The role of a second RGD motif in the 1D protein of a Namibian SAT1 foot-and-mouth disease virus for target cell attachment

by

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SUMMARY

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Foot-and-mouth disease virus (FMDV), which is a member of the *Aphthovirus* genus of *Picornaviridae*, is an economically important animal virus that infects cloven-hoofed animals. At least two families of cell-surface receptors have been identified that mediate binding of FMDV to susceptible cells, *i.e.* integrins and heparan sulfate proteoglycans (HSPG). Whereas several tissue culture-adapted FMD viruses use heparan sulfate proteoglycans for internalization, field isolates of FMDV use integrins as receptors. A conserved amino acid sequence Arg-Gly-Asp (RGD), located in the surface-exposed flexible βG-βH loop of the viral outer capsid protein 1D, participates in binding FMDV to integrin receptors on the surface of susceptible cells. Sequence analyses of the 1D-encoding genome region of a SAT1 type FMDV field isolate, NAM/307/98, indicated the presence of a second RGD sequence upstream of the conserved G-H loop RGD sequence. Since FMDV is capable of binding to RGD-binding integrin receptors, the aim of this study was to investigate whether the second RGD motif in capsid protein 1D of NAM/307/98 may function as a ligand for receptor-binding in baby hamster kidney (BHK) cells.

Towards this end, a cDNA copy of the genomic region encoding the external capsid proteins (1B-1D) of NAM/307/98 was cloned into pSAT2, a genome-length cDNA clone derived

from the SAT2 strain ZIM/7/83. Transfection of BHK-21 cells with *in vitro*-transcribed RNA derived from the chimeric pNAM/SAT2 clone resulted in the recovery of infectious chimeric virus particles. The availability of such an infectious chimeric cDNA clone greatly facilitated the introduction of specific, targeted mutations in the 1D capsid-encoding region of NAM/307/98 in order to investigate the functional role of the second RGD sequence.

Using the chimeric SAT1/SAT2 cDNA clone as template, the RGD codons in the G-H loop of NAM/307/98 were replaced with codons specifying a KGE sequence by a polymerase chain reaction (PCR)-based method of site-directed mutagenesis. The mutated DNA fragment was introduced into the pSAT2 infectious cDNA clone, transcribed *in vitro*, and the resulting RNA transfected into BHK-21 cells. The transfected cells were analyzed for cytopathic effect (CPE). In contrast to cells transfected with non-mutated RNA transcripts, from which infectious virus could be recovered, cells transfected with the mutant RNA transcripts showed cell lysis, but no CPE could be observed upon subsequent passaging of the resultant viruses on BHK cells. Notably, subsequent replacement of the KGE sequence with an RGD sequence in the mutant clones led to recovery of infectious viruses. Furthermore, RNA replication could be demonstrated with the mutant and non-mutant chimeric viruses, suggesting that virus particles were indeed present in the tissue culture supernatants of all transfected cells. Based on the results obtained during the course of this investigation, it was therefore concluded that the second RGD motif, situated upstream of the conserved RGD motif in the G-H loop of capsid protein 1D of NAM/307/98, does not function as a ligand for receptor-binding in BHK-21 cells.

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LIST OF ABBREVIATIONS

aa	amino acid
Arg (R)	arginine
Asp (D)	aspartic acid
BHK	baby hamster kidney
BME	Eagle's basal medium
bp	base pair
ºC	degrees Celsius
ca.	approximately
cDNA	complementary deoxyribonucleic acid
CHO	chinese hamster ovary
CPE	cytopathic effect
DAPSA	DNA And Protein Sequence Analysis
D-MEM	Dulbeco's minimal essential medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside-5'-triphosphate
EDTA	ethylenediaminetetra-acetic acid
e.g.	for example
EtBr	ethidium bromide
FCS	fetal calf serum
Fig.	figure
FMDV	foot-and-mouth disease virus
g	gram
Glu (E)	glutamic acid
GuSCN	guanidinium thiocyanate
Gly (G)	glycine
h	hour
HS	heparan sulfate
HSPGS	heparan sulfate proteoglycans
IB-RS-2	Instituto Biologico Renal Suino
kb	kilobase pair
LB medium	Luria-Bertani medium
Lys (K)	lysine
M	molar

MAbs	monoclonal antibodies
MBS	MES-buffered saline
MES	[*N*-morpholino]ethane-sulfonic acid
min	minute
ml	milliliter
mM	millimolar
mRNA	messenger ribonucleic acid
NEAA	non-essential amino acids
OIE	Office International des Epizooties
ORF	open reading frame
PCR	polymerase chain reaction
PK	pig kidney
pMol	picomole
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute-1640 Medium
RT-PCR	reverse transcriptase-polymerase chain reaction
s	second
SAT	South African Territories
SDS	sodium dodecyl sulphate
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TPB	tryptose phosphate broth
Tris	Tris-hydroxymethyl-aminomethane
U	units
UTR	untranslated region
µg	microgram
µl	microliter
µM	micromolar
V	Volts
v/v	volume per volume
w/v	weight per volume

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CHAPTER 1

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Foot-and-mouth disease (FMD), of which foot-and-mouth disease virus (FMDV) is the causative agent, is a highly contagious disease of cloven-hoofed animals. Its host range include domestic animals such as cattle, swine, sheep and goats, as well as more than 30 species of wild ruminants (Thomson, 1994). FMD is one of the most important infectious animal diseases, as an outbreak could have a major economic impact on the livestock industry of an affected country. In the last decade, costly FMD outbreaks have occurred in Asia (India and the Philippines) (1996), Taiwan (1997), the Republic of Korea (2000 and 2002), South Africa (KwaZulu-Natal) (2000), Japan (2000), South America (Argentina and Brazil) (2001) and the United Kingdom (2001) (Knowles and Samuel, 2003). Although mortality associated with FMD is usually low (less than 3%), the disease decreases livestock productivity and affected countries cannot participate in international trade of animals and animal products. This is particularly detrimental for developing countries, which rely on agricultural exports of animal origin for economic development. Other losses that may be incurred during an outbreak include a reduction in the production of meat, milk and other animal products (Thomson, 1994). Based on its major economic and international importance, FMDV has been categorized as a list A disease by the Office International des Epizooties (OIE) (Quinn and Markey, 2001; OIE Manual, 2004). List A diseases are transmissible diseases that have the potential for very serious and rapid spread, they have particularly serious socio-economic or public health consequences and are of major importance in the international trade of animals and animal products (OIE Manual, 2004).

FMD spreads rapidly to susceptible animals with transmission of the disease being direct or indirect. The most common mode of transmission is through direct contact of susceptible animals with infected animals that excrete the virus, since virus particles are present in all secretions and aerosols during infection (*e.g*. saliva and semen). Indirect transmission of the disease can occur by mechanical carriage of the virus on vehicles, footwear, clothing and through contact with viral-contaminated animal products such as meat, offal and milk (Quinn

and Markey, 2001). Under favorable conditions of low temperatures, high humidity and moderate wind speeds, viral aerosols may spread 10 km or more over land (Quinn and Markey, 2001). In natural infections, the main route of virus entry is via the respiratory tract. Following virus multiplication in the pharynx epithelium, which leads to the production of primary vesicles (Burrows *et al*., 1981), the virus enters the blood stream and lymphatic system and spreads rapidly to different organs and tissues, resulting in the formation of secondary vesicles in the mouth and feet of infected hosts (Sobrino *et al*., 2001).

The incubation period is generally short, ranging from two to eight days, but can last up to 13 days. Whereas the earliest clinical signs of disease include fever, dullness, loss of appetite, a fall in milk yield and the cessation of rumination, it is shortly followed by signs more related to the development of lesions such as lameness, a disinclination to stand, salivation, smacking of the lips and grinding of the teeth (Fig. 1.1) (Thomson, 1994). The severity of the disease may vary with the level of immunity, the virus dose and strain, host species and between individuals of a given species. Asymptomatic, persistent infections can be found in ruminants for periods ranging from a few weeks to several years, depending on the animal species, during which virus can be isolated from the oesophagus and throat fluids (Van Bekkum *et al*., 1959; Condy *et al*., 1985). Both naïve and vaccinated animals are capable of becoming persistently infected following an acute infection. The mechanisms that mediate virus persistence are unclear, but are likely to result from a dynamic equilibrium between the host immune system and the selection of viral antigenic variants at the mucosae of the upper respiratory tract (Gebauer *et al*., 1988; Salt, 1993).

Countries free of FMD have restrictions on the movement of animals and animal products originating from countries where FMD occurs. In the event of a FMD outbreak, the first step is to confirm clinical diagnosis by laboratory tests. A quarantine area should immediately be declared and enforced, and the extent of the spread of the disease should be established. A stamping-out policy is then followed during which infected or susceptible in-contact animals are slaughtered. The carcasses are either buried or, more commonly, burnt (Quinn and Markey, 2001). In contrast, in countries where FMD is endemic, the disease is controlled by vaccination and zoosanitary measures, *e.g.* restrictions on the movement of animals and animal products from affected areas. Vaccines can help to contain the disease quickly if it is used strategically to create barriers between infected zones and disease-free zones. However, the immunity period that a vaccine offers is normally short (6 to 12 months) and annual

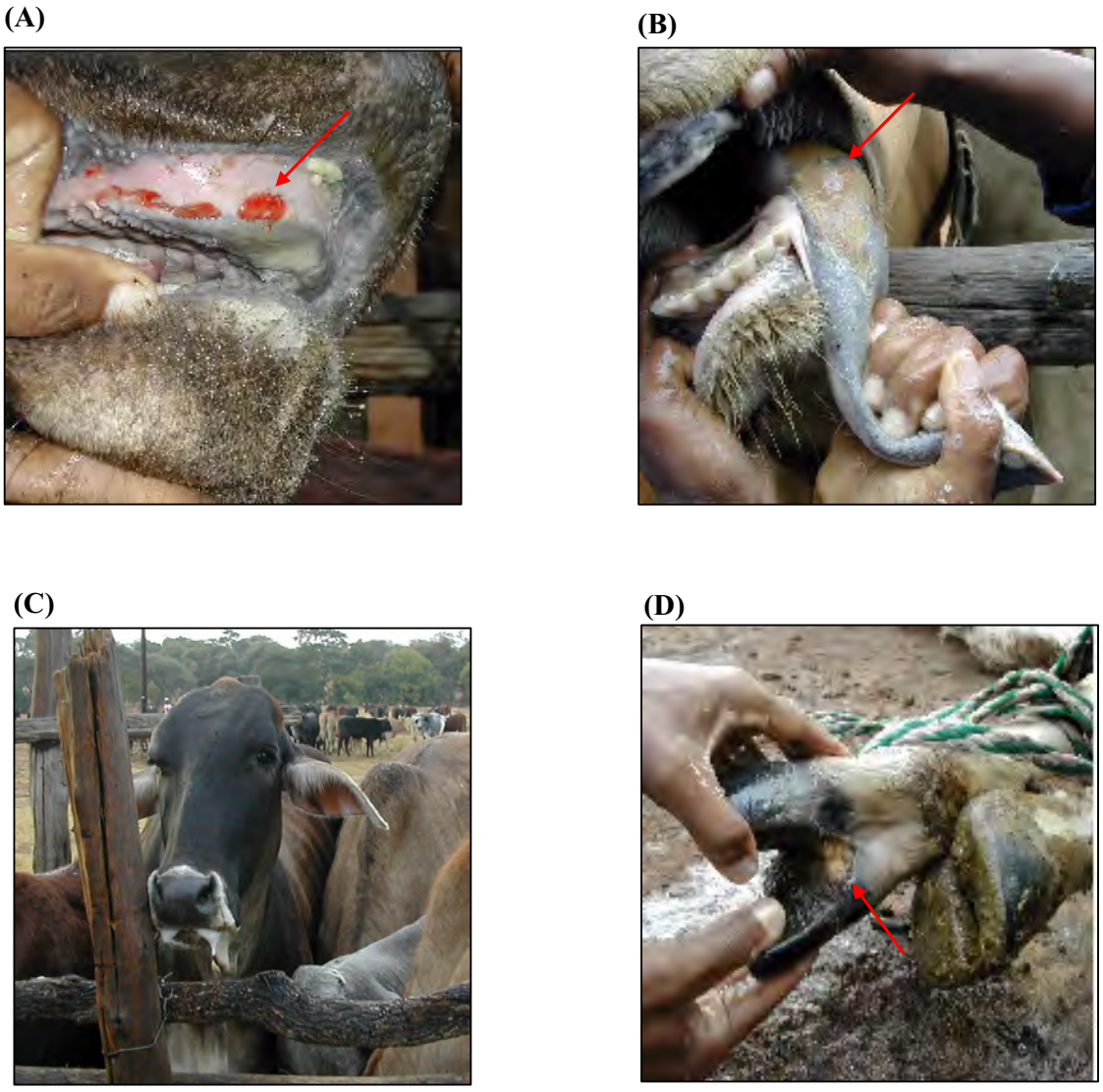

Fig. 1.1 Examples of foot-and-mouth disease lesions. (A) Raw lesions on the gum of a bovine, as indicated by a red arrow. (B) Ruptured lesion on the tongue (arrow) of a bovine that has already begun to heal. (C) A bovine salivating due to lesion within the mouth. (D) A lesion on the hoof of a bovine, indicated by a red arrow. (Pictures obtained from Dr Wilna Vosloo).

revaccination is required. A further complicating factor is that FMDV is subject to extensive antigenic variation and consequently, variants arise from which animals immunised with a particular vaccine may not be protected (Thomson, 1994). FMDV evolves independently and rapidly in different geographical areas, thus necessitating the development of vaccines that are custom-made to specific geographic areas in order for them to be effective (Doel, 2003).

Another important aspect in the control of FMD concerns the rapid and accurate diagnosis of the disease. Since FMDV spreads rapidly and is highly infectious, the handling of the virus, as well as procedures relating to diagnosis of the disease is restricted to high-security laboratories (Thomson, 1994). The diagnosis of the disease is dependent on clinical assessments, identification of the virus or viral antigens during the early stages of infection, and serology in cases where the infection has lasted longer than two weeks (Thomson, 1994). Originally, the complementation fixation (CF) test was used for the routine detection of FMDV in clinical samples. In recent years this test has been replaced in many laboratories by enzyme-linked immunosorbent assays (ELISA) and the serum neutralisation tests (SNT) (Roeder and Le Blanc-Smith, 1987; OIE Manual, 2004). However, the development of reverse transcriptase-polymerase chain reaction (RT-PCR) techniques, coupled with automated nucleotide sequencing, has become an important tool for the efficient and rapid diagnosis of FMD (Collen, 1994; Sobrino *et al*., 2001).

1.2 CLASSIFICATION OF FOOT-AND-MOUTH DISEASE VIRUS

FMDV belongs to the family *Picornaviridae* (Semler and Wimmer, 2002) and is the prototype member of the *Aphthovirus* genus. Equine rhinitis A virus is also a member of the *Aphthovirus* genus due to its genomic structure being more closely related to FMDV than to any of the other picornaviruses (Li *et al.,* 1996). Seven distinct FMDV serotypes (Type O, A, C, Asia-1 and the South African Territories [SAT] 1, 2 and 3) have been identified based on the lack of cross-protection following infection or vaccination (Domingo *et al.,* 2002). Types O and A were recognized in the 1920s in France (Vallée and Carré, 1922; Waldmann and Trautwein, 1926) and have the broadest distribution, occurring in many parts of Africa, southern Asia, the Far East (excluding type A) and South America. Type C was discovered in 1926 in Germany (Waldmann and Trauttwein, 1926) and appears to have become confined to the Indian sub-continent (Knowles and Samuel, 2003). This was followed by the discovery of the SAT types in the 1940s (Brooksby, 1958), which are generally confined to sub-Saharan

Africa, whilst the Asia-1 serotype, the last serotype to be discovered in Pakistan in 1954, is restricted to southern Asia (Brown, 2003).

1.3 GENOME ORGANIZATION

The FMDV genome is a positive sense single-stranded RNA molecule of *ca.* 8 450 nucleotides in length (Bachrach, 1977). The viral RNA genome consists of a single open reading frame (ORF) flanked by two untranslated regions (UTRs), both of which display complex secondary structure (Fig. 1.2).

Covalently linked to the 5' UTR of the genome is the virus-encoded protein VPg (viral genome-linked protein) (Beck *et al.,* 1983), which is encoded by the 3B-coding region in the viral genome. The VPg protein is thought to play a role in pathogenesis and host range determination of the virus (Mason *et al*., 2003). The first region of the 5' UTR, known as the S fragment (S), is *ca.* 360 nucleotides in length and has a sequence that is capable of folding into a long stem-loop structure, but it does not encode proteins (Newton *et al*., 1985). Following the S fragment is an RNase T1-resistant tract composed of *ca.* 90% cytosine (C) residues, known as the poly-cytidylate tract (poly (C) tract) (Brown *et al.,* 1974). Initial reports suggested that the length of the poly (C) tract played a role in virulence (Harris and Brown, 1977). However, viruses containing a poly (C) tract of only two C residues have been reported to be virulent in mice, but they display higher particle:infectious doses than viruses with a long poly (C) tract (Rieder *et al.,* 1993). A cellular poly (C)-binding protein (PCBP), together with host and viral proteins, is postulated to bring together the 5'- and 3'-ends of the poliovirus genome in a structure that is capable of regulating the switch from translation to replication during the viral infectious cycle (Barton *et al*., 2001; Herold and Andino, 2001). For FMDV, the PCBP associates with the poly (C) tract, suggesting that the poly (C) tract might play a similar role in genome circularization (Mason *et al*., 2003). The poly (C) tract separates the S fragment from the large (L) fragment. The L fragment contains a number of highly conserved secondary structures, including tandem repeats of pseudoknots (PKs), a *cis*-acting replication element (*cre*) and the internal ribosome entry site (IRES) (Clarke *et al*., 1987; Martinez-Salas, 1999; Mason *et al*., 2002). Whereas the function of the pseudoknots is not yet known (Clarke *et al*., 1987), the *cre*, a short hairpin element containing a highly conserved AAACA pentanucleotide in the loop region, is required for picornavirus genome

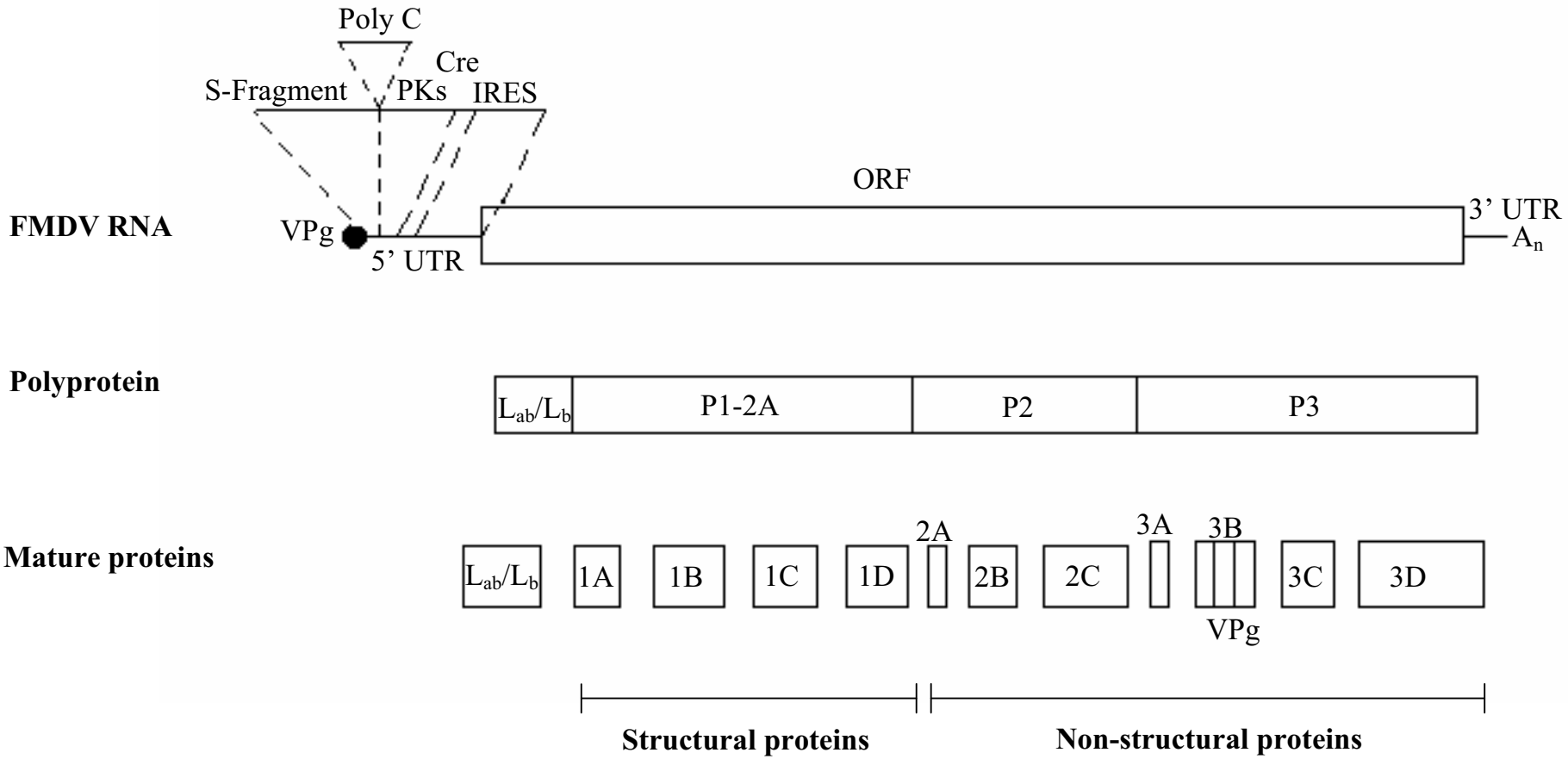

Fig. 1.2 Schematic diagram of the FMDV RNA genome. The 5' and 3' untranslated regions (UTR), the encoded polyproteins and the mature viral proteins are indicated.

replication (McKnight and Lemon, 1996; 1998). The highly structured IRES directs cap-independent translation of the viral RNA (Martinez-Salas, 1999).

The viral ORF encodes a single polyprotein (Forss *et al*., 1984), which can be divided into four different polyproteins, namely L, P1-2A, P2 and P3. The Leader proteinase (L^{pro}) is encoded by the 5'-end of the ORF (Robertson *et al*., 1985). Within the L^{pro}-encoding region of all seven FMDV serotypes are two in-frame AUG codons (Sangar *et al*., 1987), which initiate synthesis of two forms of the Leader (L) protein, L_{ab} and L_b, respectively (Beck *et al.,* 1983; Sangar *et al*., 1987). Although both proteins have been detected *in vitro* and in infected cells (Clarke *et al*., 1985), it has been suggested that L_b is the major protein synthesised *in vivo* (Cao *et al.,* 1995). Both L_{ab} and L_b catalyse their proteolytic excision from the growing polyprotein at its carboxy (C)-terminus (Strebel and Beck, 1986; Vakharia *et al.,* 1987) and initiate the cleavage of the eukaryotic translation initiation factor eIF-4G (Devaney *et al*., 1988, Medina *et al*., 1993). The L protein is not only essential for pathogenesis and permitting virus transmission in livestock hosts, but it has also been associated with virus virulence (Mason *et al.,* 2003). The P1 polyprotein is the precursor of the viral capsid proteins 1A, 1B, 1C and 1D (Domingo *et al*., 1990; Belsham, 1993). The polyprotein is rapidly cleaved into proteins 1AB, 1C and 1D by the 3C proteinase (Bablanian and Grubman 1993). Upon encapsidation of the RNA, 1AB is autocatalytically cleaved into proteins 1A and 1B (Harber *et al*., 1991; Lee *et al*., 1993).

