEAYKEFLKLAISSTYGTQMHTR
EHDV VP5      LEYVHYEDLTRHWHALGAAQEVTGRSLKEAYSEFFQLAAQIDGAGAIHQK
              ..  **.***.  * **.* **    **.* *** **....       .* .

                                                                   500
BTV4          RMIRSRAVHPIFLGSMHYDITYEALKNNAQRIVYDEELQMHILRGPLHFQ
BTV10 VP5     RMIRSRAVHPIFLGSMHYDITYEALKNNAQRIVYDDELQMHILRGPLHFQ
BTV1(SA)      RLVRSKTVHPIYLGSLHYDISFSDLRGNAQRIVYDDELQMHILRGPIHFQ
BTV1(AUS)     RLVRSKMVHPIYLGSLHYDISFLDLRGNAQRIVYDDELQMHILRGPIHFQ
BTV13 VP5     RMIRSKTVHPIYLGSMHYDITYETLKTNATEARYDEDLQMHILRGPLHFQ
BTV2 VP5      RLIRSKTVHPIYLGSLHYDISFADLRGNAQRIVYDDELQMHILRGPLHFQ
EHDV VP5      RLIRSKSYHPIYLGAMHYDIAYRELKNNALKIVNDSEVQKHLLRGPKHFQ
              *..**.   ***.**..****..  *.  **      *  ..* *.**** ***

                                    526
BTV4          RRATLGALKFGVKILGDKIDVPLFLRNA
BTV10 VP5     RRAILGALKFGVKILGDKIDVPLFLRNA
BTV1(SA)      RRAILGALKFGCKVLGDRLDVPLFLRNA
BTV1(AUS)     RRAILGALKFGCKVLGDRLDVPLFLRNA
BTV13 VP5     RRAILGALKFGVKVLGDKVDVPLFLKNA
BTV2 VP5      RRAILGALKFGCKILGDRLDVPLFLRNP
EHDV VP5      RRAILSALKDGVKLLGG-VDLAEFMRYA
              *** *.*** * *.**. .*.. *.. .
```

of disulfide bridges for protein structure. Oldfield *et al.,* 1991 commented on the importance of a conserved glycine residue, identified at the amino acid terminus of VP5 following the starting methionine residue, which may be involved in myristoylation of this terminus. The fact that this residue is also found in VP5 of BTV4 gives further weight to this proposal. Gould and Pritchard,1988 identified 4 major conserved amino acid regions (aa 1-112, 190-273, 361-420 and 478-515) in VP5 linked by 3 variable regions (aa 137-189, 280-333 and 410-475). These assignments are also applicable to BTV4 VP5.

The percentage nucleotide and amino acid similarities of VP5-genes of different Orbiviruses is presented in Table 3.2. The top row shows the nucleotide and the bottom row the amino acid similarities. The proteins of the bluetongue serotypes were much more conserved than their corresponding genes. In contrast, the proteins of EHDV in comparison with the bluetongue serotypes had approximately the same percentage of conservation as their corresponding genes. The overall direct aa similarities between the VP5 proteins of the bluetongue serotypes varied between 72% and 95% while the VP5 protein of EHDV was approximately 60% homologous to that of the bluetongue serotypes. It was clear that VP5 is not necessarily more conserved within serotypes (between SA-BTV1 and AUS-BTV1) than between serotypes. Indeed, the highest similarities occured between SA-BTV4 and US-BTV10 (80.2% homologous at nt level, 95.2% at aa level) and US-BTV2 and AUS-BTV1 (78.2 % homologous at nt level, 90.3% at aa level), whereas SA-BTV1 and AUS-BTV1 were only 77% homologous at nt level and 88.6% at aa level.

