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SELECTABLE MARKERS FOR RECOMBINANT POXVIRUSES

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ABBREVIATIONS

β-gal	beta-D-galactosidase
bp	base pair
BSA	bovine serum albumin
BTV	bluetongue virus
°C	degrees centigrade
CAM	chorioallantoic membrane
CFK	fetal calf kidney
CIP	calf intestinal alkaline phosphatase
CV-1 cells	continuous fibroblast-like cell-line derived from an African Green Monkey kidney
Da	dalton
DIG	digoxigenin
DMEM	Dulbecco's Modified Eagle's Essential Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
ds	double stranded
dTMP	deoxy-thymidine-5'-monophosphate
dTTP	deoxy-thymidine-5'-triphosphate
dUTP	deoxyuridine-triphosphate
E. coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid (disodium salt)
g	gravitational acceleration
GMP	guanylic acid or guanosine-5'-monophosphate
Gpt	xanthine-guanine phosphoribosyl transferase
IMP	inosinate or inosine-5'-monophosphate
Kb	kilobase
kDa	kilodalton
LSDV	lumpy skin disease virus
LT	lamb testes
M	molar
MDBK	Madin-Darby bovine kidney
Mol	mole
MPA	mycophenolic acid
M_r	relative molecular weight
OD_{400}	optical density at 400 nm wavelength
ORF	open reading frame
OVI	Onderstepoort Veterinary Institute
PBS	phosphate-buffered saline

poly(A)	polyadenylic acid or polyadenylate
PRPP	phosphoribosyl pyrophosphate
PSN	penicillin streptomycin neomycin
RNA	ribonucleic acid
RNAase	ribonuclease
SDS	sodium dodecyl sulphate
ss	single stranded
TAE	tris-acetate
TBE	tris-borate
TK	thymidine kinase
TMP	thymidine-5'-monophosphate
Tris	tris(hydroxymethyl)aminomethane
U	unit
UCT	University of Cape Town
UV	ultraviolet
VP	viral protein
VV	vaccinia virus
v/v	volume per volume (in ml per 100 ml)
w/v	weight per volume (in g per 100 ml)
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactosidase
XMP	xanthosine monophosphate

ABSTRACT

Poxviruses have many favourable characteristics that render them useful as vectors for the expression of genes encoding foreign antigens to be used for vaccination purposes. Several poxviral vaccines have been constructed by using vaccinia virus (VV) as vector. VV has a wide host range and may not be a suitable vector for veterinary vaccines where a vector would be preferred with a narrow host range which would be confined to the target species. Attempts to utilize lumpy skin disease virus (LSDV) as a vector with a narrow host range are the subject of the thesis.

The first phase of this project was to grow LSDV. Problems were experienced in reaching high enough LSDV-titres, although higher titres were obtained with primary cell lines than with continuous MDBK-cells. Attempts to propagate the virus in eggs were unsuccessful. These results were similar to earlier reports.

The next phase of the project was to investigate if foreign genes could be inserted into the LSDV-genome. Thymidine kinase selection cannot be used to select for recombinant LSDV, because there is no TK^- cell-line available in which the virus can be propagated. Two other methods have been used with VV for the selection of recombinant virus. Recombinants expressing β-galactosidase (β-gal) produce blue plaques; alternatively, recombinants expressing the guanine phosphoribosyl transferase (*Gpt*) gene (encoding xanthine-guanine phosphoribosyl transferase) are capable of overcoming plaque inhibition by mycophenolic acid (MPA). It was decided to adapt each of these approaches for use in LSDV.

Two plasmids were constructed, in which coding sequences for β-gal and *Gpt* respectively, were placed under the control of a promoter derived from VV and flanked by sequences from the TK-gene of LSDV (to direct homologous recombination with the TK-gene of the LSDV-genome). Restriction enzyme mapping was performed to confirm the integrity of these constructs. Transient expression assays were done to check whether the β-gal or *Gpt* inserts were functional *in vivo*. The β-gal-gene was not expressed strongly enough to be useful. The *Gpt*-gene appeared to be functional based on results obtained with indirect immunoperoxidase staining. However, no further evidence was obtained that the *Gpt*-gene was functional in this construct. Subsequent evidence showed that a mutational event occurred in the *Gpt*-gene itself which probably could effect it's functional expression.

Upon completion of these experiments, attempts were made to obtain a LSDV or a VV recombinant expressing the viral protein 7 (VP7)-gene of bluetongue virus (BTV), using *Gpt* as selectable marker-gene. This involved the construction of a plasmid (designated pGS20-VP7:*Gpt*) containing both the *Gpt*-

and the BTV4 VP7-gene separately under the control of VV-promoters. The pGS20-vector, containing VV TK sequences, was used for the construction of the pGS20-VP7:*Gpt* construct. Restriction enzyme mapping was performed on the native pGS20 vector, the pGS20 vector containing the VP7 gene and subsequently the vector containing both the VP7- and the *Gpt*-gene. This was done to determine the orientation of the inserted genes as well as the integrity of the final construct. The digest showed that a plasmid has been selected in which the VP7 insert was correctly oriented under the control of the P 7.5 promoter. A cassette containing the *Gpt*-gene, also under the control of the VV P 7.5 promoter, was inserted in the pGS20 *Hind111* site outside the TK-sequences. Restriction enzyme digests showed that the *Gpt* insert was oriented in the same direction as the VP7 insert.

LSDV-infected CFK-cells were transfected with the pGS20-VP7:*Gpt* construct and the virus obtained was subsequently passaged through selection medium for up to six times. In parallel with this, the pGS20-VP7:*Gpt* construct was transfected into VV-infected CV-1 cells and passaged through selection medium. Total DNA was extracted from the cells infected with the obtained virus (presumed to contain recombinants). The DNA was then subjected to restriction enzyme digestion and Southern blot hybridization analysis.

Recombinant LSDV could not be obtained from the heterologous transfection experiments with the pGS20-VP7:*Gpt* construct. One probable reason for the lack of LSDV-recombinant could be that the relative low level of nucleotide sequence similarity (68%) shared between the heterologous TK-genes present in the plasmid construct (VV TK) and the virus (LSDV TK) was not capable of directing recombination efficiently.

In the case of VV, however, the recombination experiment was successful. This indicated that homologous TK-sequences may direct recombination at a higher frequency than heterologous sequences do, as would be expected. A mixture of recombinant and wild type VV was obtained from the pGS20-VP7:*Gpt*-transfection experiment in VV-infected CV-1 cells, as shown by the size of the TK-containing fragment observed following *Hind111*-digestion of total DNA-extracts and Southern Blot hybridization analysis. The presence of the *Gpt*-gene indicated by the ability of the virus to overcome MPA-selection, could be verified by Southern blot hybridization analysis of the harvested DNA. The VP7 gene could also be detected in the DNA harvested from the VV-infected CV-1 cells by probing with a DIG-labelled VP7 *BamH1*-fragment. No plaque purification steps were included as this was only a preliminary screening for the presence of recombinant virus(es). The virus would need to be plaque purified and expression of the VP7-gene should be further investigated before this recombinant could be tested in animal trials for efficiency.

CHAPTER 1. LITERATURE REVIEW

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CHAPTER 1. LITERATURE REVIEW

1.1. General characteristics of poxviruses

Poxviruses are well characterised. The general characteristics of poxviruses have been reviewed extensively (Esposito and Murphy, 1989; Moss 1990 and 1991; and Piccini and Paoletti, 1986 and 1988). A summary of the aforementioned review articles and other sources of relevant literature (as cited) follows hereafter.

1.1.1. Classification of poxviruses

The poxvirus family consists of a large family of complex DNA viruses that infect both vertebrate and invertebrate hosts (Moss, 1990). Poxviruses have been classified as indicated in Table 1.1. on grounds of their morphology, serology and host range.

1.1.2. Virion structure

Poxvirus virions are oval or brick-shaped with a size of approximately 300 x 240 x 100 nm. The outer surface contains ridges that may be arranged in parallel rows or as a single continuous helix. The core, containing the DNA genome, is dumbell-shaped. Two lateral bodies of unknown function are found in the concavities of the core. A lipoprotein bilayer, the outer membrane, surrounds the core. Extracellular forms of poxviruses contain an additional lipoprotein envelope. The two types of infectious virus particles are the intracytoplasmic naked virions and the extracellular enveloped virions (Engelstad and Smith, 1993). The extracellular enveloped virions are antigenically distinct because the envelopes are derived from modified plasma membranes. This distinct antigenicity of the envelope may direct tissue tropism, pathogenicity, immunogenicity and host range properties. Intracytoplasmic naked virions (without envelopes) are produced in infected cells and are released *in vivo* through cytolysis as extracellular enveloped virions. These extra-cellular enveloped virions are involved in virus dissemination *in vivo* and *in vitro* (Engelstad and Smith, 1993). Immunity to proteins located in the outer layer of the virion appears to be more important for protection against orthopoxviruses than immunity to naked intracellular virions (Engelstad and Smith, 1993).

Protein, lipid and DNA (90%, 5% and 3.2% respectively of the dry weight) are the main components of vaccinia virus (VV). The predominant lipid components are phospholipids and cholesterol. Spermine, spermidine and only small amounts of RNA are found in VV. The virion contains more than 100 polypeptides.

TABLE 1.1 CLASSIFICATION OF FAMILY POXVIRIDEAE[*]

SUBFAMILIES	GENERA	MEMBERS	FEATURES
Chordopoxviridae (vertebrate poxviruses)	Orthopoxvirus	Buffalopox, camelpox, cowpox, monkeypox, rabbitpox, raccoon pox, tatera pox, vaccinia, variola, vole pox	Variola cause of smallpox, vaccinia used as vaccine
	Parapoxvirus	Chamois contagious ecthyma, Orf, pseudocowpox, stomatitis papulosa	Ungulate viruses, surface tubule forms regular spiral
	Avipoxvirus	Canary pox, fowlpox, junco pox, pigeon pox, quail pox, sparrow pox, starling pox, turkey pox	Viruses of birds, arthropod transmission
	Capripoxvirus	Goatpox, sheeppox, lumpy skin disease	Ungulate viruses, virions longer and narrower than vaccinia, arthropod transmission
	Leporipoxvirus	Hare fibroma, myxoma, rabbit (Shope) fibroma, squirrel fibroma	Viruses of leporids and squirrels, arthropod transmission
	Suipoxvirus	Swinepox	Limited host range
	Molluscipoxvirus	Molluscum contagiosum	Benign tumors in man, does not grow in culture
	Yatapoxvirus	Tanapox, Yaba	Benign tumors in primates
Entomopoxviridae (insect poxviruses)	A	Melontha melontha	Isolated from Coleoptera, one lateral body
	B	Amsacta moori	Isolated from Lepidoptera, indistinct lateral body
	C	Chironimus luridus	Isolated from other Diptera, two lateral bodies

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[*] Table taken from Moss 1990

1.1.3. Genome

The genome of poxviruses is composed of a single, large, linear, double-stranded DNA molecule (Keck, Baldick and Moss, 1990). The genome size of orthopoxvirus (vaccinia) is 186 kb (143-147 kb for capripoxvirus) and it has the capacity to encode between 150 and 200 proteins (Gershon and Black, 1988; Keck *et al.*, 1990; Weir and Moss, 1983). Some restriction endonuclease cleavage sites are highly conserved within genera among orthopoxviruses (Esposito and Knight, 1985) and capripoxviruses (Black, Hammond and Kitching, 1986).

The central region of the genome is more highly conserved than the termini and appear to contain most of the genes essential for productive infection. Both ends of poxvirus DNA are covalently closed by A + T-rich hairpin loops so that the two DNA strands form one single stranded circle upon denaturation (Esposito and Knight, 1985; Stuart, Ellison, Graham and McFadden, 1992). Hairpin loops are a characteristic feature of all vertebrate poxvirus genera examined thus far. Inverted terminal repeats (ITRs) of variable lengths are found at the two ends of the genome. The ITRs are subjected to transcription by virus-encoded RNA polymerases and are prone to deletions and rearrangements. The hypervariability of the ITRs suggests that this region contains mostly nonessential genes. However, they may have important roles *in vivo* by enabling the virus to evade the host immune response and to spread from the initial site of infection (Turner and Moyer, 1992). Poxvirus pathogenesis studies indicate that many genes involved in host range and virulence are located within the terminal regions of the genome (Turner and Moyer, 1992). The mutability of the terminal region may be the key for virus adaptation. Tandemly repeated sequences (TRS) are located close to the ends of the genome or within the ITRs. One way of comparing the relatedness of poxviruses may be to compare the repeated units at the termini of their genomes (Baroudy and Moss, 1982). An apparent absence of introns, relatively short promoter sequences and the relatively small sizes of many open reading frames (ORFs) account for a high gene density in the genome. The functional elements of promoter sequences are conserved between poxvirus genera. Naked poxvirus DNA is not infectious and is not transcribed by cellular RNA polymerase (Scheiflinger, Dorner and Falkner, 1992). Transcription occurs from both strands of the viral DNA.

1.1.4. Replication

The reproductive cycle of poxviruses consists of: (a) virus entry, (b) regulated gene expression, (c) DNA replication, (d) virion assembly and (e) virus dissemination. Genes required for the replication of orthopox viruses in specific cell types have been mapped close to the left-hand termini of their genomes

(Gillard, Spehner and Drillien, 1985; Gillard, Spehner, Drillien and Kirn, 1986). A restriction of the host-range phenotype has been correlated with a loss of DNA from these regions (Dumbell and Archard, 1980; Moyer and Rothe, 1980).

Poxvirus entry into cells occurs via plasma membrane fusion or via endophagocytosis. The extracellular form of VV appears to be more rapidly and efficiently adsorbed to cells than the intracellular form and is important for spread of infection in cultured cells and animals. Fusion of the virion with the outer membrane leads to uncoating. The virion core is subsequently released into the cytoplasm of the cell. The nucleoprotein complex is liberated through breaks in the core wall. A complete transcription system consisting of virus-encoded enzymes (including a multisubunit DNA-dependent RNA polymerase, capping and methylating enzymes, a transcription factor and a poly(A) polymerase) is present within the core (Keck *et al.*, 1990). Thus, protein or DNA synthesis is not required for the transcription of early genes (Keck *et al.*, 1990). Poxviruses and African swine fever virus are the only known eukaryotic DNA viruses that encode their own RNA polymerase. During the early stages of infection approximately 100 early genes are transcribed by the viral RNA polymerase. Messenger RNAs transcribed from early genes include DNA polymerase and other proteins involved in transcriptional activity of later genes. The primary transcripts are modified at the 5' end by virus-encoded capping and methylating enzymes and polyadenylated at the 3' end by the virus-encoded poly(A) polymerase (Gershon, Ahn, Garfield and Moss, 1991; Shuman, 1990). A shutdown of cellular protein synthesis occurs soon after infection. This is apparently due to viral protein(s) and/or the production of polyadenylated nontranslated viral RNAs that effect ribosome function. Cellular DNA and RNA synthesis is also inhibited.

Poxvirus replication occurs in the cytoplasm, a characteristic unique to poxviruses and African swine fever virus as far as DNA viruses are concerned. The discrete cytoplasmic foci of replication are called "virus factories". DNA replication, which plays an important role in the regulation of gene expression (Keck *et al.*, 1990), occurs at different stages of the reproductive cycle depending on the type of poxvirus. Although the replication mechanism is still not well understood, it is thought to occur through semi-conservative replication. The process presumably involves a strand displacement mechanism which commences at each end of the genome by the introduction of nicks (Pogo, O'Shea and Freimuth, 1981). Short DNA fragments, covalently linked to RNA primers, are formed. An alternative self-priming mechanism at the 3' ends, generated by the formation of nicks is another possibility. This hypothesis accounts for the formation of incompletely base-paired loops and flip-flop sequence rearrangements. It seems as if poxviruses do not have a specific origin sequence for plasmid replication. Concatemeric intermediates resolved into unit-length genomes are formed during DNA replication. These replicated DNA molecules provide the templates for the expression of intermediate and late genes.

Each temporal class of genes has its own characteristic promoter sequences that are recognized by specific viral proteins. Viral proteins are used predominantly or exclusively for replication and there is no nuclear requirement except perhaps for virus maturation (Traktman, 1990). The replication process can be divided into distinctive early and late stages, flanking the peak of DNA replication. Transcriptional switch-over occurs as viral DNA accumulates. About 100 proteins, which are primarily virion structural proteins, are produced in the late stage.

Virion morphogenesis also becomes visible during the late stage. Condensing viral genomes surrounded by precursors of the virion outer membrane and immature virions are formed. The origin of the viral membrane is apparently not associated with any cellular organelle. The majority of virus particles produced are intracytoplasmic naked virions (INV) which are released by cytolysis (Engelstad and Smith, 1993). The molecular mechanism by which mature enveloped poxvirions exit from the cell is poorly understood (Duncan and Smith, 1992a; Dales and Pogo, 1981). A small proportion of INV acquire a double layer of Golgi-derived membrane, containing several virus proteins. This enveloped particles migrate to the cell surface. The outer membrane subsequently fuses with the cell plasma membrane and extracellular enveloped virus (EEV) are released from the cell (Duncan and Smith, 1992a; Engelstad and Smith, 1993; Payne and Kristensson, 1979). Some of these enveloped particals remain secured to the cell surface and are called cell-associated enveloped virus (CEV; Engelstad and Smith, 1993). Nuclear factors, like RNA polymerase 11, are thought to be involved with the formation of infectious, mature virions. Since the EEV form of VV is important in the dissemination of virus, the loss of a gene required for EEV formation would cause attenuation of the virus. An example of this is the VV gene B5R that encodes a 42-kDa envelope protein required for the envelopment of extracellular virus (Engelstad and Smith, 1993). The SalL4R gene in the WR strain of VV, encoding EEV envelope proteins, is also required for normal plaque formation (Duncan and Smith, 1992a). There is no evidence of integration of viral DNA into the cellular genome.

1.1.5. Gene expression

Viral gene expression may be regulated at various levels. Different promoter and termination sequences are found for early and late genes. Transcription occurs bidirectionally and no particular direction is favoured by early or late RNAs. The regulatory sequences are A-T rich (Mackett, Smith and Moss, 1985). The leader sequences (30-100 bases) from early mRNAs of VV are different from one another and also from prokaryotic and eukaryotic mRNA leader sequences.

The considerable nucleotide variability in individual promoters may have a role in regulating the level of gene-expression. The functional elements of promoter sequences are conserved amongst poxvirus genera. The early promoter was divided into three regions based on the analysis of the VV 7.5 kDa early promoter: (a) a 16 bp critical region where single nucleotide substitutions has a major effect on expression, (b) an 11 bp T-rich sequence which is less critical and (c) a 5 to 7 bp region where initiation usually occurs with a purine (Davison and Moss, 1989a). The site of transcriptional initiation is specified by the critical region of the early promoter. This critical region serves as a binding site for a transcription factor (analogous to the eukaryotic TATA box) and is thus involved in transcriptional regulation (Davison and Moss, 1989a). Consensus TATA and AATA promoter sequences, separated by about 25 bp, were identified for several early and late VV genes.

The sequence TTTTTNT (N representing any nucleotide) together with an additional termination factor or protein are necessary for early termination of transcription. It appears as if the termination signal is recognized as an RNA sequence, UUUUUNU, instead of a DNA sequence. This sequence is apparently not recognized by the late transcription complex. Termination of early transcription also occurs within or near the tandemly repeated CTATTC sequence (Venkatesan, Baroudy and Moss, 1981). *In vitro* transcription studies suggest that only a free 3' hydroxyl end is required for polyadenylation of vaccinia early mRNAs. A specific virus-encoded early transcription factor (VETF), which is required for the initiation of transcription by the polymerase, was detected in VV (Broyles S.S., Yuen L., Schuman S. and Moss B., 1988; Hagler and Shuman, 1992). This transcription factor binds specifically to early promoter sequences within a 30-bp region upstream of transcription initiation sites and apparently leads to bending of the DNA template in the vicinity of the transcriptional start sites (Hagler and Schuman, 1992). The DNA-protein complex that is formed is presumably an intermediate in transcription initiation. VETF has a DNA-dependent ATPase activity and may be a functional homolog of the eukaryotic TATA-binding factor.

Late promoter sequences are dependent on DNA replication for activity. The role of DNA replication is to provide naked DNA templates for the transcription of intermediate and late genes (Keck *et al.*, 1990). Vaccinia virus gene expression are regulated by a cascade mechanism. The progeny DNA of replication serves as a template for the transcription of intermediate genes. The products of these genes then *trans*-activates the transcription of late genes (Keck *et al.*, 1990). Factors required for intermediate gene-expression are probably present in the infected cells prior to DNA replication, although they have not been identified (Keck *et al.*, 1990).

All or nearly all of the genome is transcribed during the late stage of replication (Keck *et al.*, 1990). Many late protein genes occur in clusters in the central region of the genome. Early and late genes reside close to the termini and are overlapping. Transcripts of these genes are known to be 5' capped. Late RNAs have the ability to self-anneal or anneal with early RNA to form ribonuclease-resistant hybrids. The stability of mRNA may also influence gene expression. Late mRNAs do not have defined 3' ends. This often results in size variation amongst individual mRNA species encoding a particular protein.

Sequences required for the expression of late genes are located within 100 bp of the mRNA start site (Bertholet, Drillien and Wittek, 1985). The leader sequences of most late genes are remarkably short (Rosel, Earl, Weir and Moss, 1986). A highly conserved TAAATG sequence is found at the beginning of each ORF of several late genes. Transcription is initiated within the A-triplet and translation commences from the ATG codon in the motif (Rosel *et al.*, 1986; Roseman and Hruby, 1987).

Late mRNAs have a long (25-100 base) poly(A) tract at their 5' ends which is a unique feature of poxviruses (Davison and Moss, 1989b). The poly(A) leader is important for RNA stability during the late stage after infection. The function of the capped 5' poly(A) leader may be to provide a binding site for initiation factors and the 40S ribosomal subunit. This subunit can then move unimpeded by antisense RNA to the first AUG codon where ribosome assembly and translation occur (Davison and Moss, 1989b).

A gradual decrease in the expression of a reporter gene was observed as 5' to 3' deletions were made in late promoters. Late promoters can be divided into three regions: a more or less 20 bp A-T rich upstream sequence and a highly conserved TAAAT sequence within which transcription initiates, separated by a region of about 6 bp (Davison and Moss, 1989b). The TAAAT sequence is required for promoter function and for poly(A) leader formation. Reductions in promoter strength was observed with the addition of an extra A or single nucleotide substitutions within the three A residues of the TAAAT sequence (Davison and Moss, 1989b).

It appears as if similar RNA polymerases are used for early and late transcription, or that they at least share subunits. Transcription by poxvirus RNA polymerase is controlled by cis-acting elements. Gene regulation may also occur at the translational level by suppressor proteins.

1.1.6. Immune response to poxvirus infection and vaccination

Poxviruses are transmitted by contact and by the respiratory route. Some can be transmitted by arthropods. Infection (also after vaccination) is initiated in epithelial cells at the site of virus entry. The progression of the infection is influenced by several factors like the virulence and biological characteristics of the virus and the host response. The nature of the VV proteins involved in the activation of the immune respons of the host is not yet known. Demkowicz, Maa and Esteban (1992) identified four highly antigenic VV proteins in the virus core and two in the virus envelope. According to Czerny and Mahnel (1990) orthopoxviruses can potentially stimulate the specific immune system (T and B cell-mediated) and non-specific defence mechanisms (e.g. macrophage-, interferon-, interleukin- and natural killer cell-mediated). Poxviruses have developed ways of impeding the host immune mechanisms. VV is relatively resistant to interferon in several cell-lines. This is propably due to the production of a VV factor early in infection which prevents interferon from inhibiting protein synthesis. VV can also rescue more sensitive viruses, like vesicular stomatitis virus, from the effects of interferon.

Protection and recovery from poxvirus infections are dependent on the cell-mediated immune response to viral proteins, but little is known about this response to poxviruses (Blanden, 1970; Gardner and Blanden, 1976; Kempe, 1960; Littaua, Takeda, Cruz and Ennis, 1992). The cell-mediated immune response occurs via soluble mediators, produced by T cells and specifically activated macrophages, that affect virus, infected cells and the local environment of the infection site. The B-cell response generates non-neutralizing and neutralizing antibodies in pathogenic poxvirus infections and in vaccinated individuals respectively. It appears as if specific neutralizing antibodies are not required to recover from poxvirus infections (Kempe, 1960; O'Connell, Karzon, Barron, Plaut and Ali, 1964). Specific immunity, which is T and B cell-mediated (Czerny and Mahnel, 1990) is the principal mechanism for developing resistance against poxviruses. The long-term nature of the resistance to smallpox or VV after vaccination may be due to the presence of a large number of cross-reactive memory cytotoxic T-lymphocytes which would become activated and rapidly eliminate infected cells after challenge (Littaua *et al.* 1992). It is very likely that vectored poxviruses will behave generally like their parents.

1.2. The use of poxviruses as vectors for live recombinant viral vaccines

1.2.1. Historical overview and general perspective of live viral vaccines

As early as the tenth century live-virus immunization or variolation was practiced in China and India, when previously uninfected people were inoculated with pustular fluid from a smallpox-patient. Natural smallpox infection had a mortality rate of 25%, whereas variolation caused infection of varying severity and 1-2% mortality. Edward Jenner successfully vaccinated an 8-year old boy with pustular fluid from a milkmaid's pox lesion in 1796, after he observed that people who had been previously infected with the pox from the teats of cows, failed to show characteristic symptoms following variolation. Louis Pasteur proposed the term "vaccination" instead of "inoculation" in 1881. The inoculum later became known as VV, but after it's long history through man and animals the precise derivation of VV as it is known today is unknown. VV was shown to be distinct but closely related to cowpox virus, variola virus and other naturally occurring members of the poxvirus family on the basis of DNA analysis.

The use of live-virus vaccines is recommended in cases where there is systemic invasion of the host, only a few antigenic types of the etiologic agents are present and a highly effective immunity follows a natural infection (Kucera and Myrvik, 1985). Active humoral and local cellular immunity can be induced with live virus (Czerny and Mahnel, 1990). This activation of all phases of the immune system is an important characteristic of live virus vaccines, and thus also of live recombinant viral vaccines. The result is a balanced response, systemic and local immunity, humoral and cell-mediated immunity (Murphy and Chanock, 1990). This is particularly important for infections in which cell-mediated immunity plays a role and for mucosal infections in which both local and systemic immunity are required for optimal resistance (Murphy and Chanock, 1990). An immune response is stimulated to each of the protective antigens of a virus by a live virus vaccine, whereas destruction of some of the antigens may occur during the preparation of an inactivated vaccine (Murphy and Chanock, 1990). Immunity induced by live virus vaccines is generally more durable, more effective, and more cross-reactive (due to the fact that all phases of the immune system is stimulated and that T-cells display a broader cross-recognition of related viral strains than B-cells) than that induced by inactivated vaccines (Murphy and Chanock, 1990). The latter phenomenon is particularly important for viruses such as influenza that undergo progressive antigenic variation. Even when immunity levels have declined with time, natural subclinical re-infections can serve as harmless boosters.

Through the years live viral vaccines against several diseases proved to be effective, reasonably safe and cost-effective (Murphy and Chanock, 1990). Notwithstanding its proven track record, there has been some serious problems with some live viral vaccines. These problems include the back-mutation to virulence of, for example, poliovirus vaccine; the genetic instability of 17D yellow fever virus vaccine; inadequate attenuation leading to complications or overattenuation leading to a lack of protection in the case of smallpox vaccine; the need for storage and transport of measles vaccine at low temperature and thus a short shelve life (Murphy and Chanock, 1990). The presence of defective interfering particles can modify the clinical response to the vaccine and can result in a variation in immunogenicity and level of attenuation among successive lots of vaccine (Murphy and Chanock, 1990). If vaccine progeny virus should spread from a vaccinated individual to unvaccinated individuals it may appear to be an advantage, but this progeny virus might be mutated and cannot be tested for safety and licensed for use in the general population (Melnick, 1990). This prompted research to develop more stable recombinant vaccines which are generally regarded as being safer, less costly to produce, easy to administer and have an extended shelf-life (Kucera and Myrvik, 1985).

1.2.2. General concepts regarding vaccines derived from virus vectors

The concept for the construction of a live, recombinant viral vaccine is based upon the exploitation of a non-pathogenic virus vector to express one or more antigens from a pathogen or pathogens. The immunogenic proteins are thus presented to the host immune system in an authentic way and cause the body to mount a lasting immune response to it. The aim is to genetically manipulate a virus vector to such a degree that it will only contain the genetic material for replication and lack those parts of the genome responsible for pathogenesis.

1.2.3. Poxviruses and their potential for use as vectors in live recombinant vaccines with special reference to VV

A recombinant poxvirus vaccine would offer all the advantages of a live pox vaccine and more (section 1.2.1.). Poxviruses have many favourable characteristics that render them useful as vectors for the expression of genes encoding foreign antigens to obtain vaccines. Apart from the use of VV vaccine against smallpox in humans, poxvirus vaccines have also been used in veterinary medicine for the protection of animals and birds. An example of this is the use of VV for the prevention of rabbitpox and other orthopoxvirus infections of captive and domestic animals.

It has been shown that poxvirus DNA is subject to extensive genetic recombination during its replicative cycle (Ball, 1987; Spyropoulos, Roberts, Panicali and Cohen, 1988). Analysis of the viral products of recombination has shown that both single and double crossover events occur with high frequency and the unstable tandem repeat products of single crossover events are rapidly processed via second crossover events into more stable structures (Ball, 1987; Spyropoulos *et al.*, 1988). Efficient recombination has been observed between coinfecting viral genomes as well as between viral genomic DNA and homologous sequences in transfected plasmid DNA (Fenner and Comben, 1959; Nakano, Panicali and Paoletti, 1982). In 1981 Sam and Dumbell demonstrated the occurrence of marker rescue of a fragment of VV DNA by homologous DNA recombination in cells infected with ectromelia virus or thermosensitive mutants of VV, and transfected with intact VV DNA fragments (marker$^+$ DNA). They obtained ectromelia/vaccinia and recombinant viruses. This meant that VV genes could be mapped by marker rescue and that foreign genes could be inserted into the viral genome by homologous packaging.

The aforementioned facts render poxviruses suitable as vectors for the introduction of foreign genes into mammalian cells. The insertion of the herpes simplex virus thymidine kinase gene into VV stimulated tremendous interest in inserting foreign genes into VV (Mackett, Smith, and Moss, 1982; Panicali and Paoletti, 1982).

Naked viral DNA is noninfectious when transfected into permissive cells. Therefore recombinant poxviruses were hitherto constructed by *in vivo* homologous recombination. The recombination occurred following the transfection of virus-infected cells with plasmids that shared homologous sequences with the resident virus. However, Scheiflinger *et al.* (1992) demonstrated heterologous packaging of modified orthopox (VV) genomic DNA into avipox (fowlpox) helper virus-infected avian cells. (Foreign DNA is inserted into the viral genomic DNA by direct cloning before packaging). Viable fowlpox virus is not formed in mammalian cells. Although infection can occur, it is not followed by complete replication. This provides a selection step for packaged orthopoxvirus. Heterologous packaging could be a useful technique for the construction of recombinant poxviral vaccines provided unsheared viral DNA can be purified, and that restriction sites with a single recognition sequence in a non-essential location in the genome can be identified.

Another advantage of poxviruses as a live recombinant vaccine vector, are their large genome sizes which allows the insertion of relatively large fragments of DNA. Several foreign viral genes can be inserted and expressed in the large genome of VV without significantly altering the virus's ability to replicate (Moss, 1993; Murphy and Chanock, 1990). Several non-essential loci have been identified in the vaccinia genome, presenting more that one target site for gene insertion. Viable deletion mutants of VV can also be generated from these non-essential loci, extending the ability of vaccinia to hold considerable quantities of foreign genetic material (Smith and Moss, 1983). Foreign genes can be inserted anywhere within these areas where DNA that are not essential for viral replication has been deleted. This allows the simultaneous expression of multiple foreign genes, which facilitates the construction of polyvalent vaccines (for immunization against multiple pathogens). Polyvalent recombinant vaccines can include the coordinated expression of a family of related gene products or the production of a variety of biologically active molecules from a single vaccinia infection. It is also possible to construct infectious vectors that contain genes from more than one virus, thus providing several immunizations simultaneously (Hollinger, 1990). An infectious VV recombinant, expressing multiple foreign antigens, has already been constructed. The genes encoding the influenza virus hemagglutinin, the hepatitis B virus surface antigen and the herpes virus type-1 glycoprotein D were inserted into a VV vector, yielding a triple recombinant (Perkus, Piccini, Lipinskas and Paoletti, 1985).

VV strains with a more limited host range have been produced after three host range genes were identified and deleted (Duncan and Smith, 1992b). A more limited host range is desirable to prevent inadvertent interspecies transmission of the recombinant virus. Virus attenuation can also be achieved by the identification and deletion of genes encoding glycoproteins that affect virus spread (Duncan and Smith, 1992b; Engelstad and Smith, 1993; Lee, Roos, McGuigan, Smith, Cormier, Cohen, Roberts and Payne, 1992). At high immunization doses most mutants could induce an immune response similar to that of the wild-type virus (Lee *et al.*, 1992). Two novel, highly attenuated poxvirus vectors, NYVAC and ALVAG, have been developed (Tartaglia, Cox, Taylor, Perkus, Riviere, Meignier and Paoletti, 1992). Strong immunological responses against extrinsic immunogens have been obtained with these vectors (Tartaglia *et al.*, 1992). NYVAC was derived from the COPENHAGEN VV strain by the deletion of genes implied in virus virulence. ALVAG, a canarypoxvirus-based vector system, is replication-restricted in mammalian cells. Thus, genetically engineered, stable, attenuated VV strains can be produced to serve as vectors for the construction of live recombinant vaccines.

The defined, identifiable attenuating mutations or gene constellations in these genetically engineered vaccine viruses can be monitored during the development, manufacture and utilization in humans or animals (Murphy and Chanock, 1990; Moss, 1993).

An advantage of VV as a vector for live recombinant viral vaccines is that it is not oncogenic and does not cause latent infections (Hollinger, 1990). VV vaccine is stable and induces a relatively long-lived immunity. The host range of vaccinia and other poxviruses make them suitable for veterinary and human vaccines.

Theoretically any poxvirus can potentially be used as a vector. The choice of virus may for instance depend on the host-range requirements. An example of this is the case where a raccoon poxvirus has been used as a vector for the rabies virus glycoprotein gene. This construct was developed as an oral, bait-delivered vaccine and used successfully to vaccinate raccoons against rabies (Esposito, Knight, Shaddock, Novembre and Bauer, 1988). An oral bait form of a thymidine kinase-negative VV vector, expressing the glycoprotein gene of rabies virus, has also been found to be effective for wildlife including foxes and raccoons (Blancou, Kieny, Lathe, Lecocq, Pastovet, Soulebot and Desmettre, 1986; Brochier, Kieny, Costy, Coppens, Bauduin, Lecocq, Languet, Chappuis, Desmettre, Afiademanyo, Libois and Pastoret, 1991).

Dogs developed virus-neutralizing antibody titers when vaccinated intradermally with VV recombinants expressing the rabies virus glycoprotein (Fekadu, Sumner, Shaddock, Sanderlin and Baer, 1992).

1.2.4. Disadvantages and potential hazards of live recombinant poxviral vaccines

As mentioned before (Section 1.2.1.) the motivation for making more stable recombinant vaccines is problems experienced with some of the currently available live viral vaccines. Some of the disadvantages of live viral vaccines, and also of live recombinant poxviral vaccines, is that there is always the potential of contamination with live adventitious agents, although this has rarely been a problem. Contaminating DNA in live recombinant viral vaccines has a potential tumour risk, because its integration into the cell genome can cause the inactivation of suppressor genes or the activation of proto-oncogenes (Temin, 1990; Hofschneider, 1990). An amount of 100 pg heterogeneous DNA per single dose of vaccine are accepted as tolerable (Hofschneider, 1990). It can also be assumed that injected DNA is degraded quickly and inactivated biologically *in vivo* (Hofschneider, 1990). Contamination of vaccines with transforming proteins, like growth factors, is not a long-term risk as their effect is transient and their biological half-life is short (Hofschneider, 1990).

Back-mutations and intertypic recombination between vaccine strains may lead to restoration of a varying degree of virulence (Murphy and Chanock, 1990). If vaccine progeny virus should spread from a vaccinated individual to unvaccinated individuals it may appear to be an advantage, but this progeny virus might be mutated and cannot be tested for safety and licensed for use in the general population (Melnick, 1990). The majority of buffalopox virus (BPV) outbreaks, a sporadic cause of morbidity among cattle and humans in India, may have originated from the widespread use of the smallpox vaccine before 1977 (Dumbell and Richardson, 1993). Buffalocalves were used for smallpox vaccine production in India and the virus presumably adapted to survive in partially immune or immune hosts (Dumbell and Richardson, 1993). It was also suggested that VV strains, used as smallpox vaccine in Africa, may have been maintained for at least a short period in nature by transmission (Carra and Dumbell, 1987). This virus may have resulted in the occurrence of sporadic human orthopoxvirus-infections (Carra and Dumbell, 1987).

Genetic recombination between poxvirus genomes has occurred in nature in the absence of laboratory selection or screening (Gershon, Kitching, Hammond and Black, 1989). A possible hazard could thus arise from the use of vaccines based on poxviruses. If genetic recombination should occur between a recombinant poxvirus vaccine and a naturally occuring poxvirus a novel virus with altered pathogenicity might be generated (Gershon *et al.*, 1989). This risk would be even greater if the new virus was highly transmissible with a broad host range. These hazards cast a shadow over the use of VV (an orthopoxvirus) as a vector for live recombinant vaccines (Massung and Moyer, 1991). Orthopoxviruses, with special reference to VV, are well known for their broad host range and it may be even wider than is presently realized. There is also strong evidence that orthopoxviruses can be transmitted from animals to humans (Dumbell and Richardson, 1993; Duncan and Smith, 1992b; Gershon *et al.*, 1989). Field trials with baits for the oral distribution of vaccines based on VV should hopefully cast some light on this problem.

Naturally occurring wild-type viruses may interfere with infection by a live virus vaccine (Murphy and Chanock, 1990). Existing immunity within the target population to a potential vector, or to any of the expressed antigens, may restrict replication of the vector and reduce the overall immune response to the vaccine (Littaua *et al.*, 1992; Old and Primrose, 1989). For example, VV vectors might not be effective in people who had a smallpox vaccination. To avoid this problem of immunity to the vector in previously unvaccinated people, different VV-based vaccines will have to be administered simultaneously, or a polyvalent virus should be constructed.

However, Yamanouchi, Inui, Sugimoto, Asano, Nishimaki, Kitching, Takamatsu and Barrett (1993) reported the vaccination of cattle (that were inoculated with a VV strain three weeks earlier) with a recombinant vaccine, constructed by the insertion of the rinderpest virus haemagglutinin gene into an attenuated VV strain. Upon challenge with virulent rinderpest virus the cattle appeared to be protected. Antibodies to rinderpest virus developed in the cattle. This showed that the vaccine was effective in animals with pre-existing immunity to VV. An accidental human vaccination with a recombinant VV has been reported by Jones, Ristow, Yilma and Moss in 1986. A researcher accidentally inoculated a small cut on his finger with a recombinant VV expressing the vesicular stomatitis virus (VSV) nucleoprotein. Although a non-vaccine strain of VV was involved, the researcher only experienced mild symptoms. This could be due to the fact that he had been vaccinated about 30 years before or to attenuation of the virus by the insertional inactivation of the tk gene. A high antibody titre against the VSV nucleoprotein could be detected in the serum from the researcher. This showed that a new immune response was elicited against the foreign VSV nucleoprotein in man, despite his previous vaccination (Old and Primrose, 1989). A recombinant vaccine may thus be effective in humans with pre-existing immunity to the viral vector employed.