Non-structural proteins derived from the P2- and P3-coding regions of the genome participate in RNA replication and in the folding and assembly of structural proteins. The P2 polyprotein precursor is proteolytically processed into the three mature polypeptides 2A, 2B and 2C (Rueckert and Wimmer, 1984). The 18-amino-acid peptide 2A (Robertson *et al*., 1985; Donnelly *et al.*, 1997) remains associated with the P1 polyprotein precursor following primary cleavage (Vakharia *et al.*, 1987) and appears to be an autoproteinase (Ryan *et al*., 1991). Limited research has been completed on protein 2B of FMDV and the exact function of this protein is not known. However, picornaviral proteins 2B and 2C have been implicated in virus-induced cytopathic effect and protein 2B has been implicated in enhancing membrane permeability and blocking of protein secretory pathways (Doedens and Kirkegaard, 1995; Van Kuppeveld *et al*., 1997a;b; Jecht *et al.*, 1998). Protein 2C of FMDV contains three nucleoside triphosphate-binding motifs (GXXXXGK, DXXG, NKXD) and helicase motifs (Dever *et al*., 1987; Dmitrieva *et al*., 1991). In addition, mutations that confer

resistance to guanidine hydrochloride, an inhibitor of viral RNA replication, are located in 2C, thus implying a role for this protein in viral RNA synthesis (Saunders *et al.*, 1985; Klein *et al*., 2000). Furthermore, protein 2C and its precursor 2BC are associated with cell membranes and induce cell membrane vesicle proliferation (Bienz *et al*., 1990).

The P3 polypeptide precursor is proteolytically processed to yield the four mature proteins 3A, 3B, 3C and 3D. The protein 3A of FMDV is longer than that of other picornaviruses (153 amino acids (aa) as compared with 87 aa for poliovirus) (Mason *et al*., 2003) and is proposed to be the membrane anchor for the picornavirus replication complex (Xiang *et al.,* 1997). It is associated to viral-induced membrane vesicles and contributes to the cytopathic effect and to the inhibition of protein secretion (Doedens and Kirkegaard, 1995). Three tandem, non-identical copies of the 3B gene encode three copies of the VPg protein, which participate in the initiation of RNA synthesis and in the encapsidation of viral RNA (Hogle *et al*., 1985; Xiang *et al*., 1997; Barclay *et al*., 1998). The 3B genes display highly conserved sequences and each VPg contains a Tyr-3, which is known to be involved in phosphodiester linkage to the viral RNA (Forss and Schaller, 1982). Viable viruses can be recovered from infectious RNA carrying a single copy of the 3B gene, but the level of infectivity correlates with the number of 3B gene copies present in the RNA (Falk *et al*., 1992). The 3C proteinase ($3C^{pro}$) is a serine protease (Bazan and Fletterick, 1988; Gorbalenya *et al*., 1989) and is responsible for catalyzing most of the proteolytic cleavage events necessary for polyprotein processing (Vakharia *et al.,* 1987; Clarke and Sanger, 1988), the exceptions being the cleavage of L^{pro} from P1, 2A from P1-2A, and the maturation cleavage of 1AB into 1A and 1B (Bablanian and Grubman, 1993). The $3C^{pro}$ is also responsible for the proteolytic processing of the histone protein H3, which leads to inhibition of host transcription in infected cells (Falk *et al.*, 1990; Tesar and Marquardt, 1990). As in other picornaviruses, protein 3D is the RNA-dependent RNA polymerase (Polatnick and Arlinghaus, 1967; Newman *et al*., 1979) and is highly conserved both in nucleotide and amino acid sequence among the different FMDV serotypes (Martinez-Salas *et al.*, 1985; George *et al.*, 2001).

The 3'-end of the genome contains a poly (A) tract that is separated from the 3D-encoding region by a short RNA sequence that is able to fold into a specific structure (Pilipenko *et al.*, 1992). The poly (A) tract is genetically encoded, unlike cellular mRNAs where the poly (A) tract is added post-transcriptionally (Dorsch-Hasler *et al.*, 1975). The poly (A) tract plays a role in a variety of processes required for polyadenylated mRNAs, including genome

circularization that could play a role in RNA replication (Barton *et al.,* 2001; Herold and Andino, 2001).

1.4 THE VIRUS PARTICLE

The FMD virions are *ca.* 30 nm in diameter and consist of 70% protein, 30% RNA and a limited amount of lipid (Bachrach *et al.*, 1964). FMD virions are distinguished from other picornaviruses by their lability at pH values below 6.5 (Acharya *et al*., 1989). The viral RNA genome is enclosed in a non-enveloped protein capsid of icosahedral symmetry (Bachrach, 1977). The capsid consists of 60 copies of each of the four capsid proteins, namely 1A, 1B, 1C and 1D. The surface capsid proteins (1B-1D) have an eight-stranded antiparallel β-barrel structure and are arranged in a sandwich of two four-stranded β-sheets (Fig. 1.3A). A single copy of each capsid protein assembles to produce a protomer (Fig. 1.3B), five protomers form a pentamer (Fig. 1.3C) and twelve pentamers form a complete icosahedral capsid that encloses the viral genome (Acharya *et al*., 1989) (Fig. 1.3D).

The 1D proteins are located around the icosahedral five-fold axes and contribute most to the accessible surface of the virus particle. Protein 1D also contains a highly mobile loop protruding from its surface, known as the G-H loop (Acharya *et al.,* 1989; Logan *et al*., 1993; Fry *et al*., 1999). This flexible external loop contains a highly conserved three-amino-acid-sequence Arg-Gly-Asp (RGD) at its apex, which is involved in binding of FMDV to host cells (Fox *et al*., 1989; Logan *et al*., 1993; Lea *et al*., 1994; Mason *et al*., 1994; Curry *et al*., 1996). Proteins 1B and 1C alternate around the two- and three-fold axes and it has been suggested that a histidine-rich region at the 1B/1C interface is responsible for acid-induced capsid disassembly. Protonation of these residues at a pH below 6.5 may cause electrostatic repulsive forces across the pentamer interface, thereby resulting in the capsid opening up (Acharya *et al.*, 1989; Curry *et al*., 1995; Ellard *et al*., 1999). Multiple conformational- and non-conformational-dependant immuno-important regions present on 1B-1D have been identified for types A, O and C (Kitson *et al*., 1990; Crowther *et al*., 1993; Mateu *et al*., 1995). In contrast to the above capsid proteins, protein 1A is located entirely at the inner surface of the capsid and is modified by a myristate group at its amino (N)-terminus, which has been reported to be essential for capsid assembly and stability (Chow *et al*., 1987; Acharya *et al*., 1989).

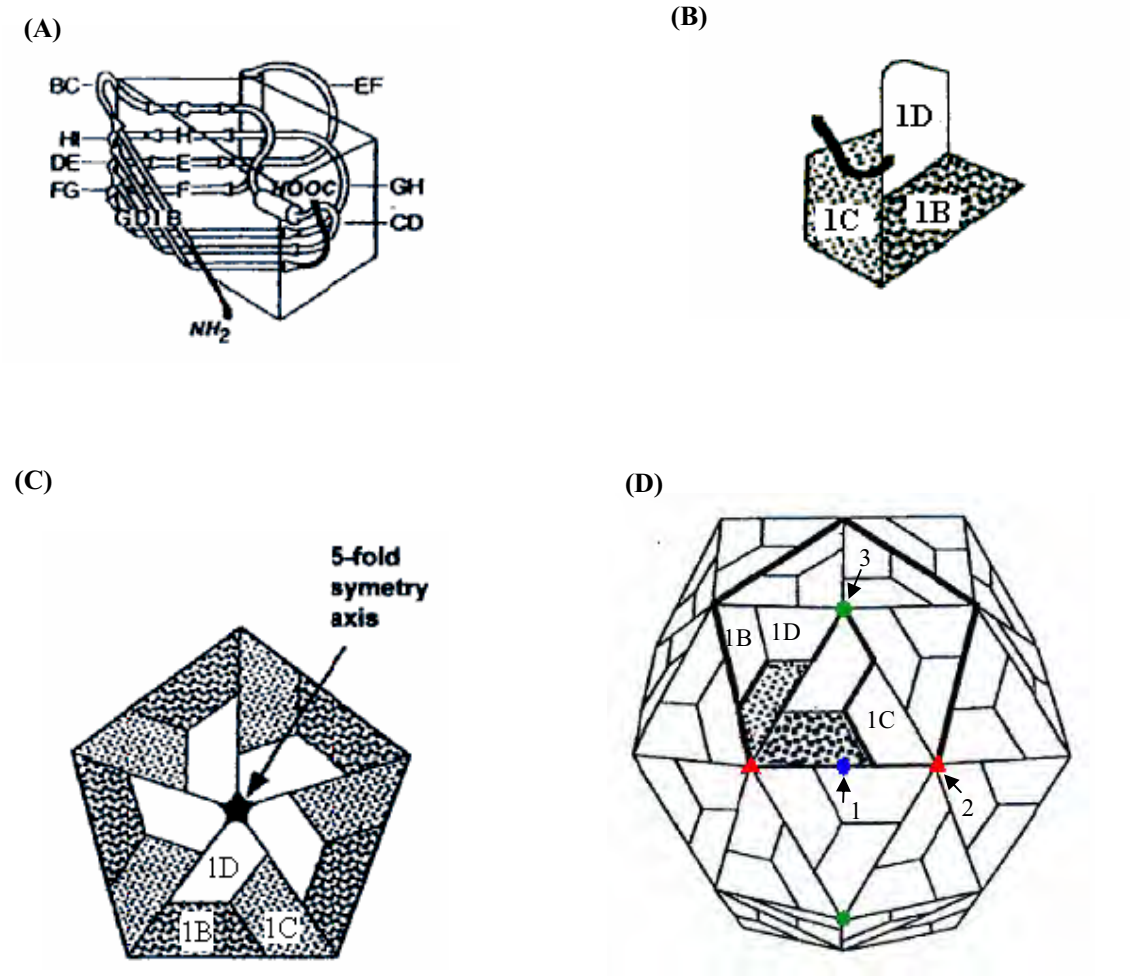

Fig. 1.3 Schematic view of the surface structure of FMDV capsid proteins, the subunits and the viral capsid. (A) Schematic representation indicating the eight-stranded β-sandwich core of proteins 1B, 1C and 1D. The β-strands are indicated as B, I, D, G, C, H, E and F and are joined through connecting loops. (B) The arrangement of the external capsid proteins (1B, 1C and 1D) in a biological protomer. The location of the carboxy- and amino–terminal domain of 1D is indicated by a black ribbon. (C) Arrangement of five protomers into a pentamer. (D) Structure of the virion capsid, consisting of 60 protomers. Each protomer is composed of one copy of 1B, 1C and 1D. The two-fold, three-fold and five-fold axes are indicated, in blue by an ellipse (1), in red by a triangle (2) and in green by a circle (3), respectively. A pentamer is outlined in the capsid, and a protomer is indicated inside the pentamer. (Taken form Saiz *et al*., 2002).

1.5 THE VIRUS REPLICATION CYCLE

Replication and translation of the FMDV RNA occurs in the cytoplasm of infected cells, and these processes are associated with cell membranes (Rueckert, 1996). The FMDV infectious cycle (Fig. 1.4) is initiated by the attachment of the virus to receptors exposed on the cell surface (Tamkun *et al*., 1986) via an RGD sequence found within the surface-exposed G-H loop of capsid protein 1D (Fox *et al*., 1989; Mason *et al*., 1994). Although the highly conserved RGD tripeptide is characteristic of the ligands of several members of the integrin family (Hynes, 1992), tissue culture-adapted FMDV strains can utilize other receptors such as heparan sulfate proteoglycans in an RGD-independent manner (Jackson *et al*., 1996; Sa-Carvalho *et al*., 1997). Cellular receptors are discussed in more detail in Section 1.6.

Following binding of the virion to the cell surface receptor, the virus-receptor complex is invaginated and internalized by endocytosis to form a clathrin-coated vesicle (endosome) (Madshus *et al*., 1984a;b). Acidification of the endosome leads to the release of protein 1A and unfolding of the hydrophobic regions buried inside the viral capsid (Curry *et al*., 1995). Fusion of the lipid bilayer with the hydrophobic regions of the exposed capsid proteins leads to the formation of a pore through which the viral RNA is transferred to the cytosol (Madshus *et al.*, 1984a;b). In the cytoplasm, the VPg protein is released from the 5' UTR of the viral RNA (Lee *et al*., 1977). The internal ribosome entry site (IRES) then forms a secondary structure, which is able to bind ribosomes and deliver them directly to the polyprotein initiation codon in a cap-independent manner. Translation initiation factors involved in cap-dependent mRNA translation, such as the eukaryotic translation initiation factor eIF-4B (Meyer *et al*., 1995) and the cellular polypyrimidine tract-binding protein (PTBP) (Niepmann, 1996), have been reported to play a role in stimulation of the internal translation initiation at the picornaviral IRES. The RNA strand directs synthesis of the viral polyprotein, which is cleaved into individual proteins as synthesis progresses.

The leader proteinase, which is the first protein to be synthesized, cleaves itself from the rest of the growing polyprotein before cleaving the eukaryotic translation initiation factor eIF-4G. Since eIF-4G is a component of the cap-binding complex required for translation of most cellular mRNAs (Meerovitch and Sonenberg, 1993), cleavage of eIF-4G thus results in the shutting-off of host cap-dependent mRNA translation (Devaney *et al.*, 1988). Shut-off of host

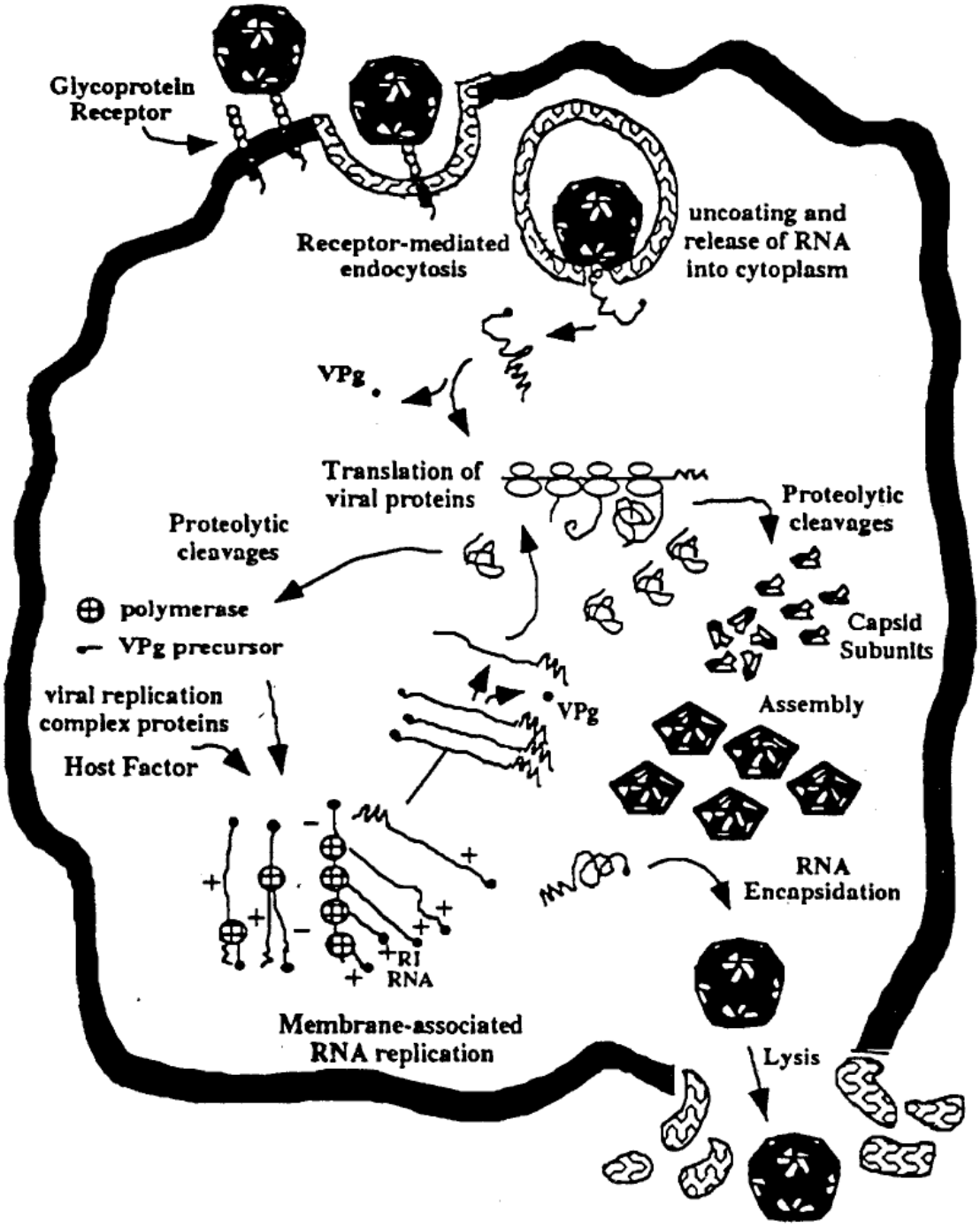

Fig. 1.4 Overview of the poliovirus infectious cycle. (Taken from Ansardi *et al*., 1996).

cell translation is normally marked by a sharp decline in protein synthesis occurring *ca.* 30 min after infection (Rueckert, 1996). Subsequently, the virus-induced decrease in cellular protein synthesis is followed by increased cap-independent synthesis of viral proteins.

The single polyprotein encoded by the viral ORF undergoes several processing events to produce the three polyprotein precursors P1-2A, P2 and P3 (Domingo *et al*., 1990; Belsham, 1993). The P1-2A polyprotein, which is the precursor for the capsid proteins, is obtained following the autocatalytic cleavage of L^{pro} from P1 (Strebel and Beck, 1986) and the 2A cleavage between P1-2A and 2B (Ryan *et al*., 1989). The P1-2A precursor is then cleaved by the $3C^{pro}$ to produce 1AB, 1C and 1D. Besides the cleavage of L^{pro} from P1, the cleavage of 2A and the maturation cleavage of 1AB to 1A and 1B, all other cleavages are performed by $3C^{pro}$ and results in several mature structural and non-structural proteins (Vakharia *et al*., 1987; Clarke and Sanger, 1988; Bablanian and Grubman, 1993). The $3C^{pro}$ protein is also responsible for the cleavage of the cellular H3 histone protein, which may contribute to inhibiting host cell transcription (Grigera and Tisminetzky, 1984; Falk *et al*., 1990; Tesar and Marquardt, 1990). Thus, the cleavage of eIF-4G and histone H3 results in an almost complete breakdown of host cell functions during viral infection.

One of the products produced by the cleavage of P3 is the RNA-dependent RNA polymerase (3D), which copies the positive sense viral RNA to produce complementary negative sense RNA. Progeny virus positive sense strands are synthesized repeatedly from these negative sense templates by a peeling-off mechanism (Joklik, 1980). The progeny positive sense RNA strands are either translated or packaged into progeny virions (Joklik, 1980; Rueckert, 1996). Virus assembly involves the formation of capsid protomers, five of which assemble into pentamers, followed by packaging of the positive sense VPg-RNA to form provirions (Guttman and Baltimore, 1977; Belsham, 1993). The final step in virion maturation involves the autocatalytic cleavage of 1AB into 1A and 1B, which is required for the formation of infectious virus particles (Harber *et al*., 1991; Lee *et al*., 1993; Knipe *et al*., 1997). The virions are then released from the host cells by lysis (Rueckert, 1996).

1.6 CELLULAR RECEPTORS FOR FOOT-AND-MOUTH DISEASE VIRUS

Over the last few years, much progress has been made in identifying cellular receptors for FMDV. This has led to an improved understanding of how FMDV targets epithelial cells, the preferred cell type infected by FMDV, and of the mechanisms underlying disease transmission. Whereas field isolates of FMDV utilize integrin receptors for cell internalization, several tissue culture-adapted viruses have a higher affinity for heparan sulfate proteoglycans (Jackson *et al*., 1996; Sa-Carvalho *et al*., 1997). Consequently, both these cellular receptors and other alternate receptors used for FMDV internalization, will be discussed in more detail.

1.6.1 INTEGRIN RECEPTORS

Integrins are a family of α/β heterodimeric transmembrane glycoproteins that contribute to various different processes, *e.g.* cell-cell and cell-extracellular matrix adhesion, as well as the induction of signal transduction pathways that regulate various processes such as cell proliferation, morphology, migration and apoptosis (Hynes, 1992; Fernandez *et al.,* 1998). Integrins consist of α and β subunits (Hynes, 1992), with each subunit possessing a large extracellular domain or ectodomain, a single transmembrane domain and a short cytoplasmic domain (Fig. 1.5) (Fernandez *et al.,* 1998). In total, 18 α and eight β integrin subunits of mammalian origin have been described, which interact non-covalently to give rise to 24 different integrins, each with its own ligand-binding specificity (Hynes, 1999). Although most integrins recognize their ligands by binding to short linear peptide sequences, several integrins do, however, bind their natural ligands via an RGD sequence (Ruoslahti, 1996).

1.6.1.1 INTEGRIN ACTIVATION

Integrins exist in one of two states: an active state, whereby they are able to bind ligands, and an inactive state, whereby they are unable to bind ligands (Springer, 1990). Conversion from an inactive to active state is thought to occur through "inside-out signaling", which comprises two different mechanisms. Whereas the first mechanism, termed avidity modulation, is mediated by clustering of heterodimers within the plane of the membrane, the second mechanism, termed affinity modulation, is mediated through conformational changes in the integrin ectodomain that allow more efficient ligand binding (Dedhar and Hannigan, 1996; Jackson *et al*., 2000a; 2002; Chan *et al*., 2000).

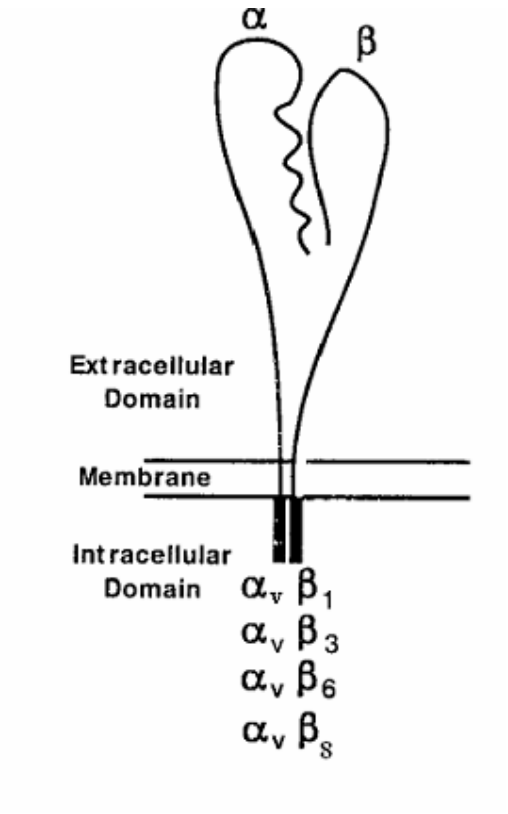

Fig. 1.5 A schematic representation of the structure of an integrin, indicating the α and β subunits, as well as the extracellular (ecto) domain, the transmembrane domain and the intracellular (cytoplasmic) domain of the integrin. FMDV is able to use the RGD-dependent integrin receptors $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$ and $\alpha_v\beta_8$ for binding to susceptible cells. (Taken from Rieder and Wimmer, 2002).

Although the molecular mechanism that regulates these processes *in vivo* has not yet been elucidated, it has been proposed that both of the integrin activation processes involve the cytoplasmic domains of the integrins, as well as cellular proteins (O'Toole *et al*., 1994; 1995; Dedhar and Hannigan, 1996; Zhang *et al.*, 1996; Hughes *et al*., 1997). The GFFKR sequence of the α subunit, located only a few amino acids after the transmembrane domain on the cytoplasmic side, is thought to maintain an inactive state of the integrin, as mutations or the complete removal of this sequence led to active integrins (Briesewitz *et al*., 1995; 1996). Another sequence thought to play an important role in controlling integrin affinity, through its involvement in inside-out signaling and its interaction with cytoskeletal components, is the conserved cytoplasmic domain sequence NPXY found within 30 residues of the transmembrane domain of many β subunits (O'Toole *et al*., 1995; Ylane *et al*., 1995).