Table 3.2 COMPARISON OF THE *NUCLEIC ACID* / PROTEIN SEQUENCE SIMILARITIES OF VP5 OF DIFFERENT ORBIVIRUS SEROTYPES

US-BTV10	**US-BTV2**	**AUS-BTV1**	**SA-BTV1**	**US-BTV13**	**EHDV1**	
80.2 95.2	*69.1* 77.7	*68.4* 75.9	*68.5* 73.2	*71.7* 81.0	*63.3* 62.1	**SA-BTV4**
	68.6 78.0	*68.0* 76.3	*68.3* 73.8	*72.3* 81.8	*61.3* 62.4	**US-BTV10**
		78.2 90.3	*76.2* 85.7	*68.5* 75.7	*61.9* 61.8	**US-BTV2**
			76.9 88.6	*68.9* 75.3	*62.1* 62.1	**AUS-BTV1**
				68.4 72.6	*61.1* 60.0	**SA-BTV1**
					63.4 62.0	**US-BTV13**

CHAPTER FOUR

LUMPY SKIN DISEASE VIRUS PROTEIN SYNTHESIS

4.1 INTRODUCTION

The potential of vaccinia virus (VV) to serve as a vector for live recombinant vaccines is widely recognised. Several drawbacks are associated with the use of VV (literature review, 1.6.2). For example its lack of species specificity is a major concern. This has prompted research on viruses which have a limited host-range, hence preventing spread to unintended species. The present study is part of a general programme to develop lumpy skin disease virus (LSDV) as a recombinant vector for use in veterinary medicine, since it has a host-range restricted to cattle, sheep and goats.

As described in Chapter two, approximately 50% of the LSDV genome has been cloned. In order to exploit LSDV as an expression vecor of foreign genes, it is necessary to identify suitable promoters. Proteins and mRNA of the virus which are expressed to high levels are indicative of strong promoters. To identify highly expressed proteins and mRNA species, some basic knowledge of the growth cycle of LSDV is required. DNA accumulation and protein synthesis are parameters for viral growth. Very little is known about the characteristics of a LSDV infection, in stark contrast to that of a VV infection. It is for example not known how soon after an LSDV infection viral DNA starts to replicate, when early protein synthesis starts and late protein synthesis ends and when progeny viruses are produced. Answers to these questions need to be established for a natural infection, before any deductions with regard to future recombinants could be made.

It has been demonstrated that LSDV can be propagated in a variety of cell cultures (Weiss, 1968), including primary and established cells. In this study, the MDBK cell line was chosen for cultivation of LSDV for DNA isolation purposes (Chapter 2). It soon became apparent, however, that this virus behaved quite differently in these cells from other poxviruses that have been described. Most notably, CPE was not evident until approximately 4 days post infection (p.i.), in contrast to that of the orthopoxviruses such as vaccinia virus, for which CPE is readily observable within a few hours after infection (Moss, 1968). LSDV cytopathology remains localised and is limited to foci, with little immediate effect on the surrounding monolayer, whereas with an VV infection, spread of the virus

soon destroys the monolayer. Low titres of LSDV (10^5 ffu/ml) in MDBK cells are characteristic and present a potential problem for animal trials.

Consequently, the aim of the work presented in this Chapter was to perform a preliminary investigation into the protein synthesis and the kinetics of LSDV in cell culture. To study the time course of accumulation of proteins in infected cells, newly synthesised proteins needed to be pulsed labelled with ^{35}S-methionine at various times post infection, followed by analysis by SDS-polyacrylamide gel electrophoresis and autoradiography. An attempt was made to identify some early and late LSDV proteins, by investigating the effect of an inhibitor of DNA synthesis on LSDV protein synthesis.

4.2 MATERIALS

4.2.1 Radio-isotope

The following radioisotope was purchased from Amersham International (UK):
L-(^{35}S)-Methionine with a specific activity of 1326 Ci/mMol and a radioactive concentration of 15mCi/ml (555MBq/ml).

4.2.3 Other materials

Item	Manufacturer
Cytosine arabinoside	Sigma
β-Mercaptoethanol (βME)	BDH Chemicals
Tetramethyl-ethylene-diamine (TEMED)	BDH Chemicals
Cronex MRF31 X-Ray film	NEN DuPont
Radioactive markers	NEN DuPont

4.3 METHODS

4.3.1 Virus and cells

Primary lamb testis (LT) cells were used for the propagation of virus and was obtained from Mr. P.A.M. Wege from the Virology Section, OVI. The LSDV vaccine strain Neethling, was used for the identification of proteins. Cell monolayers were infected with LSDV at an input multiplicity of 5 ffu/cell in Eagles medium. Adsorption was allowed to continue for 2 hr at 37°C. The monolayers were then washed and maintained in Eagles medium at 37°C until such time that labelling of proteins commenced.