Live virus vaccines, and thus also live recombinant viral vaccines are contraindicated in people with immunodeficiency diseases or undergoing immunosuppressive therapy and in their household associates, because vaccination in these cases can result in systemic infections (Dorozynski and Anderson, 1991; Redfield, Wright, James, Jones, Brown and Burke, 1987). An example of this is the case of a HIV-seropositive U.S. military recruit who developed disseminated vaccinia after routine vaccination (Redfield *et al.*, 1987).

Studies indicated that a factor(s) present in VV infected cells is capable of activating gene-expression under the direction of the long terminal repeat (LTR) of human immunodeficiency virus type 1 (HIV-1) (Stellrecht, Sperber and Pogo, 1992). The mechanism by which VV activates the HIV LTR is still unknown. It is therefore essential to identify genes in the VV genome that are responsible for virulence and HIV-1 activation.

Because of all the disadvantages related to the use of VV as vector other poxviruses, like for instance capripoxviruses and avian poxviruses, are considered for use as vectors mainly for safety reasons (see Section 1.3.9. for a discussion of the advantages of using capripoxviruses as vectors). Avian poxviruses share the advantages of other poxvirus vectors but are incapable of complete replication in mammalian cells (Cadoz *et al.*, 1992). However, early gene products are presented to the immune system (Cadoz *et al.*, 1992). A rabies glycoprotein G gene was inserted into a canarypox virus and was tested for efficacy and safety in animals and humans. This vaccine was found to be safe and well tolerated (Cadoz *et al.*, 1992). In VV recombinants pre-existing immunisation against smallpox can block the immune response. No blocking effect on responses to booster vaccination with the canarypox vaccine could be observed by pre-existing immunity to either the canarypox-virus vector or the rabies glycoprotein (Cadoz *et al.*, 1992). This proved that non-replicating poxvirus vectors can be used for the construction of live recombinant vaccines for use in humans. Non-replicating poxvirus vectors have the advantages that disseminated infection within the vaccinee or spread to contacts or the general environment should be eliminated. These vectors can also be used in immuno-compromised animals or humans. Capripoxviruses may also have potential for use as non-replicating vectors.

1.3. Capripoxviruses and their potential as vectors for live recombinant vaccines with special reference to lumpy skin disease virus (LSDV).

The general characteristics of lumpy skin disease virus (LSDV) have been reviewed extensively by Davies (1991). A summary of the aforementioned article, together with other sources of information (as cited) follows hereafter.

1.3.1. Classification.

The genus capripoxvirus consists of the species lumpy skin disease (Neethling) of cattle, sheep pox and goat pox (Gershon and Black, 1987). Based on the restriction enzyme analysis of the genomes of 26 capripoxvirus isolates, they have been divided into four types: (1) isolates only from sheep, (2) isolates only from goats, (3) isolates restricted to Africa from sheep, goats and cattle, and (4) isolates from either sheep or goats in Africa or the Middle East (Gershon *et al.*, 1989). It has been suggested that the type 4 isolates arose by genetic recombination between type 2 and type 3 isolates (Gershon *et al.*, 1989). Very little is known about the molecular biology of capripoxvirus genomes (Gershon and Black, 1987).

1.3.2. Clinical signs and diagnostic features of LSD.

LSD often occurs in epizootic form and is characterized by the eruption of nodules (5 - 50 mm or more in diameter) in the skin which may cover the whole of the animal's body. Lesions develop, which become necrotic and cause permanent damage to the hides. Infection results in spread to and lesions within internal organs (Massung and Moyer, 1991). The necrotic lesions furnish ideal oviposition sites for flies which may further the prosperity of other pests or diseases. The lesions are often also found in the mouth and respiratory tract. Systemic effects and complications include fever, pneumonia, abortion, infertility, anorexia, dysgalactia and disinclination to move. Histological characteristics of lesions of LSD as well as sheep and goat pox are oedema, hyperplasia of the epidermis and infiltration with an epithelioid cell. The important diagnostic features are thrombosis of the blood vessels in the dermis and subcutis, the cellular infiltration and typical intracytoplasmic pox inclusion bodies in cells associated with the nodules. Brick shaped poxvirus particles can be seen in tissue fragments from skin biopsies by electron microscopy, which is the most rapid way to demonstrate virus in LSD (Davies, Krauss, Lund and Taylor, 1971). Contamination of the material with herpesviruses is a recurring problem when identification of the virus particles by electron microscopy or isolation of the virus in tissue culture are attempted. Cattle recovering from the disease may remain in a very poor condition for 4-6 months.

1.3.3. Transmission and host range of LSD.

The precise mode of transmission of LSD is not known, but it has been suggested that arthropod vectors like mosquitoes, tabanids, *Culicoides* and *Glossina* species may be involved by mechanical transmitting the disease (Kitching and Mellor, 1986). Transmission by contact with infected cattle does not readily occur in insect-proofed cattle pens or under natural conditions (Kitching, 1983; Kitching and Mellor, 1986). LSD has a fairly narrow host range. Cattle of all ages are susceptible to LSD, but the severity of the disease varies between breeds and strains of cattle. Serum antibody to capripoxviruses have been found in African buffalo where LSD had been prevalent, but no antibody could be identified specifically to LSD. Natural infections have not been observed in any other ruminant species during outbreaks of LSD, except for five cases in the Asian water buffalo in Egypt. The development of LSD-like skin lesions could be produced by experimental infections in impala, Thomson's gazelle and the giraffe. Rodents and lagomorphs are refractory to experimental laboratory infection while camels are insusceptible.

1.3.4. Areas and ecotypes affected by LSD.

LSD is mainly confined to sub-Saharan Africa (including Madagascar). LSD was first identified in Zambia in 1929. Capripoxvirus isolates are usually designated by their country and species of origin, for e.g. "Kenya cattle-1" (KC-1) was isolated from infected cattle during an outbreak of LSD in Kenya in 1958 (Black and Gershon, 1987). The spread of the disease has been related to the movement of insect-vectors or the movement of cattle from areas where there have been recent outbreaks of LSD. Shortly after the importation of cattle from Somalia, where LSD had occurred, the disease appeared in Egypt in 1988 where it is now enzootic. An extension of the disease has occurred towards Israel in 1989 which was probably due to the aerial movement of infected insect vectors. Enzootic situations exist in sub-Saharan African countries in which LSD has appeared with periodic cycles of sporadic and epizootic virus activity. LSD has the ability to spread rapidly in a wide range of ecotypes anywhere and the potential exists that it may spread to North Africa, the Middle East and northern Mediterranean countries. It has already occurred in high altitude temperate grasslands, wet and dry bushed and wooded savannah, semi-desert and irrigated areas.

1.3.5. Genomic relationship between capripoxviruses.

Serological (direct and indirect fluorescent antibody and serum neutralization tests) and restriction enzyme studies failed to indicate any differences between strains of LSDV and sheep and goat pox viruses (Black *et al.*, 1986; Davies and Otema, 1981). Schumperli *et al.* (1977) calculated that if the restriction patterns of two DNAs digested with a six nucleotide recognizing restriction enzyme, contain comigrating fragments, a genomic homology of greater than 80% can be inferred (Black *et al.*, 1986). The DNAs of capripoxvirus field isolates and vaccine samples were analysed by restriction enzyme digestion with *Hind111* (a six nucleotide cutter) and agarose gel electrophoresis. This showed that the capripoxviruses are closely related and, based on the calculations of Schumperli *et al.* (1977), it can be concluded that the genome homology must be more than 80% (Black *et al.*, 1986). This conclusion was supported by the high level of sequence homology between capripoxvirus isolates detected by Southern Cross hybridization (Black *et al.*, 1986). Gershon and Black (1988) showed that even the most divergent genomes are 95.8-97.0% homologous at the nucleotide sequence level. However, a correlation between restriction profiles and the virus host type was evident, which appeared to form a sheep pox group and a goat pox group. The molecular weights of the genomes estimated for different isolates also varied from 73 to 91 MDa despite the high sequence homology (Black *et al.*, 1986). Of the capripoxvirus field isolates and vaccine strains analysed by restriction enzyme analysis, LSD and the Kenya sheep and goat pox isolate were found to be the closest related to one another (Black *et al.* 1986). Serological studies by virus-serum neutralization and immunofluorescence also did not show any differences between LSDV and Kenya sheep and goat poxvirus (Davies and Otema, 1981). It was suggested that LSD had arisen following a change in the host affinities of the Kenya sheep and goat poxvirus (Kitching, Hammond and Black, 1986a). LSDV strains also appear to be identical according to serological and restriction enzyme studies (Kitching *et al.*, 1986a). It is suspected that the sheep and cattle isolates diverged from the goat isolate prior to diverging form one another (Gershon and Black, 1988). A close epidemiological relationship was also observed between the capripoxviruses (Kitching *et al.*, 1986a; Kitching and Mellor, 1986). A major common precipitating antigen with a molecular weight of 67K was detected in four isolates of capripoxvirus (sheep pox, goat pox, sheep and goat pox and LSDV) and are probably shared by all isolates of capripoxvirus (Kitching *et al.*, 1986a). Preliminary work using detergent-treated purified virus preparations showed that this antigen was located on the outer membrane of the virus particle (Kitching *et al.*, 1986a). This casted doubt on the justification for the continued classification of the capripoxviruses into distinct types (Kitching *et al.*, 1986a). The sheep and goat pox viruses from the Middle East and elsewhere, however, display some differences from LSDV (Black *et al.*, 1986; Davies and Otema, 1981). In South Africa sheep pox has not been observed despite large numbers of sheep and outbreaks of LSD in cattle, suggesting a different host range (Dr. A-L Williamson, Department of Medical Microbiology, Medical School, UCT - personal communication).

1.3.6. Genomic relationship between capripoxviruses and orthopoxviruses.

The capripoxviruses and the orthopoxviruses appear to be closely related on grounds of their morphological and physical properties (Davies and Otema, 1981). However, no apparent relationship was evident between the *Hind111* restriction pattern of VV DNA (representative of the orthopoxviruses) and those of capripoxvirus DNA (Black *et al.*, 1986). It is thus possible to identify a capripoxvirus on the basis of its *Hind111* DNA restriction profile (Black *et al.*, 1986). Although the capripoxviruses have a smaller genome size than the orthopoxviruses, their *Hind111* DNA restriction profiles exhibit more fragments (Black *et al.*, 1986). This suggests that the capripox virus genome sequences are highly divergent from orthopox viral genomes (Black *et al.*, 1986). The size of the vaccine strain of LSDV was found to be 7 kb bigger than the size stated by Black *et al.* in 1986 (L. Perlman, Department of Medical Microbiology, Medical School, UCT - personal communication). Restriction analysis is a useful method for the identification and classification of poxvirus isolates (Muller, Wittek, Schaffner, Schumperli, Menna and Wyler, 1977). The DNA of capripoxvirus contains covalent terminal cross links. This was indicated by the presence of two rapidly re-annealing fragments in the *Hind111* digest upon denaturation (Black *et al.*, 1986).

1.3.7. Vaccines and immunity to LSD.

A Kenyan sheep and goat pox virus (KSGPV) strain and the South African Neethling strain of LSDV have been used successfully to protect cattle against LSD (Kitching, Hammond and Taylor, 1986b). Two further strains of vaccine virus have been used more recently. A Romanian sheep pox vaccine strain produced in sheep was used successfully to immunize cattle in Egypt in 1989. Sheep and goat pox virus (SGPV) strains appear to be similar to LSD and a cross-protection capacity has been observed between SGPV and LSD.

The KSGPV strain was used as a freeze dried live virus vaccine against SGPV and LSD after it was cultured in lamb testis or fetal muscle cells for 16 passages. This vaccine is absolutely safe in *Bos indicus* cattle, but on occasion mild LSD skin lesions and regional lymph node enlargements have been observed in approximately 0.02% of *Bos taurus* cattle. This complication could not be reproduced under experimental conditions and it may be due to needle transmission of virulent LSD in the field.

The South African Neethling strain of LSD proved to be a satisfactory immunogenic modified live virus vaccine, after it was passaged 60 times in lamb kidney cells and then 20 times in embryonated eggs. This vaccine is unpopular with some cattle owners, because in up to 50% of cattle it causes a granulomatous lump at the inoculation-site.

Good protection is normally obtained with an immunizing dose of approximately 10^2 tissue culture infective dose 50s ($TCID_{50}$) in the field, but $10^{3.5}$ $TCID_{50}$ is used for both vaccines. The KSGPV vaccine apparently induces immunity for at least 2 years and the South African vaccine for 3 years. It is possible that life-long immunity is induced as in the case of other poxvirus vaccines. Calves born to cows which have recovered from the disease, have a passive immunity for up to 6 months. Serological methods (indirect fluorescent antibody and virus serum neutralization tests) and/or an hypersensitivity test (developed at Kabete) can be applied to assess immunity.

No correlation could be detected between neutralizing antibody and the immune status of animals. It was suggested that a hypersensitive reaction occurs and that cell-mediated immunity contributes significantly to the development of full immunity against capripoxviruses (Kitching, 1983). Attempts to replace live capripox vaccines with inactivated (or dead) preparations have been unsuccessful. The shortage on information on the immunity to pox virus infections makes it difficult to tell whether it will be possible to use inactivated (or dead) vaccine preparations in future (Kitching *et al.*, 1986b).

1.3.8. Control and eradication of LSD.

The OIE classified LSD as a group A infectious disease and it ranks with foot and mouth disease as an economically important disease of cattle. Poxviruses are resistant viruses and may remain viable in tissue or scab for a long time. Infected animals and in-contact animals should be slaughtered and the carcases burned or buried immediately after the disease has been identified for the first time in a country. It appears as if LSD has been successfully eradicated in a country for the first time when the Israeli authorities reacted rapidly in the diagnosis of the disease and the slaughtering of the diseased and in-contact cattle. No antibiotic or chemotherapeutic agent has any effect upon the course of the disease. Control regulations for an area at risk include the restriction of animal movement, vaccination repeated after 4-6 months and close surveillance of the cattle for at least 6 months. Annual vaccinations should be maintained for 2-3 years. It is unsafe to import animals from areas where LSD occurred within the previous 3 years.

1.3.9. Advantages and disadvantages of capripoxviruses as vectors for live recombinant vaccines.

Most of the general advantages and disadvantages of using poxviruses as vectors for live recombinant vaccines (refer to Sections 1.2.3. and 1.2.4.) can also be applied to LSDV, except for, or in addition to the following:

A vaccine strain of LSDV has been used successfully to protect cattle against LSDV and it should thus be safe to use this strain as a vector for the construction of live recombinant vaccines. This vaccine is easy to use and stable in the freeze-dried form. The narrow host range of LSDV and the fact that it is not easily transmitted by contact, opposed to VV, is another big advantage since the spread of the virus to unvaccinated animals, animals of different species or humans should pose a diminished risk. It was found that the capripoxvirus vaccines was not transmitted horizontally from vaccinated to unvaccinated animals, or vertically during pregnancy (Kitching *et al.*, 1986b). A potential problem for the use of LSDV is the potential for damage to the host brain, spleen, liver and other internal organs, since infection of susceptible hosts results in spread of the virus to internal organs (Massung and Moyer, 1991). However, this is not a problem with the vaccine strain of LSDV. Recently, a recombinant capripoxvirus expressing rift valley fever virus (RVFV) glycoproteins has been obtained (J.C. DeMartini - personal communication). A single capripoxvirus recombinant vaccine for the protection of cattle against rinderpest and lumpy skin disease has also been reported (Romero *et al.*, 1993). A big disadvantage is that to date very little is known about the molecular biology of the capripoxviruses, including LSDV.

1.4. Project motivation

Due to the problems encountered with live viral vaccines and the disadvantages associated with live VV-based recombinant vaccines (as discussed in Sections 1.2.1. and 1.2.4.), it is desirable to develop LSDV as a vaccine vector. Thymidine kinase selection cannot be used for this virus, because there are no TK^- negative cell-lines available in which LSDV can be propagated. Two other methods have been used with VV for the selection of recombinant virus. Recombinants expressing β-galactosidase (β-gal) produce blue plaques; alternatively, recombinants expressing the guanine phosphoribosyl transferase (*Gpt*) gene are capable of overcoming plaque inhibition by mycophenolic acid.

It will be useful to adapt each of these approaches for use in LSDV. The first step was to construct two plasmids, in which coding sequences for either β-gal or *Gpt* were placed under the control of a promoter derived from VV. This construct was flanked by non-essential LSDV sequences from the TK-gene of LSDV and the whole incorporated into an appropriate plasmid.

The next step was to set up transfection experiments to allow ***in vivo*** recombination between LSDV and the plasmid constructs. The products of these transfections were screened for blue plaques or for growth in the presence of mycophenolic acid. Once these experiments were completed, attempts were made to make a LSDV recombinant expressing the viral protein 7 (VP7)-gene of bluetongue virus (BTV). In parallel it was also attempted to make a VV recombinant expressing the VP7-gene of BTV to test the credibility of the construct(s) made and of the methods used.

CHAPTER 2 - METHODS

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CHAPTER 2 - METHODS

2.1. Nucleic Acid Methods

2.1.1. Preparation of plasmid DNA

2.1.1.1. Small scale extraction of plasmid DNA

2.1.1.1.1. LiCl-Boiling method

The LiCl-boiling method for the small scale extraction of plasmid DNA was adapted from the method of Wilimzig (1985).

An overnight culture of *Escherichia coli (E. coli)* cells harbouring the plasmid to be isolated were harvested by centrifugation for three minutes at 11 600 g in a Beckman Microfuge E^{TM} (Beckman, Irvine, USA). The cells were suspended in 100ul TELT-buffer (50 mM Tris-HCl, 62.5 mM EDTA, 0.4% Triton X-100, 2.5 M LiCl, pH 7.5) in which lysozyme was freshly dissolved to a concentration of 1mg/ml. The tube was left at room temperature for 15 minutes so that lysis of the cells could occur. After incubation in a waterbath at 80°C for 2 minutes the tube was immediately put on ice for 5 minutes. Following centrifugation at 11 600 g for 15 minutes, the DNA in the supernatant was transferred to a fresh tube and precipitated by the addition of 3 volumes (1 volume = original volume of supernatant obtained) water and 2.5 volumes (1 volume = volume obtained after the addition of water to the supernatant) absolute ethanol. After chilling on dry ice for 15 minutes the DNA solution was centrifuged for 15 minutes at 11 600 g. The DNA-pellet was washed with 70% ethanol by spinning the tube in the opposite orientation such that the pellet migrated through the 70% ethanol. The supernatant was discarded. The pellet was then dried in a Speedivac drier (Eppendorf, Germany) for 10 to 15 minutes and dissolved in 60 µl of sterile ultra pure water (BDH Laboratory Supplies, England).

To obtain DNA preparations that were free of contaminating RNA, DNAase-free pancreatic RNAase was added to the dissolved DNA to a final concentration of 10 ug/ml. The mixture was incubated for 1 hour at room temperature. After phenol/chloroform extractions to remove the RNAase and protein-contaminants, the DNA was re-precipitated, washed with 70% ethanol and dissolved in sterile ultra pure water.

2.1.1.1.2. Alkaline DNA extraction method

The alkaline DNA extraction method for the small scale preparation of plasmid DNA was adapted from the method of Crouse, Frischauf and Lehrach (1983).

An overnight *E. coli*-culture (1.5 ml), harbouring the plasmid to be isolated, was centrifuged in a microfuge tube at 11 600 g for 10 minutes to pellet the cells. The cell-pellet was suspended in 180 µl 50 mM glucose, 10 mM EDTA and 25 mM Tris-HCl (pH 8,0). A few grains dry lysozyme was added, mixed gently and the tube put on ice for 10 minutes. Thereafter 400 µl NaOH/SDS (0,2 N NaOH; 1% SDS) was added, mixed gently and the tube was again put on ice for 10 minutes. The solution cleared at this stage due to the denaturing and dissolving of protein and DNA. The chromosomal DNA and protein was precipitated with 300 µl high salt (3 M Potassium acetate; 1,3 M Formic acid). After mixing gently it was left on ice for 15 minutes or longer, centrifuged at 11 600 g for 15 minutes and the supernatant containing the plasmid DNA transferred to a fresh tube. The DNA was extracted twice with phenol-chloroform before it was precipitated by the addition of an equal amount cold isopropanol. After thorough mixing the tube was put on dry ice for 30 minutes. The DNA was then pelleted and dissolved in sterile ultra pure water as described in section 2.1.1.1.1. Contaminating RNA was removed from the DNA preparation as described in section 2.1.1.1.1.

2.1.1.2. Large scale extraction of plasmid DNA.

The method used for the preparation of large quantities of plasmid DNA was adapted from that of Greenaway and Dale (personal communication). The incubation times were modified to produce greater yields and the chloramphenicol amplification step was omitted.

A single bacterial colony was inoculated into 10 ml Luria-broth containing an appropriate selective antibiotic. If the cells were not actively growing they were inoculated into 10 ml of Luria-broth without antibiotic and grown up for an hour at 37°C with shaking to get expression of the antibiotic-resistance gene. The appropriate antibiotic was then added and cultures were incubated for a further 4-8 hours. The 10 ml culture was then added to 200ml pre-warmed Luria-broth containing antibiotic. A large flask with a capacity of about 500-1000ml was used for this step to maximize surface aeration in order to get a good plasmid yield. It was left shaking at 37°C for about 15-18 hours or overnight.

The cells were harvested by centrifugation at 4 400 g in a Beckman centrifuge (Model J2-21; Beckman, Irvine, USA) for 15 minutes at 4°C. The cell-pellet was suspended in 2,6 ml 25% sucrose in 0,05 M Tris-HCl, pH 8.0, transferred to a chilled polypropylene centrifuge tube and put on ice. A spatula tip of dry lysozyme was added to the cell-suspension and it was swirled on ice for 5 minutes. To this was added 2.6 ml of 0.25 M Na_2EDTA (pH 8.0) to provide an isotonic solution and to chelate divalent metal-cations and it was swirled on ice for another 5 minutes. Then 4 ml Triton-x-100/DOC (1% Triton, 0.4% Sodium deoxycholate in 0.01 M Tris, 0.001 M Na_2EDTA, pH 8.0) was added rapidly with a 10 ml pipet and mixed by drawing up and expelling three times. It was left on ice for 20 to 30 minutes to allow maximal cell lysis to occur.

Centrifugation was carried out at 27 200 g in a Beckman Model J2-21 centrifuge for 45 minutes at 4°C to pellet the cell debris and chromosomal DNA. The chromosomal DNA is known to be associated with the cell-membrane and will pellet together with the membrane in a detergent solution.

The supernatant was removed and the volume made up to 10 ml with Triton-x-100/DOC. To this 9.5 g of CsCl and 0.2 ml of ethidium bromide (10 mg/ml) was added. Two Quick-Seal polyallomer tubes (Beckman), suitable for centrifugation in a Beckman VTi 65 rotor, were filled to the neck with the solution and heat-sealed. Isopycnic gradient centrifugation was carried out at 239 000 g in a Beckman L7-55 ultracentrifuge (Beckman, Irvine, USA) for 18-20 hours at 20°C.

The DNA was visualized under an ultraviolet light of 375 nm wavelength. Two bands were visible: a higher band consisting of chromosomal and nicked plasmid DNA, and a lower band consisting of covalently closed supercoiled plasmid DNA which resided at an equilibrium position corresponding to its buoyant density. Ethidium bromide intercalates less efficiently into DNA in the supercoiled configuration than into DNA in the relaxed configuration. Supercoiled DNA thus contains less ethidium bromide resulting in a higher density (ethidium bromide unwinds DNA when it intercalates), which facilitates its separation from nicked and chromosomal DNA by ultracentrifugation in CsCl (Ausubel *et al.*, 1987; Sambrook *et al.*, 1989).

Both the top and the bottom of the tube were punctured with a needle and the plasmid bands were collected under long wave UV-light through the bottom of the tube. The RNA which had collected at the bottom of the tube was eluted first. Denatured protein was sometimes visible against the side of the tube.

Ethidium bromide was removed by extraction with an equal volume of isoamylalcohol. After thorough mixing, the phases were separated by centrifugation for 2 minutes at 11 600 g. The upper organic layer was disgarded and the extraction was repeated until no pink colour could be seen in either phase.

The DNA in the remaining aqueous phase was precipitated by the addition of 3 volumes (1 volume = volume of DNA-solution obtained) of sterile distilled water and 2.5 times the volume obtained after the addition of water, of absolute ethanol. The microfuge tubes were put on dry ice for 5 minutes or overnight at -20°C. Polypropylene tubes were put on dry ice for 2 hours or overnight at -20°C. The DNA in the polypropylene tubes was pelleted by centrifugation at 3 000 g in a Beckman model J2-21 centrifuge for 45 minutes at 4°C. (The DNA in the microfuge tubes was pelleted by centrifugation at 11 600 g for 15 minutes at 4°C.) The DNA-pellet in the polypropylene tube was washed with chilled 70% ethanol by centrifugation at 3 000 g for 30 minutes at 4°C (or at 11 600 g for 15 minutes at 4°C when using microfuge tubes), dried in a Savant speedvac and resuspended in sterile distilled water.

The dialysis step (in order to remove excess CsCl-salt) as described in the original method (Greenaway and Dale - personal communication) was omitted after the removal of ethidium bromide. This reduced the loss of DNA, saved time and no adverse effects on subsequent DNA manipulations were evident. The CsCl concentration was sufficient to precipitate the DNA without the addition of any more salt.

2.1.2. Phenol/chloroform extractions.

Phenol/chloroform extractions (Ausubel, 1987; Sambrook *et al.*, 1989) were performed to remove contaminating proteins from nucleic acid preparations during purification or for the removal of enzymes after enzymatic manipulations of DNA or RNA. Deproteinization is more efficient when two different organic solvents are used instead of one. Isoamyl alcohol, used in combination with chloroform, helps to separate the organic and acqueous phases (Perbal, 1988). When nucleic acids were purified from complex mixtures such as cell lysates, additional measures were required. In these cases most of the protein was removed by digestion with proteinase K before extracting with phenol/chloroform.

A volume of phenol (equal to half that of the DNA solution) (Appendix A.11.) was added and the solution was vortexed. Chloroform:isoamylalcohol (24:1) was added at the same ratio as the phenol and the solution was mixed by vortexing. The phases were separated by centrifugation for 2 minutes at 11 600 g and the upper aqueous phase, containing the nucleic acid, was retained. These steps were repeated at least one more time or until no precipitate was visible on the aqueous/organic interphase. A subsequent extraction was sometimes carried out with an equal amount of water-saturated diethyl-ether to remove any traces of phenol, but it was not required for routine purification of DNA (Ausubel, 1987). The DNA was precipitated as described in section 2.1.3.

2.1.3. DNA precipitations.

DNA was precipitated after purification by phenol/chloroform extractions. This was usually done by the addition of LiCl to a final concentration of 0.4 M, or Na-acetate to a final concentration of 0.3 M and 2.5 volumes of ice-cold absolute ethanol (Ausubel, 1987; Sambrook *et al.*, 1989). LiCl was preferred because of its solubility in ethanolic solutions and its selectivety in precipitating DNA as opposed to RNA at this particular concentration (Sambrook *et al.*, 1989), but Na-acetate precipitated poxvirus-DNA more effectively. The DNA was kept on dry ice for 5-30 minutes or overnight at -20°C for maximum precipitation. It was then pelleted by centrifugation at 12 000 g for 15 minutes at 4°C. The pellet was washed by centrifugation with chilled 70% ethanol in the opposite direction so that the DNA migrated through the ethanol, dried in a speedivac (Eppendorf, Germany) and resuspended in sterile distilled water. The resuspended DNA was stored at 4°C.

2.1.4. Restriction endonuclease digestion.

Restriction endonuclease digestions were performed on small- and large-scale DNA preparations as recommended by the suppliers. The correct salt concentrations were used and the glycerol concentration was kept low enough to prevent inhibition of the reaction. The enzyme volumes used never exceeded one tenth of the total reaction volume.

Digestions of plasmid DNA were usually done by incubation at 37°C in a waterbath for 60 to 120 minutes, except when different incubation temperatures were recommended by the suppliers. Digestion periods were kept to a minimum for enzymes such as *EcoR1* which could exhibit star activity according to Boehringer Mannheim. Digestions of genomic- or poxviral-DNA were incubated for two to three hours at 37°C in a waterbath or overnight for about 18 hours at 37°C in a humidified incubator to minimize the evaporation-rate which would result in a change of the reaction-concentrations. Multiple digestions were done simultaneously when the temperature- and buffer-requirements of the enzymes involved were compatible. If however, the reaction conditions were not compatible, they were done sequentially (starting with the enzyme with the lowest salt requirement). In this case phenol/chloroform extractions and ethanol precipitations of the DNA were done between digestions. A small aliquot of digested DNA was routinely checked for complete digestion by agarose gel electrophoresis (Ausubel *et al.*, 1987).

The enzyme-reactions were terminated by one of the following ways: (1)phenol/chloroform extraction and ethanol precipitation; (2)heating to 75°C for 15 minutes in a waterbath; (3)adding 10 X Stop buffer (50% v/v glycerol, 100mM Na_2EDTA pH 8.0, 1% w/v SDS, 0.1% w/v bromophenol blue, 0.1% cyanol) to the reaction mixture (methods adapted from Ausubel *et al.*, 1987). The fragments generated from the digests were separated by agarose gel electrophoresis, stained with ethidium bromide and visualized on a 254 nm UV transilluminator.

2.1.5. Agarose gel electrophoresis.

Agarose gel electrophoresis was performed using a horizontal submerged slab gel system. Agarose (0.8% or 1.0%, depending on the sizes of the fragments to be separated) was dissolved in TAE (40 mM Tris-acetate; 2 mM EDTA pH 8.0; Appendix A.15.) or TBE (90mM Tris-borate; 2 mM EDTA pH 8.0; Appendix A.16.) buffer by melting in a microwave oven, and cooled to approximately 56°C before pouring. Gels were run either with ethidium bromide (0.5 μg/ml) already added to the agarose before the gel was poured or they were stained after electrophoresis with ethidium bromide at a concentration of 1 to 2 μg/ml for 30 minutes. In case of overstaining gels were destained in distilled water for 30 minutes. When very accurate size estimations of DNA were necessary, gels were stained afterwards, because the fluorescent ethidium bromide intercalates with the DNA and reduces the electrophoretic mobility of linear DNA by about 15% (Ausubel *et al.*, 1987).

Horizontal gel slabs, about 5 mm thick, were poured in a mold with the comb already in position. Once the gel had set (after about 30 minutes) the comb was removed and the gel submerged in a tank filled with TAE or TBE buffer. The samples of DNA, which were mixed with gel-loading buffer (Appendix A.6.) were loaded into the slots of the gel. A marker DNA with known sizes was also loaded into the gel (Ausubel *et al.*, 1987).

Electrophoresis of minigels (75 x 50 x 3 mm) was carried out for 30 to 60 minutes at 5-20 V/cm until the bromophenol blue and xylene cyanol FF had migrated the required distance. Electrophoresis of large analytical gels (200 mm long x 150 mm wide x 5-7 mm thick) was carried out for 5 hours at a potential difference of 4 V/cm or for 18-20 hours at a potential difference of 1.6-2.0 V/cm. A higher resolution of the bands were attained when electrophoresis was carried out at 2 V/cm for 18-20 hours at 4°C. DNA bands were visualized (due to the presence of ethidium bromide) and photographed on a 254 nm UV transilluminator.

2.1.6. Isolation of DNA fragments from agarose gels.

DNA fragments were isolated from agarose gels according to the method of Seth (1984).

An amount of DNA that would yield approximately 1 μg of the fragment of interest, was digested with a suitable restriction enzyme. The fragments were separated by agarose-gel electrophoresis and stained with ethidium bromide. The band of interest were located with a hand-held long-wavelength (375 nm) ultraviolet light (to minimize the nicking of the DNA) and cut out with a scalpel blade. The slice of agarose containing the band of interest was cut into small pieces in a petridish using a scalpel blade and transferred to a microfuge tube(s). An equal amount of phenol was added and mixed thoroughly by vortexing. It was kept on dry ice for 30 minutes or overnight at -20°C before it was centrifuged for 15 minutes at 11 600 g in a bench-top microfuge. The upper aqueous phase was transferred to a clean tube and the DNA was further purified by phenol extraction. The upper phase was again transferred to a clean tube and an equal amount of chloroform:isoamylalcohol (24:1) was added, mixed and separated by centrifugation at 11 600 g for 5 minutes. The DNA was precipitated as described in section 2.1.3. The DNA was subjected to agarose-gel electrophoresis to examine the quality and estimate the quantity.

2.1.7. Cloning

In principle, the plasmid DNA or vector was cleaved with a restriction enzyme and ligated *in vitro* to a piece of foreign DNA with compatible ends. Products of the ligation mixture were introduced into competent *E. coli* cells and transformants, harbouring the desired DNA molecule, were identified (Ausubel *et.al.*, 1987; Sambrook et.al., 1989; Perbal 1988).

2.1.7.1. Preparation of insert and vector DNA

The insert-DNA was prepared as described in section 2.1.6. The plasmid-vector was linearized with a restriction enzyme which generated the appropriate sticky ends for ligation. The linearized plasmid was phenol/chloroform extracted (to remove the enzyme), alcohol precipitated and redissolved in sterile distilled water.

A vector digested with only one restriction enzyme is capable of recircularizing under ligation conditions. This can be limited to some extent by adjusting the concentrations of the vector DNA and the foreign DNA in the ligation reaction so that they will be optimized for the formation of the "correct" ligation products. An additional method to prevent this recircularization of the vector is to remove the 5'-phosphate group of the DNA molecule, necessary for self-ligation and recircularization, by treatment with calf intestinal phosphatase (CIP). This `dephosphorylation'-reaction was performed for the preparation of the pGS20-VP7 vector cut with *Hind111* (Chapter 4.2.3.3.). Ligation to foreign DNA would still be possible, since the foreign DNA would still contain a phosphate-group. At each end only 1 of the 2 strands will be covalently joined *in vitro*, but *E. coli* can repair the single-stranded nicks *in vivo* to generate the desired DNA molecules (Ausubel *et al.*, 1987; Sambrook *et al.*, 1989).

`Dephosphorylation' of the vector was done as follows (Sambrook *et al.*, 1989): after phenol/chloroform extraction and alcohol precipitation of the linearized DNA (10-20 μg), it was resuspended in 90 μl sterile distilled water to which 10 μl of 10 x CIP buffer (10 mM $ZnCl_2$, 10 mM $MgCl_2$, 100 mM Tris.Cl pH 8.3) was added. For protruding 5' termini, one unit of CIP/100 pmoles of free ends were used and incubation was done for 30 minutes at 37°C. For blunt or recessed termini, 1 unit of CIP/2 pmoles was used and incubated for 15 minutes at 37°C, after which another aliquot of CIP was added. The reaction was then incubated for a further 45 minutes at 55°C, after which the following were added: 5 μl 10% SDS, 1 μl 0.5 M EDTA and 0.5 μl (20 mg/ml) proteinase K and mixed well. Incubation was done for 30 minutes at 56°C after which two phenol/chloroform extractions were performed. The DNA was ethanol precipitated and washed with 70% ethanol. The pellet was resuspended in sterile distilled water. (The CIP must be removed completely for the ligation reactions to be efficient.)

Short DNA fragments are more likely to circularize than large fragments, and high concentrations (20 to 100 μg/ml) should be used to favor intermolecular ligation (Ausubel et.al., 1987). Intramolecular ligation of insert fragments does not generally affect the background of unwanted colonies as the insert fragment does not contain the selectable marker.

2.1.7.2. Ligation

Samples of the vector and the insert were run on an agarose-gel to verify the integrity of the DNA and to estimate the relative concentrations.

The ligation reaction were set up so that the molar concentration of the termini of foreign DNA was approximately two- to threefold the molar concentration of the termini of the plasmid DNA to obtain the maximum yield of monomeric plasmid:insert DNA chimeras. The absolute concentration of termini should be high enough to favour intermolecular ligation, but low enough to prevent extensive oligomeric molecule formation. To the vector and insert DNA, 2 μl of 5 x ligase buffer (obtained from Boehringer Mannheim) and 1 μl of T4 DNA ligase (1 unit, Boehringer Mannheim) were added in a total reaction volume of 10 μl. The final DNA concentration in the reaction mixture should not exceed 5 ng/μl, because the PEG in the ligase buffer tends to concentrate DNA (Sambrook *et al.* 1989).

Two additional control ligation-reactions were set up in the case of dephosphorylated vector DNA, one with only dephosphorylated plasmid DNA and the other with linearized plasmid DNA that was not treated with CIP. The absence of transformants in the case of dephosphorylated DNA subjected to "self-religation", was an indication of a successful CIP treatment.

Ligation was carried out at 15°C in a waterbath overnight (18 to 20 hours), after which bacterial cells were transformed with the mixture. The ligation-temperature should be high enough for the enzyme to be active, but also low enough to permit hydrogen bonding between the sticky ends of DNA.

2.1.7.3. Preparation of competent *E. coli* cells

A single bacterial colony (*E. coli* strain JM109, LK-111 or DK-1) was picked from a minimal media plate, inoculated into 10 ml of Luria-broth (Appendix A.7.) and grown overnight at 37°C with shaking. From this culture, 100 μl was used to inoculate 100 ml of pre-warmed Luria-broth and allowed to grow to an early log phase (OD_{600} = 0.5). (All manipulations were carried out aseptically.) The cells were transferred to sterile, ice-cold polypropylene tubes, cooled on ice for 10 minutes and harvested by centrifugation at 2831 g for 10 minutes at 4°C.

The cell-pellet was carefully resuspended in 10 ml of ice-cold TSB buffer (Luria-broth containing 5% (v/v) DMSO, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 10% (w/v) PEG Mr 4 000) and left on ice for 10-15 minutes.

Competent cells were used immediately for transformation or 100 µl aliquotes were snap-frozen in a dry-ice/ethanol bath and stored at -70°C (Chung and Miller, 1988).

2.1.7.4. Transformation of competent *E. coli* cells

Approximately 50 ng DNA from the ligation mixture in a volume of no more than 5 µl, were transferred to a sterile Greiner-tube and flooded with 100 or 200 µl competent cells (immediately after they were thawed). Two control reactions were set up in parallel: one with competent cells transformed with 1 ng of pUC19 DNA and the other with only competent cells.

After gentle mixing and incubation on ice for an hour, the cells were heat shocked for 90 seconds at 42°C in a waterbath. The cells were put on ice for 2 minutes and 1 ml of 37°C Luria-broth (Appendix A.7.) was added to each tube, mixed and incubated for 1 hour at 37°C to get expression of the antibiotic (ampicillin)-resistance gene. Of each of the transformation mixtures, different aliquots were plated out onto three separate Luria-agar plates containing ampicillin (50 µg/ml), left to absorb and incubated in an inverted position at 37°C overnight. Ampicillin-resistant colonies secrete β-lactamase into the medium surrounding the colonies which inactivates ampicillin (Sambrook et.al., 1989). Therefore transformed cells should be plated out at low density and the plates incubated for no longer than 20 hours at 37°C to prevent the appearance of ampicillin-sensitive satellite colonies when selecting for ampicillin-resistance. The number of resistant colonies do not increase in a linear way to the number of cells plated out as cells killed by ampicillin might release growth-inhibiting substances (Sambrook *et al.*, 1989). When the inactivation of the β-gal gene of the pUC vector was used as marker, 250 µl of a 20 mg X-gal/ml N,N-dimethylformamide solution, 50 µl of a 23.8 mg IPTG/ml distilled water solution and 100 µl (50 mg/ml) ampicillin was added per 100 ml pre-cooled (<54°C) Luria-agar before the plates were poured.

2.1.7.5. Screening for recombinants

The three methods used to screen for bacterial colonies containing recombinant plasmids were the following: restriction analysis of small-scale plasmid-preparations; insertional inactivation of the *E. coli lacZ* gene, which encodes the glycoside hydrolase β-D-galactosidase (β-gal), and *in situ* hybridization of bacterial colonies.