A number of proteins, such as cytoplasmic proteins, calcium-binding proteins, membrane-anchored proteins, as well as a number of signaling pathways have also been proposed to play a regulatory role in the ligand binding activity of integrins (Coppolino *et al*., 1995; Hannigan *et al*., 1996; Hemler *et al*., 1996; Shimizu and Hunt, 1996; Fenczik *et al*., 1997). Contact between the cytoplasmic domain of integrins and cellular proteins is proposed to promote a structural change within the integrin that is transmitted across the plasma membrane to the extracellular domain. Integrins are also proposed to move laterally within the extracellular membrane, clustering at distinct sites known as focal contacts, thus providing a link between the extracellular matrix proteins and the acticytoskeleton. This clustering effect may play an important role in regulating ligand binding (Fernadez *et al*., 1998).

1.6.1.2 INTEGRIN BINDING

As mentioned above, most integrins recognize their ligands by binding to short linear peptides, but several members of the integrin family recognize their ligands by binding to an RGD tripeptide. The G-H loop of the capsid protein 1D, containing an RGD sequence at its apex, has been shown to be important for binding FMDV to susceptible cells. Treatment of the virus with trypsin, which removes both the G-H loop and the C-terminus of protein 1D, resulted in virus particles that were less infectious compared to untreated virus. This effect was attributed to the inability of the virus to attach to cells, presumably through the disruption of the attachment site of the virus (Strohmaier *et al.*, 1982; Wild and Brown, 1967; Wild *et al.,* 1969). The first direct evidence that the RGD motif is involved in cell receptor

recognition was provided by Fox and co-workers (1989). By making use of synthetic peptides containing an RGD sequence, it was shown that infection of susceptible cells by FMDV could be inhibited by preventing cell attachment. The importance of the RGD sequence for virus interaction with its cellular receptor was finally confirmed by reverse genetics studies, in which genetically engineered viruses lacking the RGD sequence were found to be non-infectious (Mason *et al*., 1994).

1.6.1.3 INTEGRINS UTILIZED BY FMDV

Several integrins, including $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_5\beta_1$, $\alpha_v\beta_8$ and $\alpha_8\beta_1$, bind their ligands via an RGD sequence (Hynes, 1992; Nishimura *et al*., 1994; Schnapp *et al*., 1995; Jackson *et al*., 2003). However, despite the presence of an RGD sequence on capsid protein 1D, FMDV does not use all of these RGD-dependent integrin receptors for binding to host cells. For example, integrins $\alpha_5\beta_1$ and $\alpha_v\beta_5$ are not used by FMDV to initiate infection of cultured cells (Neff *et al*., 1998; Baranowski *et al*., 2000; Jackson *et al.,* 2000b), albeit that the virus and chimeric proteins that include the RGD motif, are ligands for purified preparations of $\alpha_5\beta_1$ (Chambers *et al*., 1996; Villaverde *et al*., 1996; Jackson *et al*., 2000a). The integrins that have been reported to function as receptors for cell attachment by FMDV will be discussed in more detail in the following sections.

(A) $\alpha_v\beta_3$

Integrin $\alpha_v\beta_3$, also known as the vitronectin (VNR) receptor, was the first integrin receptor to be identified that functions as a receptor for FMDV (Berinstein *et al*., 1995). By making use of competition binding experiments, type A_{12} FMDV and the human enterovirus coxsackievirus A9 (CAV-9), which also has an RGD motif in its capsid protein 1D (Chang *et al*., 1989; Chang *et al*., 1992), were reported to both utilize $\alpha_v\beta_3$ as a receptor on LLC-MK2 cells (a rhesus monkey kidney cell line) and on BHK-21 cells (baby hamster kidney cell line). It was subsequently shown that a polyclonal anti-VNR serum and a MAb prepared against the human α_v subunit inhibited both FMDV binding and plaque formation in LLC-MK2 cells, whilst a MAb to the β_3 subunit inhibited virus binding to the cells (Berinstein *et al*., 1995). Although this report was the first to provide direct evidence that an integrin can function as a receptor for FMDV, it also suggested that FMDV might use alternative RGD-dependent integrins, since it could bind and replicate in some cells that CAV-9 could not.

In a subsequent study, Jackson and co-workers (1997) reported that representatives of six FMDV serotypes bound to purified isolated human $\alpha_v\beta_3$ in an RGD-dependent manner *in vitro*. Similarly, Neff and co-workers (1998) reported that the human erythroleukemia cell line K562 and CHO-K1 cells, both of which do not express $\alpha_v\beta_3$, could not replicate FMDV type A_{12} unless the cells were transfected with cDNA encoding human α_v and β_3 integrin subunits. These results therefore indicated that type A_{12} replication was dependent on expression of the integrin $\alpha_v\beta_3$. Notably, analysis of two genetically engineered variants of type O_1 Campos, namely vCRM4, which has a reduced virulence in cattle and binds to heparan-sepharose columns, and vCRM8, which is highly virulent in cattle and does not bind to heparan-sepharose, indicated that vCRM8, by contrast to vCRM4, replicated only in K562 and CHO-K1 cells transfected with $\alpha_v\beta_3$ (Neff *et al*., 1998). These results suggested that virulent FMDV utilizes the $\alpha_v\beta_3$ integrin as a primary receptor for infection (Neff *et al*., 1998). In contrast, adaptation of type O_1 Campos to cell culture resulted in the ability of the virus to utilize heparan sulphate as a receptor (Refer to Section 1.6.2).

The above-mentioned studies were performed with integrin $\alpha_v\beta_3$ from either simian (Berinstein *et al*., 1995) or human (Neff *et al*., 1998) origin. However, since FMDV is a livestock pathogen, Neff and co-workers (2000) subsequently investigated whether the bovine homologue of $\alpha_v\beta_3$ was efficiently utilized by FMDV. A panel of COS-1 cells were produced that expressed the human or the bovine α_v and β_3 integrin subunits, as well as cells expressing a combination of human α_v and bovine β_3 subunits and vice versa. The results obtained indicated that the bovine $\alpha_v\beta_3$ integrin receptor was more efficiently utilized by FMDV than the human homologue. Notably, the increased efficiency of the bovine receptor appeared to be associated with the bovine β_3 subunit, since cells co-transfected with a human α_v subunit and a bovine β_3 subunit yielded similar results to those obtained with cells expressing the bovine $\alpha_v\beta_3$ subunits (Neff *et al.*, 2000). Despite high levels of amino acid sequence similarity between the human and bovine α_v and β_3 subunits, the bovine sequence downstream from codon 488, encoding the C-terminal of the β_3 subunit extracellular domain, appears to be responsible for the increased efficiency of the bovine receptor. This region is rich in cysteine residues, which may contribute to the overall structure of the integrin through disulfide bonding (Carrell *et al.*, 1985). Therefore, the bovine $\alpha_v\beta_3$ may be expressed in a high-affinity ligand-binding state that permits a tighter virus binding to bovine $\alpha_v\beta_3$, thus

resulting in an increased rate of virus internalization. Furthermore, truncations, extensions or deletion of the cytoplasmic domains of the α_v or β_3 subunits have been reported not to affect the ability of the bovine $\alpha_v\beta_3$ integrin to function as a receptor for FMDV, since these integrins were utilized as well as intact integrins (Neff and Baxt, 2001).

(B) $\alpha_V\beta_6$

The second integrin to be identified as a receptor for FMDV was $\alpha_v\beta_6$ (Jackson *et al*., 2000b), which is expressed exclusively on epithelial cells (Breuss *et al*., 1993; 1995). The β_6 integrin subunit forms only a single heterodimer $\alpha_v\beta_6$, which is a receptor for the extracellular matrix proteins fibronectin (Weinacker *et al.*, 1994), tenascin (Prieto *et al.*, 1993), vitronectin (Huang *et al.*, 1998) and for latency-associated protein 1 (LAP-1) (Munger *et al.*, 1999). The pentapeptide DLXXL has been reported to be a high-affinity ligand for $\alpha_v\beta_6$ in the absence of an RGD motif (Kraft *et al.*, 1999). This peptide shares sequence similarity with the region flanking the RGD motif (RGDLXXI) found in LAP-1 (Munger *et al.*, 1999), and the residues flanking the RGD motif (RGDLXXL) of FMDV (Jackson *et al*., 2000a). Evidence indicating that $\alpha_v\beta_6$ is indeed utilized as a receptor by FMDV was provided by Jackson *et al*. (2000b). The human colon carcinoma cell line SW480, which is normally non-permissive for FMDV, became susceptible to infection by the type C virus C-S8c1 and a SAT3 isolate following transfection with the integrin β_6 subunit and subsequent expression of $\alpha_v\beta_6$ at the cell surface. Furthermore, binding of FMDV and infection of the β_6-transfected cells was inhibited (>90%) by an anti-$\alpha_v\beta_6$ MAb. In addition, an RGD-containing peptide with its sequence derived from the G-H loop of type O FMDV inhibited virus attachment and infection of the β_6-transfected cells, indicating that the virus binds to $\alpha_v\beta_6$ through an RGD-mediated interaction.

In contrast to $\alpha_v\beta_3$ integrins, the cytoplasmic domain of the β_6 subunit of the $\alpha_v\beta_6$ integrin appears to play an important role in its functioning as a cellular receptor for FMDV. Truncation of the β_6 cytoplasmic domain of the $\alpha_v\beta_6$ integrin resulted in receptors that, although expressed on the cell surface and able to bind FMDV, were unable to mediate infection (Miller *et al.*, 2001). These results suggest that the $\alpha_v\beta_6$ integrin plays an important role in events subsequent to virus attachment. The cytoplasmic domains of each of the defective receptors lacked the sequence NPXY, which functions as a signal that directs

various membrane proteins into the clathrin-coated vesicles (Chen *et al.*, 1990). The role of the β_6-subunit NPXY sequence in integrin endocytosis is, however, not well defined (Vignoud *et al.*, 1994; Van Nhieu *et al.*, 1996) and further research is needed to determine whether this motif plays a role in $\alpha_v\beta_6$-mediated FMDV infection.

Recently, Duque and co-workers (2004) analyzed the interaction of purified soluble integrins $\alpha_v\beta_6$ and $\alpha_v\beta_3$ with representative FMD viruses of serotypes A and O, immobilized on plastic plates. Soluble $\alpha_v\beta_6$ bound to the A_{24} virus, two O_1 Campos viruses (vCRM8 and vCRM4), as well as a genetically engineered type A_{12} virus chimera containing the G-H loop from O_1BFS (A/O), and inhibited viral replication significantly. In contrast, soluble $\alpha_v\beta_3$ was unable to bind to immobilized viruses of either serotype. Since the binding of the integrins in solution to immobilized ligands is a function of affinity (Smith *et al*., 1994), these results indicate that $\alpha_v\beta_6$ and $\alpha_v\beta_3$ act as high- and low-affinity receptors, respectively, for FMDV. Based on reports indicating that both these integrins serve as receptors for FMDV, Duque and co-workers (2004) proposed that the $\alpha_v\beta_6$ integrin might play a role in capturing the virus and aiding in initial virus replication within susceptible hosts, whilst the $\alpha_v\beta_3$ integrin plays a role in disseminating the virus within the host to distant replication sites.

(C) $\alpha_v\beta_1$

The third integrin to be identified as a receptor for FMDV was $\alpha_v\beta_1$ (Jackson *et al*., 2002). This finding was based on the observation that CHO-B2 cells, which are normally non-permissive for field strains of FMDV, became susceptible to FMDV infection following transfection with the human α_v integrin subunit and subsequent expression of the $\alpha_v\beta_1$ integrin at the cell surface. Furthermore, both virus attachment to and infection of the transfected cells were effectively inhibited (>98%) by function-blocking anti-α_v MAbs, and by RGD-containing peptides (Jackson *et al*., 2002). However, integrin $\alpha_v\beta_1$ was found to be an ineffective receptor in the presence of physiological concentrations of calcium and magnesium, but virus binding and infection were greatly enhanced following integrin activation by manganese (Mn^{2+}) ions or by an activating anti-β_1 antibody. In contrast to $\alpha_v\beta_1$, $\alpha_v\beta_6$ appears to be expressed in a high affinity state on transfected CHO-B2 cells, as neither virus binding nor infection was enhanced by Mn^{2+}, suggesting that different molecular mechanisms may regulate the affinities of $\alpha_v\beta_1$ and $\alpha_v\beta_6$ for FMDV (Jackson *et al.*, 2002).

(D) $\alpha_v\beta_8$

Integrin $\alpha_v\beta_8$ is the most recently reported integrin receptor for FMDV (Jackson *et al*., 2004). Based on the similarity in the amino acid sequences immediately following the RGD motif of LAP-1 (RGDLXXI), LAP-3 (RGDLXXL) and FMDV (RGDLXXL), as well as the discovery that LAP-1 is a ligand for $\alpha_v\beta_8$ (Mu *et al*., 2002), Jackson and co-workers (2004) investigated whether $\alpha_v\beta_8$ could serve as a receptor for FMDV. SW480 cells, which normally express $\alpha_v\beta_5$ and $\alpha_5\beta_1$ as their only RGD-binding integrins (Weinacker *et al*., 1994) and are non-permissive for FMDV, became susceptible for FMDV following transfection with the β_8 integrin subunit and the stable expression of $\alpha_v\beta_8$ integrin at the cell surface. Furthermore, function-blocking MAbs specific either for the $\alpha_v\beta_8$ heterodimer or the α_v subunit inhibited virus attachment to and infection of $\alpha_v\beta_8$-expressing cells by 60% and 80%, respectively. Infection of the cells was also inhibited by more than 90% with RGD-containing peptides whose sequence was derived from the FMDV RGD site.

Jackson and co-workers (2004) also investigated the role of the β-subunit cytoplasmic domain in integrin-mediated infection and showed that the replacement of the cytoplasmic domain of β_8 with the corresponding region of β_6 had no effect on the expression of the chimeric $\alpha_v\beta_8$ integrin at the cell surface or on its ability to bind FMDV and mediate infection. However, a chimeric $\alpha_v\beta_6$ integrin containing the β_8 cytoplasmic domain ($\alpha_v\beta_6/\beta_8$) resulted in an integrin that bound FMDV, but was unable to mediate infection. These results confirmed that the cytoplasmic domain of the $\alpha_v\beta_6$ integrin plays an important role in its functioning as a receptor for FMDV (Miller *et al.*, 2001), probably by maintaining the ectodomain of $\alpha_v\beta_6$ in a conformation that is necessary for high-affinity binding of FMDV (Miller *et al*., 2001; Jackson *et al.*, 2004). Since the sequences required for integrin-mediated virus internalization are missing from the cytoplasmic domain of the β_8-subunit and its primary sequence is almost completely divergent of the primary sequences found in the cytoplasmic domain of other integrin β-subunits, Jackson and co-workers (2004) proposed that $\alpha_v\beta_8$ mediates FMDV infection through a mechanism independent of its β-subunit cytoplasmic domain.

1.6.1.4 INTEGRIN SPECIFICITY

The first and fourth residues that follow the RGD motif (RGD+1 and RGD+4 residues) may be of importance for recognition of RGD-dependent integrins by FMDV (Rieder *et al.*, 1994; Mateu *et al.*, 1996). A leucine residue is most commonly found at the RGD+1 position of the European FMDV serotypes and SAT1 (Vosloo *et al*., 1995; Mateu *et al*., 1996; Jackson *et al*., 2000a), but some type A and SAT3 isolates have a methionine, whilst most Asia-1 and SAT2 isolates have an arginine residue at this position (Vosloo *et al*., 1995; Mateu *et al*., 1996; Jackson *et al.*, 2000a; Bastos *et al*., 2003). A leucine residue is highly conserved at the RGD+4 position, but an isoleucine has been noted occasionally at this position (Domingo *et al*., 1992; Mateu *et al*., 1996; Jackson *et al.*, 2000a;b).

Evidence suggesting that amino acid residues flanking the RGD triplet of FMDV may be important for differentiating between the binding specificities of $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$ and $\alpha_v\beta_8$, has been obtained by studies using different RGD-containing peptides. A short RGD-containing peptide (GRGDSP) and a longer peptide (VPNLRGDLQVLA) have been reported to inhibit virus binding and infection mediated by $\alpha_v\beta_1$ (Jackson *et al*., 2002). These peptides also inhibited FMDV binding to purified $\alpha_v\beta_3$ *in vitro* (Jackson *et al*., 1997), and for both $\alpha_v\beta_1$ and $\alpha_v\beta_3$, the GRGDSP peptide was the more potent inhibitor. However, the GRGDSP peptide was largely ineffective in inhibiting virus binding and infection mediated by $\alpha_v\beta_6$ (Jackson *et al*., 2002) and $\alpha_v\beta_8$ (Jackson *et al*., 2004). The similarity between the residues following the RGD motif of FMDV (RGDLXXL) and those of LAP-1 (RGDLXXI) and the observations that the pentapeptide DLXXL can inhibit ligand binding to $\alpha_v\beta_6$ (Kraft *et al*., 1999), suggests that the conservation of the leucine residues at the RGD+1 and RGD+4 positions in FMDV may be required for virus binding to its integrin receptors, especially $\alpha_v\beta_6$. Integrin receptor $\alpha_v\beta_3$ binds a broad range of RGD-containing ligands (Hynes, 1992) and is tolerant of several different amino acid residues immediately following the RGD motif, including those at the RGD+1 and RGD+4 positions (Springer, 1990; Healy *et al*., 1995). In contrast, integrin $\alpha_v\beta_6$ binds to relatively few ligands and has a more restricted set of amino acid residues at the RGD+1 and RGD+4 positions (Kraft *et al*., 1999). These findings have been substantiated by a recent report demonstrating that a type O virus, containing leucine residues at RGD+1 and RGD+4, bound more efficiently to $\alpha_v\beta_6$ compared with binding to integrins $\alpha_v\beta_1$ and $\alpha_v\beta_3$. In contrast, type A viruses containing various different amino acid

substitutions at RGD+1 and a leucine residue at RGD+4, utilized $\alpha_v\beta_3$ with the highest efficiency and $\alpha_v\beta_6$ to a lesser extent (Duque and Baxt, 2003).

1.6.1.5 ROLE OF INTEGRIN RECEPTORS IN THE PATHOGENESIS OF FMDV

From the preceding sections, it follows that FMDV can use four RGD-dependent integrins, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$ and $\alpha_v\beta_8$, to initiate infection of cultured cells (Berinstein *et al*., 1995; Jackson *et al*., 2000b; Jackson *et al*., 2002; Jackson *et al*., 2004). Although the various RGD-binding integrins have distinct tissue distributions, the role of the various integrin receptors in the pathogenesis of FMDV, however, remains unclear.

The primary site of FMD virus replication is thought to be epithelial cells of the oropharynx and associated lymphoid tissues (Brown *et al*., 1991; 1992; Murphy *et al*., 1999; Alexandersen *et al*., 2001). During the development of the disease, the virus is spread throughout the body, with secondary sites of replication in many epithelial tissues (Burrows *et al*., 1981; Zhang and Kitching, 2000; Alexandersen *et al*., 2001). Due to a lack of specific antibodies, little is known about the *in vivo* cell type expression or tissue distribution of $\alpha_v\beta_1$. Although it is expressed on malignant cells in smooth muscle and in the central nervous system (Friedlander *et al*., 1996; Milner *et al*., 1996; Dahm and Bowers, 1998), it is unclear what role, if any, $\alpha_v\beta_1$ might play in *in vivo* infections with FMDV. Similarly, the $\alpha_v\beta_3$ integrin may not have a major role during the initial infection of an animal, as it is only weakly expressed in bovine and porcine airway epithelium (Singh *et al*., 2001) and has a limited expression in lymphoid cells (Damjanovich *et al*., 1992; Mette *et al*., 1993). In contrast to integrins $\alpha_v\beta_1$ and $\alpha_v\beta_3$, expression of the $\alpha_v\beta_6$ and $\alpha_v\beta_8$ integrins are restricted to epithelial cells (Damjanovich *et al*., 1992; Mette *et al*., 1993; Fjellbirkeland *et al*., 2003; Cambier *et al*., 2000), and expression of $\alpha_v\beta_6$ has been observed on the epithelia of uterus, bladder, respiratory tract and salivary gland (Breuss *et al*., 1993). Expression levels of $\alpha_v\beta_6$ are moderate to low in normally healthy adult epithelia, but are rapidly up-regulated at sites of tissue injury and inflammation (Breuss *et al*., 1995; Huang *et al*., 1996). Based on their exclusive expression on epithelial cells, including sites where initial virus replication is believed to occur, $\alpha_v\beta_6$ and $\alpha_v\beta_8$ have been proposed to be the most likely receptors used by FMDV during the initial phase of infection in an animal (Jackson *et al*., 2000b; 2004; Duque *et al*., 2004).

1.6.2 HEPARAN SULFATE PROTEOGLYCAN RECEPTORS

Propagation of FMDV in cell culture may lead to the rapid selection of mutant viruses, which are characterized by multiple phenotypic alterations, including enhanced replication capacity, enhanced resistance to neutralizing MAbs, expanded cell tropism, binding to heparan and attenuation for cattle (Holland *et al*., 1991; Martinez *et al*., 1991; Baranowski *et al*., 1998; 2000). These phenotypic traits have been found to be associated with a limited number of capsid alterations that confer to FMDV the capacity to use RGD-independent methods of cell binding such as utilization of heparan sulfate proteoglycan (HSPG) as receptors (Mason *et al*., 1993; Fry *et al*., 1999; Baranowski *et al*., 2000). HSPG is widely distributed in animal tissues, occurring on almost all cell types as part of the extracellular matrix (Kjellen and Lindahl, 1991).

The FMDV type O virus O_1BFS was the first FMD virus identified as being able to use HSPG as cellular receptors (Jackson *et al*., 1996). Subsequently, several other viruses representing other FMDV serotypes have been identified that also use HS as a cellular receptor (Baranowski *et al*., 1998; Fry *et al*., 1999). Initially, it was thought that FMDV infection was initiated by a two-stage attachment step, the first contact being with a low-affinity HS proteoglycan receptor and the second with a high-affinity integrin receptor (Jackson *et al.,* 1996). The HS was thought to concentrate the virus at the cell surface by restricting movement to two dimensions, so improving the probability of binding to a second integrin receptor used for internalization (Spear, 1993; Haywood, 1994). However, a functional link between HS and integrins has never been established.

Binding to heparan sulphate, a highly sulfated polymer of disaccharide repeats carrying a negative charge (Kjellen and Lindahl, 1991; Fry *et al.,* 1999), has subsequently been reported to involve the acquisition of positively charged amino acid residues at the FMDV capsid surface. Sa-Carvalho and co-workers (1997) demonstrated that serial passaging of FMDV strains in BHK and CHO cells led to alterations of specific amino acid residues that resulted in an increase of positively charged residues at the capsid surface. In the case of type O_1BFS, these alterations comprised, amongst other, substitution of His-56 on protein 1C with an arginine. This alteration coincided with an increased affinity for HS. However, the location of the acquired positively charged residues appears to vary for different FMDV strains or even

for the same virus clone with different passage histories in cell culture (Baranowski *et al*., 1998; Escarmís *et al*., 1998).

Sulphated heparan is thought to bind to a shallow depression of the FMDV protomer, thus making contact with all three major capsid proteins. The three sides of the depression are formed by the β1 strand of protein 1C, the C-terminus of protein 1D and the αB helix of protein 1B (Fry *et al.,* 1999). The base of the depression is formed by a 3_{10} helix running in the same direction of the sugar. Notably, based on structural data, the RGD integrin recognition motif in the G-H loop of protein 1D is *ca.* 15Å from the closest sugar moiety (Fry *et al*., 1999). Thus, the integrin recognition site is unaffected by heparan binding and the two receptor binding sites therefore appear to be independent of each other. This may serve to explain why multiply passaged FMD viruses, despite having an enhanced affinity for HS as cell surface receptor, still maintain the ability to also use integrin cell receptors (Baranowski *et al*., 2000).