4.3.2 Radiolabelling of proteins

Confluent primary LT monolayers were washed twice with warm methionine-free Eagle's medium. Proteins were then labelled by the addition of (^{35}S)-methionine (60μCi/1x10^{7} infected cells)

contained in methionine-free Eagle's medium. After 2 hr of incubation at 37°C, the medium was removed and the cells from each plate harvested using glass beads. Cells were pelleted at 800xg for 15 min, resuspended in PBS and pelleted again. The cells were then lysed by the addition of 150μl of sample buffer (0.2% SDS, 5% glyserol, 0.2% β-ME, 15% urea, 50mM EDTA and 80mM Tris-HCl pH 8.7) and stored at -70°C prior to electrophoresis.

4.3.3 Gel electrophoresis and autoradiography

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of LSDV proteins was carried out using the discontinuous gel system as described by Laemmli (1970). The acrylamide:bis acrylamide ratio used was 30 : 0.8 and 15% polyacrylamide seperating gels (13cm x 15cm x 1.5mm) were used. The gels were polymerised in a buffer containing 0.375M Tris-HCl pH 8.8, 0.1% SDS, 0.04% ammonium persulphate and 15μl TEMED per 30ml of gel. A 5% stacking gel (4cm x 15cm x 1.5mm) was polymerised in a buffer containing 0.125M Tris-HCl pH 6.8, 0.1% SDS, 0.06% ammonium persulphate and 15μl TEMED per 12ml of gel. The running buffer consisted of 0.1M Tris-HCl pH 8.9, 60mM glycine and 1% SDS. Before electrophoresis, samples were mixed with 2x loading buffer (0.275M Tris-HCl pH 6.8, 0.7M β-ME, 2% SDS, 10% glycerol and 0.001% Bromophenol blue as dye marker) and denatured by incubation at 90°C for 5 min. Electrophoresis was carried out using the mini-gel Hoefer electrophoresis system, applying a current of 150V for either 2 or 3.5 hr. After electrophoresis, the gels were stained for 15 min in a solution containing 0.2% Serva blue, 50% methanol and 10% acetic acid and destained at 60°C in 4% acetic acid. Gels were then dried and autoradiographed overnight.

4.3.4 Treatment with cytosine arabinoside

Cytosine arabinoside (CAR), an inhibitor of DNA synthesis was added to cell monolayers at a concentration of 25μg/ml. This inhibited the late virus stage from progressing. The drug was added to infected cells after adsorption of viruses had occured.

4.4 RESULTS

4.4.1 Identification of LSDV proteins

Before experiments concerning viral protein kinetics or influence of inhibitors on protein synthesis could be performed, the presence of LSDV-specific proteins in infected cells needed to be demonstrated. LSDV infected cells were radio-actively labelled for a 4 hr labelling period, commencing at two different times post infection, namely at 24 hr and 42 hr p.i. After the labelling period, cells were harvested and lysed to yield a total cell-extract. The patterns of ^{35}S-methionine incorporated polypeptides of uninfected (control) and LSDV-infected cells were analysed on polyacrylamide gels and compared. Gels were electrophoresed for either 3.5 hr (Fig 4.1 A) or 2 hr (Fig 4.1 B), for the detection of both large and small polypeptides. The autoradiographs showed a complex pattern of bands which was, nevertheless, highly reproducible from experiment to experiment.

Several polypeptide bands were detected in infected cell samples that were unique and differed from polypeptides found in the uninfected cells, in mobility and hence in molecular weight. This suggested that they were virus specific. Of these, 18 of the most abundantly expressed viral proteins were identified and are indicated by a (·) in Fig. 4.1 A and 4.1 B. A summary of the proteins and their relative expressions as judged by eye, of band intensities, is given in table 4.1. A reference protein (* Fig. 4.1) indicated that approximately the same amount of protein was loaded onto the gel in the case of Fig. 4.1 A. In Fig. 4.1 B, lane 2 contained slightly less protein, which was taken into account when table 4.1 was compiled. Densitometric scanning, which allows a more precise quantitation of bands, was not feasible in this case due to inadequate resolution of these gels and the vast number of protein bands that were present.

Many of these proteins, e.g. proteins 2, 4, 10, 11 and 13 seemed to be synthesised in approximately the same amount at both times post infection. Some, however, notably 6, 7, 9, 12 and 17 have declined after 40 hr. Because of incompetent host cell protein shut-down, as is evident from the continual host protein synthesis throughout the 44 hr labelling period, the protein pattern was rather complicated. It is most probable that many of the viral proteins were masked by cellular proteins and could therefore not be identified. LSDV did however have some effect on the host cell metabolism, since some of the host proteins declined as the infection progressed (indicated by a (◦) in Fig 4.1 A and B), when compared with the reference protein (*).