A number of separate bacterial colonies were randomly picked (when blue-and-white selection could not be performed as discussed in the following paragraph) and each colony was streaked onto a grid plate with Luria-agar and inoculated into 10 ml of Luria-broth. After incubation at 37°C overnight, the grid plate was stored at 4°C for future reference and the liquid cultures were used for small-scale plasmid-preparations. The plasmid-DNA obtained was analyzed by restriction enzyme digestion and gel-electrophoresis. This was done when there was a relatively good chance of finding a recombinant within a small number of colonies, for example when the control ligation with only dephosphorylated vector-DNA yielded far less colonies than the actual ligation reaction.

When plasmids containing the β-lactamase gene from pBR (which confers ampicillin-resistance upon the host cell) were used as vectors, it allowed for the selection of transformed cells in the presence of ampicillin. A vector containing the amino-terminal fragment of the β-gal gene (with a multiple cloning site within this gene) will allow expression of β-galactosidase in host cells containing the carboxy-terminal fragment of this gene through α-complementation. IPTG (isopropyl-β-D-thiogalactopyranoside), a synthetic inducer of the lac operon, was included in the medium. The enzyme β-galactosidase hydrolyses the chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) to generate galactose and soluble indoxyl molecules (MacGregor *et al.*, 1991). The soluble indoxyl molecules are oxidized to form bromochloroindole, an insoluble blue dye which gives the colonies a blue appearance on Luria-agar plates containing ampicillin, X-gal and IPTG. When foreign DNA is cloned into the multiple cloning site, functional β-galactosidase cannot be produced and the colonies will appear white (MacGregor *et al.*, 1991; Sambrook *et al.*, 1989). The presence of inserts in white colonies was verified by subjecting DNA from minipreparations to restriction analysis.

Screening for recombinants was also done by colony hybridization (Sambrook *et al.*, 1989). Plates which had been incubated until the bacterial colonies were about 0.1-0.2 mm in diameter, were placed at 4°C for 1 to 2 hours in an inverted position. A replica plate was prepared which was stored at 4°C until needed. The bacterial colonies were transferred to a nylon-membrane (Hybond N^+, Amersham, UK) by placing the dry membrane on the surface of the agar-medium in contact with the colonies. The membrane and the underlying agar was marked in three asymmetric positions by stabbing through the filter with a needle. The wet membrane was peeled off and put, colonies facing up, on a filter paper disk (Whatman 3MM paper) saturated with 10% SDS for 3 minutes to lyse the bacteria. The agar-plate was wrapped in Saran-wrap and stored at 4°C until further use. The membrane was transferred to a second piece of filter paper saturated with denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 5 minutes. After this the membrane was transferred to a third piece of filter paper that has been saturated with neutralizing solution (1.5 M NaCl, 0.5 M Tris.Cl pH 8.0) and left for 5 minutes. The DNA was fixed to the membrane by transferring the membrane to a filter paper saturated with 0.4 N NaOH for 10 minutes. After being washed with 6 x SSC the filter was sealed in a plastic bag for hybridization. As many as 10^4 colonies per 150-mm plate can be screened using this technique

(Sambrook *et al.*, 1989). The diffusion of the plasmid DNA during denaturation and neutralization is limited by putting the membrane on filter papers wetted with the appropriate solutions. After hybridization with an appropriate non-radioactively labelled DNA probe and immunological detection (Sections 2.1.8.2. and 2.1.8.3.), the colonies harbouring recombinant plasmids could be identified by coloured precipitate formation on the nylon membrane. By re-alignment of the membrane with the agar-plate corresponding colonies were picked from a replica plate onto a new grid plate with Luria-agar for future storage. These colonies were also subjected to mini-scale DNA plasmid preparations.

2.1.7.6. Storage of bacterial cells

After identification of the colonies harbouring the recombinant plasmid, the corresponding colonies on the grid plate were used to inoculate 10 ml Luria-broth (Appendix A.7.) containing 50 μg/ml ampicillin, and the cultures were grown up for 3 to 4 hours at 37°C with shaking. From this culture, about 3 x 0.85 ml was mixed with glycerol to a final concentration of 15%. Cells were then frozen in a dry ice/ethanol bath, stored at -70°C and used as stock cultures (Maniatis *et al.*, 1982).

2.1.8. Hybridization

DNA was immobilized on nylon membranes and then hybridized to appropriate labelled nucleic acid probes to detect DNA sequences of interest. Nylon membranes were preferred because of their durability and the fact that they can withstand several rounds of hybridization, washing, stripping and reprobing at elevated temperatures.

2.1.8.1. DNA transfer to membrane

The DNA was digested with one or more restriction enzymes and the fragments separated by gel-electrophoresis in an agarose gel. This DNA was then denatured *in situ* and transferred from the gel to a nylon membrane by unidirectional Southern blotting (Perbal, 1988; Smith & Summers, 1980; method modified slightly) as follows:

After electrophoresis, the gel was photographed and cut such that only the lanes of interest were transferred. The gel was then soaked in a volume of 0.25 M HCl for 2 minutes with gentle shaking to induce chemical cleavage of the DNA. The gel was rinsed with distilled water and soaked in buffer 1 (1.5 M NaCl, 0.5 M NaOH) for 20 minutes with gentle shaking to denature the DNA fragments.

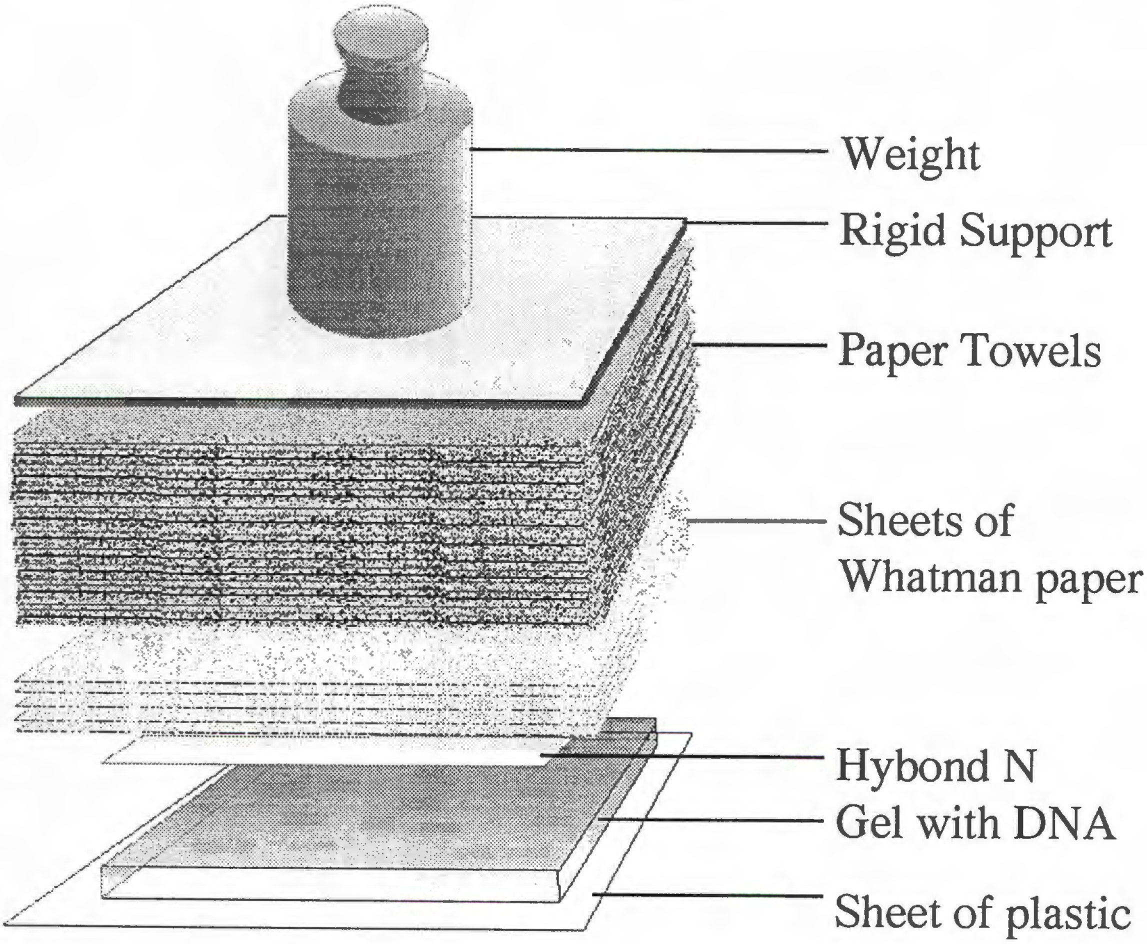


Fig. 2.1. Diagrammatic representation of Southern Blot transfer of DNA to Hybond-N/N$^+$ membrane for hybridization.

Neutralization followed after rinsing the gel with distilled water by soaking in buffer 2 (1 M Ammonium acetate, 0.02 M NaOH) with gentle shaking for at least 40 minutes.

A Southern blot was set up as demonstrated in Fig. 2.1. (Sambrook *et al.* 1989, modified). The gel was transferred to an used X-Ray film and the excess solution removed. A piece of Nylon membrane (Hybond N or Hybond N$^+$, Amersham), cut to the same dimensions as the gel, was carefully placed on top of the gel such that no bubbles were trapped between the gel and the membrane. The sides of the gel were covered with cling film so that the buffer would not be absorbed directly into the paper towels. Three pieces of Whatman 3 MM paper, also cut to the same dimensions as the gel and wetted in buffer 2, were put on top of the membrane followed by a stack of dry paper towels. A flexiglass with a 0.75 to 1.0 kg weight was put on top of this and the DNA transfer was allowed to proceed overnight. All the steps were carried out at room temperature.

Before removing the membrane from the gel, the slot positions were marked with pencil on the membrane. After rinsing the membrane briefly with 2 x SSC, it was air-dried slightly and UV-illuminated for a few seconds (1 200 x 100 microjoules) to facilitate co-valent cross-linking of the DNA to the membrane. Thereafter it was used directly for hybridization or sealed in a plastic bag and stored at 4°C until further use.

2.1.8.2. Non-radioactive labelling of DNA

Non-radioactive labelling of DNA with digoxigenin-dUTP was performed as described in the protocol supplied with the DNA Labelling Kit (Boehringer Mannheim, Cat. No. 1175 033).

2.1.8.3. Hybridization and detection

For non-radioactive labelling the membranes were prehybridized with at least 20 ml hybridization solution (50% (v/v) formamide, 5 x SSC, 2% (w/v) blocking reagent, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS) per 100 cm^2 of filter at 42°C with shaking overnight. (Protocol supplied with the Nucleic Acid Detection Kit, Boehringer Mannheim, Cat. No. 1175 041). This was replaced with about 2.5 ml of hybridization solution per 100 cm^2 of filter to which the denatured probe was added. The probe was denatured by boiling in a waterbath for 15 minutes and quickly chilled in a dry ice/ethanol bath for 5 minutes. Hybridization was carried out at 42°C overnight with gentle shaking. The membrane was washed as follows: two 5 minute washes with 2 x SSC, 0.1% SDS (w/v) and three 15 minute washes with 0.5 x SSC, 0.1% SDS (w/v) at room temperature with shaking. Hybridized DNA was detected by chemiluminescent or immunological detection.

Chemiluminescent detection was performed as described in the updated protocol using maleate buffer, provided with the DIG Luminescent Detection Kit (Boehringer Mannheim, Cat. No. 1363 514). The anti-digoxigenin-AP, Fab fragments were diluted to 150 mU/ml (1:5000) in buffer 2. Tween 20 was not added to buffer 1 for washing purposes.

Immunological detection instead of chemiluminescent detection was performed for colony blots as described in the protocol provided with the DIG Nucleic Acid Detection Kit (Boehringer Mannheim, Cat. No. 1175 041). Chemiluminescent detection was found to be more sensitive than colorimetric detection.

2.1.8.4. Autoradiography

Labelled blots, sealed in a plastic bag, were exposed to Curix RP 1 X-ray film (Agfa, West Germany) in X-ray cassettes with intensifying screens. Blots hybridized with non-radioactive labelled probes were exposed for about 30 minutes at room temperature. Autoradiographs were processed after exposure by developing for 3-8 minutes, washing for 1 minute (in a 2% acetic acid stop bath) and fixing and hardening for 5 minutes using Agfa chemicals as prescribed by the manufacturer. The X-ray was air-dried after being rinsed in running tap water for 15 to 20 minutes.

2.1.8.5. Re-use of blots

The digoxigenin-dUTP probe was removed (if the membrane was kept wet) by first washing the membrane in sterile water, then 2 x 15 minutes in 0.2 M NaOH, 0.1% SDS (w/v) at 37°C followed by a short wash in 2 x SSC (protocol supplied with the Nucleic Acid Detection Kit, Boehringer Mannheim, Cat. No. 1175 041).

The removal of the probe was tested by exposure to X-ray film and the membranes were only re-used when no signal could be detected.

2.2. Tissue Culture Methods And Virus Propagation

Tissue cultures have been applied in virology for three main purposes due to their ability to support the growth of viruses and to reveal their presence by a cytopathic effect (CPE) which are sometimes specific: (1) virus production for vaccine manufacturing purposes, (2) detection and identification of viruses and (3) the study of host-parasite relationships. The techniques used depended on the task to be performed and are varied and specialized.

2.2.1. Propagation of virus in fertilized eggs of domestic hens (*Gallus domesticus*)

Virus was propagated by a method adapted from Westwood *et al.* (1957) and McCarthy and Dumbell (1961). The virus purification method was adapted from Joklik (1962).

2.2.1.1. Preparation of chorioallantioc membranes (CAMs)

Twelve day old embryonated chicken eggs were candled to determine the position of the air-sac (on the blunt end of the egg) and a region near the main blood vessels on the dorsal side of the egg was marked. A small hole through the eggshell was made with a spring-loaded bore at the markings. A mixture of paraffin wax and petroleum jelly was melted and a drop was placed next to the hole on the dorsal side of the egg. A drop of sterile physiological saline was placed onto the hole on the dorsal side of the egg and allowed to go in between the membranes by carefully pushing the membrane down through the hole with the nib of a witch pen. An air-lock was created by breaking the shell membrane and thus allowing saline penetration. The eggs were re-candled and the air was extracted from the air-sac with a rubber suction bulb so that the chorioallantoic membrane (CAM) dropped and a new air-sac formed above it at the hole on the dorsal side of the egg. The eggs were left at 37°C for about 2 hours so that the membranes could equilibrate.

2.2.1.2. Inoculation of CAMs

An appropriate virus dilution (0.1 ml) was inoculated onto the prepared CAM through the hole on the dorsal side of the egg, using a one ml syringe with a thin short (22 gauge) needle. After this the hole was sealed with the mixture of wax and petroleum jelly. The egg was swirled around for a few seconds for the inoculum to spread evenly and incubated at 37°C for three days. The virus-inoculum was diluted in sterile physiological saline to which PSN (each antibiotic at a concentration of 200 μg/ml; Appendix A.13.) was added.

2.2.1.3. Harvesting of virus from CAMs

After three days incubation the CAMs were harvested as described below:

The egg was held so that the inoculation site pointed upwards and was cut open along the midline with a pair of sterile scissors. While holding on to the upper half of the eggshell the egg-contents were dropped into a bin containing Biodan disinfectant. The infected area of the membrane was peeled away from the eggshell with sterile forceps and washed in physiological saline. After examining it for the presence of focal lesions, the membranes were transferred to sterile pre-cooled McCarthy-bottles, containing glass beads to about a fourth of its volume, and the bottles were kept on ice to keep enzyme activity to a minimum.

One ml of pre-cooled 4 mM McIlvains buffer, pH 7.4 (Appendix A.9.), was added per membrane. A paper towel was wrapped around each bottle (for heat-isolation and to detect possible leakage) and it was vigorously shaken for 1 minute. The culture was centrifuged in a swing out rotor at 110 g for 8 to 10 minutes at 4°C and the supernatant (containing the virus) was retained on ice. A second volume of McIlvain's buffer was added to the membranes in the bottles and the process was repeated. The supernatant fluids were pooled and incubated on ice for 1,5 hours. Virus suspensions were clarified by centrifugation at 1 200 g for 15 minutes at 4°C. The virus in the supernatant was concentrated by centrifugation at 17 418 g in a swing-out rotor for 1,5 hours at 4°C. The virus was cushioned by 1 ml of 36% sucrose in TE-buffer, pH 9, (Appendix A.17.) which was added to the bottom of the tube prior to centrifugation. The virus pellet was resuspended in 1 ml of TE-buffer, pH 9, and layered onto a density gradient consisting of 1 volume of 36% sucrose and 2 volumes of 10% Dextran T10 (w/v in TE-buffer, pH 9). Centrifugation was performed at 17 418 g for 1 hour at 4°C. The viral pellet was resuspended in 1 ml of McIlvain's buffer, pH 7.4. For long term storage glycerol was added to a concentration of 80% and virus aliquots were stored at -70°C. Working stocks were stored at -20°C.

2.2.2. Preparation of primary cells

Primary cells were prepared according to the method by Freshney (1987).

The bovine fetal calf kidneys or the lamb testis were obtained from the abattoir in Maitland and processed immediately after it was aseptically removed from the animal. Embryonic tissue disperses more readily and gives a higher yield of proliferating cells than newborn or adult tissue. The kidney/testis was rinsed in physiological saline containing appropriate antibiotics and the fat, connective tissue and capsule were removed. The remaining tissue was rinsed again and cut into small 2 to 3 mm pieces with crossed scalpels. These pieces were rinsed several times in crude trypsin (0.25% trypsin in PBS; Appendix A.12) to get rid of excess blood and transferred to a sterile glass bottle. Crude trypsin was added and stirred at 4°C overnight to allow penetration of the enzyme with little tryptic activity. Crude trypsin is tolerated quite well by most cells and is effective for a variety of tissues. However, to preserve maximum viability it is better to minimize the exposure of cells to active trypsin (Freshney, 1987). The trypsin was removed and the tissue digested at 37°C for 30 minutes. Tissue culture media (DMEM; Appendix A.3.), supplemented with 0.5% PSN (Appendix A.13) and 10% fetal calf serum (FCS), was added and the cells dispersed by pipetting. Any residual tryptic activity left will be neutralized by the serum in the media (Freshney, 1987). After counting the cells with a haemocytometer as described below, the cells were diluted to 10^6 per ml in growth medium and flasks were seeded with approximately 2 x 10^5 cells per cm^2. The cells were incubated at 37°C in a CO_2-incubator until confluent. The medium was changed at regular intervals.

2.2.3. Quantitation of cells

Of a cell suspension in 10% FCS tissue culture medium, 0.1 ml was added to 0.15 ml of physiological saline and 0.25 ml of a Trypan blue solution (Appendix A.18.) and mixed. This gave a dilution factor of 5. A small amount of the Trypan Blue-cell suspension was transferred to both chambers of a haemocytometer (Neubauer, Brand, Blaubrand, Germany) with the coverslip in place. The viable cells were counted in a 1 mm center and four corner squares of both chambers separately. (Live cells exclude the dye whereas dead cells take it up to stain blue). If less than 20 or more than 50 cells were counted per square, the entire procedure was repeated adjusting the cell dilution. To calculate the viable cell-concentration per ml, the average reading per square was multiplied with the dilution factor and with 10^4 (the total volume of the 1 mm square with the cover slip in place is 0.1 µl).

2.2.4. Growth and maintenance of cell cultures

The cells were grown at 37°C under CO_2 atmosphere in medium supplemented with 10% FCS, PSN (100 units/ml Penicillin, 100 mg/ml Streptomycin and 100 mg/ml Neomycin; Appendix A.13.) and 3 mM sodium bicarbonate. The enriched medium, Dulbecco's Minimum Essential Medium (DMEM; Appendix A.3.), does not require supplementary sodium bicarbonate.

Confluent cell monolayers were maintained with the appropriate medium supplemented as above with PSN and sodium bicarbonate, but with a reduction in the FCS-concentration to 2 or 4%, depending on the cell-line. (Mitosis is not stimulated by this reduced serum-concentration in most untransformed cells. Transformed cells, however, do not block in a regulated fashion in the G_1 phase of the cell cycle and may either deteriorate or continue to divide. Continuous cell lines and some embryonic cells also deteriorate at high cell densities (Freshney, 1987). The cell-lines were not kept on maintenance medium for longer than a week.)

The cell- monolayers were grown and maintained in tissue culture flasks or in multi-well tissue culture dishes. Confluent cell-layers were subcultured by rinsing the monolayer with PBS and with activated trypsin versene (ATV; Appendix A.1.). A second small volume of ATV (3 ml/75 cm^2) was added and the flask was incubated at 37°C for about 10 minutes for the cells to dissociate. Growth medium was added to a final volume of 10 ml and the cells were dispersed by repeated pipetting for a few times. Cells were re-seeded by the addition of the appropriate amount of cells to growth medium. The medium was changed on a regular basis (about every third or fourth day) depending on the cell-density or cell-line, or when indicated by a change in the pH or signs of morphological deterioration.

2.2.5. Storage and preservation of cells

Stocks of cells were frozen away to serve as a back-up in case of accidental loss of cells due to contamination or a limited life span (Freshney, 1987; Lennette and Schmidt, 1979).

Cells were grown up to the late log phase of the cell-cycle (3+) in growth medium that was frequently changed. After trypsinization and centrifugation at 100 g for 10 minutes, the cells were resuspended in cell-freezing medium (Appendix A.2.) to a concentration of 1 x 10^6 viable cells/ml. (DMSO presumably protect the cells during freezing by binding water so that less are available to form ice-crystals and intracellular electrolyte build-up is reduced.) One ml aliquots were slowly frozen down by putting the vials at -70°C for 3 hours after which they were rapidly transferred to liquid nitrogen. Although the cooling rate could not be controlled, reasonably good viability of the cells was retained by this method. (Cells should be frozen down at a cooling rate of 1°C per minute to retain maximum viability of the cells. Ice crystals form extracellularly rather than intracellularly by slow freezing so that the cells are not disrupted.)

To thaw the frozen cells, vials were put at 37°C in a waterbath immediately after removing it from the liquid nitrogen. As soon as it was thawed the vial was swabbed with 70% ethanol, growth medium was slowly added to the cell-suspension (sudden dilution can cause severe osmotic damage to the cells) and centrifugation was carried out at 100 g for 10 minutes. The cells were resuspended in fresh growth medium, seeded into a tissue culture flask and incubated at 37°C. After 24 hours the medium was replaced.

2.2.6. Propagation of virus in mammalian cells

The mammalian cells used for the propagation of virus were prepared and maintained as described in sections 2.2.2. to 2.2.4. The most suitable cell-line for the propagation of the specific virus was selected.

Cells were grown in 150 cm^2 tissue culture flasks in DMEM, supplemented with 10% FCS and PSN (Appendix A.13.), at 37°C under CO_2 atmosphere. When the cell-sheet was about 80% confluent, the culture medium was removed. The cells were then rinsed three times with PBS (Appendix A.12.) to remove any remaining serum which may contain substances that inhibit virus infectivity (Mahy, 1985). The virus was diluted in virus diluent (Appendix A.21.) to obtain the desired multiplicity of infection and added to the rinsed cells. For the multiplication of LSDV, MDBK-cells were infected at a concentration of 1 foci forming units (FFU)/cell. After incubation at 37°C for 1 hour, the virus inoculum was replaced with DMEM supplemented with 4% FCS and PSN. Incubation was continued at 37°C under CO_2 atmosphere.

The third day after infection, or when maximum cpe (cytopathic effect) was observed, the cells were disrupted by three cycles of freeze/thawing. The disrupted cells were incubated on ice for 1 hour. The cell-debris was pelleted by low speed centrifugation in a swing out rotor at 800 g for 15 minutes at 4°C. The supernatant (containing the virus) was retained. The virus in the supernatant was concentrated by centrifugation at 14 636 g for 2 hours at 4°C, having added a 1.0 ml cushion of 36% sucrose in TE buffer (pH 9; Appendix A.17.) to the bottom of the centrifuge tube. The viral pellet was resuspended in 2 ml of McIlvain's buffer, pH 7.4. (Appendix A.9). Purification by density gradient centrifugation was not applied as this resulted in a significant reduction in virus yield. Working stocks were stored at -20°C. For long term storage, virus aliquots were stored at -70°C after glycerol was added to a concentration of 80%. A Sigma 301K centrifuge (Sigma Products, Saint Louis, United States of America) was used for the low speed centrifugation step. High speed centrifugation steps were done in a swing-out rotor in a Beckman centrifuge, Model J2-21 (Beckman, Irvine, United States of America).

Virus yield does not increase by overloading of the cells with virus. For optimum virus multiplication it is best to add sufficient virus to the cells to infect between 1% and 10% of the cells in a monolayer (Mahy, 1985). When the virus titre was unknown, this was achieved empirically by using two or three different dilutions for infection. The rate of virus multiplication in tissue culture is dependent on the particular virus strain and type of cell. Virus multiplication and release is generally accompanied by cell-degeneration, and a CPE is most readily seen in cells grown as a monolayer. With some viruses, when maximum CPE is observed the virus is completely released from the cells, while other viruses tend to remain more cell-associated. The harvest stage is crucial in some instances, since degenerating cells release nucleases which can degrade viral nucleic acids.

2.2.7. Virus Titration

(Adapted method from Paul, 1975)

Serial ten-fold dilutions of the virus to be titrated was made in virus diluent (Appendix A.21.). The growth medium was removed from 80% confluent cell-monolayers in a 24-well tissue culture dish and the cell-layers were rinsed three times with PBS to remove any remaining serum. Each well was inoculated with 0.2 ml of a virus dilution, two wells per dilution. (Dilutions in the range 10^{-2} to 10^{-7} were used for infection.) After 1 hour incubation at 37°C, the virus-inoculum was removed from the cells and they were rinsed once with PBS to remove any unadsorbed virus. To each well with infected cells, 1 ml of DMEM supplemented with 4% FCS and PSN (Appendix A.13.) was added. The cells were incubated at 37°C for 48 hours and stained with ZN Carbolfuchsin (Appendix A.5.) as follows: The medium was removed from the cells and they were covered with ZN Carbolfuchsin and left at room temperature for a period ranging from 30 to 60 seconds. Each well was held under slowly running tap water until the metallic blue-green precipitate was washed off. The water was disgarded and the wells were again filled up with tap water and left for 5 minutes. The water was disgarded and the stained cells were air-dried by leaving the tissue culture plate upside down on a paper-towel. The plaques were counted in the wells and the virus concentration was calculated as follows:

pfu (plaque forming units)/ml = average amount of plaques (for a specific dilution)/well x dilution factor x volume of inoculum per well

Vaccinia virus (VV) was titrated using this method. It was more difficult to titrate Lumpy Skin Disease Virus (LSDV) as it appeared that some cell-lines were more susceptible to the virus, for example a higher titer was observed if CFK-cells were used instead of MDBK-cells with the same virus-stock. The cytopathic effect (CPE) was also difficult to distinguish after staining with ZN Carbolfuchsin, as the virus does not form plaques like VV but causes swelling of cells which forms clumps. Due to the reasons mentioned, LSDV was titrated using CFK-cells and the sites of infection or cell-clumps (foci) were counted without staining.

Bushar and Sagripanti (1990) found that only a small percentage of virus in the inoculum is adsorbed onto the cells and thereby detected in the titration assay under conditions currently used for virus titration. No release of infective virus occurs during the 1 hour period of infection; thus, it cannot account for the presence of unadsorbed virus. They found that the volume of the inoculum is one of the most important factors and should be kept as small as possible to limit virus diffusion to the cell surface. Best results were obtained when dilutions were chosen so as to obtain between 30 and 150 plaques per well. Maximal plaque number was obtained after 1 hour of virus adsorption and little gain in sensitivity was obtained by increasing the adsorbtion time beyond 1 hour.

2.2.8. Infections and transfections of cell-cultures

(Methods adapted from Mackett *et al.*, 1985; Old and Primrose, 1989; Felgner, 1991; Singer and Berg, 1991.)

Mammalian cells have the ability to take up exogenously added DNA and to express genes encoded by this DNA. A vector that replicates independently as an extrachromosomal element, can be used to transform animal cells by infection or transfection, for example a virus genome that yields virions with recombinant genomes. These vectors are frequently unstable during the subsequent cell generations after the initial transfection, even when selective pressure is applied. However, some vectors do form stable, replicating, extrachromosomal elements in animal cells.

Infections of 80% confluent monolayers of cells with virus were performed as described in section 2.2.6. Animal cells were transfected essentially as described in the protocol provided with the Lipofectin™ Reagent (Bethesda Research Laboratories Life Technologies, Inc., Cat. No. 8292SA.). Liposomes are small single bilayer vesicles prepared from a cationic lipid, which interacts spontaneously with poly-anions such as DNA or RNA to form a Lipofectin^{TM} reagent-DNA or RNA complex. This complex transform animal cells in culture efficiently, probably by fusion with the plasma membrane (Old and Primrose, 1989) or by phagocytic uptake (Felgner, 1991). Some of the advantages of this method are the following: it is relatively simple to perform; it is unaffected by pH between 6.0-8.0; other factors like precise mixing protocols, the level of DNA and the presence of low levels of contaminants are less important; and large DNA molecules can be transfected.

For transfection, cells were infected with the appropriate wild-type virus as described in section 2.2.6 at a concentration of about 0.05 PFU/cell. (It has been found that when the wild-type virus input in the transfections are increased the total overall yield may be increased, however, the percentage of recombinants produced tends to decrease (Mackett, 1991). This will give an unnecessarily high background of wild-type virus.)

For each 30-mm dish (containing about 1 x 10^6 cells), 1 to 13 µg DNA and 24 to 32 µg Lipofectin^{TM} reagent was each diluted separately to 60 µl with ultra pure water to avoid DNA and liposome aggregation. The solutions were combined in a polystyrene tube, mixed gently and left at room temperature for 15 minutes. (Polystyrene tubes are preferred because they attract the aggregates that can form less than glassware or plastic. Optimum transfection activity occurs when the Lipofectin^{TM} reagent/DNA ratio is in the range of 2:1-10:1 (w/w), so that the net negative charge of DNA is substantially reduced (Felgner, 1991).

After infection of the cell-monolayer for an hour, the virus-inoculum was removed from the cells and the cell-layer was rinsed twice with Opti-MEM 1(GIBCO/BRL) to remove any unadsorbed virus. Opti-MEM was added to the cells, 500µl/30 mm dish, and the DNA-liposome complex was added dropwise and uniformly to this while swirling the dish gently. Incubation was performed at 37°C for 5 to 24 hours, depending on the condition of the cells which was microscopically monitored with regular intervals.

Medium, containing 4% FCS and PSN (Appendix A.13.), was added to the cells with/without selection medium after removal of the opti-MEM mixture and incubation was continued at 37°C.

To be able to determine the amount of background virus that could be expected with selection medium, controls were kept with uninfected cells and also with cells that were infected with the same concentration of wild-type virus but were not transfected.

2.2.9. Determination of cell-tolerance to mycophenolic acid (MPA) and the MPA-concentration required for virus-inhibition

Almost confluent monolayers of cells were prepared in 24-well Nunclone tissue-culture plates, cells seeded at the same concentration in all the wells. A series of different MPA-concentrations was prepared in medium containing 4% FCS. The cell-monolayers were rinsed three times with PBS and medium containing different MPA concentrations (Appendix A.10.) were added to the cells, each different MPA-concentration in duplicate.

The cells from a second plate were infected with a standard virus-concentration for an hour at 37°C. After removal of the virus-inoculum, media containing different MPA-concentrations was added as above.

Control wells with uninfected and infected cells were kept in duplicate to which medium without any MPA was added.

The cells were incubated at 37°C for several days (up to a week) during which time the cells were monitored daily by microscope for any signs of morphological changes. According to the data from this experiment the MPA-concentration which would inhibit untransformed virus without damaging the cells could be determined.

By harvesting the virus from transfected cells and passaging it on to new cells with fresh medium containing the pre-determined MPA-concentration for a few times, background untransfected virus should be eliminated to a fairly good extent.

2.2.10. Stains/assays of transfected cells

After transfection, the cells were stained at regular intervals in order to determine how successful the cells were transfected.

2.2.10.1. The X-gal stain

When the *E. coli lacZ* gene, which encodes the glycoside hydrolase, β-D-galactosidase (β-gal), was used as a reporter gene, a histochemical stain (MacGregor *et al.*, 1991) was used to detect β-gal activity in situ within cells in culture.

Subcellular localization of β-gal activity is facilitated by the deep blue colour generated by the hydrolysis of X-gal by β-gal (Section 2.1.7.5.).

The cell-layer was washed three times with PBS and fixed with 4% paraformaldehyde (Appendix A.14.) for 5 minutes at room temperature. The monolayer was covered with the substrate solution (Appendix A.14.) and left for half an hour to 18 hours at 37°C. Cells expressing β-gal would stain different grades of blue which can be visualized by the naked eye or by low-power microscopy (MacGregor *et al.*, 1991).

The pH of the system is buffered and sodium ions, which activate the β-gal enzyme, are provided by PBS. Potassium ferrocyanide and potassium ferricyanide enhance localization of the blue colour by increasing the rate of conversion of the indoxyl molecules to the indigo form. Magnesium ions act as co-factors for the enzyme and X-gal is the chromogenic indicator substrate.

This method can detect a single cell expressing β-gal within a population of non-expressing cells, although it is not as sensitive as immunocytological methods.

2.2.10.2. Assay for β-galactosidase activity in mammalian cell-extracts

The assay was done according to the method described in Sambrook *et al.* (1989), except that the volumes given were adapted for cells grown in a monolayer in a 35-mm tissue culture dish.

2.2.10.3. Indirect immunoperoxidase stain

The most sensitive and specific methods for the detection of the protein products of genes make use of antibodies. Immunohistochemistry examines individual cells which make it very suitable for analyzing transfection *in vitro.* The immunoperoxidase method is generally more sensitive than the immunofluorescence method, although the latter is easier to perform and the conjugated antibody is generally more stable and thus more reliable (Morris, 1991).

The cells were gently washed three times with PBS, fixed in cold acetone for 10 minutes at 4°C and air-dried. (The precipitating fixative, acetone, leaves the protein microenvironment maximally flexible and solubilizes or destroys the membrane barrier which allows penetration of the antibody. Cells can be stored air-dried at 4°C, but the degree of preservation of morphology might be diminished.)

The fixed cells were rinsed with PBS, 2% ovalbumin in PBS were added to the cells and blocking was performed at room temperature for 20 minutes or longer. The blocking solution was removed and the appropriate dilution of primary antibody, in PBS containing 2% ovalbumin, was added to the cells. The ovalbumin served as a carrier protein to stop adsorption of the very low concentration of antibody to the plastic of the tissue culture dish. Incubation was carried out for 2 hours at room temperature.

The cells were washed once with PBS containing 0.5% Tween 20 and twice with only PBS (5 minutes each) at room temperature. The appropriate conjugate-dilution, in PBS containing 2% ovalbumin, was added to the cells and incubation was performed at room temperature for 1 hour. The cells were again washed three times as described before.

A working solution of the substrate, diaminobenzidine tetrahydrochloride (0.5 mg DAB/ml, 0.03% H_2O_2 in PBS,filtered), was added to the cells and incubation was carried out for 3 to 10 minutes at room temperature until the desired degree of brown precipitate has formed or a general background staining started to develop. The reaction was stopped by washing with tap water.

The cell-nuclei were counterstained with haematoxylin to distinguish the cell-nuclei from the cytoplasm as follows: the cells were covered with the haematoxylin solution (Morris, 1991), left for 1 - 2 minutes and rinsed with tap water. The cells were mounted and viewed under the microscope.

2.2.11. Extraction of total DNA from infected cell-cultures

(Method adapted from Bailey and Possee, 1991.)

Almost confluent cells in tissue culture dishes (35-mm diameter) were infected with the presumed recombinant virus, which has been taken through several rounds of selection (at least five) after the transfection of cells. After one hour infection at 37°C, the virus inoculum was removed from the cells and selection medium was added. Incubation was continued for 24 hours at 37°C.

The medium was removed from the cells (about 10^6 cells/35-mm dish) and the cell-layer was rinsed three times with PBS. Lysis buffer (Appendix A.8.), 500 µl/dish, was added to the cells. Ribonuclease A was added to a final concentration of 40 µg/ml and incubation was carried out at 37°C for one hour. The lysate was transferred to an Eppendorf tube and Proteinase K was added to a final concentration of 100 µg/ml. Incubation was performed at 55°C for three to four hours or overnight at 42°C. Vigorous pipetting or shaking was avoided once the cells were lysed to prevent shearing of the DNA. The lysate was extracted twice with pre-warmed phenol (37°C) and once with chloroform:isoamyl (24:1). The DNA was precipitated by the addition of sodium acetate (to 0.3 M) and two volumes of absolute ethanol and incubated at -20°C overnight. Centrifugation was carried out at 4°C for 15 minutes at 11 600 g in a microfuge. The pellet was washed twice with cold 70% ethanol, dried and resuspended in ultra pure water at 4°C overnight.

CHAPTER 3. CONSTRUCTION OF LSDV-BASED REPORTER/SELECTABLE MARKER-GENE VECTORS AND THEIR TRANSFECTION INTO MAMMALIAN CELLS

CONTENTS

CHAPTER 3. CONSTRUCTION OF LSDV-BASED REPORTER/SELECTABLE MARKER-GENE VECTORS AND THEIR TRANSFECTION INTO MAMMALIAN CELLS

3.1. Introduction

3.1.1. Rationale for the construction of LSDV-based reporter/selectable marker-gene vectors

Lumpy skin disease virus (LSDV; as discussed in Chapter 1.3.) is a Capripoxvirus with a fairly narrow host range which includes sheep and cattle, but excludes humans (Davies, 1991). In South Africa LSDV has not been encountered in sheep even though the disease periodically occurred in cattle (personal communication - Dr. Anna-Lise Williamson). An attenuated strain has been widely used as a live vaccine for many years, and may prove to be a useful vector for the construction of recombinant veterinary vaccines for use in cattle and sheep (Davies, 1991; Kitching, Hammond and Taylor, 1986b; refer to Chapter 1.3.7. and 1.3.9.). Vaccinia virus (VV) has been used successfully as a vector in several recombinant vaccine trials, but has several disadvantages (discussed in Chapter 1.2.4.). One of the major disadvantages is the possible spread of the virus to non-vaccinated individuals due to the broad host-range of VV (Massung and Moyer, 1991), which make the construction of recombinant LSDV vaccines a desirable proposition.

Due to the low efficiency of the transfection process highly discriminatory selection procedures are required to isolate recombinants to be used as vaccines. The few commonly used selection systems rely on the use of mutant or normal recipient animal cell-lines. If the DNA, used for the transfection of cells, carries the wild type allele of a gene that occurs in a mutant form in the recipient cells, growth conditions that discriminate between the wild and the mutant type phenotype can be used to select for the desired transformants. For example, cells with a mutant thymidine kinase gene (TK^- cells) will only grow in the presence of thymidine and aminopterin if transformed by DNA containing a functional thymidine kinase gene. Aminopterin blocks the *de novo* thymidine monophosphate synthetic pathway (Fig. 3.1.), making cells dependent on the TK-gene to salvage free bases and nucleosides (thymidine) from the culture medium for the synthesis of DNA (Szybalski, 1992). Unfortunately there are no ovine or bovine TK^- cell-lines available, therefore this approach could not be applied.