1.6.3 POSSIBLE ALTERNATE RECEPTORS

FMDV can enter susceptible cells through integrin- and HS-independent pathways. For example, antibody-complexed FMDV virus has been reported to enter cells that express the immunoglobulin Fc receptor in the presence of virus-specific antibodies (Mason *et al*., 1993; Baxt and Mason, 1995). The virus can also enter cells through genetically engineered receptors containing either portions of a virus-binding antibody (Mason *et al*., 1994) or by fusing the antigen-binding domain of an FMDV-specific antibody molecule (scAB) to the cell-surface molecule intracellular adhesion molecule 1 (ICAM1) (Rieder *et al*., 1996).

Baranowski and co-workers (1998) reported that a genetically engineered derivative of a highly passaged (passaged 100 times on BHK cells) serotype C FMDV monoclonal antibody escape variant, containing an RGG-for-RGD mutation, had an enhanced affinity for HS. However, binding to cell-surface HS was not required for efficient replication in glycosaminoglycan (GAG)-deficient CHO cells. Baranowski and co-workers (2000) subsequently reported that biological selection allowed a complete reversion of the heparin-binding motif phenotype of FMDV variants that lacked the RGD motif, and that these variants infected cells via a mechanism that was independent of integrin $\alpha_v\beta_3$ or HS. These

findings suggested that a possible third mechanism for cell recognition by FMDV, which excluded the use of HS and integrins, exists.

Recently, Zhao and co-workers (2003) suggested the existence of a novel integrin- and HS-independent FMDV cell-binding site, which functions in cell culture and in animals. Genetic and biochemical characterization of a BHK tissue culture-adapted derivative (vac-O/CHA/90) of a Cathay topotype isolate of FMDV serotype O (O/CHA/90), led to the identification of several amino acid residues in the 1D viral capsid protein, including two aromatic amino acids (His-108 and Tyr-174) and two positively charged residues (Lys-83 and Arg-172), that were shown to extend the virus' host range in cell culture and were required for growth in mutant CHO cells, in which cell-surface GAG expression is defective. These residues are distant from the RGD sequence that mediate binding of FMDV to integrins and from the sequences at the 1C/1D interface within the biological protomer that has been shown to participate in binding HS to the type O_1 Campos capsid (Sa-Carvalho *et al*., 1997; Fry *et al*., 1999). Consequently, the participation of these residues in integrin-dependent cell binding was investigated by making use of genetically engineered viruses containing a non-conservative KGE-for-RGD substitution. The results indicated that the KGE-derivatives of O/CHA/90 replicated well in cell culture, maintaining the KGE sequence during passage, and was found to cause a mild disease in pigs. The virus was also shown not to be inhibited from forming plaques on BHK cells by pretreatment with RGD- and KGE-containing peptides.

Although the cell surface molecule utilized by the above-mentioned viruses has yet to be identified, Zhao and co-workers (2003) proposed that the cell surface molecules utilized could consist of one or more GAG molecules that bind the virus in a manner that is different to that previously described for type O_1 Campos (Sa-Carvalho *et al*., 1997). Alternatively, during serial cytolytic infections of the above-mentioned viruses in tissue culture cell lines, mutations could arise within the capsid of the FMDV that altered the conformation of the G-H loop, which, in turn, allowed these viruses to bind a different subset of integrin molecules present on CHO cells that are not able to interact with field viruses such as O/CHA/90.

1.7 AIM OF THE STUDY

From the literature review, it is evident that two families of cellular receptors mediate binding of FMDV to susceptible cells, *i.e.* integrins and heparan sulfate proteoglycans. Several tissue culture-adapted FMD viruses acquire an affinity for heparan sulfate and, as a consequence, use heparan sulfate proteoglycans as receptors for attachment and subsequent internalization in an integrin-independent pathway (Jackson *et al*., 1996; Sa-Carvalho *et al*., 1997). By contrast, field isolates of FMDV use RGD-dependent integrins as receptors through an interaction mediated by the conserved surface-exposed arginine-glycine-aspartic acid (RGD) tripeptide in the G-H loop of capsid protein 1D (Acharya *et al*., 1989, Fox *et al*., 1989; Logan *et al*., 1993; Mason *et al*., 1994). Several studies have reported that the RGD sequence of this loop interacts with integrins, including $\alpha_v\beta_3$ (Berinstein *et al*., 1995), $\alpha_v\beta_1$ (Jackson *et al*., 2002) $\alpha_v\beta_6$ (Jackson *et al*., 2000b) and $\alpha_v\beta_8$ (Jackson *et al*., 2004). However, the recent identification of FMDV mutants, which are able to enter cells via an integrin-independent and heparan sulfate-independent pathway (Zhao *et al*., 2003), indicates the existence of other receptor molecules and alternative mechanisms for FMDV entry into cells. It has been suggested that the use of different receptors may confer a functional flexibility on the virus that allows it to regulate its receptor usage in response to environmental modifications (Zhao *et al*., 2003).

Prior to undertaking this investigation, a SAT1 type FMDV, designated NAM/307/98, was isolated from samples obtained from a buffalo in the West Caprivi game reserve in Namibia, during a routine survey. Upon partial nucleotide sequencing of the 1D-encoding region, a second RGD motif was noted upstream of the conserved RGD triplet on the G-H loop of protein 1D (Bastos *et al*., 2001). Based on the ability of field isolates of FMDV to use RGD-dependent integrins as receptors, the potential role of this second RGD sequence in receptor recognition warranted further investigation.

Therefore, the aim of this investigation was the following:

To determine whether a second RGD sequence, located upstream of the G-H loop RGD sequence in capsid protein 1D of the SAT1 type virus NAM/307/98, can function as a ligand for binding the virus to susceptible tissue culture cells.

The research strategies for obtaining the primary objective involved the following:

- Molecular characterization of the capsid-encoding region of NAM/307/98 (Chapter 2).
- Engineering of a chimeric FMD virus by cloning the external capsid-encoding region of NAM/307/98 into a genome-length SAT2 cDNA clone, lacking the corresponding region (Chapter 2).
- Site-directed mutagenesis of the RGD sequence in the G-H loop of capsid protein 1D of NAM/307/98 and engineering of mutant vNAM/SAT2 viruses (Chapter 3).
- Investigation of the mutant and non-mutant chimeric viruses for their ability to use integrin receptors by serial passaging on BHK-21 tissue culture cells (Chapter 3).

CHAPTER 2

MOLECULAR CHARACTERIZATION OF THE CAPSID-ENCODING REGION OF NAM/307/98, A SAT1 TYPE FOOT-AND-MOUTH DISEASE VIRUS, AND ENGINEERING OF A CHIMERIC FMD VIRUS, vNAM/SAT2

2.1 INTRODUCTION

In 1998, a SAT1 type FMDV, NAM/307/98, was isolated from an African buffalo *(Syncerus caffer)* in the West Caprivi Game Reserve in Namibia. The African buffalo is known to be a maintenance host for FMDV (Thomson, 1996) and SAT type viruses are capable of persisting in an individual animal for at least five years and in an isolated herd for up to 24 years (Condy *et al*., 1985). Consequently, numerous antigenic and genetic variants arise in such persistently infected animals (Vosloo *et al*., 1996). Upon sequencing of the 1D-encoding region of NAM/307/98, for epidemiology purposes, a second RGD triplet at amino acid residues 110-112, upstream of the RGD triplet in the G-H loop (residues 149-151), was identified (Bastos *et al.*, 2001). It has been reported that field isolates of FMDV utilize RGD-dependent integrins as receptors for cell binding (Fox *et al*., 1989; Mason *et al*., 1994), whereas several tissue culture-adapted viruses have been shown to utilize heparan sulfate proteoglycans (Jackson *et al*., 1996; Sa-Carvalho *et al*., 1997). Therefore, the presence of a second RGD motif in capsid protein 1D of NAM/307/98 is of particular interest, since it might potentially serve as an alternative ligand for integrin receptors.

To investigate the functional importance of the second RGD sequence, an approach is required whereby specific mutations can be introduced into the FMDV RNA genome. Reverse genetics, a process whereby viral RNA genomes can be converted into complementary DNA copies and replicated as plasmid inserts in bacterial hosts, has provided a valuable tool for the manipulation of viral genetic material. The demonstration by Racaniello and Baltimore (1981) that RNA transcribed from a cloned full-length cDNA copy of the poliovirus genome produced infectious poliovirus particles upon transfection of permissive mammalian cell cultures, has provided the basis for all subsequent analyses of picornaviruses by reverse genetics. Infectious cDNA clones have since been engineered for several other picornaviruses, including rhinovirus (Mizutani and Colonno, 1985), hepatitis A

virus (Cohen *et al.*, 1987) and coxsackie virus (Kandolf and Hofschneider, 1985). However, the construction of infectious cDNA clones of viruses belonging to the *Cardiovirus-Apthovirus* group was initially hampered by the presence of a poly (C) tract at the 5' end of the viral genome (Deng and Wu, 1981). In the case of FMDV, this problem was overcome by Zibert and co-workers (1990) who engineered an infectious genome-length cDNA clone of type O_1 Kaufbeuren containing a synthetic poly (C) tract of 32 residues. Subsequently, several type A and O infectious genome-length cDNA clones have been engineered (Rieder *et al.*, 1993; Beard and Mason, 2000), as well as chimeric cDNA clones between the genomes of closely and distantly related FMDV types (Sa-Carvalho *et al*., 1997; Baranowski *et al*., 1998; Almeida *et al*., 1998; Van Rensburg *et al*., 2002a; 2004).

The application of reverse genetics to FMDV has provided a powerful tool for studying processes relating to viral protein synthesis and processing, virus replication and encapsidation (Rieder *et al.*, 1993; McKenna *et al.*, 1995; Beard and Mason 2000; McLinerney *et al*., 2000; Tiley *et al*., 2003), and may represent a novel approach for the production of attenuated vaccines (Almeida *et al.*, 1998; Rieder *et al*., 1994). In addition, reverse genetics has enabled investigations regarding viral proteins and sequences involved in the attachment and subsequent infection of permissive cells by FMDV. Using this approach, it has been reported that an infectious genome-length cDNA clone of the type A_{12} FMDV, containing a mutated RGD motif, was unable to bind to BHK cells (Mason *et al*., 1994). Towards determining whether the second, non-conserved RGD motif identified in the capsid protein 1D of NAM/307/98 may serve as a ligand for integrin receptors, the aims of this part of the study were (i) to molecularly characterize the capsid-encoding region of NAM/307/98, (ii) to construct a chimeric FMDV clone by insertion of the external capsid-encoding region of SAT1/NAM/307/98 into a genome-length cDNA clone of SAT2/ZIM/7/83 (Van Rensburg *et al*., 2004), lacking the corresponding region, and (iii) to determine whether viable infectious chimeric virus can be recovered following transfection of mammalian cell cultures with RNA derived from the constructed chimeric cDNA clone. The construction of such an infectious chimeric cDNA clone, would greatly facilitate subsequent mutagenesis procedures whereby the functionality of the second RGD sequence can be investigated.

2.2 MATERIALS AND METHODS

2.2.1 Virus and cell cultures

The SAT1/NAM/307/98 strain was isolated at the Exotic Diseases Division (EDD), Onderstepoort Veterinary Institute, from an oesophageo-pharyngeal specimen (probang) obtained from a buffalo (*Syncerus caffer)* in the West Caprivi Game Reserve in Namibia during a routine survey. The primary isolation was performed in primary pig kidney (PK) cells according to standard procedures. The isolated virus was passaged once on IB-RS-2 cells and the strain obtained (passage history: PK1RS1) was used in all subsequent experiments. A bovine-outbreak virus strain originating from western Zimbabwe, SAT2/ZIM/7/83 (passage history: B1BHK5B1BHK3), was included as a control. Baby hamster kidney (BHK) cells, strain 21, clone 13 (ATCC CCL-10), were maintained in Eagle's Basal Medium (BME; Invitrogen) supplemented with 10% (v/v) fetal calf serum (FCS; Delta Bioproducts), antibiotics, 1 mM L-glutamine (Invitrogen) and 10% (v/v) tryptose phosphate broth (TPB; Sigma). The Instituto Biologico Renal Suino cells (IB-RS-2; a pig kidney cell line) were maintained in RPMI medium (Sigma) containing 10% (v/v) FCS and antibiotics, and Chinese hamster ovary (CHO) strain K1 cells (ATCC CCL-61) were maintained in Ham's F12 medium (Invitrogen) supplemented with 10% (v/v) FCS and antibiotics.

2.2.2 Bacterial strains and plasmids

Escherichia coli MAX Efficiency® DH5α™ competent cells (genotype: Fϕ80d*lac*ZΔM15 Δ(lacZYA-argF)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1), obtained from Life Technologies, were used as the transformation host in cloning experiments. pSAT2, a genome-length cDNA clone of SAT2/ZIM/7/83, has been described previously (Van Rensburg *et al.*, 2004) and was used as the genetic backbone in the construction of chimeric cDNA clones.

2.2.3 Oligonucleotides

Oligonucleotides used in the cDNA synthesis, PCR amplification and nucleotide sequencing procedures were custom-synthesized by Inqaba Biotechnical Industries, Invitrogen or Gibco-BRL, and are described in Table 2.1.

Table 2.1: Oligonucleotides used in this part of the study

Oligonucleotide	♦Sequence	Reference	Purpose
WDA	5'-GAAGGGCCCAGGGTTGGACTC-3'	Beck and Strohmaier (1987)	cDNA synthesis, PCR amplification, nucleotide sequencing
L-internal	5'-GWTACGTCGATGARCC-3'	Van Rensburg (unpublished)	PCR amplification, nucleotide sequencing
VP2REV	5'-GTGGTCTGTGGGGAGTTC-3'	This study	PCR amplification, nucleotide sequencing
Seq 38	5'-GCGTCACCTACGGGTAC-3'	This study	PCR amplification, nucleotide sequencing
VP2-internal	5'-GAACTSCCCAMAGACCAC-3'	Van Rensburg (unpublished)	Nucleotide sequencing
Seq 37	5'-ACGGGCACGTGTACAAC-3'	This study	Nucleotide sequencing
VP1-internal	5'-GGGTTKRTACTTGCAG-3'	Van Rensburg (unpublished)	Nucleotide sequencing
cDNA-2A	*5'CGC**cccggg**GTTGGACTCAACGTCTCC 3'	Van Rensburg (unpublished)	PCR amplification
cDNA-NAM	*5'-CGG**aatatt**GACCACCAGCCATGGTACCACCAC-3'	This study	PCR amplification
P621	5'-GGACATATCT TGTTGCATA-3'	Van Rensburg (unpublished)	Nucleotide sequencing
P622	5'-GCACTGACACCACGTCTAC-3'	Van Rensburg (unpublished)	Nucleotide sequencing
VP1UA	5'-CCACRTATTACTTYTGTGACCT-3'	Bastos (1998)	Nucleotide sequencing

♦ Abbreviations representing ambiguities are Y = C/T, R = A/G, W = A/T, S = C/G, M = A/C and K = T/G

* In primer sequences, the restriction endonuclease sites are indicated in bold lower case letters; *Xma* I in oligonucleotide cDNA-2A and *Ssp* I in oligonucleotide cDNA-NAM

2.2.4 Plaque assay

Cell monolayers of IB-RS-2, BHK-21 and CHO-K1 cells were seeded in 35-mm-diameter wells to achieve 100% confluence following incubation overnight at 37°C in the presence of 5% CO_2. Log_{10} dilutions of the NAM/307/98 and ZIM/7/83 viruses were prepared in BME medium, containing 1% FCS, antibiotics and 25 mM HEPES buffer (Invitrogen). Following aspiration of the medium, 200 µl of each dilution was added to the cell monolayers and incubated for 1 h at 37ºC in a CO_2 incubator with shaking. Following incubation, 2 ml Tragacanth overlay (Appendix) was added to each well, and incubation was continued for 48 h in a CO_2 incubator. The cell monolayers were then stained for 3 h with 2 ml of a 1% (w/v) methylene blue stain (Appendix) to visualize plaques (Grubman *et al*., 1979). The plaque titration assays were performed in duplicate.

2.2.5 Characterization of the capsid (P1)-encoding region of NAM/307/98

2.2.5.1 RNA extraction

RNA was extracted from NAM/307/98-infected cell cultures using a modified guanidinium thiocyanate (GuSCN)/silica method, as described by Boom *et al*. (1990). Briefly, the cells of a 200-µl cell culture sample were lysed by addition of L6 buffer (Appendix) containing GuSCN and an aliquot of a 4% silica suspension. Following incubation for 5 min at room temperature, the silica-bound nucleic acid was collected by centrifugation (13 000 rpm; 15 s) and rinsed with L2 wash buffer (Appendix), 70% ethanol and acetone, respectively, before being air-dried. The nucleic acid was eluted from the silica matrix at 56°C for 2 min in a final volume of 30 µl 1 × TE buffer (10 mM Tris-HCl, 2 mM EDTA; pH 7.4) containing RNasin® ribonuclease inhibitor (40 U/µl; Promega) and stored at -70ºC.

2.2.5.2 cDNA synthesis

The viral RNA was reverse-transcribed using a mixture of random hexanucleotides, together with the antisense oligonucleotide WDA, which anneals at the 2A/2B junction (Table 2.1). The reaction mixture contained *ca.* 1 to 3 µg of RNA, 0.23 µM of oligonucleotide WDA, 4.55 µM of a random hexanucleotide mixture (Roche), 1 × AMV-RT buffer (50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM DTT), 0.34 mM of each dNTP (Roche), 2% (v/v) DMSO and 20 U RNasin® (40 U/µl; Promega). Following denaturation of the RNA by incubating at 70°C for 3 min, 40 U AMV-Reverse Transcriptase (10 U/µl; Promega) was

added and the RNA was reverse transcribed at 42ºC for 2 h. After incubation, the enzyme was inactivated by heating to 80ºC for 2 min and the reaction mixtures were stored at 4ºC.

2.2.5.3 Polymerase chain reaction (PCR) amplification of the capsid-encoding region of NAM/307/98

A cDNA copy of the capsid (P1)-encoding region of the NAM/307/98 virus was amplified as two overlapping amplicons to facilitate its nucleotide sequence determination (Fig. 2.1). In the first PCR, oligonucleotides L-internal, which anneals in the L region, and VP2REV, which anneals in the 1B region, were used to generate a 900-bp L-1B amplicon. In the second PCR, oligonucleotides Seq 38, which anneals in the 1B region, and WDA were used to generate a 2-kb 1B-2A amplicon. The PCR reaction mixtures (50 µl) contained 3 µl of the cDNA reaction mixture, 0.5 µM of each the sense and antisense oligonucleotides, 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$), 0.2 mM of each dNTP (Roche) and 2.5 U *Taq* DNA polymerase (5 U/µl; Roche). The tubes were placed in a Hybaid thermal cycler and after initial denaturation at 94°C for 2 min, the reactions were subjected to 30 cycles using the following temperature profile: denaturation for 30 s at 94°C, annealing for 30 s at 55°C (oligonuleotides L-internal - VP2REV) or 56°C (oligonucleotides Seq 38 - WDA), and elongation for 1 min at 72°C. After the last cycle, the reactions were kept at 72°C for 30 s to ensure synthesis of full-length products.

2.2.5.4 Agarose gel electrophoresis

The amplicons were analyzed by agarose gel electrophoresis on a 1.5% (w/v) agarose gel in 1 × TAE buffer (40 mM Tris-acetate, 2 mM EDTA; pH 8) at 100 V. The gel was supplemented with 0.5 µg/ml ethidium bromide (EtBr) to allow visualization of the DNA by UV fluorescence on a transilluminator. The DNA fragments were sized according to their migration in the gel, as compared to that of standard DNA molecular markers, namely a 100-bp DNA ladder (Promega) and phage lambda DNA digested with *Hind* III (Promega). The DNA bands of interest were excised from the agarose gel and purified using the Nucleospin® Extract kit (Macherey-Nagel) in accordance with the manufacturer's instructions.

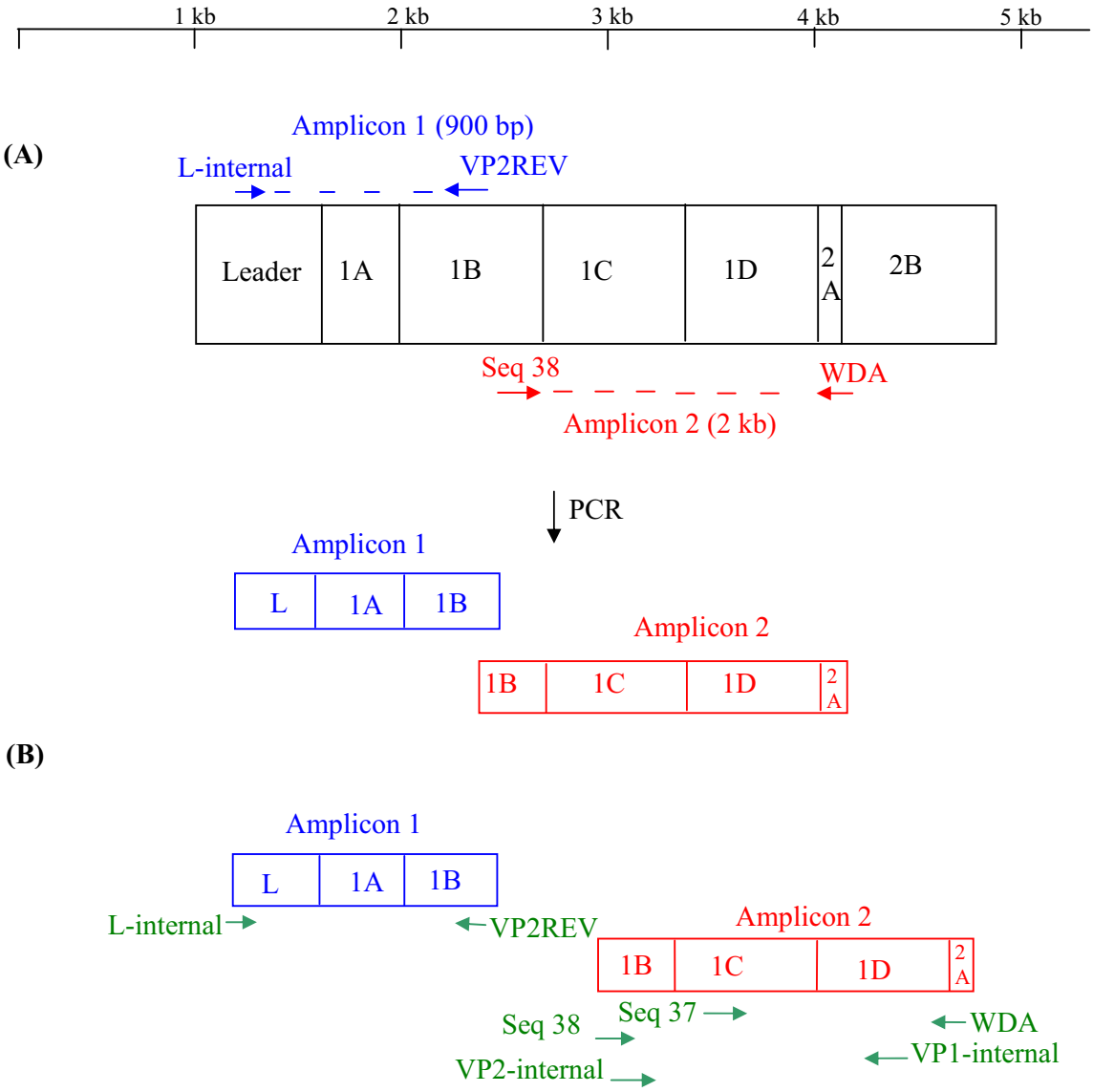

Fig. 2.1 Schematic representation of the (A) PCR amplification and (B) sequencing strategy used to obtain the complete nucleotide sequence of the capsid-encoding region of NAM/307/98. The oligonucleotide annealing positions and directions of sequencing are indicated in green. A scale bar indicating the approximate sizes of the respective protein-encoding genomic regions is indicated above the diagram.