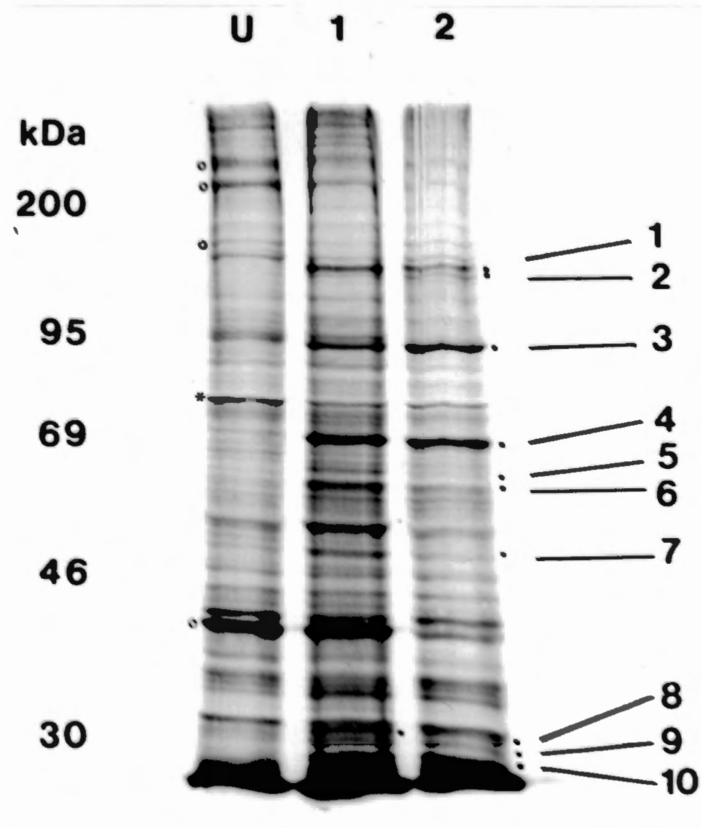


Figure 4.1 A Autoradiograph of total protein expression in LSDV-infected LT-cells. At 24 hr p.i. (lane 1) and 40 hr p.i. (lane 2) cells were labelled for 4 hr with ^{35}S-methionine. Samples were electrophoresed in a 15% polyacrylamide gel for 3.5 hr. Lane U represents an uninfected control sample. Molecular weights are indicated in kilodaltons.

Putative viral proteins ·

Reference protein *

Cellular proteins that clearly declined as the infection progressed. ∘

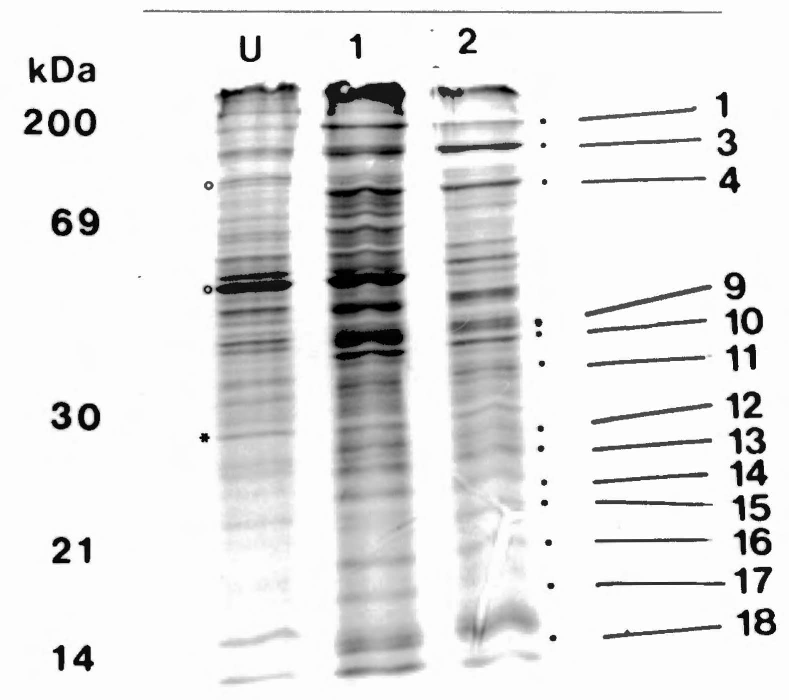


Figure 4.1 B Autoradiograph of total protein expression in LSDV-infected LT-cells. At 24 hr p.i. (lane 1) and 40 hr p.i (lane 2) cells were labelled for 4 hr with ^{35}S-methionine. Samples were electrophoresed in a 15% polyacrylamide gel for 2 hr. Lane U represents an uninfected control sample. Molecular weights are indicated in kilodaltons.