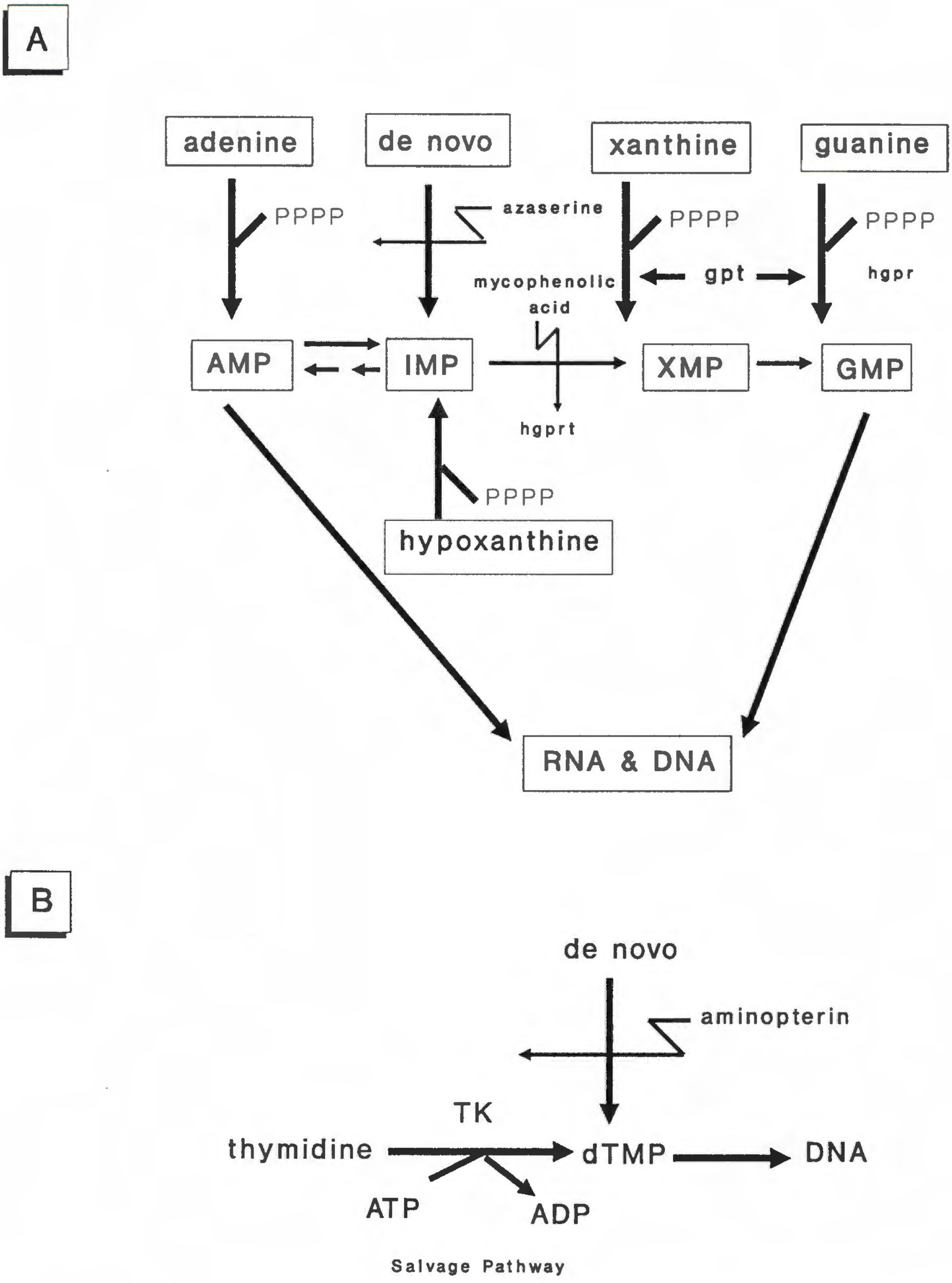


Fig. 3.1. Purine and pyrimidine salvage pathways.

A. Purine salvage pathway showing enzymes and inhibitors of interconversions (taken from Meuth and Harwood, 1991). Mycophenolic acid inhibits inosinate (IMP) dehydrogenase. This block can be overcome by the E.coli *Gpt* gene that encodes xanthine-guanine phosphoribosyltransferase. Xanthine is a poor substrate for the analogous animal enzyme hypoxanthine-guanine phosphoribosyl transferase (hgprt). PRPP is phosphoribosyl pyrophosphate.

B. The *de novo* and salvage pathways for deoxy-thymidine-5'-monophosphate (dTMP) synthesis. Aminopterin blocks *de novo* synthesis, making dTMP synthesis dependent on thymidine and thymidine kinase (TK).

A second approach relies on rendering virus multiplication in the presence of mycophenolic acid dependent on a dominant selectable transfected gene, like the *E. coli* xanthine-guanine phosphoribosyl transferase (*Gpt*) gene (Boyle and Coupar, 1988; Falkner and Moss, 1988) or by using a reporter gene of which the product can be visualized fairly easily, like the β-galactosidase (β-gal) gene (Chakrabarti, Brechling and Moss, 1985). The reporter gene should encode a product that is stable, innocuous to the cell in which it is being expressed, and readily detectable even in small quantities. The *Gpt*- and the β-gal-gene will be discussed in the following sections.

3.1.2. The xanthine-guanine phosphoribosyl transferase gene (*Gpt*-gene).

The functional dominant selectable *E. coli*-gene, *Gpt*, encodes xanthine-guanine phosphoribosyl transferase. Refer to Fig. 3.1. Cells are normally unable to synthesize guanylic acid (GMP) in a medium containing mycophenolic acid (MPA). MPA specifically inhibits inosinate (IMP) dehydrogenase preventing the formation of xanthosine monophosphate (XMP) in a manner similar to its action on VV (Boyle and Coupar, 1988). By supplying a *Gpt*-gene under the control of a suitable promoter (such as the P 7,5 promoter of VV) and xanthine, this block can be overcome. Xanthine is a poor substrate for the analogous animal enzyme, hypoxanthine-guanine phosphoribosyl-transferase, and is therefore not normally used by animal cells as a precursor to guanine nucleotides. Medium containing MPA and xanthine (without guanine) can therefore be used to selectively grow cells transformed with a functional *E. coli Gpt*-gene (Singer and Berg, 1991; Old and Primrose, 1989). By supplying guanine, this inhibition by MPA can be overcome. When the *Gpt*-gene is coupled in tandem with another gene of interest, it is possible to positively select for recombinant VV carrying both genes (Boyle and Coupar, 1988). The co-expression of *Gpt* and a gene of interest was demonstrated with the construction of recombinant VV's that expressed β-galactosidase (Falkner and Moss, 1988).

The *Gpt*-gene offers several advantages over other selectable markers which are the following:

- TK^- cell-lines are not required;
- it will operate in most if not all eukaryotic cell types and can thus be used for other host-specific poxviruses;
- it is ideally suitable for the construction of recombinants based on slow-growing host-specific poxviruses;
- it allows selection at both the recombination and subsequent steps which makes it possible to construct recombinants where the frequencies of

recombination may be low, for e.g. when inserting large fragments of foreign DNA (Boyle and Coupar, 1988);

- it provides one-step plaque isolation without the need for enrichment;
- no background of spontaneously occurring *Gpt*$^+$ virus has been observed (absence of spontaneously selectable mutants);
- MPA is nonmutagenic in the Ames test and in the related SOS test and this absence of mutagens should ensure virus stability;
- the *Gpt*-gene can be inserted into any non-essential region of the poxvirus genome as a dominant selectable marker-gene (Falkner and Moss, 1988).

3.1.3. The β-D-galactosidase gene (β-gal gene).

The *E. coli lacZ* gene (3081 bp) encodes the glycoside hydrolase, β-D-galactosidase (β-gal, refer to Chapter 2.1.7.5.). Commercially available antibodies to β-gal and a number of chromogenic and fluorogenic substrates and biochemical assays enable rapid and sensitive detection of β-gal. As little as 5 mol of β-gal can be detected in a single viable cell (MacGregor *et al.*, 1991). Falkner and Moss (1988) used plasmid constructs containing both the *Gpt*-gene and the *lacZ* gene to produce viral recombinants. The isolation of a stable, double VV recombinant containing both the Gpt- and the β-gal gene was also reported by Alexander, Moss and Fuerst (1992). All the plaques obtained under selective conditions exhibited β-gal activity, which indicated the coexpression of the selected marker gene (*Gpt*) and the gene of interest (*lacZ*). The *lacZ* gene activity is relatively independent of the orientation and of the neighbouring sequences in the virus (Falkner and Moss, 1988). Expression of the β-gal gene is a simple and reliable method for the identification of recombinant viruses and it is routinely used for the detection of VV recombinants (Rodriguez *et al.*, 1992).

3.1.4. Further requirements for the insertion of foreign genes into the LSDV genome

Some further requirements for the insertion of foreign genes into the VV genome, and thus also for the insertion of foreign genes into the LSDV genome, are the following:

- The flanking vaccinia sequences upstream of the foreign gene must contain cis-acting vaccinia or other modified transcription promoter signals so that the viral RNA polymerase can recognize and transcribe the foreign gene. The chimeric plasmids should thus be constructed so that the flanking DNA provides promoter and transcript leader sequences correctly upstream of the foreign gene (Esposito and Murphy, 1989; Piccini and Paoletti, 1988). The promoter from the VV gene encoding the 7.5 K polypeptide (referred to as P 7.5) is active early and late in poxvirus infection (Falkner and Moss, 1988). Therefore it is a very useful promoter for the expression of foreign genes in the early and late stages of infection. Evidence was reported that VV promoters work as general poxvirus promoters in that they can be controlled by LSDV trans-activating factors, Apparently non-poxvirus (heterologous) promoters are poorly transcribed by the vaccinia RNA polymerase. According to Boyle (1992) the difference in promoter function in different poxviruses may be due to small differences in promoter sequences from different poxviruses and the recognition of these sequences by the DNA-dependent RNA polymerase of the different viruses. Boyle (1992) found that there were differences in the recognition of promoters by VV and fowlpox virus (FPV). The FPV P E/L promoter expressed the highest level of product of any of the poxvirus promoters tested in FPV recombinants. The level of product expressed by the FPV P E/L promoter in VV recombinants was one-third to one-half the level expressed by the VV P L11 promoter and four to five times the level expressed by the VV P 7.5 promoter. The FPV P E/L promoter should thus be an ideal candidate for the expression of foreign genes in poxvirus recombinants (Boyle, 1992). However, Binns, Boursnell, Tomley and Campbell (1989) recommended that homologous promoters, i.e., FPV promoters in FPV vectors should be used in order to reduce the possibility of recombination occurring between different poxviruses in the environment.

- All of the sequences must be in the proper reading frame, with the stop codon appropriately followed by a poxvirus transcript terminator (in the case of early transcription). After insertion of the foreign gene into the virus genome by *in vivo* recombination, the expression of essential virus functions should not be interrupted (Esposito and Murphy, 1989; Piccini and Paoletti, 1988).
- Foreign genes must not contain introns because splicing does not occur in the cytoplasm (Moss, 1991).
- When early gene promoters are used, the cryptic termination sequences in foreign genes should be altered, because early poxvirus genes signal transcriptional termination 20 to 50 nucleotides downstream of TTTTTNT sequences in the noncoding strand (Moss, 1991). The poxvirus promoter sequences determine when and to what extent the foreign gene will be expressed. Presumably the highest amounts of protein would be obtained with late promoters, since the late poxvirus encoded proteins are the most abundant (Mackett *et al.*, 1985; Moss, 1991).

3.1.5. Proposed strategy for the insertion of foreign genes into LSDV.

3.1.5.1. Outline of general strategy

The initial phase of this project was to construct suitable LSDV-based reporter/selectable marker-gene vectors for the insertion of foreign genes into LSDV and to prove that foreign genes could be inserted successfully into the LSDV genome. This would then serve as a basis for the development of a LSDV-based recombinant vaccine.

To date very little is known about the molecular biology of LSDV. However, on the basis of morphological and physical properties, capripoxviruses are in the same family as orthopoxviruses, of which VV is the prototype. It was therefore decided to use the general approach for the insertion of foreign genes into VV as a model for the insertion of foreign genes into LSDV.

The proposed strategy for the insertion of foreign genes into LSDV is as follows (Fig. 3.2.): The isolated foreign gene of interest is cloned into a cloning vector so that non-essential LSDV sequences, for example sequences derived from the TK-gene (discussed in section 3.1.5.1.), will flank the foreign gene. These flanking LSDV sequences (approximately 100 to 1000 bp on either side of the foreign gene) are necessary to "direct" the insert to the homologous sequences present in the LSDV genome so that recombination can occur (Mackett, Smith and Moss, 1985). This construct will be introduced into tissue culture cells as follows: Cells will be infected with viable infectious LSDV, the recombinant plasmid containing the chimeric insert will then be transfected into the cells via standard procedures (Mackett *et al.*, 1985). Whole virus must be used because the DNA of poxviruses requires the enzymes and factors necessary for virus replication of the poxvirus core (Moss, 1991). In the cytoplasm, the DNA of the uncoated and replicating virus can come into contact with the DNA sequences of the recombinant plasmid and thus allow *in vivo* recombination to occur between the homologous sequences present on the viral genome and on the plasmid. The viral genome would presumably rescue the foreign gene by incorporating it into its own DNA. Replication of the recombinant DNA should then continue, followed by maturation and release of the recombinant virus (Mackett *et al.*, 1985; Sam and Dumbell, 1981).

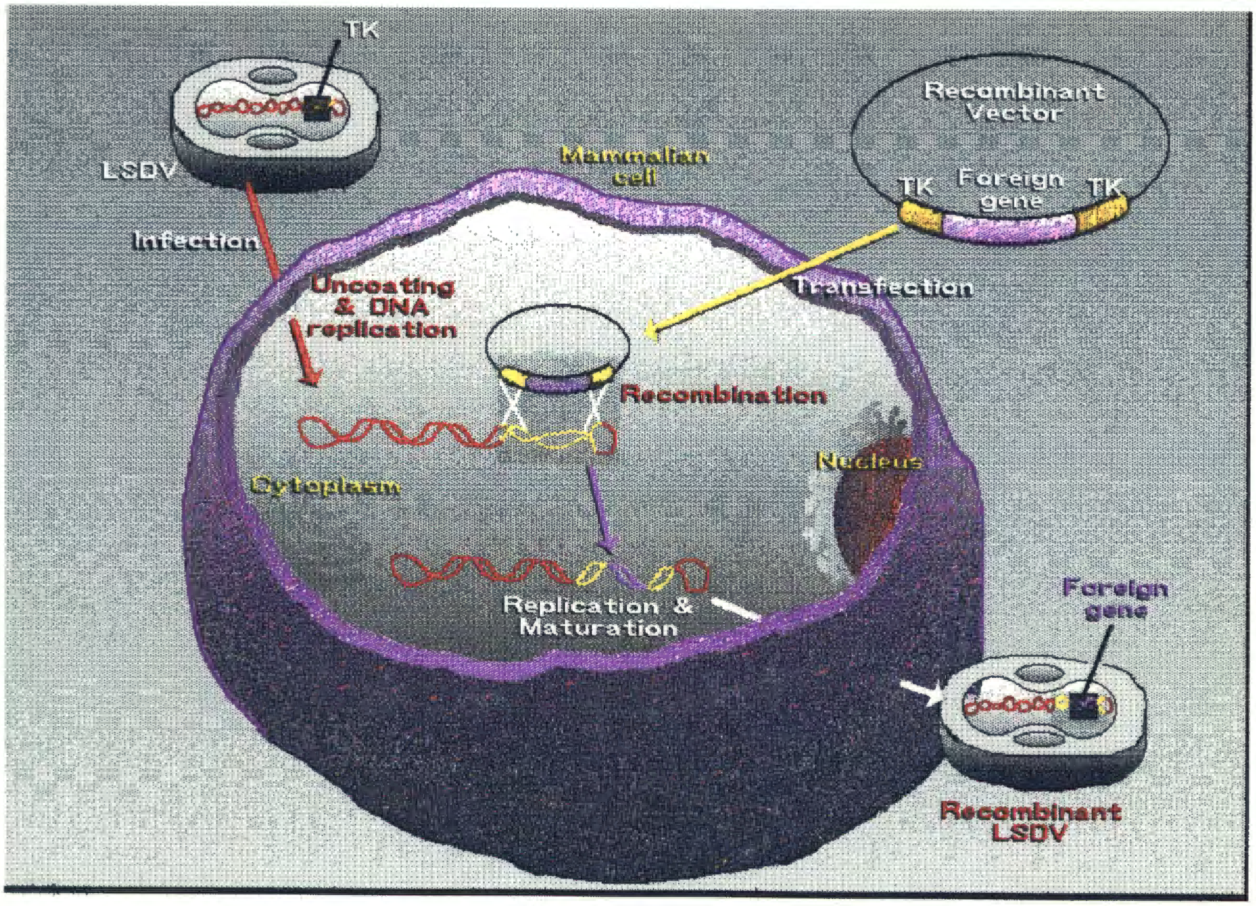


Fig. 3.2. Schematic representation of the general approach followed for the insertion of foreign genes into the lumpy skin disease virus (LSDV) genome. (Adapted from Mackett, Smith and Moss, 1985.)

3.1.5.2. Employing the thymidine kinase gene.

The thymidine kinase (TK) gene was exploited as a target-site for the insertion of foreign DNA into the LSDV genome in this chapter, as described in section 3.1.5. The enzyme TK catalyses the conversion of thymidine to thymidine-5'-monophosphate (TMP), an intermediate in pyrimidine biosynthesis (Meuth and Harwood, 1991; Singer and Berg, 1991). There are two synthetic pathways (Fig. 3.1.) for the production of deoxy-thymidine-5'-triphosphate (dTTP), a *de novo* synthetic pathway and a salvage pathway (Old and Primrose, 1989). The latter pathway requires TK. A loss of one of these two pathways is not lethal for the cells (Old and Primrose, 1989). Although the TK gene is not essential for poxvirus multiplication in tissue culture cells, inactivation of the gene causes significant attenuation of the virus although it does not seem to alter the replication efficiency (Gentry, 1992; Lee *et al.*, 1992; Moss, 1991; Taylor, Stott, Wertz and Ball, 1991).

Plasmid vectors were constructed to contain full length or partial copies of the TK-gene interrupted by multiple restriction endonuclease sites adjacent to a suitable poxvirus promoter. (The VV P 7.5 promoter is 30 times more efficient than the one associated with the TK-gene - Mackett, Smith and Moss, 1984.) A foreign gene can be inserted into any of these unique restriction sites. This plasmid can then be used to transfect cells that were pre-infected with poxvirus (Mackett, Smith and Moss, 1984). The TK-gene is present in all poxviruses studied so far (Gentry, 1992).

3.2. Materials and methods

3.2.1. Viruses, mammalian cells and eggs

The vaccine strain of LSDV was obtained from the Onderstepoort Veterinary Institute (OVI) at Pretoria. LSDV was propagated and maintained in Madin-Darby bovine kidney (MDBK) cells (Chapter 2.2.6.). Titrations were done according to the standard procedure (Chapter 2.2.7.) on foetal calf kidney (CFK) and MDBK cells. Virus propagation was also attempted on the CAM's of eggs of domestic hens according to the methods described in Chapter 2.2.1.

Primary LT and CFK cells were prepared and stocks were frozen away as described in Chapter 2.2.2 to 2.2.5. The lamb testis and foetal calf kidney used for this purpose was aseptically removed at the abattoirs in Maitland, Cape Town. The continuous MDBK cell-line is derived from a healthy bovine (*Bos taurus*) kidney (ATCC catalogue, ref. CCL 22) and was obtained from Dr. A. van Dijk, OVI, Pretoria. Fertilized eggs of domestic hens (*Gallus domesticus*) were supplied by the Golden Grove Poultry Farm, Cape Town.

3.2.2. Genes, vectors and host bacterial cells

The plasmid, p182, containing the *E. coli lacZ* (β-gal) gene under the control of the 7.5 K vaccinia promoter was provided by Dr. M. Mackett, Paterson Laboratories, Christie Hospital & Holt Radium Institute. Dr. Mackett also supplied the plasmids pox-48B, containing the β-gal gene under the control of the 4B promoter of VV, and pal-1, containing the β-gal gene under the control of the 11 K promoter of VV, used as controls in the β-galactosidase assay. The plasmid pGpt07/14 containing the *E. coli Gpt*-gene linked to the 7.5 K promoter (Boyle and Coupar, 1988) was obtained from Dr. D.B. Boyle, CSIRO, Australian Animal Health Laboratory, Geelong, Victoria, Australia.

Competent *E. coli* JM109-cells were prepared as described in Chapter 2.1.7.3. The competent *E. coli* LK111 cells were supplied by the Department of Medical Microbiology, Medical School, UCT. The pMTL25 vector was obtained from the Microbiology Technology Laboratory, Public Health Laboratory Service, Salisbury, UK. The pMTL25 cloning vector which was used in the sub-cloning steps has the β-lactamase (amp^R) gene, which allows selection of the plasmid in medium containing ampicillin, and a palindromic multiple cloning site within the β-galactosidase gene (Chambers et.al., 1988). Potential recombinant clones can be identified as white colonies on LB-agar plates containing X-gal, due to insertional inactivation of the β-gal gene of pMTL25 (Chapter 2.1.7.5.). The pMTL vectors are analogous to the high-copy-number pUC plasmids except that they are smaller by 300 bp, carry more versatile polylinker regions within the *lacZ* gene and exhibit improved plasmid stability (Chambers et.al., 1988). Dr. Anna-Lise Williamson of the Department of Medical Microbiology, Medical School, UCT, supplied the pLSDTK vector.

3.2.3. General approach for the insertion of foreign genes into lumpy skin disease virus

The pLSDTK-vector was constructed by the cloning of a 2.5 kb *Hind111*-fragment, containing the TK gene from LSDV, into the plasmid pEcoR252 by Dr. A-L. Williamson (personal communication). The *Kpn1* site within the TK gene of the pLSDTK-vector was utilized to make two further constructs into which either a β-gal or a *Gpt* gene, linked to a poxvirus promoter, was cloned. As mentioned in section 3.1.5., the TK sequences flanking the cloned gene were necessary for homologous insertional recombination with the TK gene resident in the native LSDV. These recombinant plasmids were transfected into cells infected with LSDV. The progeny were screened for *in vivo* LSDV recombinants which expressed β-gal activity or which could be positively selected for in the presence of mycophenolic acid.

3.2.4. Construction of recombinant plasmids containing the β-gal gene

The sub-cloning of the β-gal gene into pMTL25 and the subsequent cloning thereof into pLSDTK is schematically depicted in Figs. 3.3a. and 3.3b. The cassette consisting of the E. *coli* β-gal-gene, driven by the vaccinia P 7.5 promoter, was excised from the original plasmid, p182, with *EcoR1* and gel purified (Chapter 2.1.4 - 2.1.6.). The pMTL25 vector was cut with *EcoR1* and after being checked for complete digestion the enzyme was removed by phenol/chloroform extraction and ethanol precipitation (Chapter 2.1.2.-2.1.5.).

The β-gal containing *EcoR1* fragment was subsequently ligated into the *EcoR1* site of the pMTL25 plasmid (Chapter 2.1.7.2.). An equimolar ratio of vector and insert was used in the ligation reaction. Transformation of the ligation mixture into competent *E. coli* JM109 cells and screening for recombinants were performed as described in Chapter 2.1.7.4 and 2.1.7.5. Identification of the colonies harbouring the recombinant plasmid were done by restriction enzyme digestion with *EcoR1* of small-scale DNA preparations. Stock cultures of the bacterial cells harbouring the recombinant plasmid were frozen away (Chapter 2.1.7.6.). A large-scale plasmid preparations was performed on one of these identified colonies as described in Chapter 2.1.1.2. This construct was referred to as pMTL25-β-gal.

The pLSDTK plasmid was cut with *Kpn1*, phenol-chloroform extracted twice, ethanol precipitated and resuspended in ultra-pure water. The pMTL25-β-gal construct was digested with *Kpn1* and the *Kpn1*-fragment, containing the β-gal gene under the control of the VV P 7.5 promoter, was gel-purified (Chapter 2.1.4.-2.1.6.).

The β-gal containing *Kpn1* fragment was ligated into the resident *Kpn1* site inside the TK-gene of the pLSDTK plasmid (Chapter 2.1.7.2.). A colony blot was made and screened for recombinant colonies by probing with a DIG-labelled β-gal gene as described in Chapter 2.1.7.5., 2.1.8.2. and 2.1.8.3 (Fig. 3.4.). The washed membrane was subjected to immunological detection. Colonies hybridizing to the β-gal probe, were identified by coloured precipitate formation on the Hybond N$^+$. Small scale DNA preparations of identified colonies were checked for the presence of the insert by digestion with *Kpn1* followed by agarose-gel electrophoresis. The orientation of the β-gal gene in the plasmid was not checked. Stock cultures of the bacterial cells harbouring the recombinant plasmid were frozen away (Chapter 2.1.7.6.). One of the recombinants was selected for large scale DNA plasmid preparation (Chapter 2.1.1.2.). This plasmid, consisting of the pLSDTK vector harbouring the β-gal gene insert, was designated pLSD-β-gal.

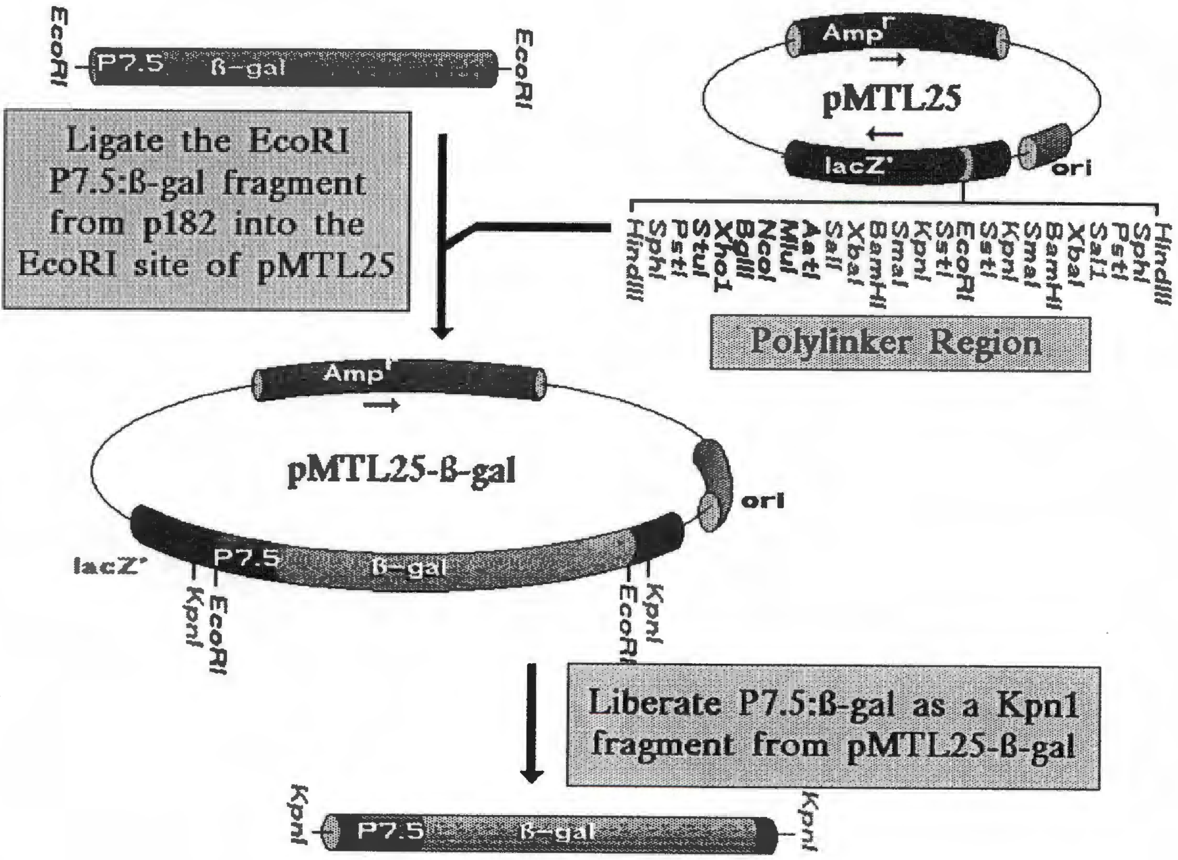


Fig. 3.3a. Schematic representation of the cloning of the *EcoR1*-fragment, containing the β-gal gene under the control of the VV P 7.5 promoter, into the pMTL25-vector (refer to Fig. 3.3b.).

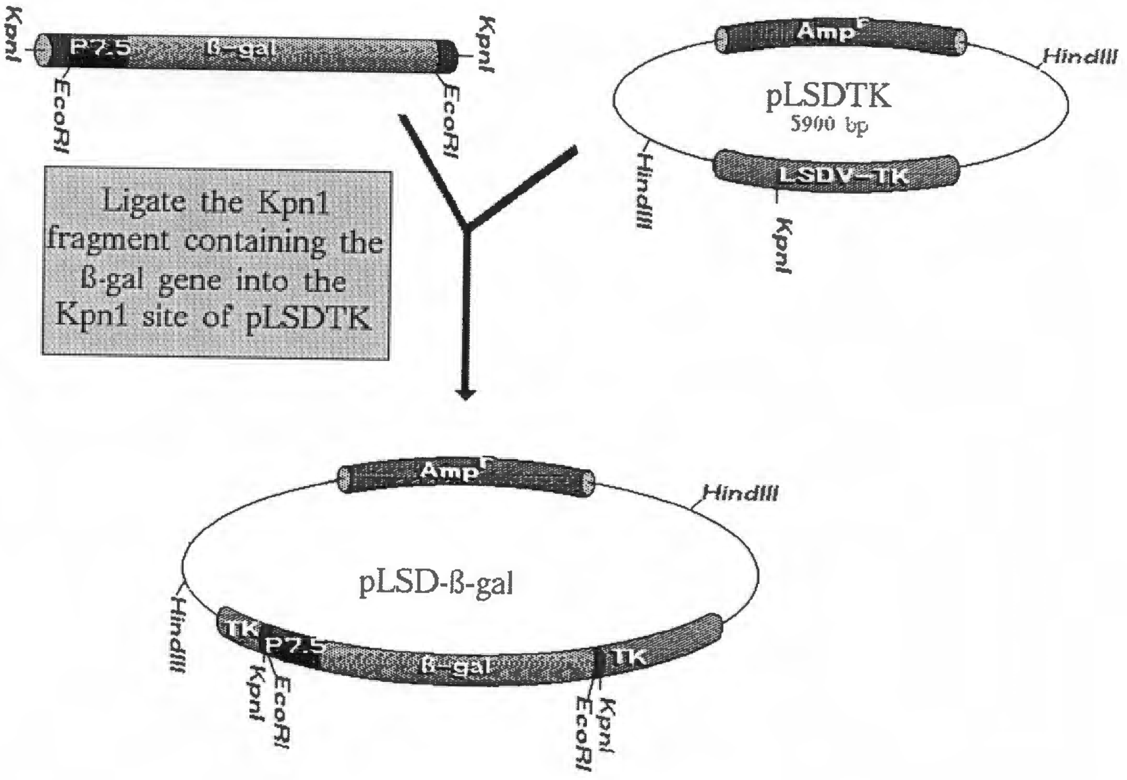


Fig. 3.3b. Schematic representation of the cloning of the *Kpn1*-fragment, containing the b-gal gene under the control of the VV P 7.5 promoter (isolated from the pMTL25-β-gal construct), into the pLSDTK-vector.

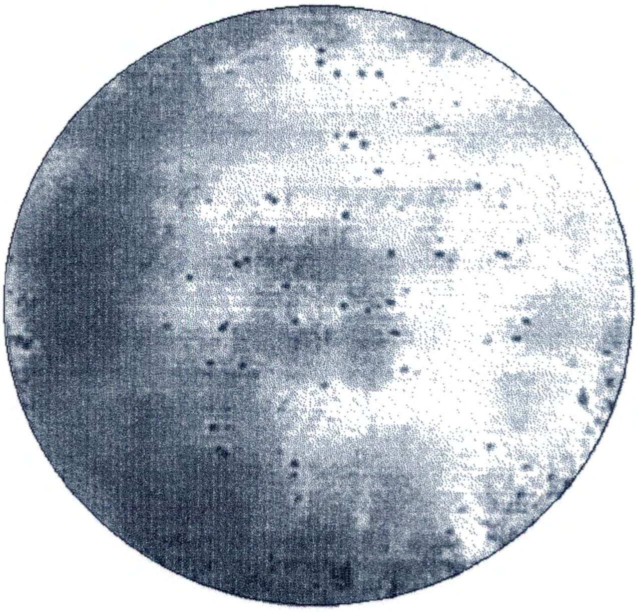


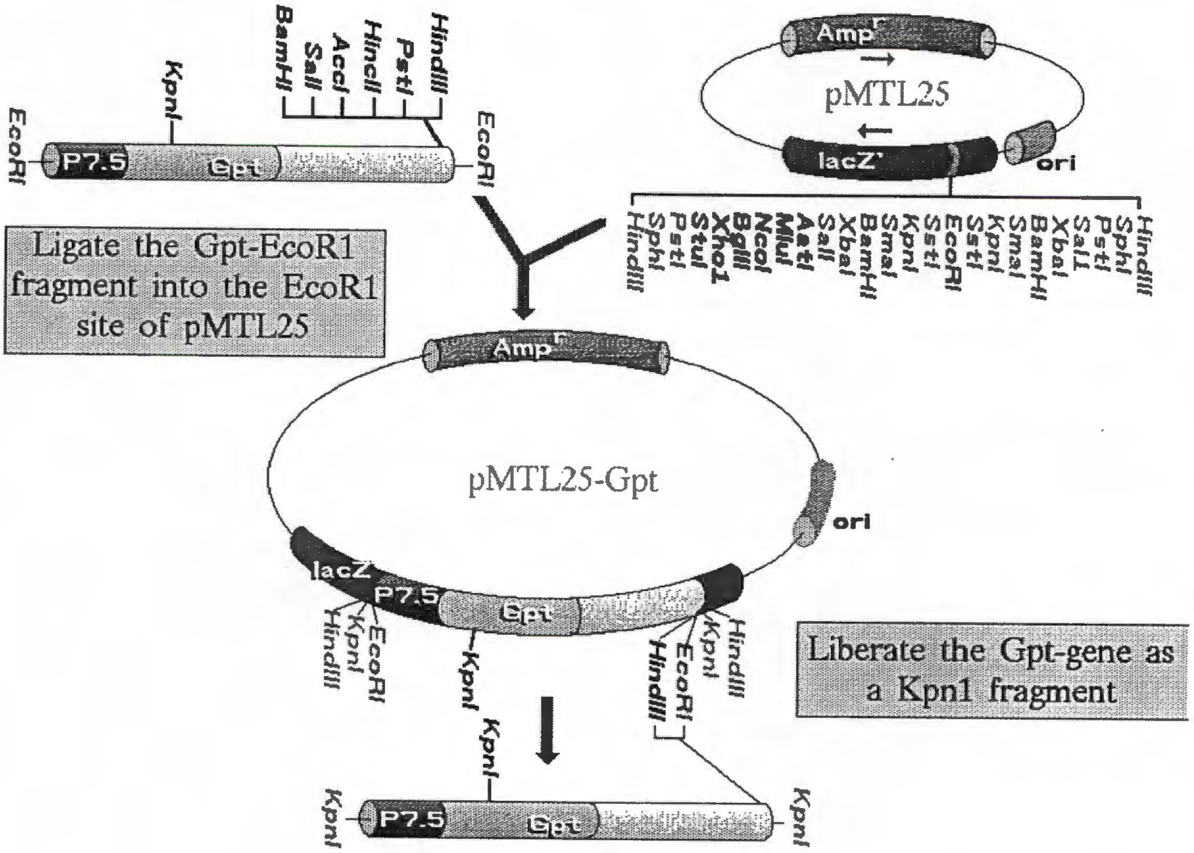
Fig. 3.4. Colony blot for detection of bacterial colonies harbouring the pLSD-β-gal construct by hybridization to a DIG-labelled β-gal gene-containing fragment.

3.2.5. Construction of recombinant plasmids containing the *Gpt*-gene

The cloning of the fragment, containing the Gpt-gene under the control of the VV P 7.5 promoter, into the various plasmids is schematically depicted in Figs. 3.5a. and 3.5b. The cassette containing the E. coli *Gpt*-gene, linked to the VV P 7.5 promoter, was excised from the pGpt07/14 plasmid (Boyle and Coupar, 1988) with *EcoR1*. The *Gpt* containing fragment was isolated and purified by agarose-gel electrophoresis (Chapter 2.1.4. - 2.1.6.). The pMTL25 vector was linearized with *EcoR1* and prepared as described for the construction of the β-gal containing vector (Section 3.2.4.).

The *Gpt*-gene containing *EcoR1*-fragment was subcloned into the *EcoR1* site of the pMTL25 vector (Chapter 2.1.7.2., 2.1.7.4. and 2.1.7.5.). The insert to vector ratio used for the ligation reaction was 3:1 and competent *E. coli* JM109 cells were used for the transformation reaction. Small scale DNA preparations from white colonies (see blue- and white selection, Chapter 2.1.7.5.) were digested with *EcoR1* and agarose-gel electrophoresis was performed with appropriate markers to verify the presence of the *Gpt*-gene. Stock cultures were prepared and frozen away of colonies harbouring the recombinant plasmid (Chapter 2.1.7.6.). A large scale DNA preparation was performed on one of these colonies (Chapter 2.1.1.2.). This recombinant plasmid, with the *Gpt*-gene fragment cloned into the *EcoR1* site of the pMTL25 vector, was designated pMTL25-*Gpt*.

Due to the presence of an internal *Kpn1* site within the Gpt-gene, the pMTL25-*Gpt* construct was partially digested with *Kpn1* and the ca. 2 kb fragment containing the intact *Gpt*-gene, linked to the P 7.5 promoter, was isolated and purified as described in Chapter 2.1.4. - 2.1.6. The pLSDTK vector was linearized with *Kpn1* and prepared for ligation as described for the construction of the β-gal construct (Section 3.2.4.). The isolated *Gpt* containing *Kpn1*-fragment was ligated into the *Kpn1* site of the pLSDTK vector (Chapter 2.1.7.2.). The insert to vector ratio used for the ligation reaction was 4:1. Competent *E. coli* JM109 cells were used for the transformation reaction (Chapter 2.1.7.4.). Screening for recombinants were performed by small scale DNA preparations on randomly picked colonies (Chapter 2.1.1.1.1. and 2.1.7.5.). The DNA was checked for the presence of the *Gpt*-gene by digestion with *Kpn1* and agarose gel-electrophoresis. Glycerol stock cultures were prepared of colonies harbouring recombinant plasmids (2.1.7.6.). A large scale plasmid preparation was performed on one of these identified recombinant colonies (Chapter 2.1.1.2.). This recombinant plasmid, consisting of the pLSDTK vector with the *Gpt*-gene containing fragment cloned into the *Kpn1* site, was designated pLSD-*Gpt*.



Ligate the Gpt-EcoR1 fragment into the EcoR1 site of pMTL25

Liberate the Gpt-gene as a Kpn1 fragment

Fig. 3.5a. Schematic representation of the cloning of the *EcoR1*-fragment, comprising the *Gpt*-gene under the control of the VV P 7.5 promoter, into the pMTL25-vector (refer to Fig. 3.5b.).