2.2.5.5 Nucleotide sequencing and analysis

The nucleotide sequence of the gel-purified amplicons was determined using 0.16 µM of the appropriate oligonucleotide (Table 2.1; Fig. 2.1) and the ABI PRISM™ BigDye™ Terminator Cycling Ready Reaction Kit v3.0 (Applied Biosystems). The extension products were resolved on an ABI PRISM™ 310 Genetic Analyser (Applied Biosystems). The nucleotide sequences obtained were assembled using DAPSA version 3.4.1 (Harley, 2001) and the deduced amino acid sequence was compared, using CLUSTALW (Thompson *et al*., 1994), with the corresponding sequence of O_1BFS (GenBank Acc. No: AY593815), a type O FMDV strain of which the three-dimensional structure of the capsid has been elucidated (Achayra *et al*., 1989). The P1 nucleotide sequence of NAM/307/98 has been submitted to GenBank under the accession number AY770519.

2.2.6 Construction of chimeric clone pNAM/SAT2

2.2.6.1 PCR amplification of the external capsid-encoding region of NAM/307/98

The antisense oligonucleotide cDNA-2A (Table 2.1), which is situated in 2A, and the sense oligonucleotide cDNA-NAM (Table 2.1), which is situated in 1B, were designed to contain *Xma* I and *Ssp* I restriction endonuclease recognition sequences, respectively, in order to facilitate subsequent cloning procedures. The cloning strategy is indicated diagrammatically in Fig. 2.2. The external capsid-encoding region (1B-1D-2A) of NAM/307/98 was amplified using the Expand™ High Fidelity PCR system (Roche). The reaction mixture (50 µl) contained 3 µl cDNA (Section 2.2.5.2), 0.3 µM of each oligonucleotide, 1 × Expand High Fidelity buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Roche) and 2.6 U of High Fidelity Enzyme (3.5 U/µl). The reaction tubes were placed in a Hybaid thermal cycler. Following initial denaturation of 2 min at 94°C, the reactions were subjected to 25 cycles of denaturation at 94°C for 15 s, annealing at 57°C for 30 s and elongation at 72°C for 2 min. After the last cycle, the reactions were kept at 72°C for 7 min to complete synthesis of all strands. For control purposes, an identical reaction mixture but lacking template DNA was also included. Aliquots of the reaction mixtures were subsequently analyzed by agarose gel electrophoresis on a 1% (w/v) agarose gel in the presence of an appropriate DNA molecular weight marker and the amplicons were purified from the gel using the Nucleospin® Extract kit (Machery-Nagel).

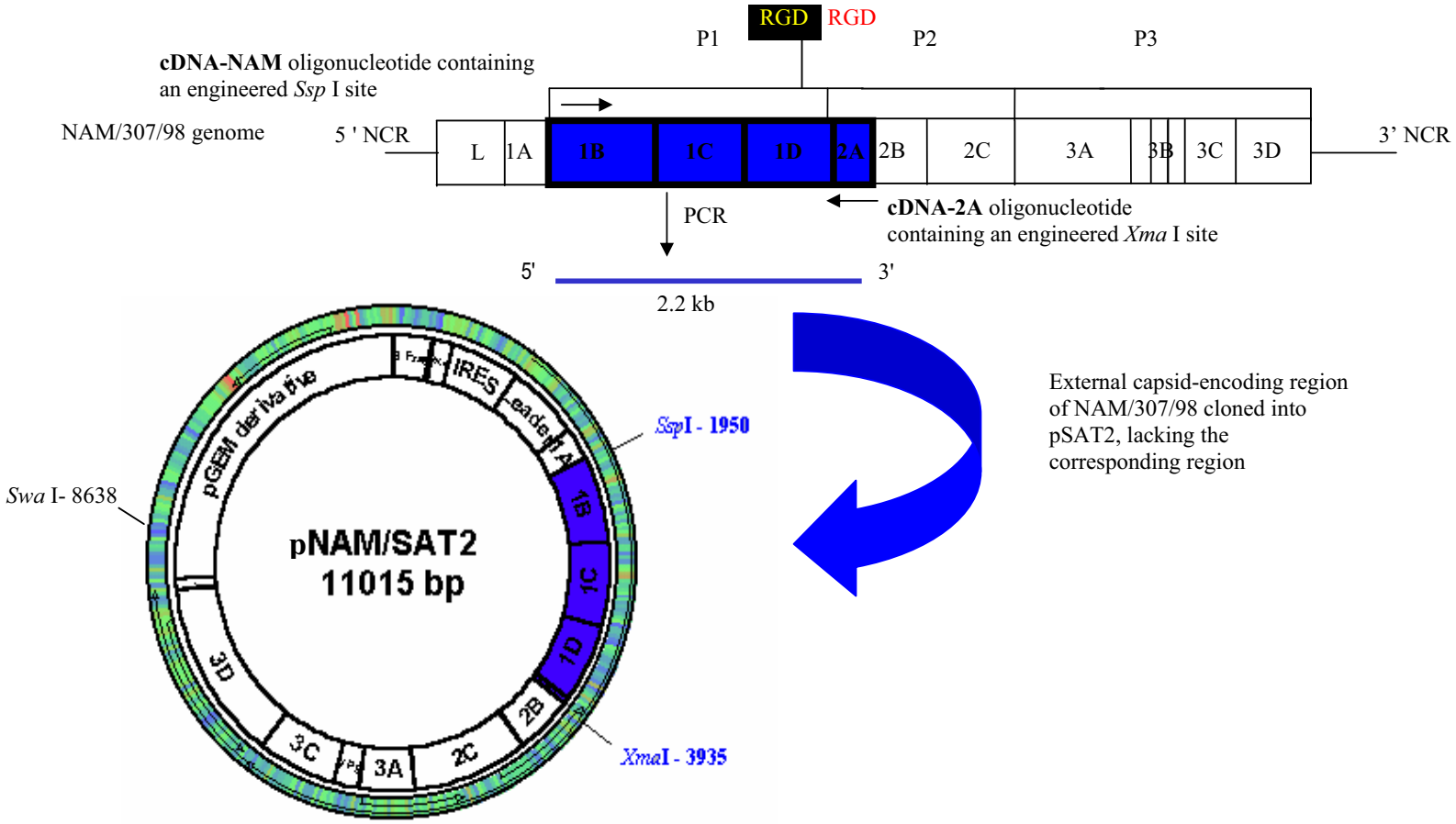

Fig. 2.2 Schematic representation of the cloning strategy used to clone the external capsid-encoding region of SAT1/NAM/307/98 into the *Ssp* I and *Xma* I sites of pSAT2, a genome-length cDNA clone derived from SAT2/ZIM/7/83. The *Swa* I restriction enzyme site, which facilitates linearization of pNAM/SAT2, is also indicated. The G-H loop RGD motif is indicated in red, whilst the second upstream RGD motif is indicated in a black box in yellow.

2.2.6.2 Restriction endonuclease digestion

Restriction endonuclease digestions were performed in sterile Eppendorf tubes and contained the appropriate concentration of salt (using the 10 × buffer supplied by the manufacturer) for the specific enzyme and 1 U of enzyme per µg of DNA. The reaction mixtures were incubated at 37°C, except for those containing *Swa* I, which was incubated at 25°C. Upon digestion of DNA with both *Xma* I and *Ssp* I, the DNA was first incubated with *Xma* I after which the salt concentration was adjusted by the addition of 50 mM NaCl and the *Ssp* I enzyme added. Following incubation, the endonucleases were inactivated by the addition of 2.5 µl RE STOP (consisting of 1 part 0.5 M EDTA, RNase A and 8 parts loading buffer; Promega) and incubated at room temperature for 5 min, after which the reaction mixture was stored at 4°C. The digestion products were typically analyzed on a 1% (w/v) agarose gel in the presence of appropriate DNA molecular markers. All restriction endonucleases were supplied by Promega or New England Biolabs.

2.2.6.3 Preparation of the pSAT2 vector DNA

pSAT2 (Van Rensburg *et al.*, 2004) was used to engineer a chimeric construct containing the external capsid-encoding region of NAM/307/98. The external capsid-encoding region of pSAT2 was removed by digestion with both *Xma* I and *Ssp* I. Following digestion, the plasmid DNA was dephosphorylated by the addition of alkaline phosphatase, as described by Turner *et al*. (1997). Briefly, the digested vector DNA was incubated at 37°C for 30 min in a 40-µl reaction volume containing 0.5 U alkaline phosphatase (1 U/µl; Roche). The *ca.* 8.8-kb DNA fragment, representing the pSAT2 backbone (excluding the external capsid-encoding region), was excised from an agarose gel following electrophoresis and purified using the Nucleospin® Extract kit (Macherey-Nagel). The amplicon resulting from PCR amplification of the external capsid-encoding region using NAM/307/98 cDNA as template (Section 2.2.6.1) was similarly digested and purified from an agarose gel.

2.2.6.4 Ligation reactions and transformation of competent cells

The purified digested PCR amplicon and the pSAT2 vector DNA were ligated for 10 min at room temperature using the Rapid DNA Ligation Kit from Roche. The reaction mixture (10 µl) contained 5 µl of 2 × T4 DNA ligase buffer, 150 ng of insert DNA, 50 ng of pSAT2 vector DNA and 1.25 U of T4 DNA ligase (2.5 U/µl). Competent *E. coli* MAX Efficiency®

DH5α™ cells were transformed using a modified heat-shock method (Sambrook and Russell, 2001). Competent cells (25 µl) were mixed with 3 µl of the ligation mixture in a sterile 1.5-ml Eppendorf tube and incubated on ice for 30 min. The cells were subsequently incubated for 40 s at 42°C and then chilled on ice for 2 min. Following addition of 175 µl pre-warmed (37°C) SOC medium (2% [w/v] tryptone, 0.5% [w/v] yeast extract, 0.05% [w/v] NaCl, 5 mM KCl, 20 mM glucose, 15 mM $MgCl_2$; pH 7), the transformation mixture was incubated at 37°C for 1 h to allow the cells to recuperate and express the ampicillin resistance gene. Transformed cells were plated on LB agar plates (1% [w/v] tryptone, 0.5% [w/v] yeast extract, 1% [w/v] NaCl, 1.3% [w/v] agar; pH 7.4) supplemented with 50 µg/ml ampicillin and the agar plates were then incubated overnight at 37°C.

2.2.6.5 Plasmid DNA extractions

To facilitate rapid screening of a large number of transformants, plasmid DNA was extracted using a robust STET-boiling minilysate method (obtained from Dr P. W. Mason, Plum Island Animal Disease Center), with the following modifications. Colonies were picked from the agar plates with sterile toothpicks and inoculated into sterile 1.5-ml Eppendorf tubes containing 1 ml LB broth (1% [w/v] tryptone, 0.5% [w/v] yeast extract, 1% [w/v] NaCl; pH 7.4) supplemented with ampicillin (50 µg/ml). Following incubation overnight at 37°C with shaking, the cells were harvested by centrifugation at 13 000 rpm for 2 min. The bacterial cell pellets were suspended in 250 µl STET buffer (Appendix) containing 1 mg/ml lysozyme, and lysed by boiling at 100°C for 1 min. The cellular debris was collected by centrifugation at 13 000 rpm for 8 min and removed using a sterile toothpick. The plasmid DNA was subsequently precipitated from the supernatants by the addition of 250 µl isopropanol. The precipitated plasmid DNA was collected by centrifugation, as above, dried under vacuum and then suspended in 20 µl 1 × TE. Plasmid DNA constructs were subsequently characterized by restriction endonuclease digestion, followed by agarose gel electrophoresis and by nucleotide sequencing, as described below. A chimeric clone, designated pNAM/SAT2, was selected and used in subsequent procedures.

2.2.6.6 Nucleotide sequencing and sequence analyses

Plasmid DNA for sequencing and *in vitro* RNA transcription reactions was extracted and purified using the QIAprep® Spin Miniprep Kit (Qiagen) according to the manufacturer's

instructions. To verify successful cloning of the external capsid-encoding region of NAM/307/98 into pSAT2, the nucleotide sequence flanking the cloning sites was determined using 0.16 µM of oligonucleotides P621 and P622 (Table 2.1), which are situated in the 2B and 1A regions, respectively, of the pSAT2 clone. The sequencing reactions and sequence analyses were performed as described under Section 2.2.5.5.

2.2.7 *In vitro* RNA synthesis

2.2.7.1 Preparation of template DNA

To facilitate RNA synthesis, 5 µg of the purified chimeric plasmid pNAM/SAT2 was linearized by restriction with *Swa* I for 10 h at 25°C (Fig. 2.2). The genome-length cDNA clone pSAT2 was included as a control and linearized by digestion with *Not* I for 10 h at 37°C. Following digestion, the reaction mixtures were incubated at 55°C for 15 min with 20 ng Proteinase K in the presence of 1 × STE buffer (10 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA; pH 8), 10 ng oyster glycogen (Sigma), 0.5 M EDTA and 0.2% (w/v) SDS. The endonucleases were subsequently inactivated by heating to 80°C for 15 min and the reaction mixtures deproteinized by a phenol and chloroform extraction. An equal volume (100 µl) of Tris-saturated phenol (pH 8) was therefore added to each sample, mixed and the organic and aqueous phases separated by centrifugation at 13 000 rpm for 2 min. The upper aqueous phase was recovered and extracted with an equal volume of chloroform, as above. The DNA was then precipitated from the aqueous phase by the addition of 2.5 volumes of 100% ethanol and incubation at -80°C for 20 min. The precipitated DNA was collected by centrifugation at 13 000 rpm for 15 min, washed with 70% ethanol and dried under vacuum before being suspended in 10 µl RNase-free water. All chemicals and reagents used in these procedures were of RNA-grade.

2.2.7.2 RNA transcription

In vitro RNA transcription reactions were performed using the MEGAScript® T7 kit (Ambion) at 39°C for 2 h. The transcription reaction mixtures contained 1 µg of purified linearized template DNA, 1 × transcription buffer, 7.5 mM of each ribonucleotide, 2 µl of the supplied enzyme mix and RNase-free water to a final volume of 20 µl. The integrity of the RNA was analyzed by electrophoresis of an aliquot of the reaction mixtures on a 1% (w/v) agarose gel in 0.5 × TBE buffer (45 mM Tris-borate, 1 mM EDTA; pH 8).

2.2.8 Recovery of infectious chimeric virus

2.2.8.1 Transfection of BHK-21 and IB-RS-2 cells with synthetic RNA

BHK-21 and IB-RS-2 cells were transfected with *in vitro*-synthesized RNA using the Lipofectamine 2000™ reagent (Invitrogen). The cells were seeded in 35-mm-diameter wells to reach 70% confluence (8.4×10^5 cells/ml) within 24 h of incubation at 37°C in the presence of 5% CO_2. For transfection, 3 µg of RNA was diluted in 250 µl serum-free D-MEM medium (Invitrogen) that lacked antibiotics. Separately, 10 µl of the Lipofectamine 2000™ reagent was diluted in 250 µl serum- and antibiotic-free D-MEM. The two solutions were then mixed and incubated at room temperature for 20 min to allow the formation of RNA-lipofectamine complexes. The cell monolayers were subsequently prepared for transfection by rinsing the cells twice with serum- and antibiotic-free D-MEM, but containing 0.1 mM non-essential amino acids (NEAA; Invitrogen). After addition of 500 µl of D-MEM medium containing 0.1 mM NEAA, the cells were overlayed with the RNA-lipofectamine complexes and incubated for 4 h at 37°C in a CO_2 incubator. For BHK-21 cell monolayers, the medium was then aspirated and replaced with 3 ml BME medium containing antibiotics, 25 mM HEPES and 1% FCS. For IB-RS-2 cell monolayers, the medium was aspirated and replaced with RPMI medium (Sigma) containing 25 mM HEPES, antibiotics and 1% FCS. Mock-transfected BHK-21 and IB-RS-2 cells, treated as above, were included as controls. The transfection plates were incubated at 37°C for 24 h in a CO_2 incubator.

2.2.8.2 Serial passaging of chimeric virus

Following incubation, the cells were lysed by freezing the transfection plates at -80°C followed by thawing on ice. Aliquots of the thawed tissue culture lysate (10% [v/v]) were subsequently used to infect BHK-21 and IB-RS-2 cell monolayers in 35-mm-diameter wells. For BHK-21 cells, the tissue culture dishes were incubated at 37°C for 1 h in a CO_2 incubator after which BME medium, containing antibiotics, 25 mM HEPES and 1% FCS, was added and the incubation continued for 48 h. For IB-RS-2 cells, the tissue culture dishes were similarly treated, except RPMI medium was added. The same procedure was used for serial passage of the viruses in BHK-21 and IB-RS-2 cells and the cells were monitored for cytopathic effect (CPE) by microscopy up to 48 h post-infection. Aliquots of the respective tissue culture lysates were stored at –80°C after each passage.

2.2.8.3 Characterization of recovered chimeric virus

To characterize the recovered chimeric viruses, RT-PCR across the cloning junction in 2A, followed by nucleotide sequencing of the obtained amplicon, were performed. RNA was extracted from the tissue culture lysates by a modified guanidinium thiocyanate/silica method (Boom *et al*., 1990) and used as template for cDNA synthesis. The viral RNA was reverse-transcribed using AMV-Reverse Transcriptase (Promega) and the antisense oligonucleotide P621. All of these procedures were performed in accordance with the methods described previously (Sections 2.2.5 and 2.2.7). Oligonucleotides P621 and VP1UA, which is situated in 1D, were used to amplify part of the 1D-2B genomic region spanning the insertion site and sequence encoding the two RGD motifs. The PCR reaction was performed as described earlier (Section 2.2.5.3), except that annealing of the oligonucleotides was performed at 55°C for 30 s. The resultant amplicon was purified from an agarose gel using the Nucleospin® Extract Kit (Macherey-Nagel) and the nucleotide sequence of the gel-purified amplicon was determined using 0.16 µM of oligonucleotide P621, as described previously (Section 2.2.5.5).

2.3 RESULTS

2.3.1 Plaque assays

Field strains of FMDV have been reported to infect permissive cells by attaching to integrin receptors, whereas tissue culture-adapted strains can also use heparan sulfate proteoglycans (Jackson *et al*., 1996; Sa-Carvalho *et al*., 1997). Thus, to determine whether the SAT1 type FMDV field isolate NAM/307/98 (PK1RS1) is capable of utilizing heparan sulfate proteoglycans, in addition to integrin receptors, plaque assays on different tissue culture cell lines were performed. For this purpose, BHK-21, IB-RS-2 and CHO-K1 cells were used in the assays. Although CHO-K1 cells normally express two RGD-binding integrins, $\alpha_v\beta_5$ and $\alpha_5\beta_1$ (Neff *et al*., 1998; You *et al*., 2001; Jackson *et al*., 2002), they are, however, non-permissive for field strains of FMDV (Mason *et al.*, 1993; Jackson *et al.*, 1997; Neff *et al.*, 1998). In contrast, field strains of FMDV are able to utilize integrin receptors $\alpha_v\beta_3$, $\alpha_v\beta_6$, $\alpha_v\beta_1$ and $\alpha_v\beta_8$ and have been reported to replicate in BHK-21 and IB-RS-2 cells (Berinstein *et al.*, 1995; Neff *et al.*, 1998; Neff *et al.*, 2001; Miller *et al.*, 2001; Jackson *et al.*, 2000b; Jackson *et al.*, 2002; Duque and Baxt, 2003). In addition to the different cell lines, a tissue culture-adapted SAT2 type FMDV, ZIM/7/83 (B1BHK5B1BHK3), which is capable of

utilizing heparan sulfate as a receptor for cell binding (Van Rensburg *et al*., 2004), was also included in these assays.

The plaque morphologies obtained following plaque titration on IB-RS-2, BHK-21 and CHO-K1 cells are indicated in Fig. 2.3. Although the NAM/307/98 and ZIM/7/83 viruses both displayed clear large plaques on IB-RS-2 cells, distinct differences were observed on the other tissue culture cell lines. Whereas ZIM/7/83 produced large clear plaques on BHK-21 cells, the NAM/307/98 virus produced small turbid plaques, suggesting a slower rate of replication in these cells. In contrast to ZIM/7/83, which produced small clear plaques on CHO-K1 cells and is characteristic of viruses that use heparan sulfate proteoglycans as cell receptors, the NAM/307/98 virus was unable to produce plaques. Since NAM/307/98 was able to infect BHK-21 and IB-RS-2 cells but not CHO-K1 cells, it was therefore concluded that NAM/307/98 is capable of using integrins, and not heparan sulfate, as cellular receptors. Furthermore, the difference in plaque morphology of NAM/307/98 on the IB-RS-2 and BHK-21 cells may be indicative of the passage history of the virus. NAM/307/98 was initially isolated in PK cells and passed once only on IB-RS-2 cells prior to performing these plaque assays. Since the virus had therefore already been "adapted" to these cells, the viral capsid was more efficient in interacting with the cells as opposed to BHK-21 cells. Consequently, large clear plaques could be seen on IB-RS-2 cells, and small turbid plaques on BHK-21 cells.

2.3.2 Nucleotide sequence and analysis of the capsid-encoding region of NAM/307/98

The nucleotide sequence of the capsid-encoding region of NAM/307/98 was determined from two overlapping amplicons through primer walking using different oligonucleotides (Table 2.1; Fig. 2.1). The sequences obtained were assembled to obtain the full-length nucleotide sequence from which the amino acid sequence of the viral proteins was deduced (Fig. 2.4).

- **Presence of two RGD motifs**

Analysis of the deduced amino acid sequence indicated the presence of a conserved RGD sequence (amino acid residues 149-151) located in a hypervariable region of the capsid protein 1D, as well as a second, upstream RGD sequence located at residues 110-112. This motif was stably retained upon passage on IB-RS-2 cells (PK1RS1) and was identical in sequence to that reported by Bastos and co-workers (2001) following primary isolation of the

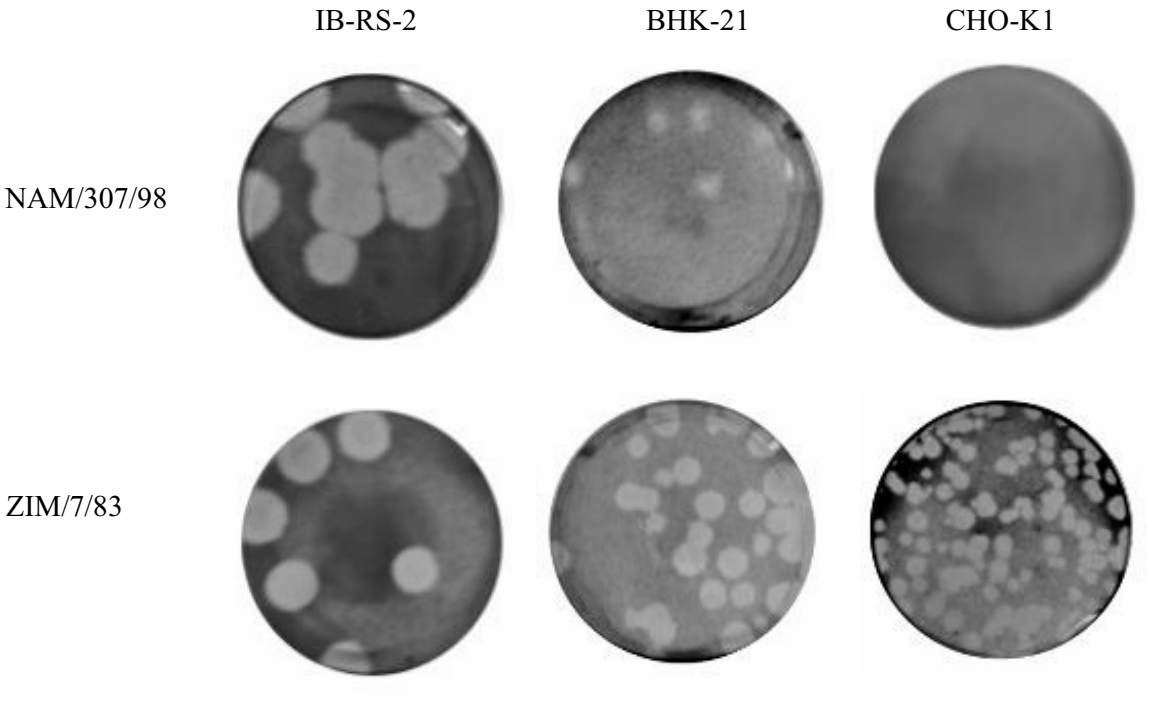

Fig. 2.3 Plaque morphology of the field strain NAM/307/98 and the tissue culture-adapted ZIM/7/83 virus isolate on IB-RS-2, BHK-21 and CHO-K1 cells.

virus in PK cells (PK1). Immediately following the conserved RGD motif in the capsid protein 1D of NAM/307/98 are leucine residues at the RGD+1 and RGD+4 positions. In contrast, the second, upstream RGD sequence is followed by tyrosine at RGD+1 and phenylalanine at RGD+4 (Fig. 2.4). Although the presence of leucine residues following the RGD sequence has been reported to be associated with a higher binding affinity to certain integrin receptors (Mateu *et al*., 1996; Jackson *et al*., 2000a), these residues are not conserved. Some type A and SAT3 isolates have a methionine, whilst most SAT2 isolates have an arginine at the RGD+1 position (Crowther *et al*., 1993; Mateu *et al*., 1996; Jackson *et al*., 1997; Jackson *et al*., 2000a; Bastos *et al*., 2003).