Putative viral proteins ·

Reference protein *

Cellular proteins that clearly declined as the infection progressed. ◦

Table 4.1 Relative expression of LSDV proteins as judged by eye, of band intensities of Fig. 4.1 A and B. Labelling of proteins commenced for 4 hours at the times indicated.

PROTEIN	24hr p.i.	40hr p.i.
1	+++	++
2	+	+
3	+++	+++
4	+++	+++
5	++	+
6	+++	+
7	++	+
8	++	+
9	++	+
10	+++	+++
11	++	++
12	++	+
13	++	++
14	++	++
15	+	+
16	++	++
17	++	+
18	++	++

Classification

+ -detectable

++ -clearly detectable

+++ -major component

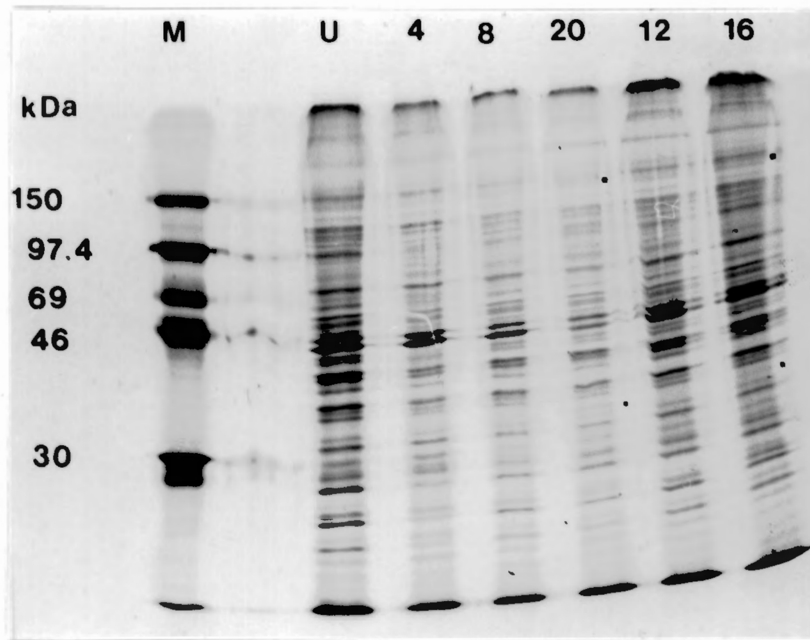
4.4.2 Kinetics of LSDV protein expression

The time course of polypeptide synthesis in infected cells was followed in experiments where infected cells were pulse-labelled for 2hr with ^{35}S-methionine at various times after infection, as indicated in Fig. 4.2 A and B. Cells were harvested after the labelling period and lysed. Samples of these total cellular extracts were then analysed by polyacrylamide gel electrophoresis and autoradiography. The total radiolabelled protein pattern of LSDV-infected cells at different times p.i., is presented in Fig 4.2 A and B. No virus-specific protein was detectable prior to 16 hr post infection. At that time 2 putative viral proteins were detected in minute amounts (Fig. 4.2 A). Between 20 and 40 hr post infection (Fig 4.2 B), other viral proteins became clearly evident and all proteins were synthesised in ever-increasing amounts throughout the labelling period. The continuation of host protein synthesis, which failed to decline significantly over the 40 hr period, may however mask viral protein synthesis that had commenced prior to 16 hr p.i.