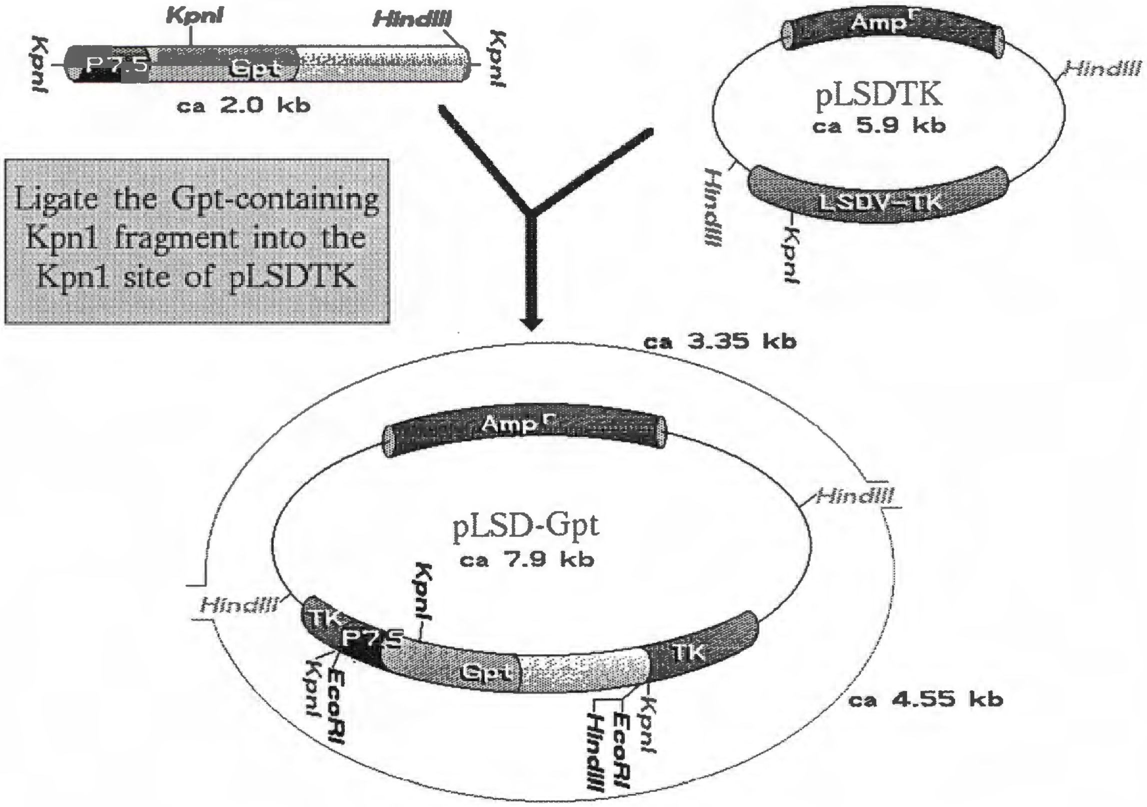


Fig. 3.5b. Schematic representation of the cloning of the *Kpn1*-fragment, containing the *Gpt*-gene under the control of the VV P 7.5 promoter (isolated from the pMTL25-*Gpt* construct), into the pLSDTK-vector.

3.2.6. Titration of MPA to determine the concentration needed for the inhibition of LSDV-growth

The tolerance of CFK-cells to different concentrations of MPA, and the concentration of MPA required to inhibit LSDV multiplication in CFK cells, were determined as described in Chapter 2.2.9.

The range of MPA concentrations tested on the uninfected cells and the virus-infected cells varied from 0.25 μg MPA/ml to 50 μg MPA/ml. For the virus-inhibition assay cells were infected with 1 PFU (plaque forming unit) of LSDV/cell before the addition of the MPA-containing medium.

3.2.7. Transfection of pLSD-β-gal into LSDV-infected LT-cells.

Infections and transfections of LT cells were performed as described in Chapter 2.2.8. with the following modifications. Monolayers of LT-cells in 35 mm tissue culture dishes that were 80% confluent, were infected with 0.1 PFU of LSDV per cell. The amount of pLSD-β-gal DNA (Section 3.2.4.) used for the transfection reaction was 2 μg/35 mm tissue culture dish. Incubation of the virus-infected cells with the Lipofectin^{TM} reagent-DNA complex was performed for 5 hours at 37°C. Control cells (cells infected with 0.1 PFU of LSDV per cell, and uninfected cells) were treated exactly the same as the cells that were transfected. After incubation for three days with DMEM supplemented with 4% FCS and PSN (Chapter 2.2.8.), the medium was removed from the cells and the X-gal stain was performed *in vivo* as described in Chapter 2.2.10.1.

A second additional assay for β-galactosidase activity was performed on cell-extracts as described in Chapter 2.2.10.2. Monolayers of CFK-cells in 35 mm tissue culture dishes that were almost confluent, were infected with LSDV at a concentration of 1 PFU/cell for 1 hour at 37°C. A tissue culture dish with uninfected cells was kept as a negative control, together with one of the dishes with LSDV infected cells. Three separate transfections were put up simultaneously with 1.7 μg of DNA of each of the following plasmids: pLSD-β-gal, pal-1 and pox-48B. The transfections with pal-1 and pox-48B were done as positive controls. The transfections were done according to the protocol described in Chapter 2.2.8., except for the following modifications. The DNA and 20 μl of Lipofectin^{TM} reagent were diluted separately for each transfection reaction, each with 250 μl of opti-MEM (Gibco/BRL). These dilutions were added together and left at room temperature for 15 minutes before adding to the cells. After 5 hours at 37°C, the DNA-containing opti-MEM was removed from the cells and replaced with DMEM, containing 4% FCS and PSN. After 48 hours incubation at 37°C, the β-gal assay (Chapter 2.2.10.2.) was performed on the cell-extracts. The reactions were incubated at 37°C for 2 hours and observed for colour development. The optical densities were spectrophotometrically determined with a Beckman DU-40 spectrophotometer (Beckman, Irvine, USA) at a wavelength of 420 nm.

3.2.8. Transfection of pLSD-Gpt into LSDV-infected CFK-cells.

Infection and transfection of CFK-cells were performed as described in Chapter 2.2.8., except for the following modifications. Almost confluent monolayers of CFK-cells in 35 mm tissue culture dishes, were infected with 0.1 PFU of LSDV per cell and transfected with 2 μg of pLSD-*Gpt* DNA (Section 3.2.5.) per dish. The cells were incubated for 5 hours at 37°C with the Lipofectin™ reagent-DNA complex. The DNA-containing opti-MEM was removed from the cells and DMEM, supplemented with 4% FCS and PSN, was added to the cells. After 24 hours the medium was replaced with selection medium (Appendix A.10.) containing 2.5 μg MPA/ml and incubation was continued for 48 hours. Following this the medium was removed from the cells and used for the infection of new, almost confluent cells. After 1 hour infection, selection medium was added to the newly infected cells and incubation was carried out at 37°C for three days. The control cells (cells infected with LSDV but incubated with/without MPA-containing selection-medium) were treated in exactly the same way. An *in vivo* indirect immunoperoxidase stain (Chapter 2.2.10.3.), using sera harvested from cattle previously infected with LSDV, was performed on the virus-infected cells, as well as the control cells, at passage no. 6 after 3 days incubation with/without selection medium.

3.3. Results

3.3.1. Virus propagation

Primary LT- and CFK-cells were successfully established and stocks were frozen away at -70°C. LSDV, which was propagated in MDBK-cells, was harvested at the third day after infection. MDBK-cells were used for the propagation of LSDV because primary cell-lines were still being established at the time. As mentioned before (Chapter 2.2.7.), LSDV does not form plaques but causes swelling of cells which forms clumps. It was assumed that a focus of cells, just as a plaque, was caused by one virus-particle or plaque-forming unit (PFU). Many virus foci or cell-clumps could be seen in the MDBK cell-monolayer (in contrast to uninfected cells) at the third day after infection. The LSDV titre obtained (of the virus-stock used in the transfection-reaction) was 3×10^6 PFU/ml when CFK cells were used for the titration-reaction. A titre of 1×10^6 PFU/ml was obtained with the same virus stock when MDBK-cells was used for the titration. The titre obtained with the CFK-cells was used for the calculation of the virus-concentration required for the transfection-experiment. The attempt to propagate

LSDV on the CAM's of domestic hens' eggs was unsuccessful. Small pox-like lesions could be seen on the CAM's when it was harvested, but no virus could be isolated. The pox-like lesions could have been due to other unknown causes.

3.3.2. Construction of recombinant plasmids

3.3.2.1. Construction of the pLSD-β-gal plasmid

The original plasmid, p182, containing the β-gal gene under the control of the VV P 7.5 promoter, digested with *EcoR1* is shown in Fig. 3.6. lane 2. (The expected fragment sizes are indicated in Table 3.1.).

The lower band, comprising the β-gal gene linked to the P 7.5 promoter, was isolated and purified and this *EcoR1*-fragment can be seen in lane 1 of the same figure. The β-gal-containing *EcoR1*-fragment was subcloned into the unique *EcoR1* site of the pMTL25-vector. The *EcoR1* linearized pMTL25-vector is shown in Fig. 3.6. lane 4. An *EcoR1* digest of the aforementioned clone is shown in Fig. 3.6 lane 5. Two bands, one corresponding to the size of the linearized pMTL25 vector (ca. 2.8 kb) and the other corresponding to the size of the β-gal containing insert (ca. 5.3 kb) were obtained. This result served to verify that the gel-purified *EcoR1*-fragment, encompassing the β-gal gene linked to the P 7.5 promoter, was cloned successfully into the *EcoR1* site of the pMTL25 vector. This construct, designated pMTL25-β-gal (Figure 3.3a.), was digested with *Kpn1* (Fig. 3.6. lane 6) and the top band encompassing the β-gal gene linked to the P 7.5 promoter, was isolated and purified. The pLSDTK-vector was linearized with *Kpn1* (Fig. 3.6 lane 7). The gel-purified β-gal containing *Kpn1*-fragment was then cloned into the unique *Kpn1* site of the pLSDTK vector. *Kpn1* digested DNA of this clone obtained is shown in lane 8 of the same figure. Two bands, one corresponding to the size of the linearized pLSDTK vector (ca. 5.9 kb) and the other band corresponding to the size of the β-gal containing *Kpn1*-fragment (ca. 5.3 kb) were evident. This served to confirm the successful cloning of the *Kpn1*-fragment, comprising the β-gal gene linked to the P 7.5 promoter, into the unique *Kpn1* site of the pLSDTK-vector. The construct was designated pLSD-β-gal (Fig. 3.3b.). *Hind111* digestion of the pLSD-β-gal plasmid (Fig. 3.6 lane 9) resulted (as expected) in two bands of sizes ca. 3.3 kb and ca.7.8 kb respectively, which served as further confirmation of the integrity of this construct.

TABLE 3.1. Expected fragment sizes of plasmid constructs digested with different restriction enzymes.

Plasmid construct	Restriction enzyme(s)	Expected fragment size(s) (bp)#
pMTL25	*EcoR1*	2800
	Kpn1	2800
pLSDTK	*Kpn1*	5900
p182	*EcoR1*	7600 + 5300
pMTL25-β-gal	*EcoR1*	5300 + 2800
	Kpn1	5300 + 2800
pLSD-β-gal	*Kpn1*	5900 + 5300
	Hind111	3350 + 7850
pGpt07/14	*EcoR1*	4300 + 2000
pMTL25-Gpt	*EcoR1*	2800 + 2000
	Kpn1	2800 + 1500 + 500
pLSD-Gpt	*Kpn1*	5900 + 1500 + 500
	Hind111	3350 + 2275 + 2275

Fragment sizes shown were estimated by restriction mapping

LEGENDS

Fig. 3.6. Gel-electrophoresis of DNA employed in the construction of pLSD-β-gal. **Lane 1**, *EcoR1*-fragment, containing the β-gal gene under the control of the VV P 7.5 promoter, isolated from p182. **Lane 2**, p182 digested with *EcoR1*. **Lane3**, λ DNA digested with *Styl*. **Lane 4**, pMTL25 digested with *EcoR1*. **Lane 5**, pMTL25-β-gal digested with *EcoR1*. **Lane 6**, pMTL25-β-gal digested with *Kpn1*. **Lane 7**, pLSDTK digested with *Kpn1*. **Lane 8**, pLSD-β-gal digested with *Kpn1*. **Lane 9**, pLSD-β-gal digested with *Hind111*. **Lane 10**, λ DNA digested with *Hind111*.

Fig. 3.7. Gel-electrophoresis of DNA employed in the construction of pLSD-Gpt.

(a) **Lane 1**, λ DNA digested with *Styl*. **Lane 2**, Original plasmid, containing the *Gpt*-gene under the control of the VV P 7.5 promoter, digested with *EcoR1*. **Lane 3**, *EcoR1* fragment isolated from the original plasmid and containing the *Gpt*-gene under the control of the VV P 7.5 promoter. **Lane 4**, pMTL25 digested with *EcoR1*. **Lane 5**, pMTL25-*Gpt* digested with *EcoR1*. **Lane 6**, pMTL25-*Gpt* digested with *Kpn1*. **Lane 7**, pLSDTK digested with *Kpn1*. **Lane 8**, λ DNA digested with *Hind111*.

(b) **Lane 1**, λ DNA digested with *Styl*. **Lane 2**, pLSD-*Gpt* digested with *Hind111*. **Lane 3**, pLSD-*Gpt* digested with *Kpn1*.

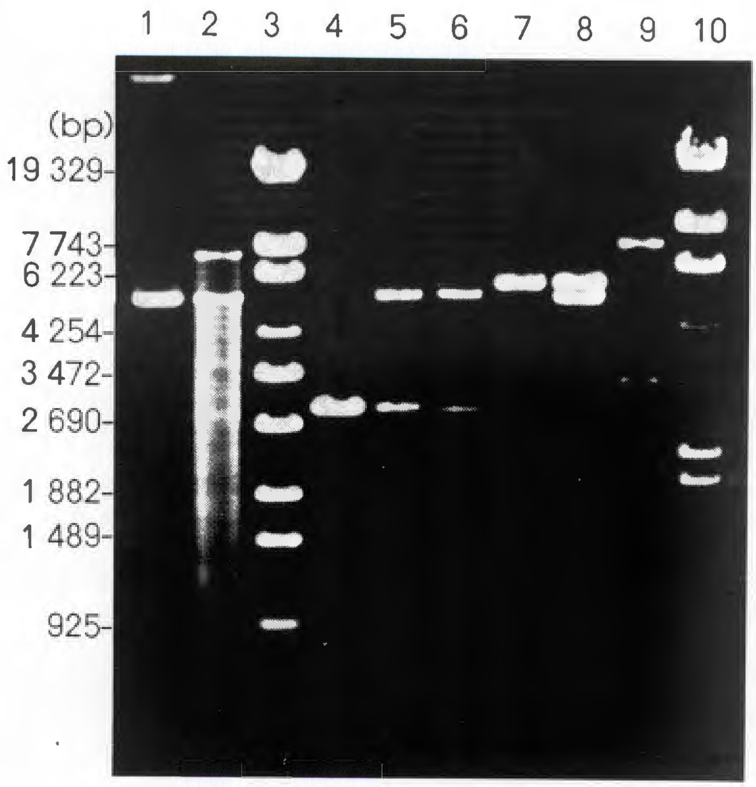


Fig. 3.6.

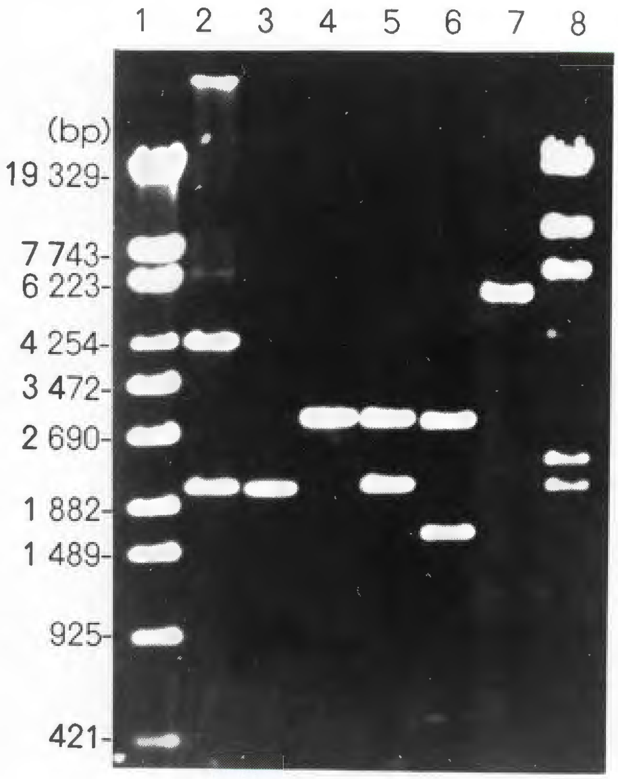


Fig. 3.7a.

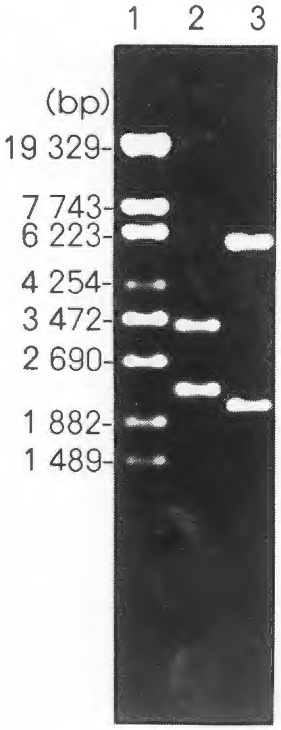


Fig. 3.7b.

3.3.2.2. Construction of the pLSD-*Gpt* plasmid

EcoR1-digestion of the pGpt07/14 plasmid containing the *Gpt*-gene under the control of the VV P 7.5 promoter (Boyle and Coupar, 1988), resulted in a ca. 4.3 kb band representing the native plasmid and a ca. 2 kb fragment (comprising the *Gpt*-gene linked to the VV P 7.5 promoter - Fig. 3.7a. lane 2). This ca. 2 kb-fragment was gel-purified (Fig. 3.7a. lane 3) and sub-cloned into the unique *EcoR1* site of the pMTL25-vector. (In Fig.3.7a. lane 4 the *EcoR1*-linearized pMTL25-vector can be seen as a ca. 2.8 kb fragment). *EcoR1*-digestion of the pMTL25-*Gpt* construct (Fig. 3.5a.) resulted in a ca. 2.8 kb fragment representing the native pMTL25-plasmid and a ca. 2 kb *Gpt*-containing fragment (Fig. 3.7a. lane 5). Due to the presence of an internal *Kpn1* site within the *Gpt*-gene, the pMTL25-*Gpt* plasmid (Fig. 3.5a.) was partially digested with *Kpn1* and the ca. 2 kb fragment (comprising the intact *Gpt*-gene linked to the P 7.5 promoter) was gel-purified. In Fig. 3.7a. lane 6 the pMTL25-*Gpt* plasmid digested with *Kpn1* can be seen as a ca. 2.8 kb fragment representing the native pMTL25-plasmid and two additional fragments of ca. 0.5 kb and 1.5 kb respectively. In Fig. 3.7a. lane 7 the *Kpn1* linearized pLSDTK-vector is shown as a ca. 5.9 kb fragment.

The ca. 2 kb *Gpt*-containing *Kpn1*-fragment was cloned into the unique *Kpn1* site within the pLSDTK-vector. The integrity of the resulting pLSD-*Gpt* plasmid construct was determined by restriction enzyme analysis. The pLSD-*Gpt* plasmid (Fig. 3.5b.) digested with *Kpn1* (Fig. 3.7b. lane 3) showed two bands, one corresponding to the size of the ca. 5.9 kb linearized pLSDTK-vector and the other band corresponding to the size of the intact (ca. 2 kb) *Gpt*-containing fragment. This verified the presence of the 2 kb *Gpt*-containing fragment in the pLSDTK-vector. However, it appeared as if the *Gpt*-gene within this construct had suffered a mutation resulting in the loss of the internal *Kpn1* site. Digestion of the pLSD-*Gpt* plasmid construct with *Hind111* yielded fragments of the expected sizes as follows: a ca. 3.35 kb fragment representing the native pEcoR252 plasmid used in the construction of the pLSDTK vector, and a doublet band consisting of two fragments of approximately 2.27 kb each (Fig. 3.7b. lane 2). Although the *Gpt*-containing fragment flanked by the TK sequences (Fig. 3.5b) would be expected to yield a 4.55 kb fragment, this fragment is essentially cleaved in half by *HindIII* due to the presence of a *HindIII* site immediately downstream of the *Gpt* fragment. The latter *HindIII* site is derived from the original pGpt07/14 construct (Boyle and Coupar, 1988).

3.3.3. Transfection of pLSD-β-gal into LSDV-infected LT cells

When performing the X-gal stain *in situ* on LT-cells infected with wild-type LSDV and transfected with the pLSD-β-gal construct, blue-gray foci developed in the cells indicating the presence of β-galactosidase activity (Fig. 3.8a.). Slight blue foci developed in the control cells (LSDV-infected and uninfected cells). Refer to Figs. 3.8b. and 3.8c. However, the cells had to be left for a relatively long period of time (ca. 12 - 24 hours at room temperature) with the substrate solution (refer to the protocol in Chapter 2.2.10.1.) to obtain a clear difference between the positives and the negatives. Therefore it was decided to perform a second assay to quantitate the expression of β-galactosidase from the P 7.5 promoter and also to compare the expression of the gene to other genes in order to show that the gene in pLSD-B-gal is active. This second assay with a soluble substrate was a transient expression assay designed to test VV promoter activity in LSDV.

During the second assay of cell-extracts for β-galactosidase activity, a yellow colour developed in reactions with extracts from cells infected with LSDV and transfected with pLSD-β-gal or pox-48B after a few minutes. The enzyme β-galactosidase hydrolysis the substrate, *o*- nitrophenol-β-*D*-galactosidase (ONPG), to yield *o*-nitrophenol and galactose (MacGregor *et al.*, 1991).

The optical density of *o*-nitrophenol can be determined spectrophotometrically. The reaction with the extracts of cells infected with LSDV and transfected with pal-1 formed an immediate intense yellow colour. No yellow colour development could be observed with the negative controls (extracts from uninfected and LSDV infected cells). This assay was done in duplicate. All the reactions were incubated for 2 hours at 37°C, during which time no further change in colour could be observed. The optical densities of the reactions were read at a wavelenght of 420 nm after the addition of Na_2CO_3. The values obtained were as indicated in Table 3.2.

According to the protocol the linear range for β-galactosidase activity is 0.2 - 0.8 OD_{420nm} (Sambrook *et al.*, 1989). Therefore the β-gal gene in the pLSD-β-gal construct appeared to be functional. However, a much higher level of β-galactosidase activity was observed with cell-extracts from LSDV-infected CFK-cells transfected with the pal-1 construct.

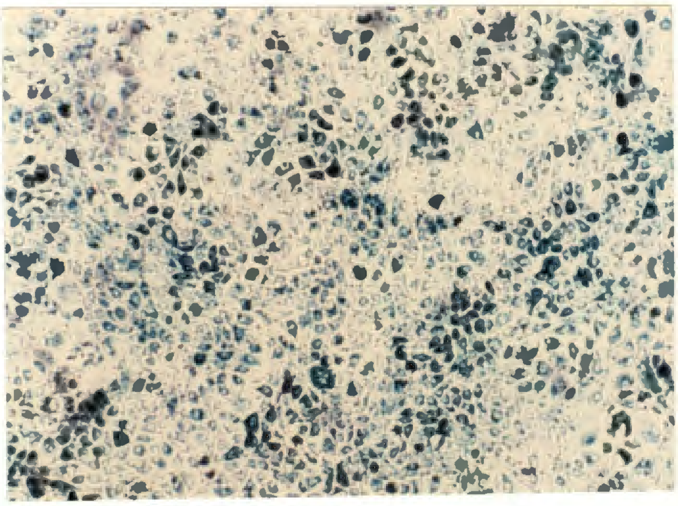


Fig. 3.8a. X-gal stain of LT-cells infected with LSDV and transfected with the pLSD-β-gal construct.

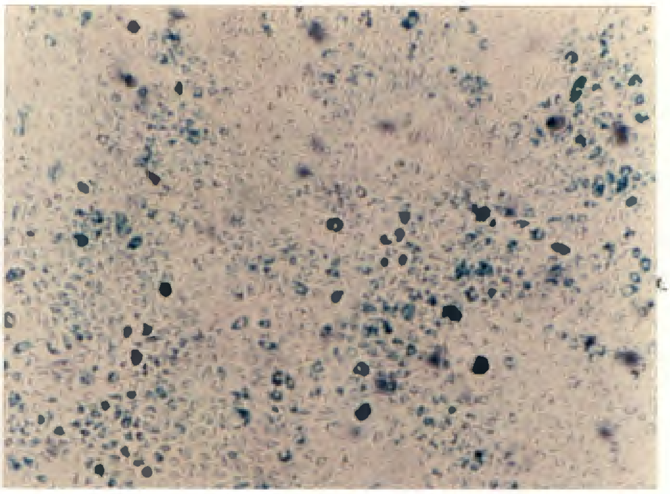


Fig. 3.8b. X-gal stain of LT-cells infected with LSDV.

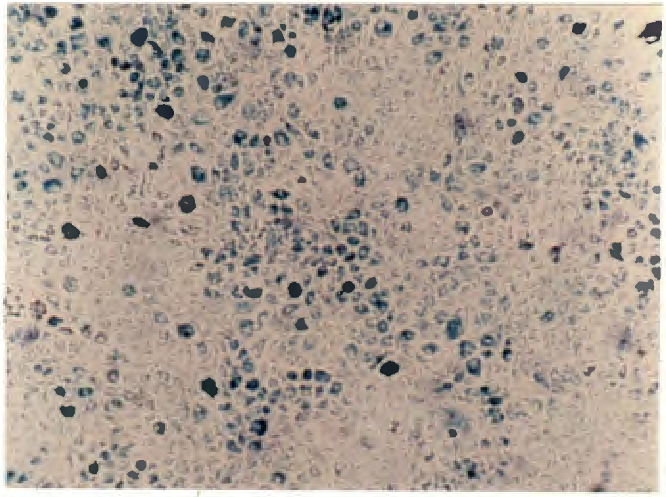


Fig. 3.8c. X-gal stain of uninfected LT-cells.

Table 3.2. Assay for β-galactosidase activity in extracts of CFK-cells.

	OD_{420nm}	
Cells	Assay 1	Assay 2
Uninfected CFK-cells	0.074	0.074
LSDV-infected CFK-cells	0.075	0.041
LSDV-infected CFK-cells transfected with pLSD-β-gal	0.232	0.224
LSDV-infected CFK-cells transfected with pox-48B	0.347	0.346
LSDV-infected CFK-cells transfected with pal-1	1.979	1.980

3.3.4. Determination of the MPA concentration required for virus-inhibition

The concentration of MPA required to inhibit the proliferation of wild-type virus in order to select for recombinant virus containing the *Gpt*-gene, without having a harmful effect on the cells, was determined. During the monitoring period the selection medium with which the cells were incubated was not replaced with fresh medium.

A slight difference between uninfected CFK cells, incubated with different concentrations of MPA, could only be observed from 2 days onwards (results not shown). A reciprocal relationship between MPA concentration and cell-growth rate could be observed.

It appeared as if the maximum MPA concentration that could be used was 30 μg/ml for the specific batch of cells.

Two days after infection of cells with LSDV (Table 3.3.), nearly 50% of the cells without selection medium had died, whereas the infected cells with 0.25 μg MPA/ml were still surviving although they were heavily infected as indicated by CPE. Virus multiplication thus seemed to be slowed down by MPA. No definite foci could be observed in the infected cells incubated with a MPA-concentration of 1,5 μg/ml and higher after 2 days. After three days definite virus foci could be observed in cells infected with virus and incubated with MPA concentrations of up to 2 μg/ml. However, although definite virus foci could not be observed in cells incubated with MPA-concentrations of 2.5 μg/ml and higher after 3 days there seemed to be infected cells present. It appeared as if the percentage infected cells decreased reciprocally with rising MPA-concentrations. The control infected cells without selection medium died off completely after 3 days.

After 6 days definite virus foci could be detected in all the wells with cells which had been incubated with different MPA-concentrations.

Table 3.2. Determination of MPA concentration required for LSDV inhibition

[MPA] µg/ml	Day1	Day2	Day3	Day4	Day5	Day6
0.00	+++	+++, 50% ✝	✝	✝	✝	✝
0.25	±	+++	+++, 50% ✝	✝	✝	✝
0.50	±	++	+++	+++, 50% ✝	✝	✝
0.75	±	+	++	+++	+++, 50% ✝	✝
1.00	-	+	+	++	+++	+++
1.50	-	±	+	++	+++	+++
2.00	-	-	+	++	+++	+++
2.50	-	-	±	+	++	+++
3.00	-	-	±	+	++	+++
3.50	-	-	±	+	++	+++
4.00	-	-	±	+	++	+++
4.50	-	-	±	+	+	+++
5.00	-	-	±	+	+	+++
>5.00	-	-	-	±	+	++

+	Some definite viral foci present in cells
++	Moderately infected cells
+++	Many viral foci present
±	Possible (but not definite) viral foci
✝	Cells died

3.3.5. Transfection of pLSD-Gpt into LSDV-infected CFK-cells

Cells infected with LSDV and incubated without selection-medium exhibited many virus-foci as demonstrated by indirect immunoperoxidase staining with anti-LSDV serum (Fig. 3.9a.). The nuclei of the cells were counterstained with haematoxylin which gave it a blue appearance (not clearly visible on photographs). This made it possible to distinguish between the cytoplasm and the nuclei. The virus foci, which stained dark brown, could be seen in the cytoplasm of the cells. This was expected as poxviruses multiply within the cytoplasm of mammalian cells. Some cells exhibited diffused staining, but most appeared to be viral factory-stains.

Fig. 3.9a. Indirect immunoperoxidase stain of CFK-cells infected with LSDV without MPA-selection.

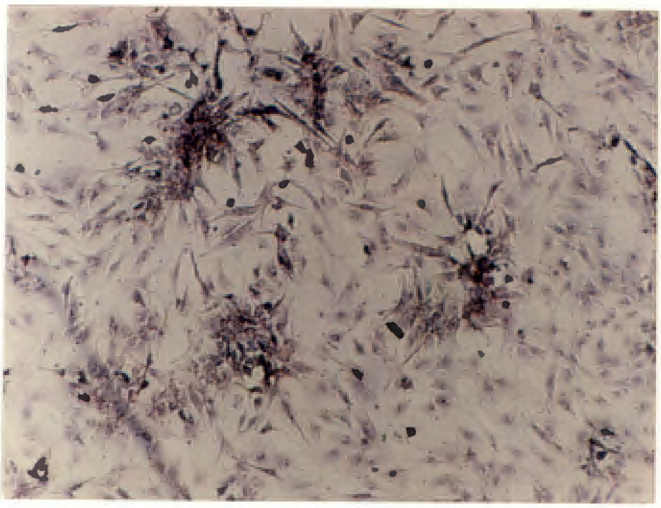


Fig. 3.9b. Indirect immunoperoxidase stain of CFK-cells infected with LSDV with MPA-selection.

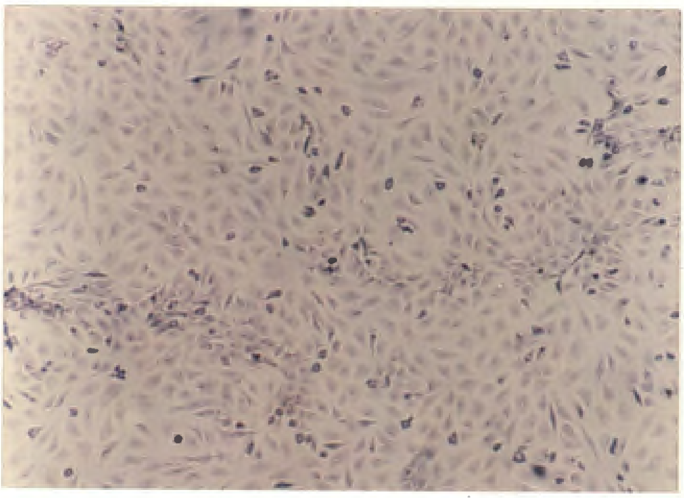
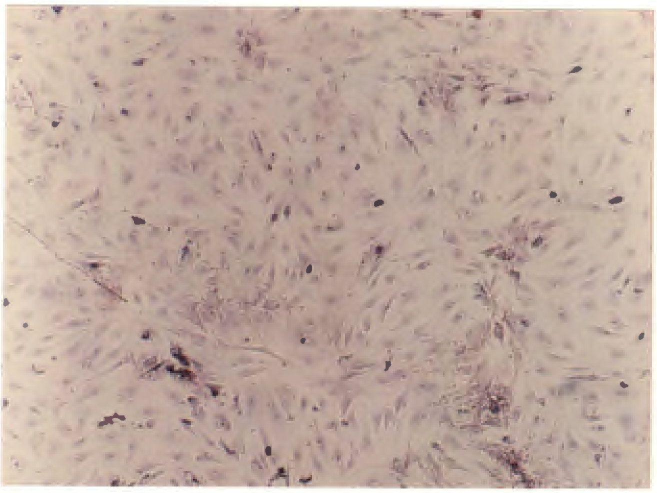


Fig. 3.9c. Indirect immunoperoxidase stain of CFK-cells infected with virus (harvested originally from cells infected with LSDV and transfected with the pLSD-*Gpt* construct) with MPA-selection.



However, no definite virus foci could be detected in the control cells infected with LSDV-virus (that had been passaged six times through selection medium) and incubated with selection medium (Fig. 3.9b.). The abscence of indirect immunoperoxidase staining in these cells also indicated that there was no detectable endogenous peroxidase activity present in the cells. Normal uninfected cells should not exhibit dark brown staining in the cytoplasm after indirect immunoperoxidase staining. Virus, obtained from cells that were infected with LSDV and transfected with the pLSD-*Gpt* construct, was passaged six times through selection medium. Cells infected with this virus (presumed to contain recombinants) at passage no. 6 were subjected to *in situ* indirect-immunoperoxidase staining (Fig. 3.9c.) which confirmed the presence of viral-foci.

3.4. Discussion and conclusions

3.4.1. Virus propagation

In order to obtain a successful transfection reaction, it is necessary to start of with a high enough virus concentration (at least 0.05 FFU/cell), since *in vivo* recombination in viruses occurs at a low frequency. *In vivo* recombination in VV occurs with a frequency of 0.1% (Piccini and Paoletti, 1988). Therefore several attempts were made to obtain a good stock of pure LSDV with a high titer. Due to the unavailability of CFK- or LT-cells at the time (as previously mentioned) LSDV was propagated in MDBK-cells. Several attempts had to be made to get a good virus-titre, since most LSDV-titers obtained with MDBK-cells were in the region of 1×10^3 - 1×10^4 PFU/ml, which was too low. Very good virus-yields are obtained with VV when propagation of the virus are done on the CAM's of eggs of domestic hens. Therefore it was decided to try and propagate LSDV on CAM's. However, no virus could be isolated. Few strains of capripoxvirus will grow on the CAM of the developing chick embryo (Kitching *et al.*, 1986). According to Davies (1991) LSDV will grow in embryonated hens eggs, but the system is insufficiently sensitive for primary virus isolation.

A higher titre was obtained when the virus stock, obtained by propagation in MDBK-cells, was titrated on CFK-cells instead of MDBK-cells. This could be attributed to a higher susceptibility of the CFK-cells to the virus. However, more titrations on both cell-lines would be required to make a definite conclusion, since different batches of cells can differ in their susceptibility to the virus. The focal cytopathic changes caused by LSDV in CFK-cells tended to remain localized and had to be searched for carefully. Plowright and Witcomb (1959) found that LSDV grew better in LT-cells than CFK-cells. They could usually obtain virus titres approximately one log unit higher in testis cells than in kidney cells. According to Plowright and Witcomb (1959) the amount of free virus was always less than the virus present in the cells throughout virus-proliferation. They concluded that the virus was probably released slowly and continuously from the cells so that it did not lead to rapid death and detachment of a large proportion of the infected cells. Therefore to obtain maximum yields the virus should not only be harvested from the infected medium, but also from infected cells.

3.4.2. Construction of recombinant plasmids

The β-gal gene-containing fragment, as in the case with the *Gpt*-gene containing fragment, were cloned into the pLSDTK vector to enable the insertion of each of these genes into the LSDV-genome by homologous recombination between the TK-segments. Both genes were placed under the control of the P 7.5 promoter. The integrity of these construct were determined by restriction enzyme analysis. The *Gpt*-gene appeared to have suffered a mutation resulting in the loss of the internal *Kpn1*-site.

3.4.3. Transfection of pLSD-β-gal into LSDV-infected LT-cells

Whether transfection be judged by transient expression or stable recombination, the frequency of recombination in general varies with the conditions and multiplicity of infection, the amount and form of plasmid DNA introduced and the interaction between homologous sequences (Spyropoulos *et al.*, 1988). The frequency of recombination is significantly increased when the plasmid and intact viral genomic DNAs are coprecipitated onto infected cells (Spyropoulos *et al.*, 1988).

Efficiency of transient and stable transfection varies widely between different cell types, cell lines and promoters used (Ray and Gage, 1992). The lipofectin method was used in the transfection procedures because it was found to be better than calcium phosphate precipitation and electroporation methods in terms of higher transfection efficiency and convenient use (Ray and Gage, 1992). Liposome-mediated transfection is efficient for the delivery of DNA to the cell cytoplasm (the site for LSDV-proliferation) although it is inefficient for nuclear delivery (Rodriguez *et al.*, 1992). Although incubation of the mammalian cells with DNA/Lipofectin mixture can be done for up to 24 hours (according to the protocol supplied with the Lipofectin-reagant), it was found that incubation with CFK-cells and LT-cells could be performed for a maximum time of 5 hours at 37°C. Even with an incubation-time of 5 hours some cell-death of the CFK-cells could be observed. This would lead to a lower transfection efficiency.

The LT-cells transfected with the pLSD-β-gal construct displayed higher levels of β-galactosidase activity than the uninfected/untransfected control cells when the X-gal stain was performed *in situ* (refer to Figs. 3.8a., 3.8b. and 3.8c.). This indicated that the β-gal-gene was getting expressed under the control of the P 7.5 promoter and could be an indication that such cells contain a recombinant construct harbouring the β-gal gene. The observed activity could, however, also be the result of transient expression of the β-gal gene. Moderate background β-galactosidase activity was observed in uninfected/untransfected control cells. According to MacGregor *et al.* (1991), certain tissues like the kidney and testes exhibit higher endogenous b-galactosidase activity than others. This is apparently due to the presence of lysosomal b-galactosidases. The cells also had to be incubated in the substrate solution of the X-gal stain for quite a long period of time to obtain a clear difference between the positives and negatives. The choice of the right promoter for each virus type is very important for optimal expression of the gene. It is possible that expression of the β-gal gene is not sufficient enough under the control of the P 7.5 promoter to give easily distinguishable blue foci (Boyle, 1992). An advantage of the X-gal stain is that expression of β-galactosidase can be detected in individual cells, although it is not as sensitive as immunocytological methods (MacGregor *et al.*, 1991). Expression of the gene in a high proportion of the cells is thus not a prerequisite for detection.