- **Location of two RGD motifs**

In all FMDV serotypes, the RGD sequence located in the G-H loop of the capsid protein 1D is highly exposed to the surface (Baxt and Becker, 1990; Parry *et al*., 1990; Mason *et al*., 1994; Jackson *et al*., 1997). To determine the location of the second, non-G-H loop RGD sequence on the viral capsid, the P1 polyprotein amino acid sequence of NAM/307/98 was aligned with that of type O_1BFS using CLUSTALW. Whereas the RGD sequence located in the G-H loop was conserved, the upstream RGD sequence in NAM/307/98 aligned with a KAP sequence in type O_1BFS (Fig. 2.5A). Based on the previously elucidated crystal structure of the type O_1BFS virion (Acharya *et al.*, 1989), the KAP sequence is located on a short surface-exposed loop between βF and βG (Fig. 2.5B). Thus, it was concluded that both RGD sequences in the capsid protein 1D of NAM/307/98 would be exposed to the surface and could therefore potentially interact with RGD-dependent cellular receptors.

- **Protease ($3C^{pro}$) cleavage sites**

The capsid proteins form part of the P1-2A polyprotein precursor, which is cleaved by the 3C proteinase ($3C^{pro}$) at the 1AB/1C, 1C/1D and 1D/2A junctions to yield proteins 1AB, 1C and 1D (Palmenberg, 1990; Bablanian and Grubman, 1993). Protein 1AB is subsequently cleaved by autocatalysis into the mature capsid proteins 1A and 1B (Harber *et al*., 1991; Lee *et al*., 1993). Since one of the aims was to engineer a chimeric SAT2 FMD virus containing the external capsid-encoding region of NAM/307/98, the P1-2A-encoding region of the NAM/307/98 isolate was also analyzed for 3C proteinase cleavage sites to determine their potential impact on the correct processing of the capsid proteins by the SAT2 $3C^{pro}$. Although the dipeptide Gln-Gly is most prevalent at the $3C^{pro}$ cleavage sites (Seipelt *et al*., 1999), the

FMDV $3C^{pro}$ has been reported to also cleave different sites (Hanecak *et al*., 1982; Palmenberg, 1990) and it has the ability to cleave proteins of heterologous serotypes (Ryan *et al*., 1989).

For NAM/307/98 (Fig. 2.4), the 1AB/1C cleavage site is Gln-Gly, which is identical to other SAT viruses (Van Rensburg *et al*., 2002b). At the 1C/1D junction, the cleavage site is Gln-Thr, which is the same for most FMDV serotypes, with type O_1 Kaufbeuren having a Glu-Thr cleavage site (Clarke and Sanger 1988; Van Rensburg *et al*., 2002b). At the 1D/2A junction, the cleavage site is Gln-Leu, which is the same for most FMDV serotypes (Van Rensburg *et al*., 2002b). The recognition of these processing sites is dependent on their position within the polyprotein and the regions flanking these $3C^{pro}$ cleavage sites may play an important role in the recognition process. A proline residue near to the cleavage site has been noted as being important in both FMDV and encephalomycarditis virus (EMCV) (Palmenberg *et al*., 1984; Van Rensburg, 2002b). In most FMDV serotypes, there is a conserved proline residue in the –4 position from the cleavage site. Some exceptions have been observed in types O and C, which have an alanine residue in this position at the 1C/1D cleavage site. A valine residue has also been observed in the –4 position at the 1D/2A cleavage site in SAT2 viruses (Palmenberg *et al*., 1984; Van Rensburg *et al*., 2002b). A proline residue was observed in the –4 position from all the $3C^{pro}$ cleavage sites in the P1-encoding region of NAM/307/98. Based on these analyses, it was concluded that the NAM/307/98 virus capsid proteins would be processed efficiently by the $3C^{pro}$ from the SAT2 virus ZIM/7/83.

1A

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 G   A   G   Q   S   S   P   A   T   G   S   Q   N   Q   S   G   N   T   G   S   I   I   N   N   Y   Y  26
GGA GCA GGT CAG TCG TCG CCA GCC ACA GGG TCA CAA AAC CAA TCT GGT AAC ACT GGC AGC ATC ATC AAC AAC TAC TAC

 M   Q   Q   Y   Q   N   S   M   D   T   Q   L   G   D   N   A   I   S   G   G   S   N   E   G   S   T  52
ATG CAG CAG TAC CAG AAC TCA ATG GAC ACC CAG CTT GGT GAC AAC GCC ATC TCG GGT GGT TCC AAT GAG GGG TCG ACC

 D   T   T   S   T   H   T   N   N   T   Q   N   N   D   W   F   S   K   L   A   Q   S   A   F   S   G  78
GAC ACC ACC TCG ACC CAC ACC AAC AAC ACT CAG AAC AAT GAC TGG TTT TCC AAA TTG GCG CAA TCG GCT TTC TCC GGA

                     1B
 L   V   G   A   L   L   A   D   K   K   T   E   E   T   T   L   L   E   D   R   I   M   T   T   S   H 104
CTT GTT GGC GCG CTT TTG GCC GAC AAG AAA ACG GAG GAG ACC ACT CTG CTT GAA GAC CGT ATC ATG ACC ACC AGC CAT

 G   T   T   T   S   T   T   Q   S   S   V   G   V   T   Y   G   Y   A   L   T   D   K   F   L   P   G 130
GGT ACC ACC ACG TCA ACC ACA CAG AGT TCG GTG GGC GTC ACC TAC GGG TAC GCC CTC ACT GAC AAG TTT CTC CCC GGT

 P   N   T   N   G   L   E   T   R   V   E   Q   A   E   R   F   F   K   H   K   L   F   D   W   T   L 156
CCA AAC ACC AAT GGA CTG GAG ACA AGA GTG GAA CAA GCA GAG AGG TTC TTT AAA CAC AAG CTT TTT GAT TGG ACA CTT

 E   Q   Q   F   G   T   T   Y   V   M   E   L   P   T   D   H   K   G   I   Y   G   Q   L   V   D   S 182
GAA CAA CAA TTT GGC ACA ACT TAC GTG ATG GAA CTC CCC ACA GAC CAC AAG GGT ATC TAC GGG CAG CTG GTT GAC TCT

 H   A   Y   I   R   N   G   W   D   V   Q   V   S   A   T   A   T   Q   F   N   G   G   C   L   L   V 208
CAC GCG TAC ATC CGC AAC GGA TGG GAC GTC CAG GTC TCT GCC ACT GCC ACC CAA TTC AAC GGA GGC TGC CTC CTG GTG

 A   M   V   P   E   L   C   K   L   G   E   R   E   K   Y   Q   L   T   L   F   P   H   Q   F   L   N 234
GCC ATG GTA CCC GAG CTC TGC AAA TTG GGT GAG AGG GAG AAA TAC CAA CTC ACT CTC TTC CCA CAC CAG TTC TTG AAC

 P   R   T   N   T   T   A   H   I   Q   V   P   Y   L   G   V   D   R   H   D   Q   G   T   R   H   K 260
CCC CGC ACC AAC ACC ACG GCA CAC ATC CAA GTA CCG TAC TTG GGT GTC GAC AGA CAC GAC CAG GGG ACT CGC CAC AAA

 A   W   T   L   V   V   M   V   L   A   P   Y   T   N   D   Q   T   I   G   S   T   K   A   E   V   Y 286
GCG TGG ACT CTA GTT GTG ATG GTG CTG GCG CCA TAC ACC AAT GAC CAG ACC ATA GGA TCT ACA AAA GCC GAG GTC TAC

                                                                 1C
 V   N   I   S   P   T   N   V   Y   V   A   G   E   K   P   S   K   Q   G   I   F   P   V   A   V   S 312
GTG AAC ATT TCA CCA ACC AAT GTT TAC GTG GCC GGT GAG AAG CCC AGC AAG CAA GGG ATT TTC CCC GTG GCC GTC TCC
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 D   G   Y   G   G   F   Q   N   T   D   P   K   T   S   D   P   I   Y   G   H   V   Y   N   P   A   R  338
GAC GGT TAC GGC GGC TTC CAA AAT ACT GAC CCC AAA ACT TCG GAC CCC ATT TAC GGG CAC GTG TAC AAC CCG GCG CGC

 T   L   Y   P   G   R   F   T   N   L   L   D   V   A   E   A   C   P   T   L   L   D   F   N   G   V  364
ACG CTG TAC CCC GGC AGG TTC ACA AAC TTG CTG GAC GTG GCA GAA GCG TGC CCC ACA CTG CTT GAT TTC AAT GGG GTG

 P   Y   V   Q   T   Q   N   N   S   G   S   K   V   L   T   C   F   D   L   A   F   G   H   K   N   M  390
CCA TAC GTC CAA ACC CAG AAC AAC TCT GGT TCA AAG GTT CTC ACA TGT TTT GAT TTG GCA TTT GGA CAC AAA AAC ATG

 K   N   T   Y   M   S   G   L   A   Q   Y   F   A   Q   Y   S   G   T   L   N   L   H   F   M   Y   T  416
AAA AAC ACA TAC ATG TCT GGT CTG GCC CAG TAC TTT GCA CAG TAC AGC GGC ACC CTC AAT CTT CAC TTC ATG TAC ACC

 G   P   T   N   N   K   A   K   Y   M   V   A   Y   I   P   P   G   T   N   P   L   P   K   T   P   E  442
GGC CCC ACC AAC AAC AAG GCC AAG TAC ATG GTG GCA TAC ATC CCA CCC GGC ACA AAC CCT CTC CCC AAA ACA CCG GAG

 M   A   S   H   C   Y   H   A   E   W   D   T   G   L   N   S   T   F   T   F   T   V   P   Y   I   S  468
ATG GCA TCA CAC TGC TAC CAC GCC GAG TGG GAC ACT GGA CTG AAC TCG ACC TTC ACC TTC ACC GTG CCG TAC ATC TCG

 A   A   D   Y   A   Y   T   Y   A   D   E   P   E   Q   A   S   V   Q   G   W   V   G   V   Y   Q   I  494
GCC GCG GAC TAC GCC TAC ACC TAC GCT GAC GAG CCT GAA CAG GCT TCA GTG CAA GGT TGG GTT GGT GTG TAT CAA ATT

 T   D   T   H   E   K   D   G   A   V   V   V   T   V   S   A   G   P   D   F   E   F   R   M   P   I  520
ACT GAC ACA CAC GAG AAG GAC GGG GCC GTC GTC GTC ACC GTG AGT GCC GGC CCC GAC TTT GAG TTC AGG ATG CCC ATT

                   ↓1D
 S   P   L   R   Q   T   T   S   A   G   E   G   A   E   P   V   T   T   D   A   S   A   H   G   G   S  546
AGC CCA TTG CGC CAG ACA ACC TCT GCA GGT GAA GGC GCG GAA CCA GTT ACC ACA GAC GCC TCC GCA CAC GGA GGC AGT

 A   R   T   A   R   R   A   H   T   D   V   T   F   L   L   D   R   F   T   L   V   G   K   T   K   D  572
GCC AGG ACT GCA CGG CGG GCC CAC ACC GAC GTG ACA TTC CTT CTT GAC CGG TTT ACC CTG GTT GGG AAG ACC AAA GAC

 N   K   L   V   L   D   L   L   S   T   K   E   K   T   L   V   G   A   L   L   R   A   A   T   Y   Y  598
AAC AAA CTG GTC CTG GAC CTC TTG AGC ACC AAG GAG AAA ACG CTG GTC GGC GCA CTC CTG CGC GCG GCC ACG TAC TAC

 F   S   D   L   E   V   A   C   V   G   T   N   A   W   V   G   W   T   P   N   G   S   P   V   L   T  624
TTC TCT GAC CTG GAG GTG GCG TGT GTT GGG ACC AAC GCG TGG GTG GGC TGG ACT CCC AAC GGC AGT CCA GTG CTG ACG
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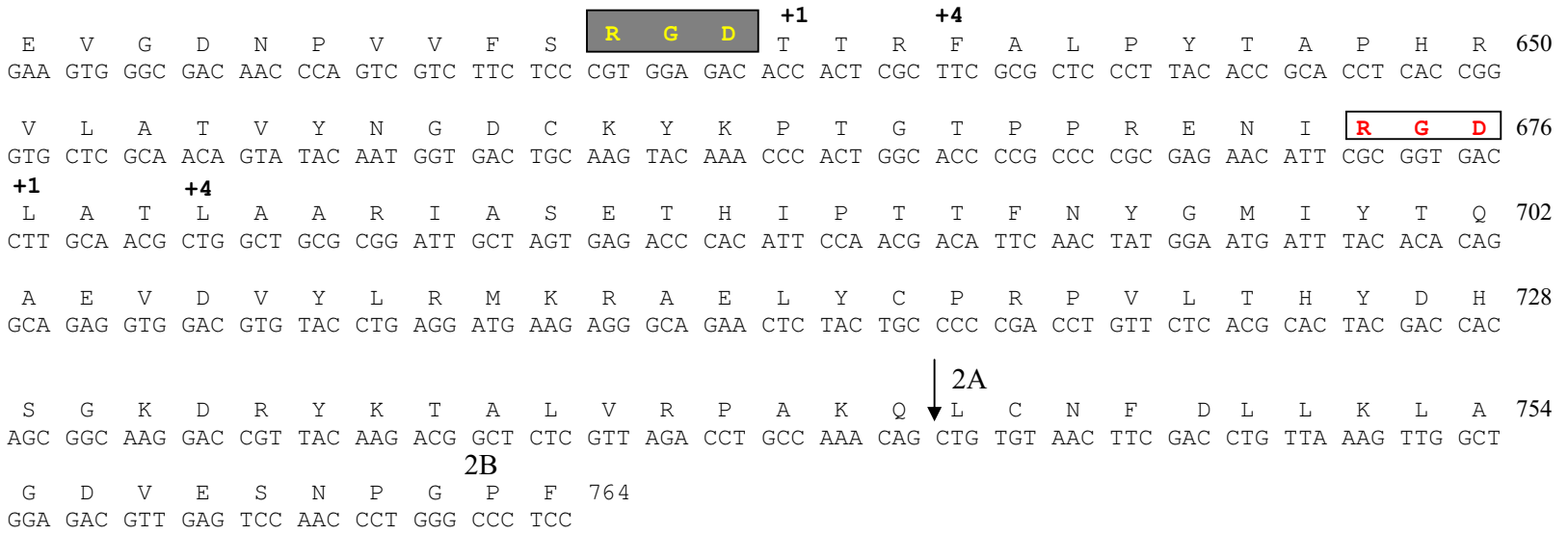
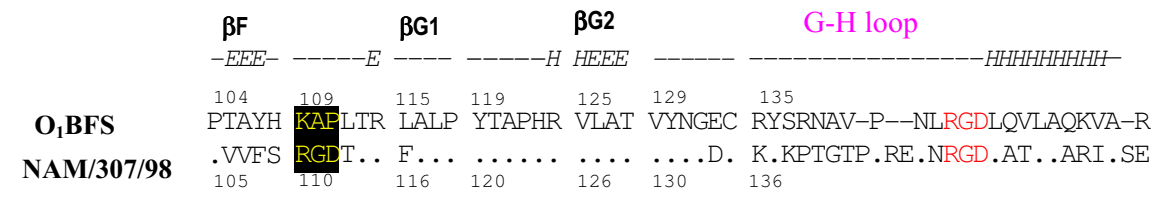


Fig. 2.4 Nucleotide and deduced amino acid sequence of the capsid (1A-1D), 2A- and part of the 2B-encoding genomic regions of NAM/307/98. The $3C^{pro}$ cleavage sites are indicated by arrows. The RGD motif in the G-H loop of protein 1D is indicated in red, whilst a second, upstream RGD is indicated in yellow. The RGD+1 and RGD+4 positions for both RGD motifs are indicated in bold.

(A)



(B)

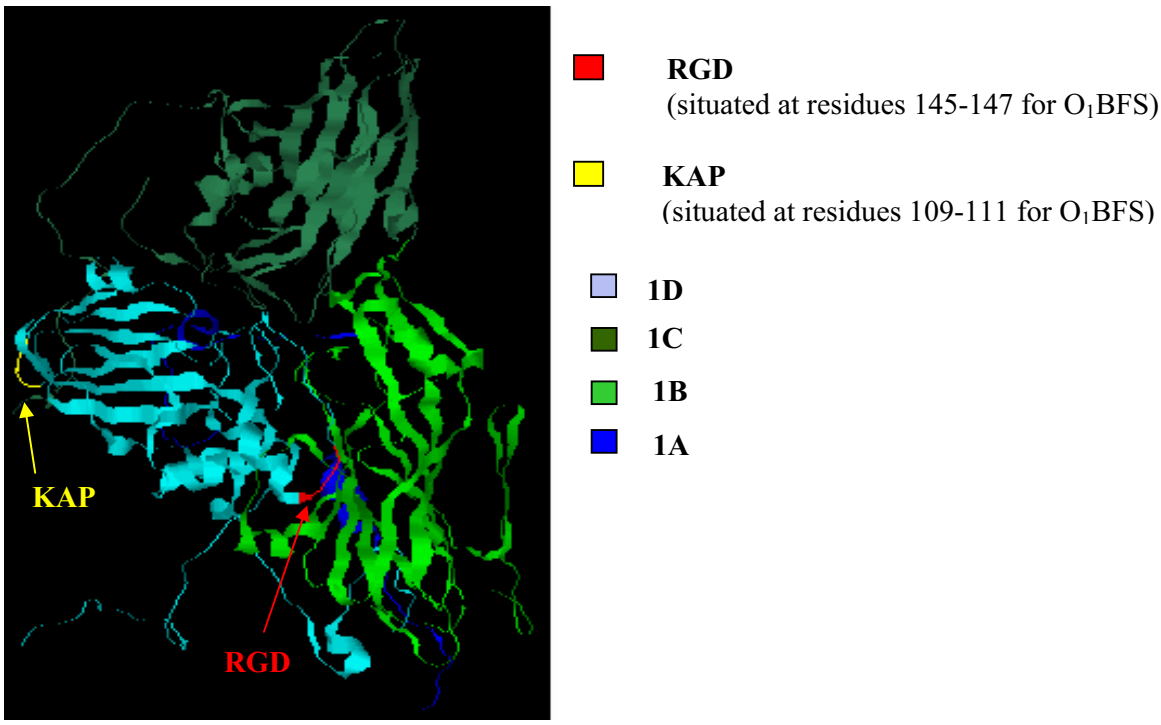


Fig. 2.5 (A) Amino acid sequence alignment between the C-terminus half of the capsid protein 1D of NAM/307/98 and that of O_1BFS. The RGD motif in the G-H loop of 1D is indicated in red and the second RGD, which aligned to the KAP sequence of O_1BFS, is indicated in yellow. The predicted secondary structure is indicated above the sequence with 'H' = α-helix, 'E' = β-strand. The loops connecting the β-strands, corresponding to that determined for O_1BFS, are also indicated. (B) Crystal structure of the O_1BFS capsid viewed in Rasmol, indicating the position of the G-H loop RGD motif, as well as the KAP sequence that aligned to the second RGD sequence of NAM/307/98.

2.3.3 Construction of chimeric clone pNAM/SAT2

2.3.3.1 *Cloning of the external capsid-encoding region of NAM/307/98 into pSAT2*

Towards investigating the functional significance of a second, non-G-H loop RGD sequence in the capsid protein 1D of NAM/307/98, a chimeric clone containing the external capsid-encoding region of NAM/307/98 in a stable SAT2 FMDV genetic backbone was constructed. Oligonucleotides cDNA-NAM (containing an *Ssp* I site) and cDNA-2A (containing an *Xma* I site) were used to PCR amplify the external capsid-encoding region from NAM/307/98 cDNA, as described under Materials and Methods (Section 2.2.6.1). To minimize misincorporation errors during PCR amplification, a high fidelity DNA polymerase with 3'→5' exonuclease proofreading activity was used. An aliquot of the reaction mixture was analyzed by agarose gel electrophoresis and a single discreet amplicon of the expected size (*ca*. 2.2 kb) was obtained (Fig. 2.6, lane 3). In contrast, no amplification products were observed in the negative control in which template DNA was omitted (Fig. 2.6, lane 2).

To construct the chimeric clone pNAM/SAT2, plasmid pSAT2 was digested with both *Ssp* I and *Xma* I to yield two DNA fragments of *ca.* 8.8 kb and 2.2 kb, respectively. The larger of the two DNA fragments, corresponding to the pSAT2 genetic backbone lacking the external capsid-encoding region, was purified from the agarose gel, dephosphorylated and used in subsequent ligation reactions. The PCR amplicon was digested with both *Ssp* I and *Xma* I, gel-purified and ligated to the prepared pSAT2 vector DNA. Following transformation of competent *E.coli* DH5α cells, plasmid DNA was extracted from ampicillin-resistant transformants and analyzed by agarose gel electrophoresis and restriction enzyme digestion.

Digestion of recombinant plasmid DNA with *Sac* I, which cuts once in 1B of NAM/307/98 and once in both 3C and 3D of pSAT2 (Fig. 2.7A), yielded DNA fragments of the expected sizes, corresponding to *ca.* 6.2, 3.8 and 1 kb, respectively (Fig. 2.7B, lanes 3 and 4). Non-recombinant plasmid DNA, however, yielded DNA fragments corresponding to *ca.* 10 kb and 1 kb (Fig. 2.7B, lanes 2 and 5). To furthermore confirm the successful construction of the recombinant plasmid, the plasmid DNA was digested with *Kpn* I, which cuts twice in 1B of NAM/307/98 and once in the IRES of pSAT2 (Fig 2.7A). In contrast to non-recombinant plasmid DNA, which was linearized (Fig. 2.7C, lanes 2 and 5), agarose gel electrophoresis of the digestion products from recombinant plasmid DNA indicated the presence of three DNA fragments of 9.6, 1 and 0.3 kb (Fig. 2.7C, lanes 3 and 4). The sizes of these DNA fragments

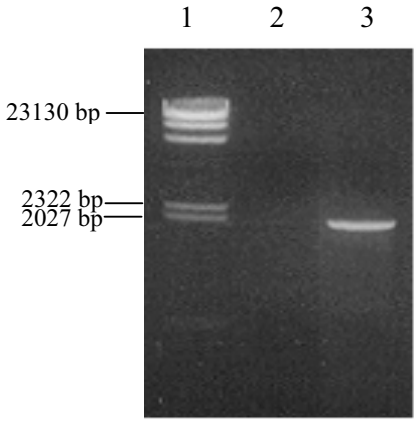


Fig. 2.6 Agarose gel electrophoretic analysis of the amplicon obtained by RT-PCR amplification of the external capsid-encoding region of NAM/307/98. Lane 1, DNA molecular weight marker; Lane 2, negative control reaction lacking template DNA; Lane 3, sample of the reaction mixture following RT-PCR. The sizes of the DNA molecular weight marker, phage λ DNA digested with *Hind* III, are indicated to the left of the figure.