4.4.3 Identification of early and late LSDV proteins

The proteins synthesised in cells infected with poxviruses can be divided into two classes, as was demonstrated for vaccinia virus (Joklik *et al.*, 1967), i.e. early proteins being made before, and late proteins after virus DNA synthesis. To identify early and late LSDV proteins, cytosine arabinoside (CAR) was used as an inhibitor of virus DNA synthesis. This inhibitor has been used for the identification of early and late viral proteins of other viruses, e.g. vaccinia virus (Holowczak and Joklik, 1967, Pennington, 1974) and swinepox virus (Massung and Moyer, 1991b). The concentration of the drug used in this experiment (25μg/ml) was reported to inhibit virus DNA synthesis in infected cells by 94% (Pennington, 1974).

For identification of early proteins, CAR (25μg/ml) was added to a cell monolayer upon infection with LSDV. At 18 hr p.i., these infected cells were labelled with ^{35}S-methionine for a period of 14 hr. CAR was included throughout the labelling period. For comparison, the late viral phase was analysed by infecting a cell monolayer with LSDV, with no addition of CAR and labelling it at 40 hr p.i. for a 6 hr period. Uninfected cells, both in the absence and presence of CAR, were labelled as controls.



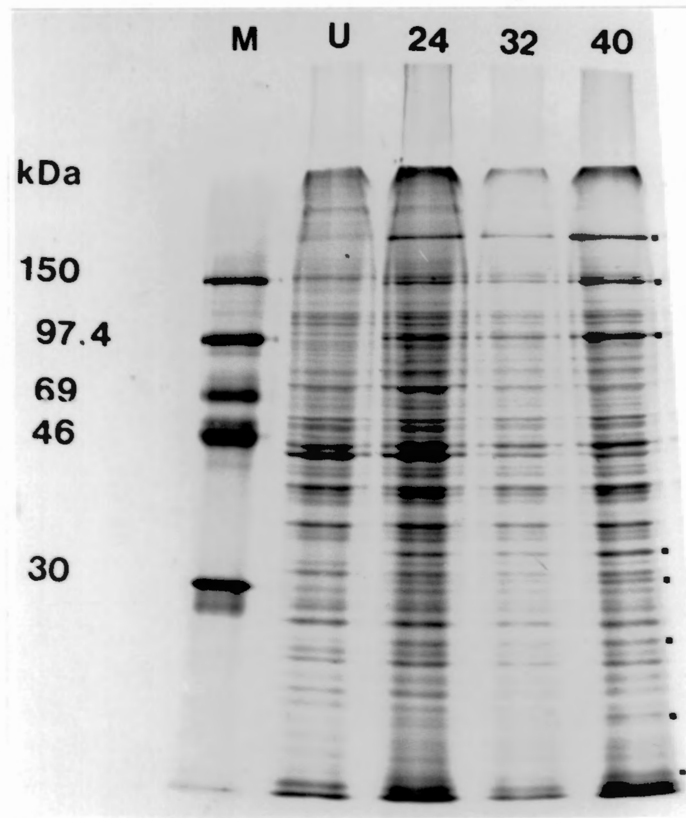


Figure 4.2 A and B Autoradiographs of protein kinetics in LSDV-infected LT-cells. At the times indicated, cells were pulsed-labelled for 2 hr with ^{35}S-methionine. Samples were electrophoresed in 15% polyacrylamide gels for 2 hr. Lane U represents an uninfected control sample. Molecular weights are indicated in kilodaltons.

Putative viral proteins are indicated by (·).

An autoradiograph of results obtained is shown in Fig. 4.3. Three classes of proteins could be distinguished in the infected cell samples (Fig. 4.3, lanes 3 and 4) namely :

1. those which were present in samples both treated and untreated with CAR (~), indicative of early proteins present in both the early and late virus phases.
2. those which were present only in the sample treated with CAR (•), indicative of exclusively early proteins.
3. those which were present only in the sample not treated with CAR (*), indicative of late proteins.

Differences between the protein profiles of the CAR-treated and -untreated LSDV-infected sample and the uninfected control samples, made identification of some early and late viral proteins possible. Eight early LSDV proteins were identified (indicated by • and ~ in Fig. 4.3, lanes 3 and 4). Of these, five (~) occured in both the CAR-treated (containing only early proteins, lane 3) and untreated (containing early and late proteins, lane 4) samples. Three occured only in the CAR-treated sample (• lane 3). The fact that some of the early proteins were found in substantial amounts in the CAR untreated sample is indicative that at 40 hr p.i. unsynchronised replication is occuring. Some progeny virions are therefore already engaged in subsequent rounds of early protein synthesis, thus accounting for the presence of both early and late LSDV proteins.