A different assay was subsequently performed to yield more informative data on the suitability of β-gal as a reporter-gene. This assay, performed on cell-extracts, is based on the conversion of o-nitrophenol-β-D-galactosidase (ONPG) to yield o-nitrophenol and galactose. The chromophore o-nitrophenol can be spectrophotometrically detected by measuring of the absorbance at 420 nm (MacGregor *et al.*, 1991). This is a sensitive assay which can be applied to quantitate the activity of β-galactosidase in cell-extracts. However, expression of β-galactosidase is needed in a higher percentage of cells than required for the X-gal stain. A possible explanation for the failure to detect endogenous β-gal activity with this assay in cell-extracts from uninfected CFK-cells, is that the activity may have occurred at a too low level to be detected. β-gal activity was detected in the cells transfected with the pLSD-β-gal construct (Table 3.2.), which provided evidence that the gene in the pLSD-β-gal construct was functional, although at a low level. The high level of β-galactosidase activity that was detected in the extracts from cells transfected with the pal-1 construct was probably due to more efficient expression of the gene under the control of the late 11 K promoter of VV. It was found that higher levels of proteins, expressed from foreign genes, are obtained with late or early and late poxvirus-promoters. Based on the specific activity of pure β-galactosidase (300 000 u/mg), the bacterial enzyme produced under the control of the P 11 promoter by VV-infected cells is more than 3% of the total cellular protein (Falkner and Moss, 1988). This amount should be easily detectable by appropriate staining methods. A lower expression of the *lacZ* gene is however obtained with the P 7.5 promoter indicating that the P 11 promoter is more efficient than the P 7.5 promoter. Unfortunately late promoters cannot be used in selection experiments where DNA replication is dependent on the selectable marker-gene (like for example the *Gpt*-gene), since the early expression of the gene selected for are required. However, other genes/recombinant proteins (like the β-gal gene) could be expressed more efficiently under the control of the 11 K promoter. Further work should be done, since this was only a preliminary experiment. Another reporter gene should be co-expressed with the β-gal gene to "normalise" the system for differences in transfection. This will allow more accurate assessment of the results. If verification of the data confirm poor expression of the β-gal gene under the VV P 7.5 promoter, another promoter should be found for the expression of genes in LSDV. The fowlpox virus (FPV) early and late promoter (P E/L) seemed to be an ideal candidate for the expression of foreign genes in VV recombinants (Boyle, 1992; discussed in section 3.1.4.) and may be a promising candidate for the expression of genes in LSDV.

The β-gal gene was not expressed strongly enough under the control of the P 7.5 promoter. It was therefore not a useful reporter gene for the selection of LSDV recombinants in this system. Another disqualifying factor of the β-gal-gene was the endogenous expression of the enzyme detected by X-gal staining.

3.4.4. Determination of the MPA-concentration necessary for virus-inhibition

The CFK-cells seemed to be quite tolerant to the different MPA-concentrations applied, although it appeared as if the MPA-concentration should not be raised above 30 µg/ml without having a harmful effect on the cells. LSDV-propagation could be reasonably successfully inhibited for a period of up to 3 days with MPA-concentrations of 2.5 µg/ml and higher. However, virus-inhibition by MPA was not complete at any of the concentrations after six days. After 4 days definite virus foci could be observed in all the cells incubated with different MPA-concentrations. This may be caused by MPA-depletion/break down in the medium after 3 days. It has been speculated that cellular break-down may also cause the release of some kind of substance which may enable the virus to overcome the MPA-block to some extent (Prof. K. Dumbell - personal communication). This might cause some problems with back-ground virus in the transfection experiment, since the MPA-concentration could not be raised without damaging the cells. Fresh selection medium should thus be added to the cells every third day. Most of the wild type virus should be eliminated with subsequent passages through selection medium. The virus-titre used in the transfection-experiments (0.1 PFU of LSDV per cell) were also much lower than the titre used for the determination of the MPA-concentration required for virus-inhibition (1 PFU of LSDV per cell). Problems with background virus may thus be eliminated.

3.4.5. Transfection of pLSD-Gpt into LSDV-infected CFK-cells

After indirect immunoperoxidase staining of cells, incubated with MPA-containing selection medium and infected with LSDV that had been passaged six times through selection medium (Fig. 3.9b.), no definite viral foci could be observed. In contrast to this, lots of viral foci could be observed in the LSDV-infected cells that had been incubated without selection medium (Fig. 3.9a.). This indicated that selection against wild-type LSDV was complete providing the virus had been passaged for several times through selection medium. The *in situ* indirect immunoperoxidase stain indicated definite viral foci in CFK-cells infected with virus obtained from cells originally transfected with the pLSD-*Gpt* construct (Fig. 3.9c.). This ability of the virus to propagate in the presence of MPA indicated that the LSDV may have acquired the *Gpt*-gene through homologous recombination in the TK-region. It has been reported that plasmid replication occurs in poxvirus-infected cells (De Lange and McFadden, 1986 - as referred to by Stellrecht, Sperber and Pogo, 1992), but transient expression of the *Gpt*-gene would not be expected at such a high level after the virus has been passaged for several times through selection medium, although the presence of some plasmid could not be ruled out completely. Unsuccessful attempts to purify adequate quantities of viral DNA for analysis obstructed verification of the results. The mutational event which occurred within the *Gpt*-gene in the pLSD-*Gpt* construct could effect it's functional expression. Virus stored at -20°C for later use lost its viability. Therefore transfection experiments with the pLSD-*Gpt* construct and subsequent DNA isolation were repeated in parallel with the pGS20-VP7:*Gpt* construct as described in Chapter 4.2.5. When *Gpt* is coupled in tandem with another gene of interest, positive selection of LSDV carrying both genes, should be possible. These results were considered as an indication that *Gpt* would be suitable as a selectable marker-gene for the selection of LSDV recombinants in this system.

CHAPTER 4. CONSTRUCTION OF A BLUETONGUE VIRUS VIRAL PROTEIN 7 POXVIRUS RECOMBINANT

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CHAPTER 4. CONSTRUCTION OF A BLUETONGUE VIRUS VIRAL PROTEIN 7 POXVIRUS RECOMBINANT

4.1. Introduction

4.1.1. Rationale for the construction of a poxvirus recombinant encoding a BTV protein

Bluetongue virus (BTV) belongs to the genus *Orbivirus* of the *Reoviridae* family (Knudson and Monath, 1990; Oldfield, Adachi, Urakawa, Hirasawa and Roy, 1990). Twenty-four distinct serotypes of the virus have been identified which vary in some characteristics. However, they all have antigens in common and cause the characteristic bluetongue disease that allow them to be grouped together (Oldfield *et al.*, 1990; St George, Alexander and Gard, 1991).

The virus is transmitted by arthropod vectors (*Culicoides* species) and causes disease in sheep and cattle (domestic and wild ruminants) with serious economic consequences (Knudson and Monath, 1990; Oldfield *et al.*, 1990; Roy, 1992). Besides the loss of domestic stock due to bluetongue, it is also a critical barrier to the international trade in livestock and livestock products. The virus is not readily transmitted by semen and embryos, since properly washed embryos obtained from infected cattle do not transmit the virus and infected bulls only shed virus in semen while viraemic (St George *et al.*, 1991). The disease occurs over an extensive area that includes Africa, America, Australia, the Middle East, South Eastern Asia and the Caribbian islands. It appears as if orbivirus infection of ruminants exists in most of the tropical zone of the world, with or without concurrent disease (Loudon, Hirasawa, Oldfield, Murphy and Roy, 1991; St George *et al.*, 1991).

Multivalent vaccines consisting of live attenuated BTV strains have been used widely due to the plurality of serotypes (24 serotypes - Kowalik and Li, 1991; Knudson and Monath, 1990; St George *et al.*, 1991). There are several problems associated with live attenuated BTV vaccine usage, like for e.g. teratogenicity, insect transmission and the potential for gene reassortment. There is thus a pressing need for more stable, reliable and safe BTV vaccines. Theoretically these problems could be overcome by the construction of a recombinant vaccine. The construction of a BTV poxvirus recombinant could also serve as a model for studying recombinant poxvirus vaccines. Several genes encoding BTV proteins are suitable candidates for the construction of such recombinant vaccines. The major group-specific core antigen, VP7, of BTV has several characteristics which renders it highly suitable for the construction of a recombinant vaccine. The properties of this antigen and its potential use will be discussed in the following sections.

4.1.2. The major group-specific core antigen VP7 of bluetongue virus.

4.1.2.1. Virion structure and the position of VP7 within the virion.

BTV, like other members of the Reoviridae family, has a double-capsid structure and lacks an envelope (Knudson and Monath, 1990; Oldfield *et al.*, 1990). The viral genome consists of 10 discrete double stranded RNA (dsRNA) segments which are located in the inner-core.

The outer shell is composed of two major proteins, VP2 and VP5 which are loosely bound to the inner shell, forming an ill-defined outer structure (refer to Fig. 4.1.). The genes encoding VP2 and VP5 are the most variable in BTV (Knudson and Monath, 1990; Roy, 1992). The highly diverse outer capsid protein, VP2, induces the production of neutralizing antibodies in animals, plays a role in hemagglutination and has serotype-specific epitopes (Huismans, Van der Walt, Cloete and Erasmus, 1987a; Yang, Johnson, Mecham, Tam and Li, 1992). The outer shell can be easily removed from the single-shelled particle enclosed within during purification procedures (Loudon and Roy, 1991; Oldfield *et al.*, 1990; Prasad, Yamaguchi and Roy, 1992).

The single-shelled virion (core or BTV particle without the outer shell) is composed of two major proteins, VP3 and VP7, and three minor proteins, VP1, VP4 and VP6 (Huismans, Van Dijk and Els, 1987; Prasad *et al.*, 1992). The diameter of this spherical single-shelled virion is 69 nm (Roy, 1992). The three-dimensional structure of the single-shelled virion exhibits icosahedral symmetry with a triangulation number of 13 in a left-handed configuration (Prasad *et al.*, 1992). The single-shelled virion's three dimensional structure can be divided into two concentric layers of protein surrounding the innermost core or subcore (Prasad *et al.*, 1992; Roy, 1992).

The outer layer of this single shelled-virion has 260 prominent knoblike capsomeres which can be located at all the local and strict threefold axes. These knoblike capsomeres consist of trimers of VP7, giving a total of 780 molecules of VP7 per virion (Eaton, Gould, Hyatt, Coupar, Martyn and White, 1991; Loudon and Roy, 1991; Prasad *et al.*, 1992). Conclusive evidence for the trimers formed by VP7, was derived from crystallographic studies, together with solution studies (Basak, Stuart and Roy, 1992; Roy, 1992).

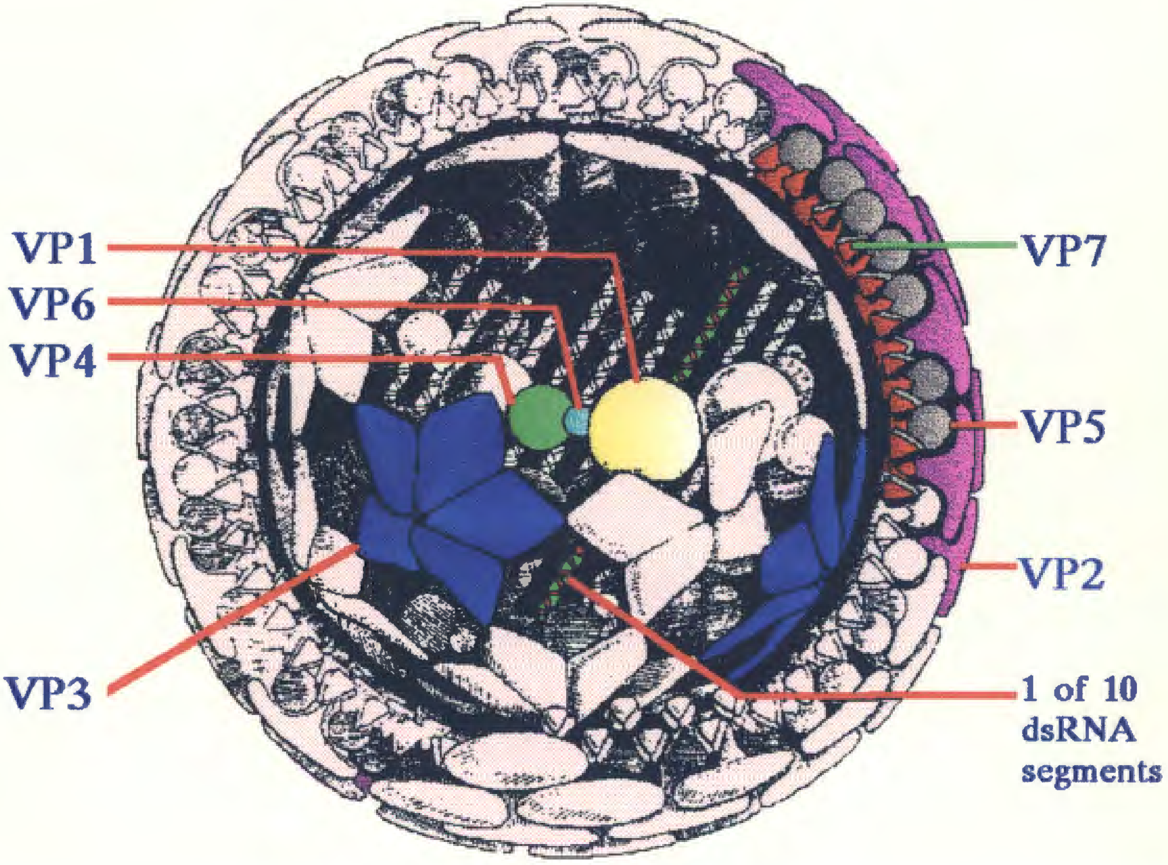

Fig. 4.1. Schematic diagram of the BTV particle (adapted from Roy, 1992). The outer shell is composed of two major proteins, VP2 and VP5. Knoblike capsomeres, consisting of trimers of VP7, are located on a smooth bed of pentameric VP3 molecules. These two major core proteins, VP7 and VP3, form two concentric layers of protein which surround the innermost core or subcore. The innermost core contains three minor proteins, VP1, VP4 and VP6, as well as ten discrete segments of genomic dsRNA.

The VP7 structures are the sites for the deposition of the two proteins, VP2 and VP5, of the outer shell of the virus (Roy, 1992). The capsomeres are arranged in such a way that there are 132 aqueous channels at all the five- and six-coordinated positions (Prasad *et al.*, 1992). Some channels penetrate the inner layer and they may provide pathways for the transportation of metabolites and mRNA during transcription (Hewat *et al.*, 1992).

The inner layer is a smooth bed upon which the capsomeres are located. This layer is about 70 nm thick and consists predominantly of the major protein, VP3 (60 molecules per core), which are arranged as 12 pentamers (Prasad *et al.*, 1992; Roy, 1992). The gene encoding VP3 is conserved between serotypes (Knudson and Monath, 1990). Except for the indentations in register with the channels of the outer layer, the surface of the inner layer is featureless. Some channels terminate inside the inner layer in small indentations at a radius of 25 nm, while others cross this layer into the subcore.

It seems as if VP7 requires close interaction with VP3, since VP7 expressed on it's own does not form icosahedral structures (Prasad *et al.*, 1992). VP3 forms a scaffold for the assembly of the VP7 capsomeres (Loudon and Roy, 1991). Correct VP7 protein-protein interactions may be unable to form in the absence of VP3, which may lead to non-specific aggregation due to hydrophobic interactions between VP7 molecules (Oldfield *et al.*, 1990). However, if VP7 and VP3 are co-synthesized in cells infected with a recombinant virus containing both genes, empty core-like particles (CLPs) are formed (French and Roy, 1990; Hewat *et al.*, 1992; Loudon *et al.*, 1991; Oldfield *et al.*, 1990). It has been concluded that the presence of the three minor core proteins, the dsRNA genome or the four non-structural proteins are not required for their formation (Prasad *et al.*, 1992; Roy, 1992). According to Huismans *et al.* (1987) parental double-shelled virions are converted to single-shelled particles of 470S shortly after infection. These 470S single-shelled particles are converted to smaller particles containing VP3 and other minor proteins (VP1, VP4 and VP6). The smaller particles are supposedly composed of the inner layer and the subcore and consist predominantly of VP3 (Huismans *et al.*, 1987; Loudon and Roy, 1991). In single-shelled virions VP7 is more abundant than VP3 and accounts for 36% of the core protein (Basak *et al.*, 1992; Roy, 1992).

The subcore is enclosed by these two layers and consist of the three minor proteins, VP1, VP4 and VP6, and the ten segments of genomic dsRNA (Loudon and Roy, 1992; Prasad *et al.*, 1992). At least four different, highly conserved, non-structural proteins (NS1, NS2, NS3 and NS3A) have been identified in BTV-infected cells, but their significance is unresolved (He, Wang, Doi, Maia, Osburn and Chuang, 1991; Roy, 1992; Thomas, Booth and Roy, 1990). Viral synthesis and maturation occurs in the cytoplasm of infected cells (Knudson and Monath, 1990).

4.1.2.2. VP7

4.1.2.2.1. The VP7 gene

The major core protein VP7 is the sole translational product encoded by the small RNA segment 7 (S7) of the BTV genome (Eaton *et al.*, 1991; Knudson and Monath, 1990; Kowalik and Li, 1991; Oldfield *et al.*, 1990; Roy, 1992). Kowalik and Li (1991) designated the VP7-encoding fragment as S1. In this chapter, however, it will be referred to as S7 which appears to be preferred by most other authors. The nucleotide and deduced amino acid sequences of the S7 segments of five United States BTV serotypes (BTV-2, -10, -11, -13 and -17) have been determined (Kowalik and Li, 1991). Each segment is 1156 bp long and contains an open reading frame (ORF) encoding the 349-amino acid VP7 protein, followed by a single termination codon. No deletions or insertions were found in any of the S7 segments.

Of the total nucleotide residues, 46.3 - 47.0% consists of G and C residues (Kowalik and Li, 1991). The VP7 proteins are well-conserved with 0 - 22 amino acid differences between the serotypes. Comparison of the African horsesickness virus serotype 4 (AHSV-4) VP7 sequence with that of BTV serotype 10 revealed an overall similarity of 44%, with the amino- and carboxy-terminal regions exhibiting the greatest levels of similarity (Roy, Hirasawa, Fernandez, Blinov and Sanchez-Vixcain Rodrique, 1991). An evolutionary rate of approximately 2.2×10^{-3} nucleotide substitutions/site/year is predicted for BTV (Kowalik and Li, 1991). This evolutionary rate implies that there is no proof-reading activity associated with the BTV RNA polymerase. No polymerase proofreading functions are known in single-strand RNA (ssRNA) viruses (Kowalik and Li, 1991). A stable stem-loop structure can be formed near the translational termination codon in the segment S1 coding strand. The last few codons and part of the 3' noncoding region are included in this stem-loop. The significance of the stem-loop structures is not known but they may be common in RNA viruses (Kowalik and Li, 1991).

4.1.2.2.2. Protein structure and properties

Most of the VP7 proteins consist of 25 acidic, 27 basic and 6 histidine residues, which result in a slight positive charge (+1.0 to +1.5) at pH 7.0 (Kowalik and Li, 1991). Hydrophobic and hydrophilic stretches found throughout the central regions of the protein may assume a structural role. A putative eight-stranded β-barrel is maintained by the VP7 protein from BTV-2, -11 and -17 (Kowalik and Li, 1991). This eight-stranded antiparallel β-barrel (the primary structural motif) is present in the cores of all ssRNA viruses with known three-dimensional structures, suggesting a common origin of ssRNA viruses found in plants and animals. The presence of this structure in BTV VP7 supports the contention that dsRNA and ssRNA viruses may have a common origin (Kowalik and Li, 1991).

The protein may exist in the cytosol of infected cells as an oligomer of which the constituent VP7 molecules are not linked together via intermolecular disulfide bonds (Eaton *et al.*, 1991). A striking feature of the BTV VP7 protein is that it contains only one lysine residue (K 255) which is conserved amongst the sequenced BTV serotypes (Roy, 1992). This positively charged residue at position 255 may have a particular structural role. Three cysteine residues are found at positions 15, 65 and 154. The cysteines at positions 15 and 65 may be linked via an intramolecular disulfide bond (Eaton *et al.*, 1991).

The hydrophobic nature of the VP7 protein makes it possible to purify the protein (M_r = 38,550 kDa) by a low percentage ammonium sulphate precipitation as the first step and subsequent ion-exchange chromatography (Oldfield *et al.*, 1990; Prasad *et al.*, 1992; Roy, 1992). Material with a purity of at least 95% can be obtained with this method.

4.1.2.2.3. Antigenic properties

The fact that VP7 is highly conserved amongst BTV serotypes and at least partly conserved amongst three serogroups of orbiviruses, contributes to its suitability as an antigen for vaccination (Oldfield *et al.*, 1990). Purified VP7 antigen is furthermore highly immunogenic. It cross-reacts with the antisera of all 24 BTV serotypes, as well as an antiserum raised to African horsesickness virus type 4 (AHSV-4), a member of another serogroup of the orbiviruses (Oldfield *et al.*, 1990). A monospecific antiserum raised against the expressed VP7 protein, reacted with two serotypes of the most closely related orbivirus to BTV, epizootic haemorrhagic disease virus (EHDV), in addition to it's reaction with the other BTV serotypes (Oldfield *et al.*, 1990). Sequence data obtained for the VP7 proteins of BTV, AHSV and EHDV indicate that the viruses are closely related at the genetic level and derived from a common ancestor (Iwata, Chuma and Roy, 1992). It has been suggested that BTV and EHDV should be considered as members of the same serogroup (Iwata *et al.*, 1992). AHSV and BTV have much in common with referral to pathogenesis, pathology and vectors (Oldfield *et al.*, 1990; St George *et al.*, 1991).

An additional advantage of VP7 for the construction of recombinant vaccines is that the protein is accessible on the surface of bluetongue virus particles. The surface layer or outer shell of BTV can be considered as a net which only partially obscures the underlying core particle (Eaton *et al.*, 1991). The serogroup-reactive epitope was located in the amino-terminal half of the VP7 protein (Eaton *et al.*, 1991). There is strong evidence that the epitope may be located in the VP7 protein near or between the cysteine residues at positions 15 and 65 which are linked via a disulfide bond (Eaton *et al.*, 1991). It was suggested that the amino-terminal portion of VP7 may be exposed and not located on the inside of the core particle (Eaton *et al.*, 1991). The major reactive epitopes of VP7 are apparently conformational, since cross-reactivity between VP7 proteins were observed in ELISA and immunoprecipitation tests, but not under denaturing conditions in Western blot analysis (Iwata *et al.*, 1992; Kowalik and Li, 1991).

4.1.3. Aim of this study

The aim of this study was to construct a recombinant LSDV/VV virus containing the BTV4 VP7 gene under the control of the VV P 7.5 promoter. This recombinant virus could be tested as a potential vaccine against BTV and LSDV (in the case of a LSDV-recombinant). It would also serve as a model for studying recombinant poxviral vaccines. Employment of the *Gpt*-gene as a dominant selectable marker for the selection of recombinant poxviruses should be possible. It was also attempted to compare the effectiveness of homologous and heterologous TK-sequences to direct recombination with the viral TK-gene. Therefore two different constructs, one containing LSDV TK-sequences and the other VV TK-sequences, was each transfected separately into both LSDV and VV. This was done to determine if heterologous VV TK-based constructs could be used for the construction of LSDV-recombinants, since many VV vectors encoding foreign proteins are available. Using existant constructs would save time and progress on projects involving the construction of LSDV-recombinants would thus be quicker.

4.2. Materials and methods

4.2.1. Viruses, mammalian cells and domestic hens' eggs

The vaccine strain of LSDV was obtained and maintained as described in Chapter 3.2.1. Vaccinia virus, a South African strain, was supplied by Prof. K.R. Dumbell, Department of Medical Microbiology, Medical School, UCT. Vaccinia virus was propagated in eggs of domestic hens (Chapter 2.2.1.) and titrated on CV-1 cells (Chapter 2.2.7.).

The CFK-cells were prepared and maintained as described in Chapter 3.2.1. The CV-1 cell-line, derived from an African Green Monkey kidney, is a continuous fibroblast-like cell line (ATCC Catalogue, ref. CCL 70) and were supplied by the Department of Medical Microbiology, Medical School, UCT. All the cells were grown in DMEM, supplemented with 10% FCS and 0.5% PSN (Appendix A.13.). The cells were maintained in DMEM supplemented with 0.5% PSN and 2 or 4% FCS. Selection medium consisted of maintenance medium supplemented with 250 μg Xanthine/ml, 14 μg Hypoxanthine/ml and MPA at the required concentration.

The eggs of domestic hens, used for the propagation of VV was obtained as described in Chapter 3.2.1.

4.2.2. Genes, vectors and host bacterial cells

The Bluetongue virus serotype 4 viral protein 7 (BTV4VP7)-gene was obtained from Dr. A. van Dijk, Onderstepoort Veterinary Institute (OVI), Pretoria. This gene was supplied as a pMC50-construct which consisted of the BTV4VP7-gene cloned into the *Pst1* site of pUC-4K. The pGS20 vector was obtained from Dr. M. Mackett, Paterson Laboratories, Christie Hospital and Holt Radium Institute. The competent *E. coli* LK111 cells were supplied by the Department of Medical Microbiology, Medical School, UCT. Refer to Chapter 3.2.2. for the suppliers of the xanthine-guanine phosphoribosyl transferase (*Gpt*)-gene and pLSDTK vector.

4.2.3. Construction of a plasmid containing the VP7- and the *Gpt*-gene

4.2.3.1. General strategy

The BTV VP7-gene was cloned into a suitable plasmid vector for the construction of a lumpy skin disease virus (LSDV; discussed in Chapter 1.3.) - or a vaccinia virus (VV; discussed in Chapter 1.2.) - recombinant vaccine. The plasmid insertion vector used for this purpose, pGS20 (Mackett, Smith and Moss, 1984), has unique *BamH1* and *Sma1* sites for the insertion of foreign DNA adjacent to the VV 7.5 K gene promoter (P 7.5, Chapter 3.1.4.). The *EcoR1* fragment of pGS20, containing P 7.5 and the *BamH1* and *Sma1* sites, is flanked on both sides by segments of the VV thymidine kinase (TK) gene. These sequences can direct homologous recombination with VV DNA, and presumably also with other poxvirus-DNA, to insert the foreign DNA into the TK gene of the viral genome. The similarity at the nucleotide/amino acid levels between the capripoxvirus TK-gene and the TK-gene of VV was found to be 68.4/64.6% (Gershon and Black, 1989). Therefore the similarity should be sufficient to allow for recombination between the two mentioned TK-genes, since as few as 4 matching base pairs are sufficient to support intermolecular strand transfer (Shuman, 1992).

To enable selection of recombinant virus containing the foreign gene of interest (VP7), the transient dominant selection (TDS) strategy (Fig. 4.2.) described by Falkner and Moss (1990) was employed.

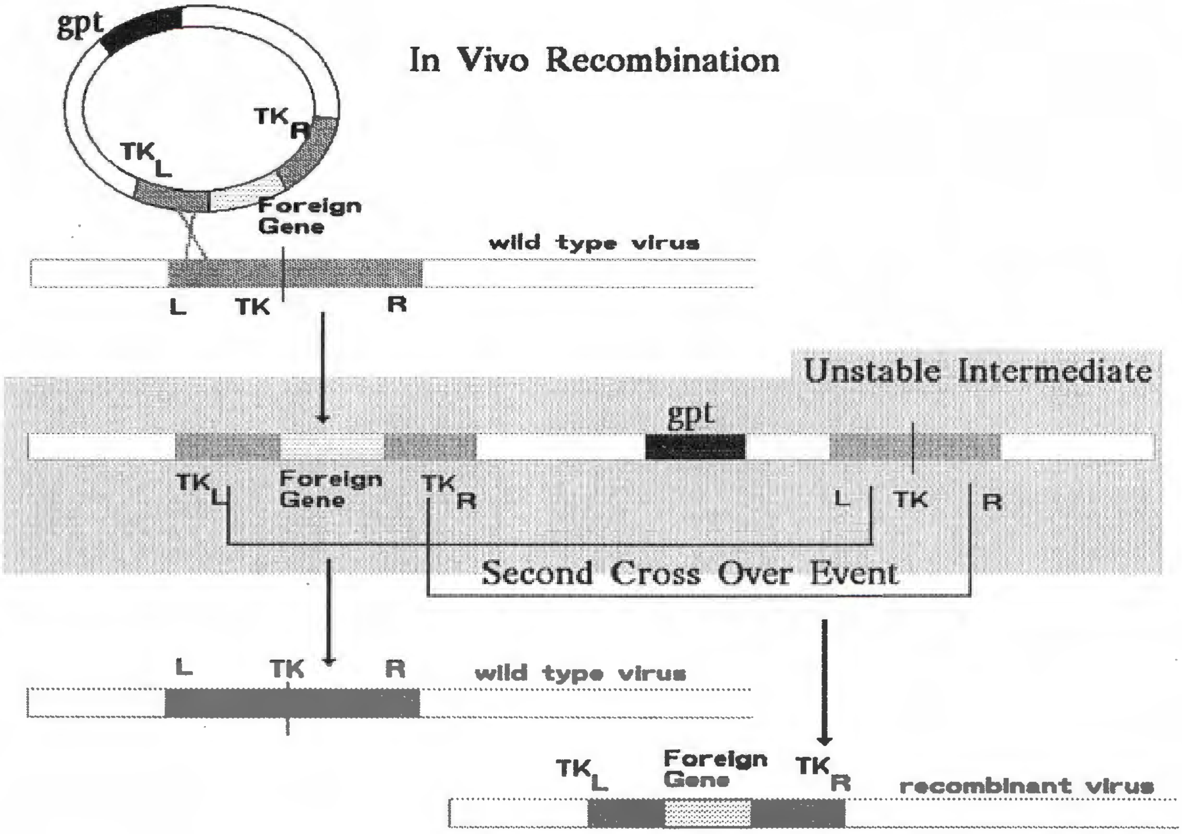

Fig. 4.2. Schematic outline of the transient dominant selection (TDS) strategy (adapted from Falkner and Moss, 1990). The foreign gene of interest is cloned into a plasmid so that it is flanked by TK-sequences. The dominant selectable marker-gene, *Gpt,* is inserted into the plasmid outside the TK gene. When the plasmid is transfected into a cell that has been infected with wild type virus, recombination can occur. A single crossover event between the TK-sequences present in the plasmid as well as the virus would result in the insertion of the full-length plasmid into the virus-genome. A second cross-over event between direct repeats will result in the formation of either wild type virus or recombinant virus containing the foreign gene of interest.

The dominant selectable marker-gene *Gpt* (Chapter 3.1.2.), under the control of the P 7.5 promoter, was cloned into the *Hind111* site of the pGS20-vector. The marker gene was thus not flanked by VV DNA and would not be stably integrated into the virus-genome by double crossover or gene conversion events. A single, crossover event between the TK-sequences present in the circular insertion vector (pGS20) as well as the poxvirus genome, should result in the insertion of the full-length plasmid into the virus genome. Selection for virus containing the *Gpt*-gene (mycophenolic acid resistance) could then be applied. After a single crossover event, the *Gpt*-gene would be flanked by an uninterrupted TK-gene on the one side and an interrupted TK-gene on the other side. The *Gpt*-gene would thus be lost if a second crossover event should occur between these TK sequences, resulting in the formation of either wild-type virus or recombinant virus containing the foreign gene (VP7 in this case).

4.2.3.2. Cloning of the BTV4VP7-gene into pGS20

A large scale DNA extraction (Chapter 2.1.1.2.) was performed on the pMC50-clone containing the BTV4VP7-gene (referred to as VP7, Section 4.2.1.). The VP7 gene was excised from this construct as a *BamH1* fragment which was isolated and purified by agarose gel-electrophoresis (Chapter 2.1.4. - 2.1.6.).

The pGS20 vector was linearized with *BamH1*, phenol/chloroform extracted twice, ethanol precipitated and resuspended in ultra-pure water (Chapter 2.1.2. - 2.1.4.). The VP7 *BamH1* fragment (1160 bp) was ligated into the *BamH1* site of pGS20 (ca 7500 bp) as described in Chapter 2.1.7.2. (Fig. 4.3a.). Competent LK111 cells were used for the transformation reaction (Chapter 2.1.7.4.). Small scale DNA preparations of randomly picked colonies were performed to screen for colonies harbouring the recombinant plasmid (Chapter 2.1.1.1.2., 2.1.7.5.). The DNA was digested with *BamH1* and analyzed by agarose-gel electrophoresis (Chapter 2.1.4. - 2.1.5) to verify the presence of the VP7 insert. Liquid cultures of colonies identified as harbouring the recombinant plasmid, were frozen away as stock (Chapter 2.1.7.6.).

The published map of pGS20 (Mackett, Smith and Moss, 1984) does not show all the restriction enzyme sites. Therefore mapping by single and multiple restriction digest-patterns were performed on pGS20 and recombinant plasmids harbouring the VP7 gene. This was done to determine the orientation of VP7, relative to P 7.5, in pGS20. The vector pGS20 was subjected to single digests with *BamH1, Hind111, Sma1* and *Sal1*. Multiple digests of pGS20 were performed with *BamH1 + Hind111, Hind111 + Sal1, BamH1 + Sal1, Sma1 + Hind111, Sma1 + Sal1* and *BamH1 + Hind111 + Sal1*. The digests were performed as described in Chapter 2.1.4. A restriction map of pGS20 based on information from these digests was compiled.

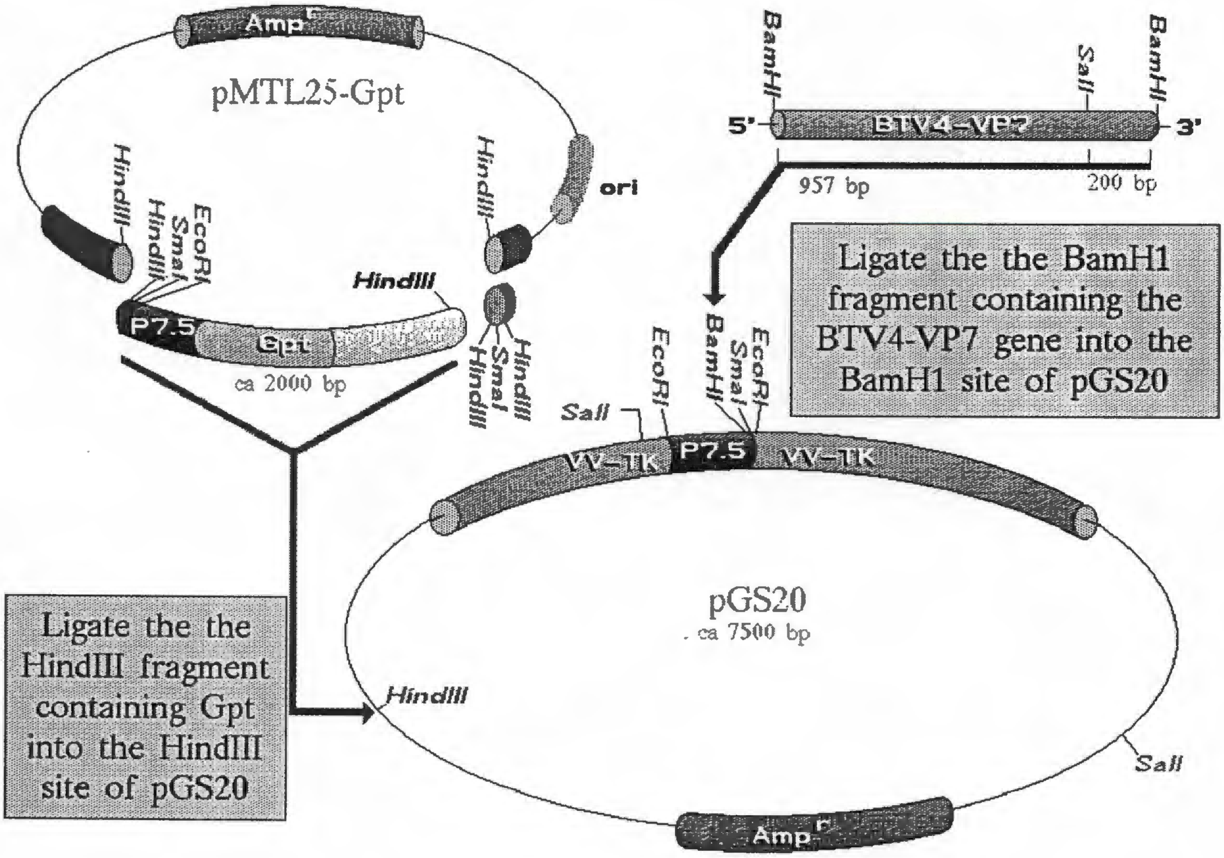

Fig. 4.3a. Schematic representation of the cloning of the BTV4VP7-gene containing *BamH1*-fragment into the pGS20 vector, followed by the cloning of the *Hind111*-fragment comprising the *Gpt*-gene under the control of the VV P 7.5 promoter into the pGS20-VP7 construct (refer to Fig. 4.3b).

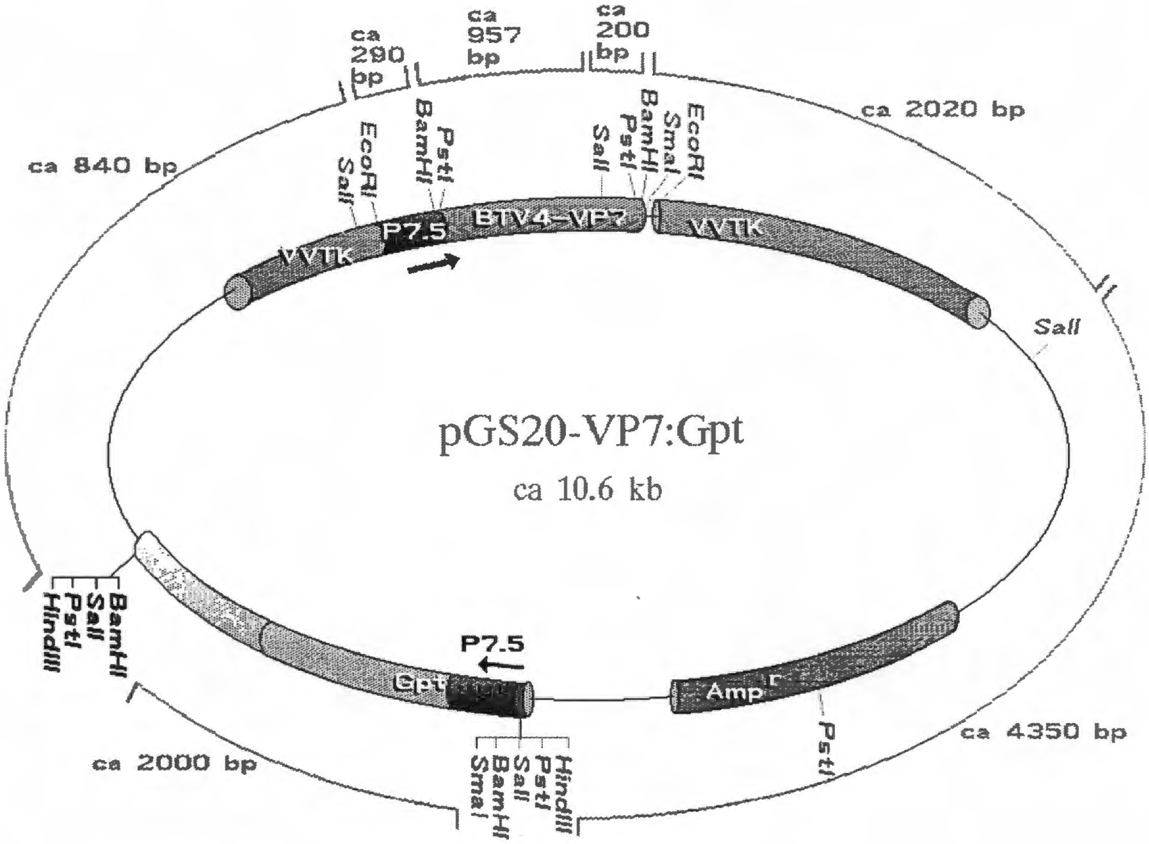

Fig. 4.3b. Schematic representation of the pGS20-VP7:*Gpt* construct, with the BTV4VP7-gene cloned into the *BamH1* site and the *Gpt*-gene (under the control of the VV P 7.5 promoter) cloned into the *Hind111* site of the pGS20 vector.

Five recombinants harbouring the VP7 gene were selected and digested with *Sal1*, *Sma1* + *Sal1* and *Hind111* + *Sal1*. One of these recombinants, with the VP7 gene in the sense orientation relative to P 7.5 in the pGS20 plasmid (designated pGS20-VP7) was used for a large scale preparation (Chapter 2.1.1.2.) of recombinant DNA. This DNA was used for all the subsequent manipulations.