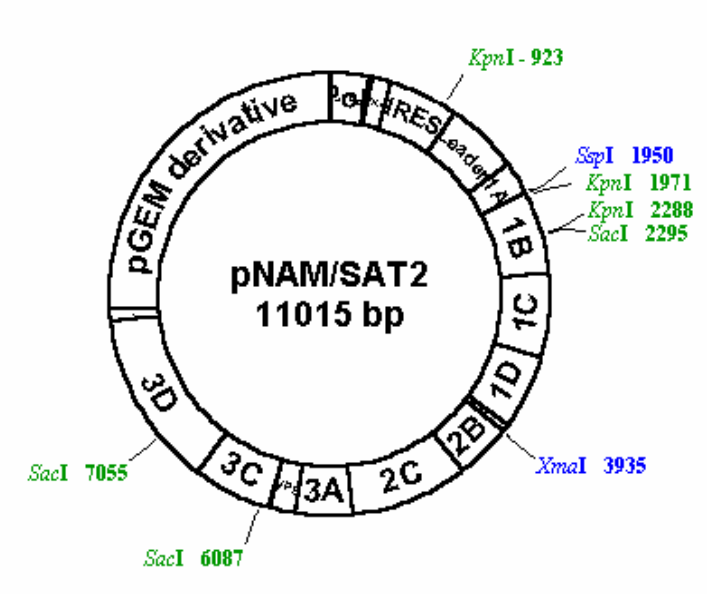


Fig. 2.7A Plasmid map of the chimeric construct pNAM/SAT2.

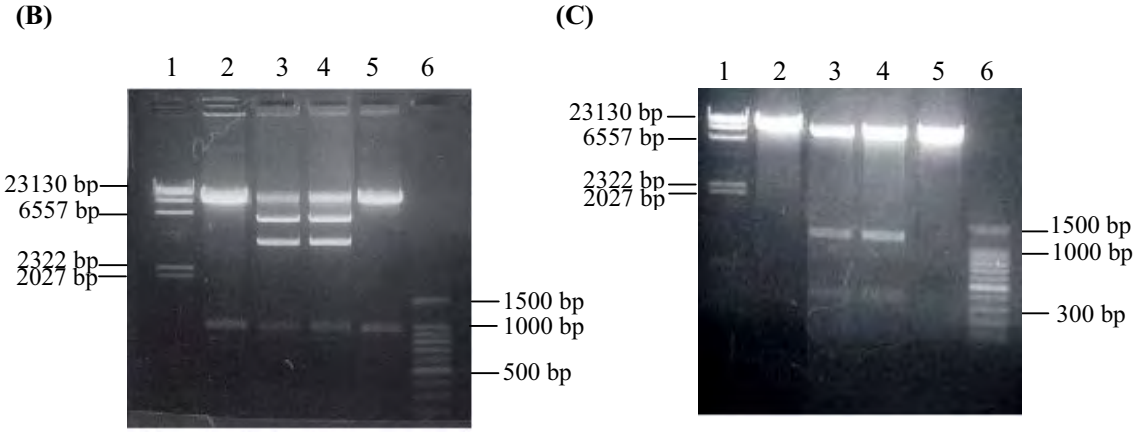


Fig. 2.7B,C Agarose gel electrophoretic analysis of plasmid DNA from ampicillin-resistant transformants following digestion with (B) *Sac* I and (C) *Kpn* I. Lanes 1 and 6, DNA molecular weight marker; Lanes 2 and 5, restriction endonuclease-digested non-recombinant plasmid DNA; Lanes 3 and 4, restriction endonuclease-digested recombinant plasmid DNA. The sizes of the molecular weight markers, phage λ DNA digested with *Hind* III (lane 1) and a 100-bp DNA ladder (lane 6), are indicated to the left and right of the figure, respectively.

were in agreement with those predicted from a map of the chimeric plasmid DNA (Fig. 2.7A). These results therefore indicated that the external capsid encoding region of NAM/307/98 had been cloned successfully into the pSAT2 vector backbone. One of these chimeric clones, designated pNAM/SAT2, was selected and characterized by nucleotide sequencing.

2.3.3.2 *Nucleotide sequencing and analysis*

The integrity of the cloned DNA in the chimeric construct pNAM/SAT2 was verified by determining the nucleotide sequence through the cloning regions using automated DNA sequencing procedures. To facilitate insertion of the external capsid-encoding region of the NAM/307/98 strain into the SAT2 genetic backbone, the external capsid-encoding region of NAM/307/98 was amplified by PCR utilizing oligonucleotide cDNA-NAM, which had been engineered to incorporate an *Ssp* I site, and oligonucleotide cDNA-2A, engineered to incorporate an *Xma* I site. The engineered *Ssp* I site, codons 14 to 15 of protein 1B, introduces a silent mutation (Ile [ATC] to Ile [ATA]) at codon 14, and an amino acid change (Met [ATG] to Leu [TTG]) at codon 15. Oligonucleotide cDNA-2A containing a *Xma* I site was added by silent mutation (Pro [CCT] to Pro [CCC]) at the highly conserved sequence at codon 17 of protein 2A. Analysis of the nucleotide sequences (Fig. 2.8) and deduced amino acid sequence (Fig. 2.9) indicated that the sequences encoding the two RGD sequences in the capsid protein 1D of NAM/307/98 were retained in the chimeric clone. Except for the expected nucleotide changes introduced by the *Ssp* I site and *Xma* I sites, there was an additional nucleotide change at codon 60 of protein 1B that resulted in a silent mutation (Arg [AGG] to Arg [AGA]) (results not shown).

2.3.4 Recovery of infectious vNAM/SAT2 chimeric viruses

The chimeric pNAM/SAT2 clone was linearized and used as template for *in vitro* RNA synthesis reactions using T7 RNA polymerase. BHK-21 and IB-RS-2 cell monolayers were subsequently transfected with the derived synthetic RNA using Lipofectamine reagent. As a control, identically prepared RNA transcripts derived from pSAT2 were also used to transfect these cells. The supernatants obtained from the transfected cells were used to infect monolayers and monitored for signs of cytopathic effect (CPE) by comparison to mock-infected cell monolayers. The results that were obtained are summarized in Table 2.2.

(A)

```
pNAM/307/98P1  CTGCTTGAAGAC CGTATCATG ACCACCAGCCAT GGTACCACCACGTCA
pNAM/SAT2      CTGCTCGAGGAC CGAATATTG ACCACCAGCCAT GGTACCACCACGTCA
                            ••
```

(B)

```
pNAM/307/98P1  CTGTTAAAGT TGGCTGGAGA CGTTGAGTCC AACCCTGGGC CC--------
pNAM/SAT2      CTGTTAAAGT TGGCTGGAGA CGTTGAGTCC AACCCCGGGC CCTTCTTCTT
                                                •
```

Fig. 2.8 Nucleotide sequence alignment of the P1 region of pNAM/307/98 and the newly constructed pNAM/SAT2 chimeric clone. The *Ssp* I (A) and *Xma* I (B) restriction endonuclease sites used in cloning are indicated in italics. Partial nucleotide sequences are indicated; the pSAT2 sequence is indicated in blue and the P1 sequence of NAM/307/98 in red. Nucleotide differences to facilitate the introduction of the restriction enzyme sites are indicated by a black dot (•).

```
                                                                    686
pNAM/307/98P1 GDNPVVFS***RGD*** TTRFALPYTA PHRVLATVYN GDCKYKPTGT PPRENI***RGD***L ATLAARIAS
pNAM/SAT2     GDNPVVFS***RGD*** TTRFALPYTA PHRVLATVYN GDCKYKPTGT PPRENI***RGD***L ATLAARIAS
                                                    **************************
                                                          G-H loop
```

Fig. 2.9 Amino acid sequence alignment of part of the 1D protein of the chimeric pNAM/SAT2 clone indicating the presence of the two RGD sequences, underlined and in bold italics. The amino acid position is indicated to the right of the sequence, numbering starts with the first amino acid in the P1 region (as previously indicated in Fig. 2.4). The G-H loop is indicated by *, corresponding to amino acid residues 661-687 (residues 136-162 of 1D).

The BHK cells transfected with RNA derived from pSAT2 displayed 100% CPE after 48 h of incubation upon the first passage, which is in agreement with the results reported previously for the resultant vSAT2 virus (Van Rensburg *et al*., 2004). In contrast, no CPE was initially observed for BHK cells transfected with RNA derived from the chimeric pNAM/SAT2 clone, but subsequent passaging of the vNAM/SAT2 virus in BHK cells resulted in more severe CPE being observed. CPE of 90% was observed after 48 h of incubation on the third passage. Transfection of IB-RS-2 cells with RNA derived from pSAT2 and pNAM/SAT2, followed by infection of fresh cells with tissue culture lysate indicated a slower rate of recovery when compared to the results obtained in BHK-21 cells. Nevertheless, 100% CPE was observed for vSAT2 after 48 h of incubation on the third passage, whilst 90% CPE was observed for vNAM/SAT2 after the same number of passages. As no CPE was observed for mock-infected BHK-21 and IB-RS-2 cells, it was concluded that the observed cell lysis might be due to infectious viral particles having been generated from the RNA derived from the pSAT2 and the chimeric pNAM/SAT2 clones.

The above results are in agreement with those presented in Fig. 2.3, in which the wild-type NAM/307/98 virus was shown to form plaques on both BHK-21 and IB-RS-2 cells. These results thus indicated that the characteristics conferred by the external capsid-encoding region of the wild-type NAM/307/98 virus are present in the chimeric vNAM/SAT2 virus.

2.3.5 Confirmation of recovered vNAM/SAT2 chimeric viruses

To verify that a chimeric virus between the SAT1 and SAT2 type viruses had indeed been recovered successfully, viral RNA was extracted from the vNAM/SAT2-infected tissue culture lysates (BHK-21 and IB-RS-2; passage 3) and reverse-transcribed. Oligonucleotides P621 and VP1UA were used to amplify an *ca*. 0.6-kb product comprising the region flanking the *Xma* I cloning site at the 3' end of the cloned external capsid-encoding region. The nucleotide sequence of the gel-purified amplicons was determined and analysis of the sequences confirmed the presence of the capsid-encoding region of NAM/307/98 in the SAT2 genetic background, as well as the presence of the two RGD motifs in the capsid protein 1D of the chimeric viruses recovered in both BHK-21 and IB-RS-2 cells (Fig. 2.10).

Table 2.2: Cytopathic effect (CPE) observed during serial passage of the chimeric vNAM/SAT2 virus and vSAT2 virus in BHK-21 and IB-RS-2 cells

Cells and passage	*CPE observed		
	Mock-infection	**vNAM/SAT2**	**vSAT2**
BHK p1 - 48 h	-	-	++++
BHK p2 - 48 h	-	+	++++
BHK p3 - 48 h	-	+++	++++
IB-RS-2 p1 - 48 h	-	-	-
IB-RS-2 p2 - 48 h	-	-	++
IB-RS-2 p3 - 48 h	-	+++	++++

*- = No CPE observed

+ = 10-30% CPE; ++ = 40-60% CPE; +++ = 65-90% CPE; ++++ = 100% CPE

```
pNAM/307/98P1      GTNAWVGWTP NGSPVLTEVG DNPVVFSRGD TTRFALPYTA PHRVLATVYN 657
vNAM/SAT2(BHK)     GTNAWVGWTP NGSPVLTEVG DNPVVFSRGD TTRFALPYTA PHRVLATVYN
vNAM/SAT2(IB-RS)   GTNAWVGWTP NGSPVLTEVG DNPVVFSRGD TTRFALPYTA PHRVLATVYN

pNAM/307/98P1      GDCKYKPTGT PPRENIRGDL ATLAARIASE THIPTTFNYG MIYTQAEVDV 707
vNAM/SAT2(BHK)     GDCKYKPTGT PPRENIRGDL ATLAARIASE THIPTTFNYG MIYTQAEVDV
vNAM/SAT2(IB-RS)   GDCKYKPTGT PPRENIRGDL ATLAARIASE THIPTTFNYG MIYTQAEVDV
                   ******** ********** **********
                            G-H loop
```

Fig. 2.10 Amino acid sequence alignment of part of the 1D protein of NAM/307/98 and the recovered chimeric virus vNAM/SAT2 on BHK-21 (BHK p3) and IB-RS-2 (IB-RS-2 p3) cells, indicating that the two RGD motifs (bold) were maintained during passaging. The G-H loop is indicated by *, corresponding to amino acid residues 661-687 (residues 136-162 of 1D).

2.4 DISCUSSION

Many viruses initiate infection by attaching to cell surface molecules that are normal components of the plasma membrane. The elucidation of the crystal structure of several FMDV types (Acharya *et al*., 1989; Lea *et al*., 1994; 1995; Curry *et al*., 1996) has contributed greatly to the understanding of the molecular basis of the binding of FMDV to cellular receptors. The three-dimensional structures have revealed the presence of a surface protrusion comprised of a loop between the βG and βH strands of the capsid protein 1D (Acharya *et al*., 1989; Logan *et al*., 1993; Fry *et al*., 1999), which not only contains the major immunodominant epitopes of the virion (Baxt and Becker, 1990; Jackson *et al*., 1997) but also a highly conserved RGD sequence (Fox *et al*., 1989; Mason *et al*., 1994; Curry *et al*., 1996). Several approaches, *e.g.* inhibition of virus adsorption by RGD-containing synthetic peptides (Fox *et al*., 1989; Baxt and Becker, 1990; Jackson *et al*., 1997; 2002) and mutagenesis of the RGD-coding nucleotide sequences from infectious genome-length cDNA clones of FMDV (Mason *et al*., 1994; McKenna *et al*., 1995), have indicated that this loop interacts with RGD-binding integrins. FMDV has subsequently been reported to utilize multiple RGD-dependant integrins to initiate infection, including $\alpha_v\beta_3$ (Berinstein *et al*., 1995), $\alpha_v\beta_6$ (Jackson *et al*., 2000b), $\alpha_v\beta_1$ (Jackson *et al*., 2002) and $\alpha_v\beta_8$ (Jackson *et al*., 2004). However, FMDV can also enter cells through non-integrin-mediated pathways (Mason *et al*., 1993; Baxt and Mason, 1995; Rieder *et al*., 1996) and tissue culture-adapted viruses can use heparan sulfate as a receptor to bind to cells (Jackson *et al*., 1996; Baranowski *et al*., 1998; Fry *et al*., 1999). More recently, it has become apparent that FMDV may also use receptors of unknown identity that are neither integrin nor heparan sulfate (Baranowski *et al*., 1998; 2000; Zhao *et al.*, 2003).

To determine whether the SAT1 field isolate NAM/307/98 used in this investigation is capable of utilizing RGD-dependent integrins or heparan sulfate cellular receptors for cell binding, a plaque assay on different cell lines was performed. In contrast to BHK-21 and IB-RS-2 cells, NAM/307/98 could not infect and form plaques on CHO-K1 cells. However, CHO-K1 cells were permissive for a SAT2 type virus ZIM/7/83 that has been adapted for growth in cultured cells and uses heparan sulfate proteoglycans as receptors (Van Rensburg *et al.*, 2004). Thus, heparan sulfate could be ruled out as a receptor for NAM/307/98 and it was concluded that the virus uses integrin receptors for cell binding. A further extension of this line of thought includes the possibility that the two RGD-binding integrins $\alpha_v\beta_5$ and $\alpha_5\beta_1$

have no role in infection of NAM/307/98 of cultured cells, as the virus could not replicate in CHO-K1 cells, which normally express these two integrins (Neff *et al*., 1998; You *et al*., 2001; Jackson *et al*., 2002). These results are in agreement with those previously reported in the literature indicating that CHO-K1 cells are non-permissive for field strains of FMDV (Mason *et al.*, 1993; Jackson *et al.*, 1996; Neff *et al.*, 1998).

The nucleotide sequence of the capsid-encoding region of the NAM/307/98 strain was determined. Analysis of the deduced amino acid sequence indicated the presence of a highly conserved RGD sequence located in the G-H loop of capsid protein 1D (residues 149-151), as well as a second, upstream RGD sequence (residues 110-112) that is located in a short surface-exposed protrusion made up of a loop between the βF and βG strands of the capsid protein 1D. The residues that follow the RGD motif have been suggested to be of importance for recognition of certain RGD-dependent integrins by FMDV. Across the FMDV serotypes, the viruses have either a leucine, methionine or arginine residue immediately following the RGD motif (RGD+1), and a leucine residue at the RGD+4 position (Domingo *et al*., 1992; Vosloo *et al*., 1995; Mateu *et al*., 1996; Jackson *et al*., 2000a;b; Bastos *et al*., 2003). Although leucine residues are located at the RGD+1 and RGD+4 positions following the conserved RGD sequence in the G-H loop of NAM/307/98, the upstream RGD sequence contains tyrosine and phenylalanine, respectively, at these positions. Whereas tyrosine, like arginine, is a polar hydrophilic residue, phenylalanine, like leucine, is non-polar hydrophobic. Furthermore, the different integrins used by FMDV also appear to differ in their ligand-binding specificity. For example, the $\alpha_v\beta_3$ integrin is a multifunctional receptor that binds a broad range of RGD-containing ligands (Jackson *et al.*, 1996) and ligand-binding to $\alpha_v\beta_3$ has been shown to tolerate several different amino acids flanking the RGD, including those at the RGD+1 and RGD+4 positions (Hynes, 1992; Springer, 1990; Healy *et al.*, 1995). In contrast, $\alpha_v\beta_6$ binds to relatively few ligands and serves as a high affinity receptor for LAP-1 (RGDLXXI) of which the residues following the RGD motif are similar to those following the conserved RGD motif of FMDV (RGDLXXL) (Kraft *et al*., 1999). In addition, a short RGD-containing peptide (GRGDSP) has been reported to drastically inhibit FMDV binding and infection mediated by the $\alpha_v\beta_1$ integrin (Jackson *et al.*, 2002). Based on the above, it seems possible that the surface-exposed non-G-H loop RGD sequence may serve as a receptor-binding site for NAM/307/98, despite the lack of leucine residues at the RGD+1 and RGD+4 positions, and thus warranted further investigation.

Towards investigating whether the second, non-G-H loop RGD sequence in the capsid protein 1D of NAM/307/98 is indeed utilized as a cell receptor binding site, a reverse genetics strategy was used to construct an infectious chimeric cDNA clone. The availability of such a chimeric cDNA clone would greatly facilitate the introduction of specific targeted mutations or deletions in the 1D capsid-encoding region of NAM/307/98 whereby the functional importance of the second RGD motif could be investigated. This approach has been used successfully to indicate that the RGD motif of type O_1 and A_{12} viruses is essential for integrin-mediated infection of cultured cells (Mason *et al.*, 1994; Neff *et al.*, 1998). To this extent, the external capsid-encoding region of NAM/307/98 was cloned into pSAT2, a genome-length cDNA clone of the stable, tissue culture-adapted SAT2 type virus ZIM/7/83 (Van Rensburg *et al.*, 2004), of which the corresponding region had been removed. As the assembly of infectious chimeric virus particles is dependent on the efficient processing of the SAT1/NAM/307/98 external capsid proteins by the SAT2/ZIM/7/83-encoded 3C protease ($3C^{pro}$), the $3C^{pro}$ cleavage sites in the external capsid-encoding region of NAM/307/98 was compared to those of ZIM/7/83. The results from this comparative analysis indicated that the cleavage sites at the lAB/1C, 1C/1D and 1D/2A junctions are conserved between these viruses. Using the chimeric pNAM/SAT2 construct as template, *in vitro* synthesized RNA transcripts were transfected into BHK-21 and IB-RS-2 cells. This resulted in the recovery of viable vNAM/SAT2 chimeric virus that was capable of replication in these cells, as evidenced by an increase in CPE upon passaging. However, differences in the recovery rates were observed (Table 2.2). This may have been due to differences in the concentration of RNA used in the transfections, differences in the efficiency of transfection and the condition of the cells. Sequence analysis of the external capsid-encoding region of chimeric viruses obtained from the highest passage cells confirmed that this region originating from NAM/307/98 was stably maintained in the SAT2 genetic backbone, and that both of the RGD motifs were present in the capsid protein 1D.

The stable maintenance of the two RGD sequences is of particular interest, since a previous study has indicated that amino acid residues located at positions 110-112 of a SAT1 type FMDV are subject to mutations (Botha, 2002). Following infection of impala (*Aepyceros melampus*) and giraffe (*Giraffa camelopardalis*) with a tissue culture-adapted SAT1 vaccine strain SAR/9/88/1, a NRG-for-HRR substitution was observed at positions 110-112 in the capsid protein 1D of the viruses isolated from impala and giraffe. Subsequent infection of BHK-21 and IB-RS-2 cell monolayers with the wild-type and mutant viruses indicated that

distinct mutations were present at amino acid positions 110 to 112, depending on the cell culture used. In this study, both RGD sequences were maintained after passage of the NAM/307/98 virus isolate once in IB-RS-2 cells and by passing the chimeric vNAM/SAT2 virus three times on BHK-21 and IB-RS-2 cells, respectively, in spite of this mutational "hot spot" region spanning residues 110-112 (Bastos *et al*., 2001). Based on it being stably maintained, these results lend further support to the hypothesis that the second, non-G-H loop RGD sequence may be utilized by RGD-binding integrin receptors.

CHAPTER 3

A SECOND, NON-G-H LOOP RGD MOTIF IN CAPSID PROTEIN 1D OF THE SAT1 TYPE FOOT-AND-MOUTH DISEASE VIRUS NAM/307/98 DOES NOT MEDIATE VIRUS ATTACHMENT TO BHK-21 CELLS

3.1 INTRODUCTION

The tripeptide Arg-Gly-Asp (RGD), originally identified as the sequence that mediates cell attachment of fibronectin (Yamada and Kennedy, 1984), is a major determinant in the interaction of a number of protein ligands with cell surface receptors of the integrin superfamily (D'Souza *et al*., 1991; Haas and Plow, 1994). In addition, the RGD motif has been reported to mediate recognition by cells and infectivity of a variety viruses, amongst other, adenovirus 2 (Wickham *et al*., 1993), coxsackievirus A9 (Roivainen *et al*., 1994), echovirus 22 (Stanway *et al*., 1994) and foot-and-mouth disease virus (FMDV) (Fox *et al*., 1989; Baxt and Becker, 1990; Mason *et al*., 1994; Berinstein *et al*., 1995). The use of synthetic RGD peptides (Ruoslahti, 1988) and protein ligand mutants (Dennis *et al*., 1993) not only demonstrated the primary role and high specificity of the RGD motif in ligand binding to several integrins, but also provided new tools for identifying and characterizing the surface receptors of other matrix proteins.

FMDV is able to attach to cells via a conserved RGD motif located at the tip of the surface-exposed βG-βH loop of the capsid protein 1D (Fox *et al*., 1989; Mason *et al*., 1994). The RGD sequence is recognized by one of four RGD-binding integrins, *i.e.* $\alpha_v\beta_3$, $\alpha_v\beta_6$, $\alpha_v\beta_1$ and $\alpha_v\beta_8$ (Berinstein *et al*., 1995; Jackson *et al*., 2000b; Jackson *et al*., 2002; Jackson *et al*., 2004). The integrin receptor and RGD motif interact at the cell surface via a ligand-binding domain that is composed of several regions of both the α and β integrin subunits (Fernandez *et al*., 1998). The role and importance of the RGD motif in binding FMDV to susceptible cells has been confirmed by blocking and competition-binding studies using synthetic RGD-containing peptides (Berinstein *et al*., 1995; Mateu *et al*., 1996; Neff and Baxt, 2001; Jackson *et al*., 1997; 2000b; 2002; 2004) and by function-blocking antibodies against different integrins (Berinstein *et al*., 1995; Jackson *et al*., 2000b; 2002; 2004), as well as by site-directed mutagenesis of the RGD sequence (Mason *et al*., 1994; McKenna *et al*., 1995; Mateu

et al., 1996). The results of these investigations have indicated that replacement of Arg or Asp reduced the infectiousness of FMDV to a significant level, whilst the Gly residue appeared to be more tolerable of substitutions. Furthermore, Mason and co-workers (1994) showed for A_{12} FMDV that deletion of the RGD sequence led to non-infectious viral particles. In addition to the RGD motif, flanking amino acid residues, especially the RGD+1 and RGD+4 residues, which are located on the same face of the short helix neighbouring the RGD turn, have been proposed to participate in the specific recognition of some integrin receptors through modulation of the RGD triplet conformation (Acharya *et al*., 1989; D'Souza *et al*., 1991; Rieder *et al*., 1994; Mateu *et al*., 1996; Jackson *et al*., 2000b).