Only three late viral proteins (*), unique to the CAR-untreated infected sample, could be identified. It is unclear why no more could be distinguished. One explanation may be masking of other late proteins by the inefficient shut-down of host protein synthesis.

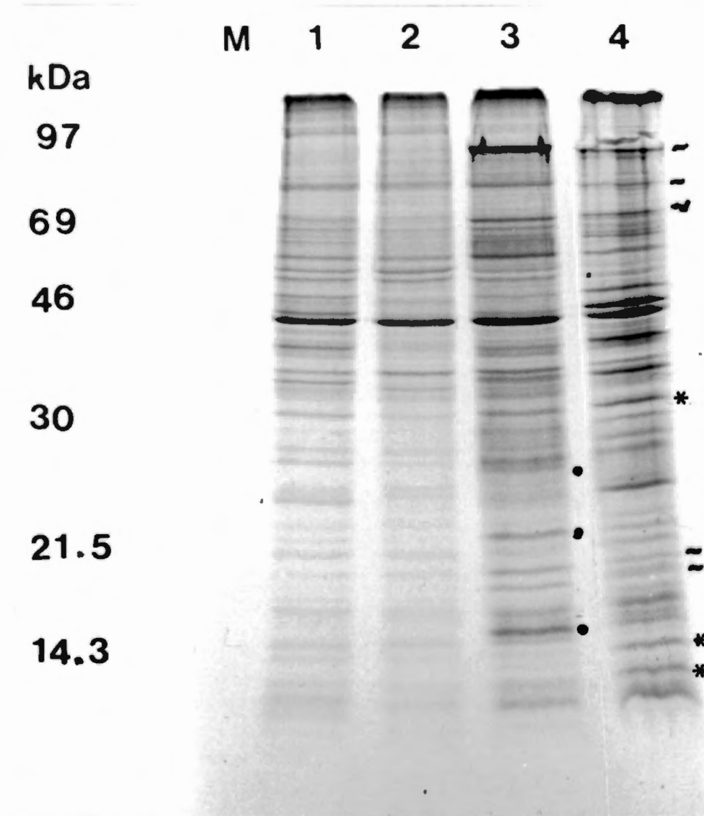


Figure 4.3 Autoradiograph demonstrating the effect of cytosine arabinoside (25µg/ml) on virus polypeptide synthesis. LSDV-infected CAR treated cells were labelled with ^{35}S-methionine for a 14 hr period, commencing at 18 hr p.i. (lane 3). CAR untreated infected cells were labelled for a 6 hr period at 40 hr p.i (lane 4). Uninfected cells, both in the absence (lane 1) and presence (lane 2) of CAR was included as controls. Samples were electrophoresed for 2 hr in 15% acrylamide gels. Molecular weights are indicated in kilodaltons.

~ Putative early LSDV proteins, present in both the early and late viral phase.

• Putative early LSDV proteins, present only in the early viral phase.

* Putative late LSDV proteins, present only in the late viral phase.

4.5 Discussion

Protein synthesis of various viruses, including vaccinia (Esteban and Metz, 1973) and swinepox viruses (Massung and Moyer, 1991b) has been analysed by the ^{35}S-methionine labelling of infected cells followed by SDS-polyacrylamide gel electrophoresis of the polypeptides. This preliminary study demonstrated that the method was also applicable for analyses of LSDV proteins.

From autoradiographs, at least 18 ^{35}S-methionine labelled polypeptide bands were detected in LSDV-infected cells, that differed in mobility from those in uninfected cells. They were therefore assumed to be viral induced. The fact that some of these proteins may be processed host proteins, can, however, not be excluded. Only the major proteins were identified and although minor proteins were present, no attempt was made towards their identification. The number of polypeptides detected was limited by the single dimensional nature of the polyacrylamide gels and masking by host proteins. Doubtless many other LSDV components remain to be detected. If the whole of the Lumpy skin viral genome (approximately 143x10^{6} bp) encodes proteins, then some 180 polypeptides of avarage molecular weight 40 000 D could be expressed. Such a large amount of polypeptides demonstrates the complex nature of LSDV. Elucidation of its biochemical aspects will be no small task.