4.2.3.3. Cloning of the *Gpt*-gene into the pGS20 vector containing the BTV4VP7 gene.

The *E. coli Gpt*-gene, under the control of P7.5, was cloned into the *EcoR1* site of the pMTL25 vector (refer to Chapter 3.2.5.). From this construct, designated pMTL25-*Gpt* (Chapter 3 Fig. 3.5a.), the *Gpt*-containing fragment was excised with *Hind111* and purified by agarose-gel electrophoresis (Chapter 2.1.5. - 2.1.6.). The recombinant construct pGS20-VP7 (Section 4.2.3.2.) was linearized with *Hind111* and subsequently treated with calf intestinal phosphatase (CIP, Chapter 2.1.7.1.).

The *Gpt*-containing *Hind111* fragment (ca. 2000 bp) was ligated (Chapter 2.1.7.2.) into the *Hind111* site of the linearized, dephosphorylated pGS20-VP7 vector (Figs. 4.3a. and 4.3b.). Competent *E. coli* LK111-cells were used for the transformation-reaction (Chapter 2.1.7.4.). Twenty-four transformant colonies were picked randomly from plates containing cells that were transformed with the pGS20-VP7:*Gpt* ligation mixture. These were subjected to small scale plasmid DNA extractions (Chapter 2.1.1.1.2.). The DNA was checked for the presence of the VP7- and the *Gpt*-gene by agarose-gel electrophoresis after double digestion with *BamH1* and *Hind111*. Stock glycerol cultures of identified recombinant colonies were frozen away as described in Chapter 2.1.7.6. A large scale DNA preparation (Chapter 2.1.1.2.) was performed on one of the identified recombinant colonies (designated pGS20-VP7:*Gpt*), harbouring the recombinant pGS20 vector containing the VP7- and the *Gpt*-gene (Figs. 4.3b.). The orientation of the *Gpt*-gene in the pGS20-VP7:*Gpt* plasmid was determined by restriction analysis using *Sma1*.

4.2.4. Titration of MPA to determine the concentration needed for the inhibition of LSDV and VV growth

The tolerance of the CFK cells to different concentrations of MPA and the MPA concentration required to inhibit LSDV multiplication in CFK cells were determined as described in Chapter 3.2.6. Determination of the tolerance of CV-1 cells to different concentrations of MPA and the MPA concentration required to inhibit VV multiplication in CV-1 cells were performed as described in Chapter 2.2.9. with the following modifications: The cells were infected with a virus-concentration of 1 PFU of VV/cell. The MPA concentrations that were tested ranged from 0.1 μg MPA/ml to 5 μg MPA/ml. The cells were constantly monitored for any signs of cell-deterioration or virus multiplication during the incubation period with the different MPA concentrations. After 48 hours the cells were stained with ZN Carbolfuchsin (Chapter 2.2.7.) to check for plaque-formation due to virus growth. The MPA concentration used in the transfection experiment was determined based on the results obtained.

4.2.5. Transfections into mammalian cells

4.2.5.1. Transfection of LSDV-infected CFK-cells

Infections and transfections of cells were performed as described in Chapter 2.2.8., except for the following modifications. Almost confluent monolayers of CFK-cells were prepared in 35 mm-diameter tissue culture dishes. The cells were infected with 0.05 PFU of LSDV per cell for 80 minutes at 37°C. (A low virus-input titre was used in order to minimize the amount of background virus yielded by the transfection reaction.) The DNA/Lipofectin^{TM} reagent complex consisted of 3 μg of the recombinant pGS20-VP7:*Gpt* plasmid (Section 4.2.3.3.), and 40 μg Lipofectin^{TM} reagent/35 mm tissue culture dish. A second transfection was performed as described for the first one, except that the DNA-Lipofectin complex consisted of 3 μg of the recombinant pLSD-*Gpt* plasmid (refer to Chapter 3.2.5.) and 50 μg Lipofectin^{TM} reagent/35 mm dish. After an incubation period of 5 hours at 37°C, the DNA-containing opti-MEM was replaced with maintenance medium (section 4.2.1.), supplemented with 2% FCS, 1 μg MPA/ml, 250 μg Xanthine/ml and 14 μg Hypoxanthine/ml. Of each transfection performed, control cells were kept with maintenance medium, containing 2% FCS but no MPA. LSDV infected cells (0.05 PFU/cell) were also kept as controls with selection and no selection medium, together with uninfected cells.

After an incubation period of 2 days at 37°C, the virus was passaged onto fresh cells (prepared in 35 mm tissue culture dishes and almost confluent) as follows. The transfected and control cells were microscopically screened for any signs of infection or deterioration of the cell-layer. After freeze-thawing the cells once to release more virus from the cells, the supernatant was used for the infection of fresh cells (Chapter 2.2.8.). Selection medium and non selection medium was added as before and incubation was continued at 37°C This passaging of the virus onto new cells was repeated every third day.

Total DNA extractions were performed separately on cells infected with the possible recombinant virus, cells infected with the wild type virus and uninfected cells at passage nos. 4, 5 and 6. Extractions were performed 48 hours after infection of the cells as described in Chapter 2.2.11. with the following modifications. The lysate was incubated with proteinase K for 5 hours at 55°C. After the first addition of pre-warmed phenol, the lysate-phenol mixture were left at room temperature for 15 minutes, while inverting the tube every now and again, before the two phases were separated by centrifugation. The DNA pellets were resuspended in ultra pure water at 4°C overnight.

Of the total DNA harvested from the cells in a 35 mm tissue culture dish, 40% was digested with 1u/µl *Hind111* (Chapter 2.1.4.) in a total volume of 100 µl for 24 hours in an incubator at 37°C. After digestion, the volume was made up to 500 µl with ultra pure water and two phenol/chloroform extractions were performed (Chapter 2.1.2.). The DNA was precipitated with 0.3 M Sodium-acetate and 2 volumes absolute ethanol at -20°C for 18 hours (Chapter 2.1.3.). The pellet was resuspended in 10 µl ultra pure water at 4°C for 6 hours.

The digested DNA was loaded onto an 0.8% agarose-gel together with appropriate markers and electrophoresis was carried out in 1 x TAE-buffer at room temperature for 18 hours at 25 volts. After ethidium bromide staining and photographing of the gel, it was cut smaller to a size of 183 x 110 mm so that it only contained the lanes with nucleic acid bands of interest. The DNA was transferred to a nylon membrane (Hybond N, Amersham) by overnight Southern blotting (Chapter 2.1.8.1.). The membrane was prehybridized for 48 hours at 42°C with shaking, as described in Chapter 2.1.8.3. for non-radioactive labelling.

The DNA was screened for the presence of the *Gpt*- and the VP7-genes respectively by means of separate non-radioactive hybridizations (Chapter 2.1.8.3.). The first probe was prepared by the non-radioactive labelling of the fragments obtained by *Hind111*-digestion of 0.8 µg of the pLSD-*Gpt* clone (Chapter 3.2.5.). The second probe was prepared by the non-radioactive labelling of the fragments obtained by *Hind111*-digestion of 0.8 µg of the pGS20-VP7 clone (Section 4.2.3.2.). Non-radioactive labelling of DNA with digoxigenin-dUTP (DIG-labelling) was performed as described in Chapter 2.1.8.2. The reactions were scaled up according to the amount of DNA used and the denaturing steps were performed at 95°C for 15 minutes.

After hybridization with the first probe, the washed membrane was subjected to chemiluminescent detection (Chapter 2.1.8.3.). Exposure to Curic RP 1 X-ray film (Agfa, West Germany) was done for 30 minutes at room temperature (Chapter 2.1.8.4.). The membrane was prepared for reprobing (Chapter 2.1.8.5.) and put up to prehybridize at 42°C for 48 hours with hybridization solution containing 5% blocking reagent (Chapter 2.1.8.3.). After hybridization with the second probe, the membrane was again subjected to chemiluminescent detection and exposure to X-ray film as described above.

4.2.5.2. Transfection of VV-infected CV1-cells

About 80-90% confluent monolayers of CV1-cells were prepared in 35 mm-diameter tissue culture dishes on a 6-well Nunclone tissue culture plate. The infection- and transfection- reactions were performed as described in Chapter 2.2.8., with the following modifications: The cells were pre-treated by incubation with selection medium (DMEM, containing 2% FCS, 0.5%PSN, 250 µg Xanthine/ml, 14 µg Hypoxanthine/ml and 1 µg MPA/ml), at 37°C overnight. The cells were infected with 0.05 PFU of VV per cell for 80 minutes at 37°C. The DNA, consisting of the recombinant pGS20-VP7:*Gpt* plasmid (Section 4.2.3.3.; 3 µg DNA/35 mm-diameter tissue culture dish) was diluted to 62 µl with ultra pure water, mixed with 60 µg of Lipofectin™ reagent and left at room temperature for 20 minutes. The DNA/Lipofectin reagent complex was added dropwise to the cells after the addition of 500 µl opti-MEM/35 mm-diameter dish to the cells. A second transfection was performed as previously described, except that the DNA was replaced with the recombinant pLSD-*Gpt* plasmid (Chapter 3.2.5.) at a concentration of 3 µg DNA/35 mm-diameter dish. Incubation was performed at 37°C for 5 hours 30 minutes. The DNA containing opti-MEM was removed from the cells and selection medium (Appendix A.10.) containing 1 µg MPA/ml was added. Maintenance medium without MPA was added to a control dish with transfected cells. Vaccinia virus infected cells (0.05 PFU/cell) and uninfected cells were kept as separate controls, each with selection medium and non-selective medium respectively. The controls were further treated exactly as the transfected cells.

After 48 hours incubation at 37°C, the virus were passaged onto new CV1-cells as follows. Almost confluent monolayers of CV1 cells were prepared in 35 mm tissue culture dishes, which were not pre-treated prior to infection. The medium, into which virus would have been released from the cells, was removed from the transfected cells and 500 µl/35 mm dish was used for the infection of fresh CV1 cells. After 1 hour incubation at 37°C, the virus-inoculum was removed and selection/non-selection media were added after which incubation was continued at 37°C. (The MPA-concentration in the selection medium was lowered to 0.3 µg/ml after the first passage, because the CV-1 cells appeared to be very sensitive to the MPA and deteriorated to quickly at the higher concentration.) The virus-infected control cells was subjected to the same treatment. The cells, from which the medium was removed, was stained with ZN-Carbolfuchsin (Chapter 2.2.7) and compared with respect of the number of plaques observed in each dish. After 3 days incubation of the newly infected cells, the released virus was again passaged to freshly prepared cells as described above. This step was repeated for a few times and after each passaging step the cells were stained and compared.

Separate total DNA extractions were performed (Chapter 2.2.11.) on cells infected with recombinant virus, cells infected with wild-type virus and uninfected cells at passage 5, 6, 7 and 8 with the following modifications. The lysate was incubated with proteinase K at a concentration of 100 µg/ml at 55°C for 2 hours, after which incubation was left to proceed overnight at 42°C. After the first addition of prewarmed phenol, incubation was performed at 37°C for 1 hour with regular mixing of the two phases, before the separation of the two phases by centrifugation. The second phenol-extraction was done as usual without any incubation steps.

The total DNA, extracted as described previously, was digested with *Hind111* as follows: Forty percent of the total DNA harvested from a 35 mm tissue culture dish (except where indicated differently) was digested with *Hind111* (1 u/µl) in a total volume of 100 µl for 24 hours at 37°C. Some more digests were also performed with *Pst1* and *Sal1* similarly to the *Hind111* digest. After digestion, the reaction-volume was made up to 500 µl with ultra pure water and phenol/chloroform extracted (Chapter 2.1.2.). The DNA was ethanol-precipitated (Chapter 2.1.3.) and the pellet was resuspended overnight in 10 µl of ultra pure water at 4°C. The digested DNA was loaded onto an 0.8% agarose gel in 1 x TAE-buffer, together with appropriate markers. The DNA was run into the gel at 100 volts for 15 minutes, after which electrophoresis was continued at room temperature for 18 hours at 23 volts. The gel was stained with ethidium bromide, photographed and cut smaller so that it only contained the lanes with nucleic acid bands to be transferred. Several gels were run with different combinations of digested DNA. The DNA was transferred to a nylon membrane (Hybond N, Amersham) by Southern blotting (Chapter 2.1.8.1., acid method) overnight.

The DNA was screened for the presence of the *Gpt*- and the VP7-genes by probing of the membranes with DIG-labelled probes as follows: The first probe was prepared by DIG-labelling of 0.8 µg pGS20-VP7:*Gpt* DNA digested with *Sma1*. Two additional probes were prepared by the non-radioactive labelling of 0.2 µg *BamH1*-fragment (the BTV4 VP7-gene) and 0.2 µg *Hind111*-fragment (containing the *Gpt*-gene under the control of the VV P 7.5 promoter) separately. DIG-labelling was performed as described in the protocol supplied with the DNA Labelling Kit (Boehringer Mannheim, Cat. No. 1175 033) with the following modifications. The reaction-volumes were scaled up according to the amount of DNA used. Denaturation steps were performed at 95°C for 15 minutes in a waterbath. The labelling reaction was left to proceed in an incubator at 37°C for 24 hours. (The probe was not precipitated afterwards in the case of the first probe.) Labelled probes were stored at -20°C until needed.

Pre-hybridization and hybridization were performed as described in Chapter 2.1.8.3. The blocking reagent were used at a concentration of 3% in the hybridization solution. After hybridization to the probe of choice, the washed membrane was subjected to chemiluminescent detection (Chapter 2.1.8.3.). The membrane was kept wet and the probe was removed (Chapter 2.1.8.5.) so that it could be re-probed with a different probe.

4.3. Results

4.3.1. Construction of the pGS20-VP7:*Gpt* plasmid

4.3.1.1. Cloning of the VP7-gene into the pGS20-vector

The BTV4VP7-gene was isolated as a 1.160 kb *BamH1*-fragment (Fig. 4.4a. lane 1) from the original plasmid pMC50. This VP7-containing fragment was cloned into the *BamH1*-site (adjacent to the P 7.5 promoter) of the pGS20-vector. Recombinant plasmids were identified by restriction enzyme analysis with *BamH1* (Fig. 4.4a. lane 4) which were expected to yield a 7.5 kb and a 1.160 kb fragment respectively. The 7.5 kb fragment corresponded with the size of the *BamH1*-linearized pGS20-vector (Fig. 4.4a. lane 11) and the 1.160 kb fragment with the size of the VP7-gene (Fig. 4.4a. lane 1).

It was necessary to compile a restriction map of pGS20 (based on restriction enzyme analysis) in order to determine the orientation of the VP7-gene relative to the P 7.5 promoter in the pGS20-vector. The pGS20-vector was subjected to single digests with *BamH1* (Fig. 4.4a. lane 11), *Hind111* (Fig. 4.4a. lane 12), *Sma1* (Fig. 4.4a. lane 13) and *Sal1* (Fig. 4.4a. lane 14). Multiple digests of pGS20 were performed with *BamH1* + *Hind111* (Fig. 4.4b. lane 10), *Hind111* + *Sal1* (Fig. 4.4b. lane 11), *BamH1* + *Sal1* (Fig. 4.4b. lane 12), *Sma1* + *Hind111* (Fig. 4.4b. lane 13), *Sma1* + *Sal1* (Fig. 4.4b. lane 14) and *BamH1* + *Hind111* + *Sal1* (Fig. 4.4b. lane 15). The fragment sizes yielded for pGS20 by each of the aforementioned restriction enzyme digestion-reactions are shown in Table 4.1. A restriction map of pGS20 based on these results, was compiled.

The *Sal1* site within the VP7-gene was exploited to determine the orientation of the VP7-gene relative to the P 7.5 promoter of the pGS20-vector as follows: Five recombinant plasmids (identified by restriction enzyme analysis with *BamH1*) were digested separately with *Sal1*; *Sma1* + *Sal1*; and *Hind111* + *Sal1*. The orientation of the VP7-gene could be determined based on the sizes of the restriction enzyme fragments obtained with the aforementioned digest-reactions. (The expected fragment sizes for the sense- and non-sense orientation are indicated in Table 4.1.) One of these recombinants (designated pGS20-VP7), with the VP7-gene in the sense orientation relative to the P 7.5 promoter in the pGS20-vector, was selected. The sense orientation of the VP7-gene relative to the P 7.5 promoter was verified by the fragment-sizes yielded upon restriction enzyme analysis with *Sal1* (Fig. 4.4a. lane 7), *Sma1* + *Sal1* (Fig. 4.4a. lane 8) and *Hind111* + *Sal1* (Fig. 4.4a. lane 9). The aforementioned fragment sizes correspond with the sizes expected for the sense orientation as indicated in Table 4.1.

TABLE 4.1. Expected fragment sizes of plasmid constructs digested with different restriction enzymes.

Plasmid construct	Restriction enzyme(s)	Expected fragment size(s) (bp)[#]
pGS20	*BamH1*	7500
	Hind111	7500
	Sma1	7500
	Sal1	2310 + 5190
	BamH1 + *Hind111*	1130 + 6370
	Hind111 + *Sal1*	840 + 2310 + 4350
	BamH1 + *Sal1*	2020 + 5190 + 290
	Sma1 + *Hind111*	6370 + 1130
	Sma1 + *Sal1*	290 + 2020 + 5190
	BamH1 + *Hind111* + *Sal1*	840 + 290 + 2020 + 4350
pGS20-VP7	*BamH1*	1160 + 7500
	Hind111	8657
	Sma1	8657
	Sal1	[*]sense orientation: 1247 + 2220 + 5190 [*]non-sense orientation: 490 + 2977 + 5190
	Sma1 + *Sal1*	[*]sense orientation: 1247 + 200 + 2020 + 5190 [*]non-sense orientation: 490 + 957 + 2020 + 5190
	Hind111 + *Sal1*	[*]sense orientation: 840 + 1247 + 2220 + 4350 [*]non-sense orientation: 840 + 490 + 2977 + 4350
pGS20-VP7:*Gpt*	*BamH1*	1160 + 6370 + 2000 + 1130
	Hind111	2000 + 8657
	Sma1	6370 + 4287
	Sal1	[*]sense orientation: 1247 + 2220 + 4350 + 2000 + 840 [*]non-sense orientation: 490 + 2977 + 4350 + 2000 + 840
	Sma1 + *Hind111*	6370 + 2000 + 2287

[#] Fragment sizes shown were estimated by restriction mapping.

[*] Orientation of the VP7-gene relative to the P 7.5 promoter in the pGS20 vector.

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Fig. 4.4. Gel-electrophoresis of DNA employed in the construction of pGS20-VP7:*Gpt*.

(a) **Lane 1,** *BamH1*-fragment containing the BTV4VP7-gene. **Lane 2,** *Hind111*-fragment containing the *Gpt*-gene under the control of the VV P 7.5 promoter. **Lane 3,** λ-DNA digested with *Sty1*. **Lane 4,** pGS20-VP7 digested with *BamH1*. **Lane 5,** pGS20-VP7 digested with *Hind111*. **Lane 6,** pGS20-VP7 digested with *Sma1*. **Lane 7,** pGS20-VP7 digested with *Sal1*. **Lane 8,** pGS20-VP7 double-digested with *Sma1* and *Sal1*. **Lane 9,** pGS20-VP7 double-digested with *Hind111* and *Sal1*. **Lane 10,** λ-DNA digested with *Sty1*. **Lane 11,** pGS20 digested with *BamH1*. **Lane 12,** pGS20 digested with *Hind111*. **Lane 13,** pGS20 digested with *Sma1*. **Lane 14,** pGS20 digested with *Sal1*

(b) **Lane 1,** *BamH1*-fragment containing the BTV4VP7-gene. **Lane 2,** *Hind111*-fragment containing the *Gpt*-gene under the control of the VV P 7.5 promoter. **Lane 3,** λ-DNA digested with *Sty1*. **Lane 4,** pGS20-VP7:*Gpt* digested with *BamH1*. **Lane 5,** pGS20-VP7:*Gpt* digested with *Hind111*. **Lane 6,** pGS20-VP7:*Gpt* digested with *Sma1*. **Lane 7,** pGS20-VP7:*Gpt* digested with *Sal1*. **Lane 8,** pGS20-VP7:*Gpt* double-digested with *Sma1* and *Hind111*. **Lane 9,** λ-DNA digested with *Sty1*. **Lane 10,** pGS20 double-digested with *BamH1* and *Hind111*. **Lane 11,** pGS20 double-digested with *Hind111* and *Sal1*. **Lane 12,** pGS20 double-digested with *BamH1* and *Sal1*. **Lane 13,** pGS20 double-digested with *Sma1* and *Hind111*. **Lane 14,** pGS20 double-digested with *Sma1* and *Sal1*. **Lane 15,** pGS20 triple-digested with *BamH1*, *Hind111* and *Sal1*.

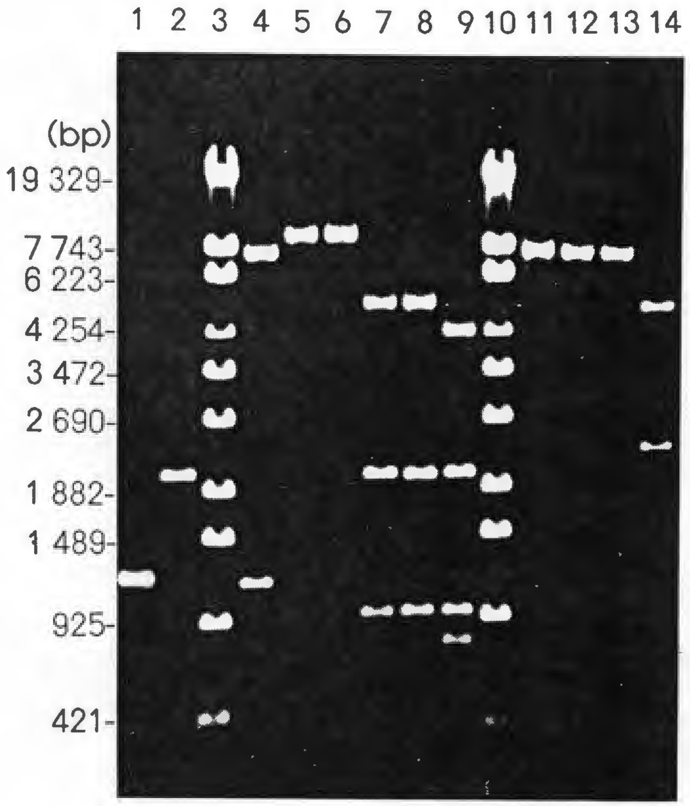

Fig. 4.4a.

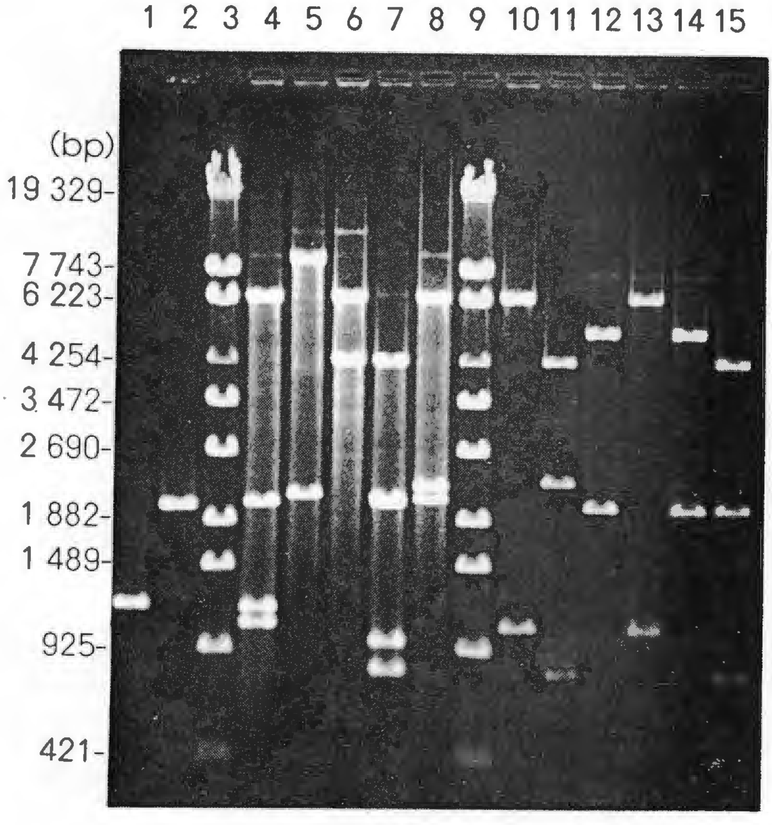

Fig. 4.4b.

4.3.1.2. Cloning of the *Gpt*-gene into pGS20-VP7.

The ca. 2 kb *Hind111*-fragment (containing the *Gpt*-gene under the control of the P 7.5 promoter) isolated from the pMTL25-*Gpt* plasmid is shown in Fig. 4.4b. lane 2. The cloning of this fragment into the *Hind111* site of the pGS20-VP7 plasmid was verified by restriction analysis of the resulting plasmid (designated pGS20-VP7:*Gpt*). Restriction enzyme analysis of pGS20-VP7:*Gpt* with *BamH1* (Fig. 4.4b. lane 4), *Hind111* (Fig. 4.4b. lane 5), *Sma1* (Fig. 4.4b. lane 6), *Sal1* (Fig. 4.4b. lane 7) and *Sma1* + *Hind111* (Fig. 4.4b. lane 8) all generated the expected fragment sizes (deduced from the compiled restriction map of pGS20) as indicated in Table 4.1.

The orientation of the *Gpt*-gene in the pGS20-VP7:*Gpt* plasmid could be deduced from the fragment-sizes generated upon digestion of the plasmid with *Sma1*. The original *Gpt*-containing *EcoR1*-fragment (which was cloned into the *EcoR1* site of the pMTL25-vector to construct the pMTL25-*Gpt* plasmid, Chapter 3.2.5.) contained a *Hind111* site within a multiple cloning site downstream of the *Gpt*-gene (Boyle and Coupar, 1988). Refer to Chapter 3 Fig. 3.5a. The *Gpt*-gene, linked to the P 7.5 promoter, was excised from pMTL25-*Gpt* with *Hind111* to be cloned into the *Hind111* site of pGS20-VP7. Due to the presence of the additional *Hind111* site downstream of the *Gpt*-gene (derived from the original fragment), only one *Sma1* site from the multiple cloning site of the pMTL25-vector (upstream of the *Gpt*-gene) should be retained within the isolated *Hind111*-fragment. The following *Sma1* restriction fragments would thus be expected (deduced from the compiled restriction map of pGS20-VP7:*Gpt*, Fig. 4.3b.), if the two promoters present in the plasmid were in the same orientation relative to one another: ca. 6370 + ca. 4287 bp. If the promoters were present in the opposite orientation relative to one another, the following fragments would be expected: ca. 8370 + ca 2287 bp. Fragments obtained with *Sma1* digestion of pGS20-VP7:*Gpt* (Fig. 4.4b. lane 6) indicated that the two promoters were present in the same orientation (as schematically depicted in Fig. 4.3b).

4.3.2. Virus propagation and the determination of the MPA concentration necessary for virus inhibition

The propagation of LSDV, the tolerance of the CFK-cells to different concentrations of MPA and the MPA-concentration necessary for LSDV-inhibition were as described in Chapter 3.3.1. and 3.3.4.

Good yields of VV, with a titre of 2.5 x 10^7 PFU/ml, were obtained when propagated on the CAM's of domestic hens. Monolayers of CV-1 cells were used for the titration of VV. Plaques formed by VV can be counted after staining of the cell-monolayer with ZN-Carbolfuchsin for the determination of the titre.

The CV-1 cells were found to be very sensitive to MPA. Even with a concentration as low as 0.5 μg MPA/ml a harmful effect on the cells could be detected with incubation for up to 3 days. After the third day of incubation with 1.0 μg MPA/ml the cells began to deteriorate and die off quickly. It appeared as if the concentration to be used should not exceed 0.3 μg MPA/ml. The propagation of VV in CV-1 cells appeared to be inhibited relatively effectively by MPA for up to 3 days, even at concentrations as low as 0.1 μg MPA/ml. However, with prolonged incubation some virus plaques started to appear in the wells incubated with MPA concentrations of up to 0.3 μg MPA/ml, from the third day onwards. It was not possible to test the inhibitory effect of MPA concentrations higher than 0.5 μg/ml on the propagation of VV for prolonged periods, because of the deleterious effect of the MPA on the cells as mentioned before.

4.3.3. Transfection of LSDV-infected CFK-cells

Virus stocks presumed to contain recombinants (originally obtained from cells infected with LSDV and transfected with the pGS20-VP7:*Gpt* or pLSD-*Gpt* construct) were passaged six times through MPA-containing selection medium as described in section 4.2.5.1. Wild type LSDV was passaged in parallel through selection medium. CFK-cells infected with wild type LSDV and virus presumed to contain recombinants were subjected to total DNA-extractions at passage nos. 4, 5 and 6. Total DNA was also harvested from uninfected cells. Viral foci could be observed in all the cells infected with virus up to passage no. 6. As expected, viral foci were not detected in the uninfected control cells.

The total DNA extracts were digested with *Hind111* and subjected to agarose-gel electrophoresis. A smear of digested DNA was observed in each lane (Fig. 4.5a.). This was expected as the extracts contained genomic DNA. The Southern-blot of this gel was probed with a DIG-labelled probe consisting of *Hind111*-fragments of the pLSD-*Gpt* clone. These fragments contained the *Gpt*-gene linked to the P 7.5 promoter and LSDV TK-gene sequences (Fig. 3.5b.). No recombinant virus could be detected (Fig. 4.5b.). However, the TK-gene without an insert (no band shift) could be detected in all the DNA samples harvested from cells infected with LSDV. This indicated the presence of wild type virus. Selection was thus not complete. In the DNA sample harvested from the uninfected control cells no signal could be detected. This was expected due to the abscence of virus. By reprobing of the blot with a DIG-labelled (*Hind111*-linearized) pGS20-VP7 fragment (containing the VP7-gene, the P 7.5 promoter and VV TK-gene sequences) a single faint band, in the same position as the band detected when probed with the pLSD-*Gpt* probe (described earlier), could be detected in all the DNA samples harvested from cells infected with LSDV (results not shown).

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Fig. 4.5a. Agarose gel-electrophoresis of total DNA extracted (*Hind111*-digested) from CFK-cells. **Lane 1** (DNA molecular weight marker), λ-DNA digested with *Hind111*. **Lane 2,** *pGS20-VP7:*Gpt* (#6). **Lane 3,** *pLSD-*Gpt* (#6). **Lane 4,** †LSDV (#6). **Lane 5,** *pGS20-VP7:*Gpt* (#4). **Lane 6,** *pLSD-*Gpt* (#4). **Lane 7,** †LSDV (#4). **Lane 8,** *pGS20-VP7:*Gpt* (#5). **Lane 9,** *pLSD-*Gpt* (#5). **Lane 10,** †LSDV (#5). **Lane 11,** uninfected CFK-cells.

* *CFK cells were pre-infected with LSDV and transfected with the indicated plasmid construct. The presumptive recombinant virus was passaged several times through MPA-selection medium. Total DNA was subsequently extracted from the infected cells at the indicated passage number (#) after transfection and digested with Hind111.*

† *LSDV was passaged several times through MPA-selection medium on CFK-cells. Total DNA was subsequently extracted from the infected CFK-cells at the indicated passage number (#) through selection medium and digested with Hind111.*

Fig. 4.5b. Southern blot of the agarose-gel shown in Fig. 4.5a. The blot was probed with DIG-labelled *Hind111*-digested pLSD-*Gpt* fragments (refer to legends of Fig. 4.5a. for descriptions of lanes).

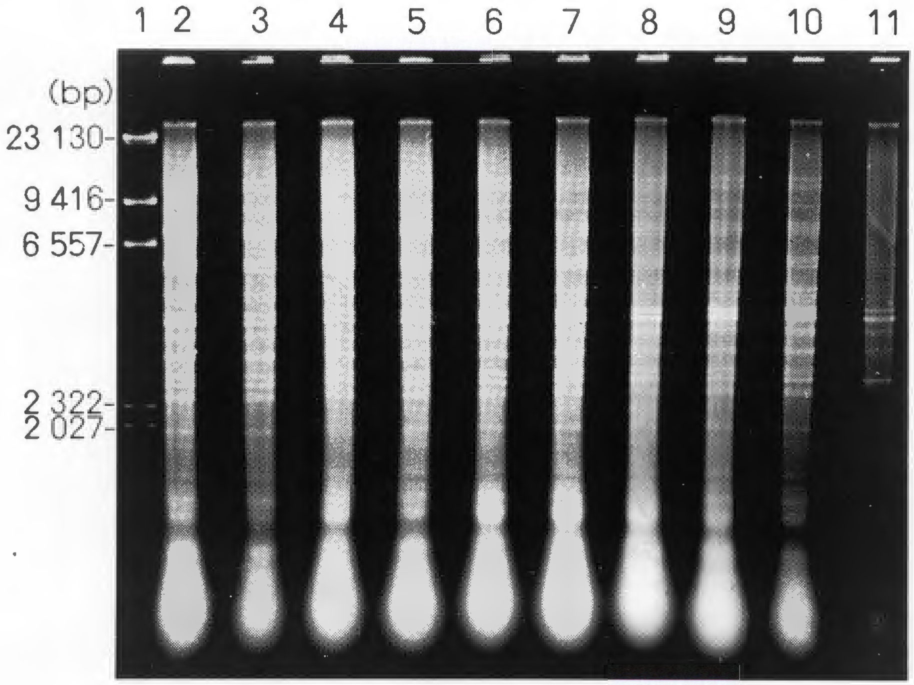

Fig. 4.5a.

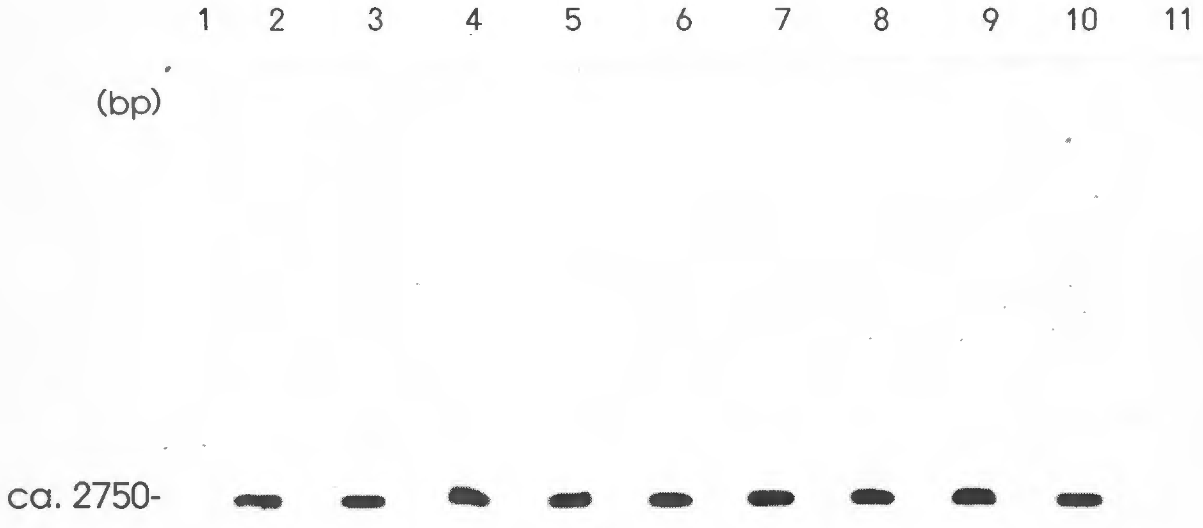

Fig. 4.5b.

4.3.4. Transfection of VV-infected CV-1 cells

In order to obtain VV recombinants, VV-infected CV-1 cells were transfected with the pGS20-VP7:*Gpt* and the pLSD-*Gpt* constructs separately as described in section 4.2.5.2. Virus harvested from these cells was passaged several times through MPA selection medium. At passage nos. 5, 6, 7 and 8 total DNA extractions were performed on the virus-infected CV-1 cells. This process was repeated in parallel with untransfected wild type VV. Total DNA-extractions were also performed on uninfected CV-1 cells.

The total DNA-extracts were digested with different restriction enzymes and subjected to agarose-gel electrophoresis. A smear of digested DNA could be observed in each lane due to the presence of genomic DNA (Figs. 4.6a., 4.7a.I., 4.7a.II. and 4.8a.). A Southern blot of the gel shown in Fig. 4.6a. was probed with a DIG-labelled pGS20-VP7:*Gpt Sma1* digested plasmid.

Bands corresponding to the size of the intact VV *Hind111* J-fragment (a 5 kb fragment encompassing the TK-gene - Mackett *et al.*, 1982), could be observed in the lanes containing DNA harvested from VV-infected cells (Fig. 4.6b.). However, these bands could also be observed in lanes containing DNA harvested from cells infected with VV and transfected with the pGS20-VP7:*Gpt* construct or the pLSD-*Gpt* construct. This indicated the presence of non-recombinant virus (i.e. viruses with an intact TK-gene). No further bands could be observed in lanes containing DNA harvested from cells transfected with the pLSD-*Gpt* construct and it can be deduced that no recombination took place between the LSDV TK-gene and the VV TK gene. Apart from the 5 kb band, two additional bands of ca. 6.5 kb and 3 kb respectively could be observed in lanes containing DNA harvested from cells transfected with the pGS20-VP7:*Gpt* construct.

The 6.5 kb band corresponded approximately to the size of the 5 kb VV *Hind111* J-fragment containing the ca. 1.2 kb VP7-insert, which indicated the presence of a recombinant virus. The presence of the VP7-gene in the 6.5 kb *Hind111* band observed was confirmed by probing of the Southern blot (of the lanes shown in Fig. 4.7a.I. and Fig. 4.7a.II.) with a DIG-labelled VP7 *BamH1*-fragment. A signal corresponding to a size of ca. 6.5 kb could be detected in the lanes containing *Hind111*-digested DNA harvested from cells transfected with the pGS20-VP7:*Gpt* construct (Fig. 4.7b.I.). No signal could be detected in lanes containing DNA harvested from VV-infected cells or uninfected cells (Fig. 4.7b.II.).

The presence of the 3 kb band (Fig. 4.6b.) could not be readily explained, since the size did not correspond to the size of the expected 2 kb *Gpt Hind111*-fragment.

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Fig. 4.6a. Agarose gel-electrophoresis of total DNA (*Hind111*-digested) extracted from CV1-cells. **Lane 1** (DNA molecular weight marker), λ-DNA digested with *Hind111*. **Lane 2,** [*]pGS20-VP7:*Gpt* (#8). **Lane 3,** [*]pGS20-VP7:*Gpt* (#5). **Lane 4,** [*]pLSD-*Gpt* (#8). **Lane 5,** [*]pLSD-*Gpt* (#5). **Lane 6,** [†]VV (#8). **Lane 7,** [†]VV (#5). **Lane 8,** total DNA from uninfected CV1-cells.

[*] *CV1-cells were pre-infected with VV and transfected with the indicated plasmid construct. The presumptive recombinant virus was passaged several times through MPA-selection medium. Total DNA was subsequently extracted from the infected cells at the indicated passage number (#) and digested with Hind111.*

[†] *VV was passaged several times through MPA-selection medium on CV1-cells. Total DNA was subsequently extracted from the infected CV1-cells at the indicated passage number after transfection (#) and digested with Hind111.*

Fig. 4.6b. Autoradiograph of the Southern blot of the agarose-gel shown in Fig. 4.6a. The blot was probed with a DIG-labelled (*Sma1*-digested) pGS20-VP7:*Gpt* plasmid (refer to legends of Fig. 4.6a. for descriptions of lanes).

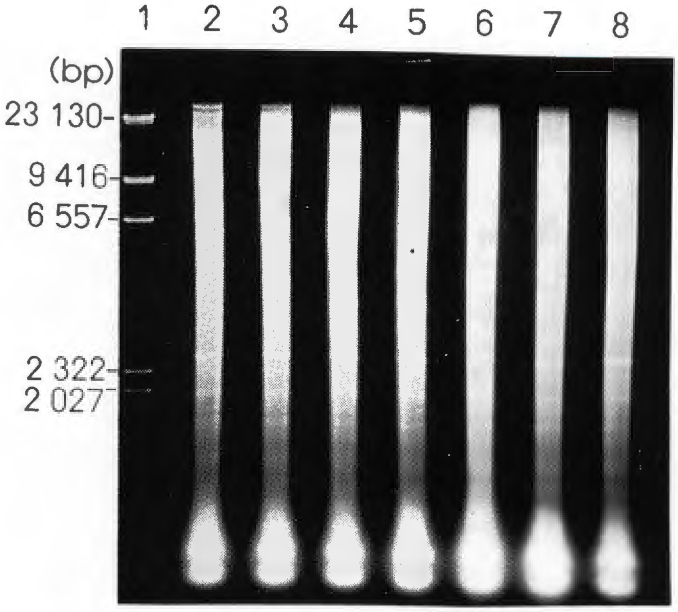

Fig. 4.6a.

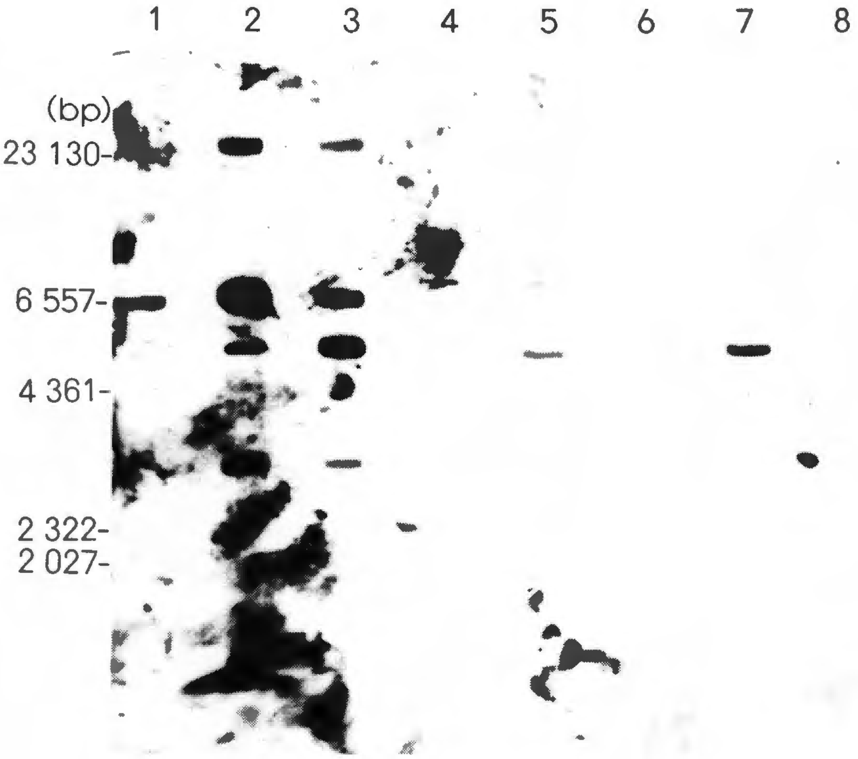

Fig. 4.6b.

LEGENDS

Fig. 4.7a. Agarose gel-electrophoresis of total DNA (*Hind111*-digested) extracted from CV1-cells.

I. **Lane 1** (DNA molecular weight marker), l-DNA digested with *Hind111*. **Lane 2,** *pGS20-VP7:*Gpt* (#8). **Lane 3,** *pGS20-VP7:*Gpt* (#7).

II. **Lane 4,** † VV (#8). **Lane 5,** total DNA from uninfected CV-1 cells **Lane 6,** PGS20-VP7:*Gpt* plasmid digested with *BamH1*.

* *CV1-cells were pre-infected with VV and transfected with the indicated plasmid construct. The presumptive recombinant virus was passaged several times through MPA-selection medium. Total DNA was subsequently extracted from the infected cells at the indicated passage number after transfection (#) and digested with Hind111.*

† *VV was passaged several times through MPA-selection medium on CV1-cells. Total DNA was subsequently extracted from the infected CV1-cells at the indicated passage number through selection medium (#) and digested with Hind111*

Fig. 4.7b. I & II. Southern blot of the agarose-gel shown in Fig. 4.7a. The blot was probed with a DIG-labelled *BamH1*-fragment containing the VP7-gene (refer to legends of Fig. 4.7a. for descriptions of lanes).

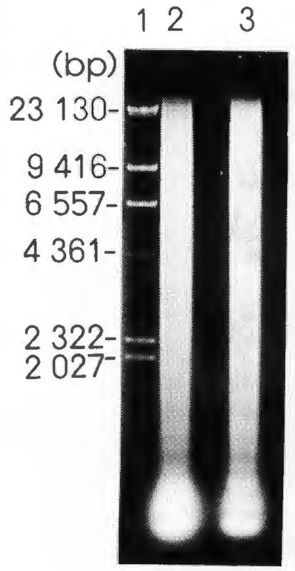

Fig. 4.7a.I.

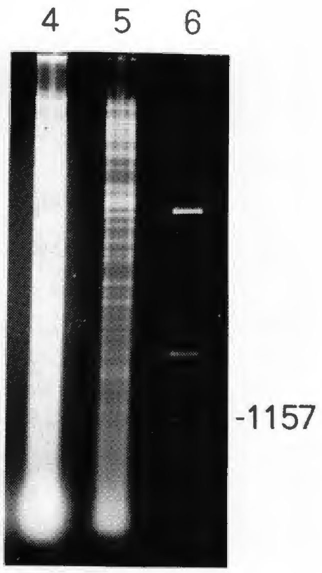

Fig. 4.7a.II.

Fig. 4.7b.I.

Fig. 4.7b.II.

LEGENDS

Fig. 4.8a. Agarose gel-electrophoresis of total DNA (digested with different restriction enzymes as indicated) extracted from CV1-cells. **Lane 1** (DNA molecular weight marker), λ-DNA digested with *Hind111*. **Lane 2,** [*]pGS20-VP7:*Gpt* (#9) digested with *Hind111*. **Lane 3,** [*]pGS20-VP7:*Gpt* (#9) digested with *Sal1*. **Lane 4,** [*]pGS20-VP7:*Gpt* (#9) digested with *Pst1*. **Lane 5,** [*]pLSD-*Gpt* (#8) digested with *Hind111*. **Lane 6,** [†]VV (#9) digested with *Hind111*. **Lane 7,** [†]VV (#9) digested with *Sal1*. **Lane 8,** [†]VV (#9) digested with *Pst1*. **Lane 9,** pLSD-*Gpt* digested with *Kpn1*.

[*] *CV1-cells were pre-infected with VV and transfected with the indicated plasmid construct. The presumptive recombinant virus was passaged several times through MPA-selection medium. Total DNA was subsequently extracted from the infected cells at the indicated passage number after transfection (#).*

[†] *VV was passaged several times through MPA-selection medium on CV1-cells. Total DNA was subsequently extracted from the infected CV1-cells at the indicated passage number through selection medium (#).*

Fig. 4.8b. Southern blot of the agarose-gel shown in Fig. 4.8a. probed with a DIG-labelled *Hind111*-fragment containing the *Gpt*-gene under the control of the VV P 7.5 promoter (refer to legends of Fig. 4.8a. for descriptions of lanes).

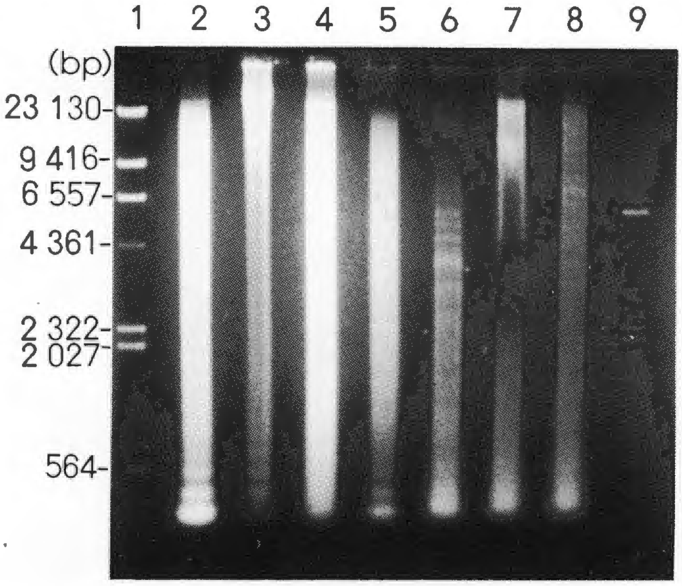

Fig. 4.8a.

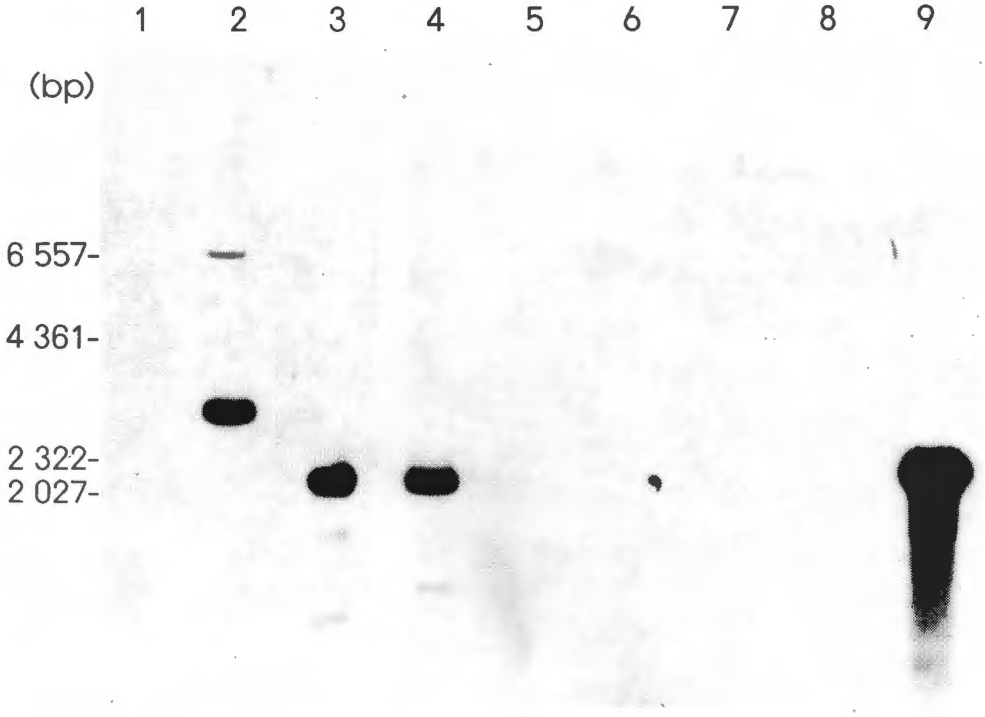

Fig. 4.8b.

After probing of the Southern blot of the gel shown in Fig. 4.8a. with a DIG-labelled *Hind111*-fragment (containing the *Gpt*-gene under the control of the VV P 7.5 promoter), the following signals were observed (Fig. 4.8b.): A strong signal corresponding to the size of a band of ca. 3 kb was detected in the lane containing *Hind111*-digested DNA harvested from VV-infected cells transfected with the pGS20-VP7:*Gpt* construct. This confirmed the presence of the *Gpt*-gene in the 3 kb *Hind111* band. Digests of DNA harvested from VV-infected cells transfected with the pGS20-VP7:*Gpt* construct with *Pst1* and *Sal1* yielded the expected fragment-size of ca. 2 kb (Figs. 4.3b and 4.8b.). No signal could be detected in the lanes containing DNA harvested from VV-infected cells or uninfected cells. A signal corresponding to the size of the expected 2 kb *Gpt*-gene was observed in the positive control lane containing *Kpn1*-digested pLSD-*Gpt* plasmid.

A possible explanation for the presence of the *Gpt*-gene in the ca. 3 kb *Hind111*-fragment detected is that a *Hind111* site could have been lost on either side of the *Gpt*-gene during the recombination event. Restriction enzyme analysis indicated that both *Hind111* sites were present in the original clone (Fig. 4.4b. lane 5). Upon examination of the autoradiographs shown in Fig. 4.6b. and 4.8b., it was found that the lost *Hind111* site was probably the site situated between the *Gpt*-gene and the 5' end TK-fragment of the pGS20 plasmid in the pGS20-VP7:*Gpt* construct (Fig. 4.3b.). If this interpretation is correct, recombination probably took place close to the *Gpt*-gene in the fragment stretching from the *Gpt*-gene to the VP7 gene (encompassing the 5' end of the TK-gene) of the pGS20-VP7:*Gpt* construct. A loss of the *Hind111* site upstream of the *Gpt*-gene would have lead to the presence of a ca. 6350 bp *Gpt*-containing fragment in a Southern blot of *Hind111*-digested recombinant DNA (Fig. 4.3b.), assuming recombination occurred between the TK-sequences. This fragment size was not obtained.

4.4. Discussion and conclusions.

4.4.1. Construction of the recombinant plasmid

A plasmid (designated pGS20-VP7:*Gpt*) was constructed so that it contained the VP7-gene (under the control of the P 7.5 promoter and flanked by VV TK-sequences), as well as the *Gpt*-gene (under the control of a separate P 7.5 promoter). The pGS20-vector, containing VV TK-sequences, was used for the construction of the pGS20-VP7:*Gpt* plasmid. Restriction enzyme mapping was performed on the native pGS20 vector, the vector containing the VP7-insert and subsequently the vector containing both the VP7- and the *Gpt*-inserts to determine the orientation of the inserted genes. A plasmid was selected in which the VP7-gene was inserted in the sense orientation, relative to the P 7.5 promoter in the pGS20-vector, to ensure expression in poxvirus-infected mammalian cells. The orientation of the VP7-gene relative to the P 7.5 promoter could easily be determined by restriction enzyme analysis due to the presence of a *Sal1*-site within the VP7-gene. The orientation of the two genes relative to one another was not important, since each gene was linked to a P 7.5 promoter. The orientation of the *Gpt*-gene could, however, be determined based on the sizes of the fragments generated by *Sma1* digestion of the pGS20-VP7:*Gpt* plasmid. The orientation of the two inserts, *Gpt* and VP7, and their respective P 7.5 promoters were found to be in the same direction relative to one another. In general it would be better to have the promoters back to back or two different promoters in the same direction to avoid recombination between direct repeats resulting in the loss of DNA. Recombination can thus occur between the two P 7.5 promoters resulting in the loss of the *Gpt*-gene. In this case, however, it is not important to retain the *Gpt*-gene in the final recombinant virus, since it is only required for the selection of the recombinants.

4.4.2. Virus propagation and the determination of the MPA concentration necessary for virus inhibition

The propagation of LSDV, the tolerance of the CFK-cells to different concentrations of MPA and the MPA-concentration necessary for LSDV-inhibition were as discussed in Chapter 3.4.2. and 3.4.4. Good yields were obtained when VV was propagated on the CAM's of domestic hens. The CV-1 cells appeared to be much more sensitive to MPA than the CFK-cells. With incubation of up to 3 days at 37°C harmful effects could be detected in CV-1 cells with concentrations as low as 0.5 μg MPA/ml, whereas concentrations up to 30 μg MPA/ml could be used with CFK-cells. Lower concentrations of MPA was required for VV-inhibition in CV-1 cells for up to 3 days than for LSDV-inhibition in CFK-cells (refer to section 4.3.2. and Chapter 3.4.4.).

4.4.3. Transfection of LSDV-infected CFK-cells

CFK-cells were infected with LSDV and transfected separately with the pGS20-VP7:*Gpt* and the pLSD-*Gpt* constructs. Transfection with the pGS20-VP7:*Gpt* construct was performed to obtain a recombinant LSDV virus containing the *Gpt*- and/or the BTV VP7-gene. The pGS20 vector contains VV TK-gene sequences. The capripoxvirus TK-gene and the TK-gene of VV are 68% similar on the nucleic acid level (Gershon and Black, 1989). This level of similarity was considered to be sufficient for heterologous recombination between the two genes (Schuman, 1992). Transfection with the pLSD-*Gpt* construct, containing LSDV TK-gene sequences (Chapter 3.2.8.), was repeated to obtain a LSDV recombinant containing the *Gpt*-gene. Virus harvested from the transfected cells were passaged six times through MPA-selection medium in parallel with wild type virus.

Total DNA was extracted from the following sources:

- Uninfected CFK cells
- CFK cells infected with LSDV at passage nos. 4, 5 and 6.
- CFK cells infected with virus that have presumably acquired the VP7 and/or the *Gpt* gene(s) through recombination at passage nos. 4, 5 and 6.

The extracted DNA was digested with *Hind111* and subjected to agarose-gel electrophoresis and Southern blotting. No recombinant LSDV could be detected by probing of the blot with a DIG-labelled pLSD-*Gpt* probe (containing the *Gpt*-gene under the control of the P 7.5 promoter and LSDV TK-gene sequences), as described in sections 4.2.5.1. and 4.3.3.

A signal detected in all the lanes containing DNA harvested from LSDV-infected cells, corresponded in size to the intact TK-gene of LSDV (Fig. 4.5b.). According to Gershon and Black (1989) the TK-gene is located in the capripoxvirus DNA *Hind111* fragment S. The *Hind111* fragment S of LSDV was found to be 2.75 kbp in size (Black *et al.*, 1986). Hybridization presumably occurred between the TK-sequences shared by the probe and the LSDV-DNA. No difference was apparent between DNA from cells containing native LSDV and DNA from cells containing putative recombinant virus. This implied that no recombination had occurred between the resident LSDV TK gene and the TK sequences present in the pLSD-*Gpt* and pGS20-VP7:*Gpt* constructs. No signal was detected in lanes containing DNA from uninfected cells. Wild type virus was thus still present even after several passages through selection medium. After the first transfection experiment no wild type LSDV could be detected by indirect immunoperoxidase staining with anti-LSDV serum after six passages of the virus through selection medium (Chapter 3 Figs. 3.9a., 3.9b. and 3.9c.). The only possible explanation for this observation could be that a lower MPA-concentration (1 μg MPA/ml) was used for the selection of recombinant LSDV in the second transfection experiment than for the first experiment (2.5 μg MPA/ml). The concentration were lowered because a lower virus input titre were used in the second transfection experiment with the pLSD-*Gpt* construct and to allow the *Gpt*-gene to get expressed sufficiently in order to overcome inhibition by MPA. If recombinant virus was present it occurred at a much lower concentration than the wild type virus, which prevented detection by hybridization.

The poor hybridization signal observed when linearized pGS20-VP7 plasmid (containing the VP7-gene, P 7.5 promoter and VV TK sequences) was used as a probe, could be attributed to the low degree of homology (68%) between the VV TK-sequences (of the pGS20 plasmid) and the LSDV TK gene.

A probable reason for the failure to obtain a recombinant LSDV virus is that the virus titre used in the transfection experiments was too low. Another possible explanation may be that the mutation, resulting in the loss of the internal *Kpn1* site of the *Gpt*-gene in the pLSD-*Gpt* construct, may have resulted in the gene being less effective even though it appeared to be functional. Recombination is a rare event even under favourable circumstances.

4.4.4. Transfection of VV-infected CV-1 cells

The construction of a recombinant VV containing a *Gpt*- and/or a BTV4 VP7-gene under the control of the VV P 7.5 promoter, was pursued as described in section 4.2.5.2. Two constructs were transfected separately into VV-infected CV-1 cells. The first construct, pGS20-VP7:*Gpt*, contained VV TK-sequences, the BTV4 VP7-gene and the *Gpt*-gene. Each of the latter two genes were under the control of a P 7.5 promoter (Fig. 4.3b.). The second construct, pLSD-*Gpt*, contained LSDV TK sequences, as well as the *Gpt*-gene linked to the P 7.5 promoter (Chapter 3 Fig. 3.5b.). Transfection studies with both the constructs were performed in parallel. Transfection with the second construct was performed to test the feasibility of heterologous recombination between the TK-genes of VV and LSDV.

Virus presumed to contain recombinants was harvested from the cells and passaged several times through selection medium. Total DNA extractions were performed at passages 5, 6, 7 and 8. Total DNA was also extracted from uninfected cells and CV-1 cells infected with wild type VV by an identical method. The DNA was subjected to non-radioactive Southern analysis using three distinct DIG labelled probes.

No recombinant virus was obtained from the transfection experiment with the pLSD-*Gpt* construct. It thus appears that heterologous recombination did not occur between the LSDV TK and the VV TK. However, in the experiment using the pGS20-VP7:*Gpt* construct homologous recombination did occur as anticipated, resulting in a recombinant virus.

The VP7 probe hybridized to a *HindIII* fragment of approximately 6.5 kb. It can be assumed that a single homologous recombinational event occurred between the VV TK sequences shared by the virus and the pGS20-VP7:*Gpt* construct. The observed 6.5 kb fragment is thus in fair agreement with the size of 6.2 kb expected for the *HindIII* fragment consisting of the VP7 gene (ca 1.2 kb) flanked by VV TK gene sequences (5 kb - Gershon and Black, 1989; Figs. 4.3b. and 4.7b.I.).

A DIG-labelled *Gpt* probe hybridized to *HindIII*-digested DNA to reveal a strong signal in the ca 3 kb position. This was rather unexpected, since the *Gpt*-containing *HindIII*-fragment was expected to occur in the 2 kb position. It appeared as if a *Hind111* site on one side of the *Gpt*-gene cassette (Fig. 4.3b.) was lost during the recombination event, since digests with *Pst1* and *Sal1* yielded the expected fragment-size of 2 kb for the excised *Gpt*-insert (Fig. 4.8b.). Both *Hind111* sites were present in the original clone (Fig. 4.4b. lane 5). Interpretation of poxvirus recombination experiments is not a trivial matter, since the molecular mechanism of homologous DNA recombination in poxvirus infected cells is still poorly understood (Ball, 1987; Fathi *et al.*, 1991; Spyropoulos *et al.*, 1988).

It is likely that a single recombinational event occurred, as multiple or double recombinational events would theoretically result in a variety of fragment sizes (including *Gpt*-containing bands in the 2 kb position). This is due to the fact that the recombinational events can theoretically occur anywhere in the TK region shared by VV and the construct.

No plaque purification steps were included as this was only a preliminary screening for the presence of recombinant virus(es). It was thus not surprising that a mixture of viruses would be present.

Southern blot analysis using a DIG-labelled pGS20-VP7:*Gpt* probe (containing VV TK sequences) revealed a 5 kb band which corresponded to the size of the intact VV *Hind111* J-fragment (Fig. 4.6b.). This was an indication of the presence of wild type virus, which meant that selection was not complete. However, a selection advantage in favour of the recombinant viruses was evident. The signal corresponding to the intact 5 kb TK gene appeared weaker with subsequent passages whereas the signal associated with the recombinant virus progressively became more pronounced. This selection advantage of the recombinant virus over the wild type virus showed that the *Gpt*-gene can be employed successfully as a dominant selectable gene in the construction of recombinant poxviruses, even though selection was not complete.

Additional restriction enzyme analysis and sequencing of the viral DNA region spanning the TK-gene are required to determine the exact nature of the recombination event. The virus would need to be plaque purified and expression of the VP7-gene should be further investigated before this recombinant could be tested in animal vaccine-trials for efficiency.

The results obtained with the transfection of the pGS20-VP7:*Gpt* construct into the LSDV-infected CFK-cells (heterologous recombination) or the VV-infected CV-1 cells (homologous recombination), indicated that homologous TK-sequences may direct recombination at a higher frequency. The VV and the LSDV TK-genes exhibit a relatively low level (68%) of nucleotide sequence similarity (Gershon and Black, 1989). Theoretical predictions do, however, not rule out the possibility of obtaining recombinants between heterologous TK sequences exhibiting sequence similarity at this level (Shuman, 1992). According to G. Viljoen of the OVI, Pretoria (personal communication), they had succeeded in inserting the BTV-VP7 gene into LSDV by heterologous recombination between VV-TK and LSDV-TK sequences. A recombinant capripoxvirus expressing Rift Valley Fever Virus (RVFV) glycoproteins was obtained by heterologous recombination between capripoxvirus TK- and VV TK-sequences (J.C. DeMartini - abstract, IXth International Congress of Virology, 1993).

Romero *et al.* (1993) reported the construction of a recombinant capripoxvirus vaccine that protects cattle against rinderpest and lumpy skin disease. Construction of the aforementioned capripoxvirus recombinant was achieved by exploiting a capripoxvirus TK to direct homologous recombination with the Pirbright Capripoxvirus vaccine TK.

At the moment LSDV is investigated as a vector for the construction of veterinary vaccines. An interesting possibility for future research, however, may be to investigate the potential of highly attenuated LSDV as a non-replicating vector in humans, as was reported for avian poxvirus by Cadoz *et al.*, 1992. LSDV-based vectors may provide an important basis for the development of safe and effective future recombinant vaccines against a wide spectrum of antigens.

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CONTENTS OF APPENDIXES

APPENDIX A. BUFFERS, MEDIA AND SOLUTIONS

A.1. Activated Trypsin Versene (ATV)

Sterile glass-distilled water	88.5 ml
Trypsin Base (section A.20.)	10 ml
Trypsin stock (5%; section A.19.)	1 ml
PSN (section A.13.)	0.5 ml

The above were mixed aseptically and stored at 4°C (Freshney, 1987).

A.2. Cell-freezing media

FCS	10% (v/v)
PSN (section A.13)	0.5% (v/v)
HEPES (section A.4.)	0.02 M
DMSO	7.5%
DMEM (section A.3.)	

DMEM, PSN and the HEPES buffer (1 M stock solution) was filter-sterilized separately by filtering through an 0.2 μm filter. FCS was supplied sterily and DMSO was sterilized by autoclaving. The mentioned ingredients were mixed together aseptically and the media was stored at 4°C (Freshney, 1987; Lennette and Schmidt, 1979).

A.3. Dulbecco's Modified Eagle's Essential Medium (DMEM)

DMEM, obtained from Highveld Biologicals, Pretoria, South Africa, was made according to the manufacturer's specifications. Ten liter medium was made up and sterilized through an 0.2 μm filter when needed. Fetal calf serum and antibiotics (PSN) were added as required.

A.4. HEPES Buffer (1 M)

HEPES	23.8 g
NaOH (0.3 M)	80 ml

The HEPES powder was dissolved in the 0.3 M NaOH and the pH was adjusted to 7.2. Distilled water was added to a final volume of 100 ml and the solution was sterilized by filtration through an 0.2 μm filter. The buffer was kept at 4°C.

A.5. ZN-Carbolfuchsin stain

Basic fuchsin	5 g
Phenol (crystalline)	25 g
Alcohol (95%)	50 ml
Water	500 ml

The fuchsin was mixed with the phenol and heated in boiling water for 5 minutes. The alcohol was added to the solution after it was mixed. The solution was filtered through a Whatman No.1 filter before it was used

A.6. Loading buffer (10 x concentrated)

Ficoll (Type 400, Pharmacia)	20%
Na_2EDTA (pH 8.0)	0.1 M
SDS	1%
Bromophenol blue	0.25%
Xylene Cyanol	0.25%

The above mentioned ingredients were mixed with distilled water to the required volume. The solution were dispensed into 10 ml aliquots and kept at room temperature (Ausubel et al., 1987).

A.7. Luria media

Bacto Tryptone	10 g
Bacto Yeast extract	5 g
NaCl	5 g
Agar	15 g

For the preparation of Luria broth the tryptone, yeast extract and NaCl were dissolved in 800 ml distilled water. After adjusting the pH to 7.5 with 10 M NaOH, distilled water was added to a final volume of 1 000 ml. Luria agar was prepared as follows: The agar was dissolved separately in 500 ml distilled water by heating in a microwave. The tryptone, yeast extract and NaCl were dissolved in 300 ml distilled water. The two solutions were mixed and the pH was adjusted to 7.5 with 10 M NaOH. Distilled water was added to a final volume of 1 000 ml. The Luria-broth and -agar were sterilized by autoclaving and kept at room temperature. Antibiotics were added to the media after autoclaving and cooling to 50°C as required (Sambrook et al., 1989).

A.8. Lysis buffer

Tris-HCl (1 M, pH 7.8)	25 ml
NaCl (5 M)	5 ml
EDTA (0.5 M)	2.5 ml
SDS (10%)	25 ml

Distilled water was added to a final volume of 250 ml. The buffer was sterilized by filtering through an 0.2 µm filter or by autoclaving and kept at room temperature in 10 ml aliquots.

A.9. McIlvain's buffer, pH 7.4

Solution A:	0.1 M Citric acid
Solution B:	0.2 M $Na_2HPO_4.12H_2O$

Of solution A, 1.83 ml was mixed with 18.7 ml of solution B. Distilled water (800 ml) was added and the pH was adjusted to 7.4 with either solution A or B. Distilled water was added to a final volume of 1 000 ml. The buffer was sterilized by autoclaving and kept at 4°C.

A.10. Mycophenolic Acid (MPA)-containing selection media

Stock solutions:
MPA (10 mg/ml)

MPA	100 mg
NaOH (0.1 N) solution	10 ml

The MPA was dissolved in the NaOH solution, filter sterilized through an 0.2 µm filter and kept in the dark at 4°C.

Xanthine (10 mg/ml)

Xanthine	100 mg
NaOH (0.1 N) solution	10 ml

The xanthine was dissolved overnight at room temperature in the NaOH solution. After filter-sterilizing through an 0.2 µm filter, it was kept at room temperature.

Hypoxanthine (10 mg/ml)

Hypoxanthine	100 mg
NaOH (0.1 N) solution	10 ml

The hypoxanthine was dissolved overnight at room temperature in the NaOH solution and filter sterilized through an 0.2 µm filter. The solution was kept at room temperature.

Selection medium:

DMEM (Section A.3.)	93 ml
PSN stock (Section A.13)	0.5 ml
FCS	4 ml
Hypoxanthine (10 mg/ml)	2,5 ml
Xanthine (10 mg/ml)	140 µl
MPA (10 mg/ml)	10 - 25 µl (as indicated in text)

The selection medium was freshly prepared when required.

A.11. Phenol

Commercial phenol was liquidified at 56°C. Hydroxyquinoline was added to a final concentration of 0.1%. Several subsequent extractions were done with an equal volume of 1 M Tris (pH 8.0) buffer until the pH of the aqueous phase was higher than 7.8. The phenol was stored in 5 ml aliquots under an equal volume of 1 M Tris (pH 8) buffer containing 2% β-mercaptoethanol at -20°C (Sambrook et al., 1989).

A.12 Phosphate Buffered Saline (PBS)

NaCl	8.0 g
KCl	0.2 g
KH_2PO_4	0.12 g
Na_2HPO_4 (anhydrous)	0.91 g
OR	
$Na_2HPO_4.2H_2O$	1.14 g
OR	
$Na_2HPO_4.12H_2O$	2.28 g

The above ingredients were dissolved in 900 ml of distilled water and the pH was adjusted to 7,5. Distilled water was added to a final volume of 1 000 ml and the solution was dispensed into 100 ml volumes. PBS was sterilized by autoclaving and stored at room temperature.

A.13. Penicillin Streptomycin Neomycin (PSN) Solution

Penicillin	2×10^6 Units
Streptomycin	2 g
Neomycin	2 g

The above mentioned antibiotics were reconstituted in 100 ml of physiological saline. After filter-sterilization through an 0.2 μm filter, the solution was stored at -20°C in 2.5 ml aliquots. PSN was used at a final concentration of 100 U Penicillin, 100 μg Streptomycin and 100 μg Neomycin per ml (0.5 ml stock PSN solution/100 ml).

A.14. Solutions used for X-gal-based histochemical assay

The solutions were prepared according to MacGregor et al. (1991).

Stock solutions:

Stock solutions of 50 mM potassium ferricyanide ($K_3Fe(CN)_6$) and 50 mM potassium ferrocyanide ($K_4Fe(CN)_6$) were prepared in double-distilled water (ddH_2O) and stored in foil-wrapped glassware at 4°C for up to 3 months.

Stock solutions of 1 M $MgCl_2$, 1 M Na_2HPO_4 and 1 M NaH_2PO_4 were prepared in ddH_2O and stored at room temperature.

The 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) was dissolved in *N,N*-dimethyl formamide (DMSO) at a concentration of 20 mg/ml and stored in a glass-container in the dark at -20°C.

Paraformaldehyde (4%) was prepared in a fume hood (gloves and a mask should be worn when handling paraformaldehyde) as follows: 8 g of powder was dissolved in 150 ml of 0.1 M sodium phosphate, pH 7.3 (77 mM Na_2HPO_4, 23 mM NaH_2PO_4) by stirring and heating to 60°C. To this 10 M NaOH was added at a rate of 1 drop/minute until the solution cleared. The volume was adjusted to 200 ml with 0.1 M sodium phosphate, pH 7.3, and the solution was stored at 4°C for up to 1 month.

PBS was prepared as in section A.12.

Working solution:

X-gal	1 mg/ml
$K_3Fe(CN)_6$	5 mM
$K_4Fe(CN)_6$	5 mM
$MgCl_2$	2 MM
PBS	

The working solution was freshly prepared in PBS, filtered through an 0,2 µm filter and used immediately.

A.15. Tris-acetate (TAE) Buffer

50 x concentrated:

TRIS	242 g
Glacial acetic acid	57.1 ml
EDTA (0.5 M, pH 8.0)	100 ml

The above mentioned ingredients were mixed together in distilled water to a final volume of 1 000 ml, autoclaved and stored at room temperature (Sambrook et al., 1989).

A.16. Tris-borate (TBE) Buffer

5 x concentrated:

Tris base	54 g
Boric acid	27.5 g
EDTA (0.5 M, pH 8.0)	20 ml

The above mentioned ingredients were mixed together in distilled water to a final volume of one litre, autoclaved and stored at room temperature. The buffer were disgarded if a precipitate formed after prolonged storage (Sambrook et al., 1989).

A.17. TE Buffer (pH 8 or 9)

TRIS (1 M, pH 9.0)	10 ml
EDTA (0.5 M)	0.5 ml

The TRIS and EDTA were mixed with 600 ml of distilled water and the pH was adjusted to pH 8 or 9. Distilled water was added to a final volume of 1 000 ml and the buffer was sterilized by autoclaving.

A.18. Trypan Blue Solution

Trypan Blue powder	5 g
Physiological saline	100 ml

The powder was dissolved in the saline solution and filtered through Whatman number 1 paper. The dye was sterilized by filtering through a 0.2 μm filter, dispensed into 2 ml amounts and stored at 4°C.

A.19. Trypsin Stock

Trypsin powder	5 g
Trypsin base	10 ml
Distilled water	90 ml

The trypsin base was added to the water and concentrated HCl was added to acidify the solution to a bright yellow colour. The trypsin powder was sprinkled onto the surface of this solution and left to dissolve slowly (without stirring or agitation). The solution was filter-sterilized through an 0.2 μm filter and stored at -20°C in 1 ml aliquots.

A.20. Trypsin Base

NaCl	30 g
KCl	2 g
KH_2PO_4	1.2 g
Na_2HPO_4	9.1 g
OR	
$Na_2HPO_4.2H_2O$	11.2 g
OR	
$Na_2HPO_4.12H_2O$	22.8 g
Phenol (0.4% solution)	25 ml
Glucose	5 g
EDTA	2 g

The EDTA was dissolved separately in 100 ml of distilled water. After the rest of the chemicals were dissolved in 700 ml of distilled water, the EDTA solution was added to it. The pH was adjusted to 7.8 with 1 N NaOH. Distilled water was added to a final volume of 1 000 ml and the solution was filter-sterilized through an 0.2 µm filter. The trypsin base was stored at -20°C in 20 ml aliquots.

A.21. Virus diluent

HEPES Buffer (1 M; section A.4.)	1 ml
PSN (section A.13)	0.5 ml
DMEM (section A.3)	98.5 ml

The HEPES buffer and PSN was added to the media (without fetal calf serum) and the pH was adjusted to 7.4 with HCl or NaOH. The diluent was filter sterilized through an 0.2 µm filter and stored at 4°C.

APPENDIX B. MATERIALS

The endonuclease restriction enzymes *BamH1, EcoR1, Hind111, Kpn1, Pst1, Sal1* and *Sma1*, together with the appropriate buffers and bovine serum albumin (BSA, special quality for molecular biology) were supplied by Boehringer Mannheim, Mannheim, Germany and Amersham International, Amersham, United Kingdom (UK).

Proteinase K, the DNA Labelling Kit (Cat. No. 1175 033), the DIG Luminescent Detection Kit (Cat. No. 1363 514) and the DIG Nucleic Acid Detection Kit (Cat. No. 1175 041) were supplied by Boehringer Mannheim, Mannheim, Germany. The nylon membranes (Hybond N and Hybond N^+) and the Nick Translation Kit were obtained from Amersham International, Amersham, United Kingdom. Gamma labelled ^{32}P ATP was supplied by the Department of Biochemistry, University of Cape Town (UCT), South Africa (S.A.).

Sigma Chemicals, St Louis, United States of America (USA) supplied the following: Cyanol, bromophenol blue, HEPES, hypoxanthine, ribonuclease A, SDS, trypan blue and xanthine.

The following chemicals were obtained from BDH, Poole, England: β-mercaptoethanol, citric acid, EDTA, glycerol, HCl, KH_2PO_4, $MgSO_4$, Na-acetate, $NaHCO_3$, NaH_2PO_4, Na-n-lauryl sarcosinate and sucrose.

Merck, Darmstadt, Germany supplied the following: acetic acid, acetone, concentrated HCl, chloroform, ethanol (absolute), ethidium bromide, formaldehyde, glacial acetic acid, glycerol, isoamylalcohol, KCl, methanol, NaCl, phenol, TRIS salt and xylol.

Ilford, Cheshire, England was the supplier of the black and white negative film and the developer and autoradiographs used with it. The X-ray film, developer, fixer and hardener were obtained from May Baker, Port Elizabeth, South Africa.

Agarose, Dextran T10 and Sephadex G50 were obtained from Pharmacia, Fine Chemicals, Upsala, Sweden. Luria-agar/-broth, tryptone broth and ZN Carbolfuchsin stain were supplied by the Department of Medical Microbiology, UCT, S.A.. Unilab Saarchem, Krugersdorp, S.A., supplied the Haematoxylin stain.

The enriched medium of Dulbecco,s Modified Eagle's Essential Medium (DMEM), used for the maintenance and growth of mammalian cells, was obtained from Highveld Biologicals, Pretoria, S.A.. The fetal calf serum was supplied by Delta

Bioproducts, Kempton Park, S.A., and the State Vaccine Institute, Pinelands, S.A.. Glaxo Pharmaceuticals, Wadeville, S.A., and Scherag, Isando, S.A., supplied the antibiotic powders Penicillin, Streptomycin and Neomycin. Trypsin powder was obtained from Difco Laboratories, Detroit, USA. Fluka BioChemika, Buchs, Switzerland supplied the Mycophenolic Acid (MPA).

The tissue culture flasks and multi-well plates were obtained from Falcon, Oxnard, USA; Nunclone, Roskilde, Denmark and Sterilin, Hounslow, UK.

APPENDIX C. SIZES OF DNA-FRAGMENTS USED AS MARKERS

Lambda DNA digested with *Hind111*

(bp)
23 130
9 416
6 557
4 361
2 322
2 027
564
125

Lambda DNA digested with *Sty1*

(bp)
19 329
7 743
6 223
4 254
3 472
2 690
1 882
1 489
925
421
74