An interesting observation, that contributed to the small number of proteins detected, was the inability of LSDV to effectively shut off host protein synthesis. This differs markedly from reports of vaccinia virus infections, where host protein synthesis is rapidly and completely inhibited (Moss, 1968). A possible explanation in this case, may be the low number of inoculating Lumpy skin disease virions (5 ffu/cell), which was used since low titres of virus were obtained during cultivation (1x10^{5} ffu/cell). In the experiments where CAR was used as an inhibitor, host proteins were very prominent when compared to viral proteins, since CAR has no effect on host protein synthesis. The inefficient host shut-down may however be intrinsic to the virus itself. In a recent report on swinepox virus infections, host protein synthesis continued until between 24 and 32 hr post infection (Massung and Moyer, 1990b). Growth characteristics of a LSDV infection, namely a CPE which is not evident until approximately 4 days p.i. and foci which remains localised, favour this explanation. These properties were also found for SPV, considered to be unusual among poxviruses, because of its slow DNA, RNA and protein kinetics (Massung and Moyer, 1991b).

Although the expression of host proteins complicated interpretation of autoradiographs, an attempt was made to examine the kinetics of LSDV protein synthesis. In this experiment infected cells were

pulse labelled with ^{35}S-methionine at various times post infection. The first indication of viral protein expression was found at 16 hr p.i., when two viral proteins were detected in small amounts (Fig. 4.2 A). By 24 hr p.i. the expression of others were clearly detected (Fig. 4.2 B). Protein synthesis for all detectable viral proteins continued to increase throughout the 42 hr labelling period of this experiment. Results of the initial experiment, where the presence of LSDV viral proteins were indicated (Fig. 4.1 A and B), demonstrated that expression of various proteins seemed to have declined when labelled from 42-46 hr p.i. (see table 4.1). This would imply that the peak of viral protein synthesis occured at approximately 42 hr p.i., whereafter it reached a plateau and started to decline.

It is highly unlikely that early viral synthesis starts as late as 16 hr p.i. as these results indicated, when all previous reports of poxvirus synthesis is taken into account. For example, for vaccinia virus, early protein synthesis commences before 2 hr p.i. Late protein synthesis can already be observed at 4-6 hr p.i. (Moss and Salzman, 1968). The growth characteristics of LSDV however, differs starkly from vaccinia virus. It can be compared to swine pox virus (SPV) in that both are slow growers, with CPE being detected 4 days post infection. Analysis of SPV protein kinetics as had been performed in a study (Massung and Moyer, 1991b), allowed the detection of SPV proteins at 24 hr p.i. Immunoprecipitation in contrast, proved to be a more sensitive method, allowing SPV early proteins to be detected 4 hr p.i. and late proteins 10-12 hr p.i. (Massung and Moyer,1991b). LSDV protein kinetics therefore needs to be examined by immunoprecipitation before any conclusions can be drawn.

A preliminary investigation into the effect of a DNA inhibitor, cytosine arabinoside (CAR) on virus growth, which inhibits the late virus stage from progressing, allowed the detection of eight apparent early and three putative late LSDV proteins. Because only two times p.i. were investigated, no conclusion on length of the early or late viral stages could be made. This experiment needs to be extended to include pulse chase labelling of viral proteins. The effect of CAR on protein synthesis, added to the LSDV infected cells for different lengths of time, therefore needs to be performed. It was interesting to note that many of the early proteins that were identified still occured in the late LSDV stage, i.e. in the 40-46 hr p.i. labelling period, where viral growth was not inhibited by CAR. If only one round of viral DNA replication occured at 40 hr p.i., these early proteins were therefore not processed and stayed intact untill the late viral stage was reached. It is however more likely that these proteins are indeed processed as usual, but have accumulated at 40 hr p.i. because LSDV has gone through several cycles of replication.

One major consideration that might have influenced all of these results, is the relative low multiplicity of infection that was used in these experiments (5 ffu/cell). This low infectivety was used because of the low titres ($5x10^5$ ffu/ml) of LSDV obtained in MDBK cells. Recently LSDV has been cultivated in Vero cells in our laboratory where it reached higher titres ($5x10^8$ ffu/ml). This would allow for a higher input of infective viruses in future experiments.

Although no concrete conclusions were reached in this study, some of the difficulties that have to be overcome in future research, have been pointed out. These include the fact that a higher input of infective viruses has to be used for a more complete host protein shut-down, stressing the need for higher titres to be obtained in cell culture.

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