Although cell adhesive activities of many different proteins have been linked to the presence of a RGD sequence, in other instances the RGD sequence appears to be functionally silent (D'Souza *et al*., 1991). The availability of an infectious cDNA clone of the SAT2 type FMDV ZIM/7/83 (Van Rensburg *et al*., 2004) provides a useful tool to investigate whether a second, non-G-H loop RGD motif present in the capsid protein 1D of the SAT1 type FMDV NAM/307/98 may serve as a ligand for integrin receptors. Therefore, the aims of this part of the study were (i) to alter the RGD motif on pNAM/SAT2 generated in Chapter 2 through a polymerase chain reaction (PCR)-based method of site-directed mutagenesis and (ii) to investigate the resulting mutant chimeric viruses for their ability to infect susceptible cells through serial passaging on BHK-21 cells.

3.2 MATERIALS AND METHODS

3.2.1 Cell cultures

Baby hamster kidney (BHK) cells, strain 21, clone 13 (ATCC CCL-10), were maintained in Eagle's Basal Medium (BME; Invitrogen) supplemented with 10% (v/v) fetal calf serum (FCS; Delta Bioproducts), antibiotics, 1 mM L-glutamine (Invitrogen) and 10% (v/v) tryptose phosphate broth (TPB; Sigma).

3.2.2 Bacterial strains and plasmids

Escherichia coli MAX Efficiency® DH5α™ competent cells (genotype: Fϕ80d*lac*ZΔM15 Δ(lacZYA-argF)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96

*rel*A1), obtained from Life Technologies, were used as the transformation host in cloning experiments. Plasmids pSAT2, an infectious genome-length cDNA clone of the SAT2 type FMDV ZIM/7/82, has been described previously (Van Rensburg *et al*., 2004) and pNAM/SAT2, an infectious chimeric cDNA clone containing the external capsid-encoding region of NAM/307/98 in the stable genetic backbone of pSAT2 (Chapter 2), were used in plasmid constructions.

3.2.3 Oligonucleotides

Oligonucleotides used in cDNA synthesis, PCR amplification, nucleotide sequencing and mutagenesis procedures were custom-synthesized by Inqaba Biotechnical Industries, Invitrogen or Gibco-BRL, and are described in Table 3.1.

Table 3.1: Oligonucleotides used in this part of the study

Oligonucleotide	Oligonucleotide sequence	Reference	Polarity and purpose of oligonucleotide
NAMmut1*	5'-CGAGAACATT**aaa**GGTGA**g**CTTGCAACGC-3'	This study	Sense, substitution
NAMmut3*	5'-CGAGAACATT**cgc**GGTGA**c**CTTGCAACGC-3'	This study	Antisense, substitution
cDNA-2A#	5'-CGCcccgggGTTGGACTCAACGTCTCC-3'	Van Rensburg (unpublished)	Antisense, situated in 2A, PCR amplification
cDNA-NAM#	5'-CGGaatattGACCACCAGCCATGGTACCACCAC-3'	This study	Sense, situated in 1A, PCR amplification
2B	5'-GACATGTCCTCCTGCATCTG-3'	Vangrysperre and De Clercq (1996)	Antisense, situated in 2B, cDNA synthesis
VP1UB	5'-CCACGTACTACTTYTCTGACCTGGA-3'	Bastos (1998)	Sense, situated in 1D, PCR amplification
P621	5'-GGACATATCT TGTTGCATA-3'	Van Rensburg (unpublished)	Nucleotide sequencing
P622	5'-GCACTGACACCACGTCTAC-3'	Van Rensburg (unpublished)	Nucleotide sequencing
WDA	5'-GAAGGGCCCAGGGTTGGACTC-3'	Beck and Strohmaier (1987)	cDNA, PCR amplification, Nucleotide sequencing

* Mispaired bases are shown in bold lower case letters

\# The restriction endonuclease sites are underlined in lower case letters; *Xma* I in oligonucleotide cDNA-2A and *Ssp* I in oligonucleotide cDNA-NAM

3.2.4 Site-directed mutagenesis of the capsid protein 1D of NAM/307/98

The RGD sequence located in the G-H loop of the capsid protein 1D of NAM/307/98 (residues 149 to 151 of 1D) was mutated to KGE by a method in which three oligonucleotides and two PCR reactions were used (Landt *et al*., 1990). The "megaprimer" strategy used for introducing the site-specific mutations is indicated in Fig. 3.1, while the sequence of the oligonucleotides used, is shown in Table 3.1. The first PCR reaction (50 µl) contained 50 ng of pNAM/SAT2 as template DNA, 0.3 µM of the 3'-specific antisense oligonucleotide cDNA-2A, 0.3 µM of the sense mutagenic oligonucleotide NAMmut1, 1 × Expand High Fidelity buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Roche) and 2.6 U High Fidelity Enzyme (3.5 U/µl; Roche). The tubes were placed in a Hybaid thermal cycler and following initial denaturation at 94°C for 2 min, the reactions were subjected to 25 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 20 s and elongation at 72°C for 30 s. After the last cycle, the reactions were kept at 72°C for 7 min to complete synthesis of all strands. Following electrophoresis on a 1% (w/v) agarose gel, in the presence of appropriate DNA molecular weight markers, the 270-bp amplicon was purified from the agarose gel using the Nucleospin® Extract Kit (Machery-Nagel) according to the manufacturer's instructions. The purified amplicon (1 µg) was used as a "megaprimer" along with 0.3 µM of the 5'-specific sense oligonucleotide cDNA-NAM in the second PCR reaction, as described above. The resultant 2.2-kb amplicon was used to replace the corresponding capsid-encoding region of pNAM/SAT2 to generate pmutNAM/SAT2 (Section 3.2.5). By making use of a similar approach, the mutant chimeric clone was subsequently used as template in a PCR to substitute the newly introduced KGE sequence for the RGD integrin-binding sequence. For this purpose, 0.3 µM of the sense mutagenic oligonucleotide NAMmut3 (Table 3.1) was used in the first PCR reaction. The PCR conditions used for both PCR reactions were identical to those indicated above.

3.2.5 Construction of chimeric clones pmutNAM/SAT2 and pBmutNAM/SAT2

All molecular cloning techniques employed in the construction of mutant and back-mutant chimeric clones were performed according to the procedures described previously (Chapter 2, Section 2.2.6). The two oligonucleotides cDNA-2A and cDNA-NAM were designed with *Xma* I and *Ssp* I restriction endonuclease cleavage sites, respectively, to facilitate cloning of the amplified mutated external capsid-encoding region into the infectious chimeric cDNA

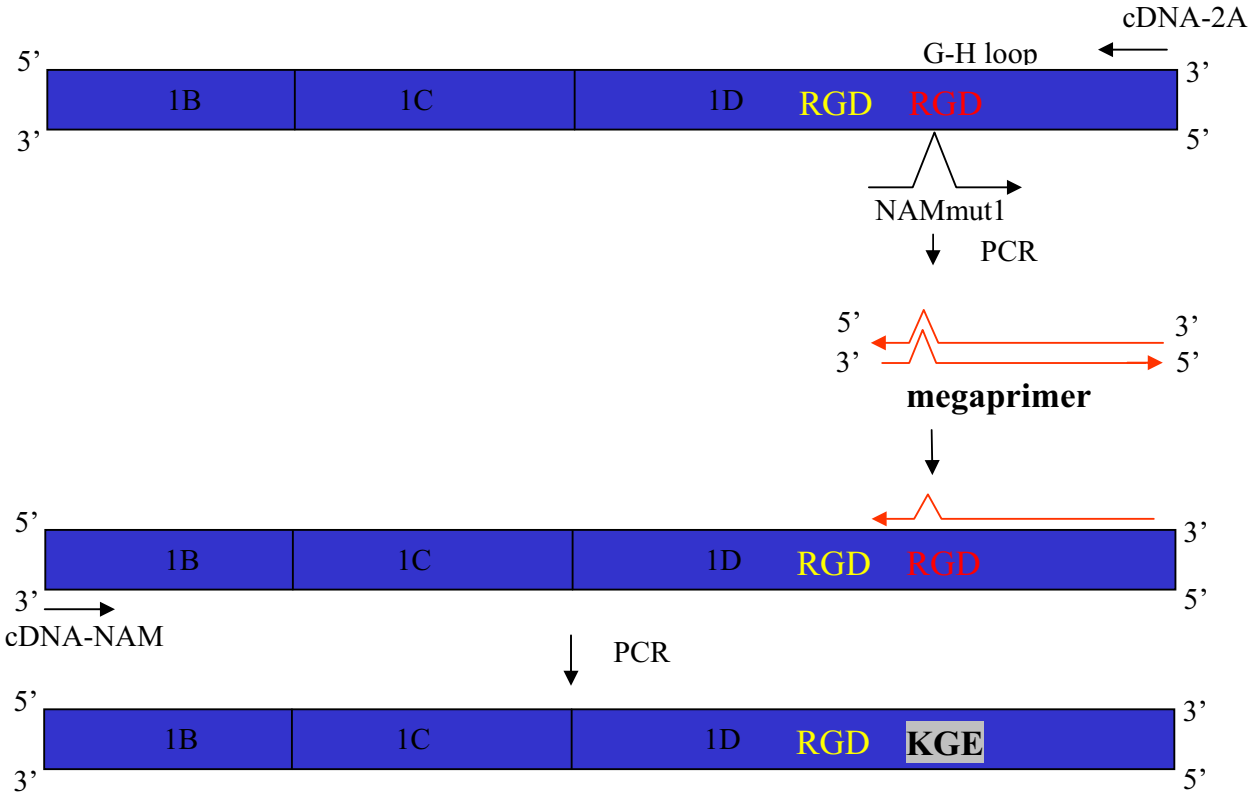


Fig. 3.1 The megaprimer method of mutagenesis, which consists of two successive PCR reactions and utilizes three different oligonucleotides, was used to introduce point mutations into the external capsid-encoding region of NAM/307/98. In the first PCR reaction, oligonucleotide cDNA-2A and the mutagenic oligonucleotide NAMmut1 were used to produce a mutant amplicon ("megaprimer"). The megaprimer along with oligonucleotide cDNA-NAM was used in a second PCR reaction to produce an amplicon representing the external capsid-encoding region of NAM/307/98 containing the desired mutations (RGD→ KGE).

clone pNAM/SAT2, lacking the corresponding region (Section 2.2.6). Following restriction enzyme digestion of both the purified amplicon representing the RGD→KGE mutated external capsid-encoding region and plasmid pNAM/SAT2, the latter of which was subsequently dephosphorylated by the addition of alkaline phosphatase (Roche), ligation reactions were performed using the Rapid DNA ligation kit from Roche. An aliquot of the ligation reaction mixture (3 µl) was transformed into 20 µl competent *E. coli* MAX Efficiency® DH5α™ cells. The plasmid DNA from several randomly selected ampicillin-resistant transformants was extracted using the STET-boiling minilysate method and characterized by restriction enzyme digestion with *Sac* I and *Kpn* I. The resultant recombinant clones were designated pmutNAM/SAT2. A similar approach was followed in the cloning of the back-mutated (KGE→RGD) external capsid-encoding region, except that pmutNAM/SAT2 was used as source for the construction of the recombinant plasmid DNA. The resultant recombinant clones were designated pBmutNAM/SAT2. Prior to verifying the presence of the newly introduced mutations, the recombinant plasmid DNA was isolated using the QIAprep® Spin Minprep Kit (Qiagen) according to the manufacturer's instructions. The nucleotide sequences across the 5' and 3' cloning junctions were determined using an ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit v3.0 (Applied Biosystems) and 0.16 µM of oligonucleotides P621 and P622, which are situated in the 2B and 1A regions, respectively, of the pSAT2 clone (Table 3.1). The extension products were resolved on an ABI PRISM™ 310 Genetic Analyser (Applied Biosystems) and the sequencing data analyzed using DAPSA version 3.4.1 (Harley, 2001).

3.2.6 Generation of mutant vNAM/SAT2 viruses

3.2.6.1 In vitro RNA synthesis

To facilitate RNA synthesis, 5 µg of the purified plasmids pNAM/SAT2, pmutNAM/SAT2 and pBmutNAM/SAT2 were linearized by restriction with *Swa* I (New England Biolabs) for 10 h at 25°C. The genome-length cDNA clone pSAT2 was included as a control and linearized by digestion with *Not* I (New England Biolabs) for 10 h at 37°C. Following digestion, the reaction mixtures were treated with Proteinase K and then deproteinized by a phenol and chloroform extraction (Section 2.2.7.1). *In vitro* RNA transcription reactions were performed using the MEGAScript® T7 kit (Ambion) and the reaction mixtures were incubated at 39°C for 2 h. The reaction mixtures contained 1 µg of purified linearized

template DNA, 7.5 mM of each ribonucleotide, 2 µl of the supplied enzyme mix and 1 × transcription buffer. The integrity of the RNA was analyzed on a 1% (w/v) agarose gel following electrophoresis in 0.5 × TBE buffer (45 mM Tris-borate, 1 mM EDTA; pH 8).

3.2.6.2 Transfection of BHK-21 cells and virus recovery

BHK-21 cells, seeded in 35-mm-diameter wells to reach 70% confluence within 24 h of incubation at 37°C in the presence of 5% CO_2, were transfected with *in vitro*-synthesized RNA using the Lipofectamine 2000™ reagent (Invitrogen), as described previously (Section 2.2.8.1). Briefly, the synthetic RNA and the lipofectamine reagent were each diluted separately in D-MEM medium (Invitrogen) without antibiotics or FCS, mixed and then incubated at room temperature for 20 min. The RNA-lipofectamine complexes were overlayed on the BHK cell monolayers, which had been pre-washed twice with D-MEM without antibiotics or FCS, but containing NEAA. Following incubation for 4 h at 37ºC in a CO_2 incubator, the medium was aspirated and replaced with 3 ml BME medium containing antibiotics, 25 mM HEPES and 1% FCS. The transfection plates were incubated for 24 h at 37°C in a CO_2 incubator after which 10% (v/v) of the supernatant was used to infect fresh BHK-21 monolayers in 35-mm-diameter wells. The tissue culture dishes were incubated at 37°C for 1 h in a CO_2 incubator with shaking whereafter BME medium, containing antibiotics, 25 mM HEPES and 1% FCS, was added and incubation continued for 48 h. Following incubation, the cells were lysed by freezing the tissue culture dishes at -80°C followed by thawing on ice and 10% of the lysate was used to infect fresh BHK-21 cells. The same procedure was used for further passaging of the viruses in BHK-21 cells. Cells were monitored for cytopathic effect (CPE) up to 48 h post-infection.

3.2.7 Characterization of recovered viruses

An experiment was designed to not only characterize the recovered mutant chimeric viruses, but also to determine whether viral particles were present in cell monolayers and in tissue culture supernatants. Using the procedures described above, BHK-21 cell monolayers were transfected with *in vitro*-synthesized RNA derived from the respective mutant and non-mutant chimeric clones and the resultant viruses were passaged four times on BHK-21 cells. These experiments were performed in duplicate and one set of the tissue culture plates were

used to monitor the cells for CPE, while the duplicate plates were used to prepare samples, as described below.

3.2.7.1 Sample preparation

Samples were prepared from the culture supernatant and cell monolayers as follows. The tissue culture lysate of the transfected cells was collected and clarified by centrifugation at 2 000 rpm for 5 min and the resulting supernatant stored at 4°C. The remaining cell pellets were rinsed twice with 3 ml BME medium prior to being suspended in 1 ml of the same medium and then lysed by a single freeze-thaw cycle. The tissue culture supernatant of cells used in the first passage was removed and stored at 4°C. Since FMDV is susceptible to low pH-induced capsid disassembly (Curry *et al*., 1995), the cell monolayers used in the first passage were rinsed once with 3 ml MBS buffer (1 M MES, 1.45 M NaCl, pH 5.5) to remove cell-associated extracellular viral particles. The cells were then rinsed twice with 3 ml BME medium after which 3 ml of the same medium was added and the cells lysed as described above. The cell cultures used in subsequent passaging of the virus were lysed by freezing and thawing. An aliquot (10% [v/v]) of each sample obtained from the cell monolayers and supernatants, was incubated at room temperature for 45 min with 10 µl RNase A (10 mg/ml; Roche) and 1 µl RNase T1 (1×10^6 U/µl; Roche) to degrade endogenous cellular RNA and unpackaged viral RNA. The RNase enzymes were subsequently inhibited by the addition of 1 × TE buffer containing 20 U RNasin® ribonuclease inhibitor (40 U/µl; Promega).

3.2.7.2 RNA extraction

RNA was extracted from the RNase-treated samples using Trizol (Gibco-BRL). To each of the RNase-treated samples in 2-ml sterile Eppendorf tubes, 750 µl of Trizol was added, vortexed briefly and incubated for 5 min at room temperature. Following incubation, 200 µl chloroform was added and the samples were vortexed for 15 s and then incubated at room temperature for a further 10 min. Following incubation, the samples were centrifuged for 15 min at 12 000 rpm to separate the organic and aqueous phases. The aqueous phases were transferred to clean 2-ml Eppendorf tubes and the RNA was purified and concentrated using a silica suspension (Section 2.2.5.1) prior to cDNA synthesis.

3.2.7.3 cDNA synthesis

Viral RNA was reverse-transcribed using the antisense oligonucleotide 2B, which anneals in the 2B region (Table 3.1), together with a mixture of random hexanucleotides. The reaction mixture contained *ca.* 1 to 3 µg of RNA, 0.23 µM of oligonucleotide 2B, 4.55 µM of a random hexanucleotide mixture (Roche), 1 × AMV-RT buffer (50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM DTT), 0.34 mM of each dNTP (Roche), 2% (v/v) DMSO and 20 U RNasin® (40 U/µl; Promega). Following denaturation of the RNA by incubating at 70°C for 3 min, the RNA was reverse-transcribed at 42ºC for 2 h by the addition of 40 U AMV-Reverse Transcriptase (10 U/µl; Promega). The enzyme was subsequently inactivated by heating to 80ºC for 2 min and the reaction mixtures were stored at 4ºC.

3.2.7.4 PCR amplification and nucleotide sequencing

Oligonucleotides WDA, which anneals at the 2A/2B junction, and VP1UB, a sense oligonucleotide situated in 1D (Table 3.1), were used to amplify part of the 1D genomic region inclusive of the sequence encoding the two RGD motifs. The PCR reaction mixtures (50 µl) contained 3 µl of the cDNA reaction mixture, 0.5 µM of each the sense and antisense oligonucleotides, 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$), 0.2 mM of each dNTP (Roche) and 2.5 U *Taq* DNA polymerase (5 U/µl; Roche). The tubes were placed in a Hybaid thermal cycler and after initial denaturation at 96°C for 20 s, the reactions were subjected to 32 cycles using the following temperature profile: denaturation for 20 s at 96°C, annealing for 30 s at 56°C and elongation for 30 s at 70°C. Reaction mixtures containing pNAM/SAT2 as template DNA and reaction mixtures lacking template DNA were also included as controls. The amplicons were analyzed by electrophoresis on a 1.5% (w/v) agarose gel and the DNA bands of interest were excised and purified using the Nucleospin® Extract kit (Macherey-Nagel). The nucleotide sequence of the gel-purified amplicons was determined using 0.16 µM of oligonucleotide WDA, as described previously (Section 3.2.5).

3.3 RESULTS

3.3.1 Construction of chimeric clones containing mutated 1D-encoding regions of NAM/307/98

3.3.1.1 Construction of pmutNAM/SAT2

The RGD sequence situated in the G-H loop of capsid protein 1D of NAM/307/98 was mutated to KGE, since it has been reported previously that BHK-21 cells transfected with RNA containing this mutation produced FMD viral particles that although indistinguishable from wild-type virus, were unable to adsorb to and infect BHK cells (Mason *et al*., 1994). It was therefore reasoned that should the derived mutant virus be capable of infecting BHK cells, it may indicate that a second, upstream RGD motif located in capsid protein 1D of NAM/307/98 serves to bind the virus to these cells.

Site-directed mutagenesis of the conserved RGD sequence in capsid protein 1D of NAM/307/98 was performed in a two-step PCR process, as indicated in Fig. 3.1. Using pNAM/SAT2 as template, and the PCR reaction conditions described in Section 3.2.4, an amplicon of *ca.* 2.2 kb was synthesized of which the size was in agreement with that of the external capsid-encoding region of NAM/307/98. Since each of the external flanking oligonucleotides used in the PCR contained an unique restriction endonuclease site, the amplicon was digested with both *Xma* I and *Ssp* I and cloned into pNAM/SAT2, thus resulting in clones in which the wild-type NAM/307/98 external capsid-encoding region was replaced with that corresponding to the mutated external capsid-encoding region. Plasmid DNA extracted from a number of ampicillin-resistant transformants was subsequently analyzed by restriction enzyme digestion and agarose gel electrophoresis.

The recombinant plasmid DNA was digested with *Sac* I, which cuts once in 1B of the cloned insert DNA and twice in the pSAT2 genetic backbone (Fig. 3.2A). Despite the presence of partially digested DNA, agarose gel electrophoresis of the digestion products indicated the presence of three DNA fragments of 6.2, 3.8 and 1 kb (Fig. 3.2B, lanes 2 and 3). Subsequent digestion of the recombinant plasmid DNA with *Kpn* I, which cuts twice in 1B of the cloned insert DNA and once in the IRES of pSAT2 (Fig. 3.2A), yielded DNA fragments of 9.6, 1 and 0.3 kb (Fig. 3.2C, lanes 2 and 3). The latter band was not clearly visible on the agarose gel, possibly due to diffusion of the small DNA fragment from the agarose gel during

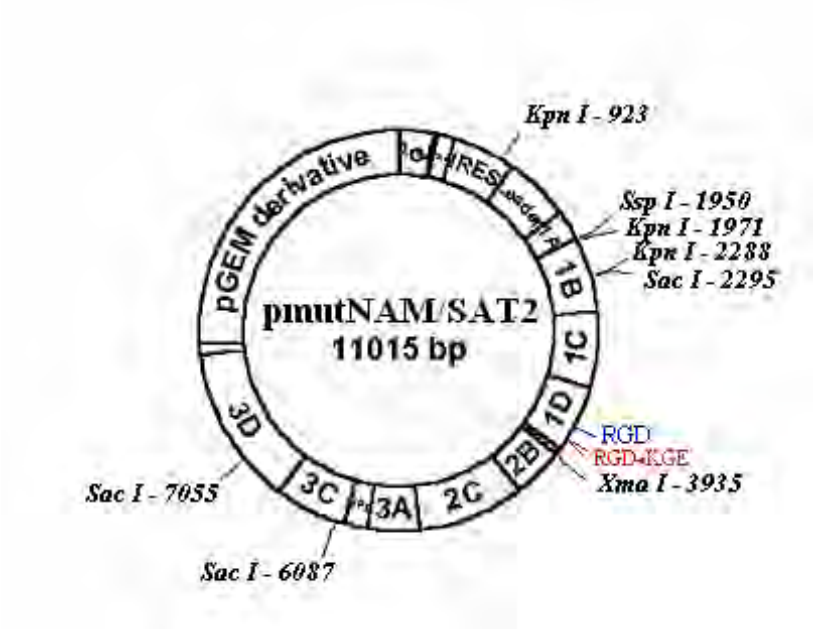


Fig. 3.2A Plasmid map of pmutNAM/SAT2. The newly introduced RGD→KGE mutation in the G-H loop of capsid protein 1D is indicated in red, whilst a second, upstream RGD motif is indicated in blue.

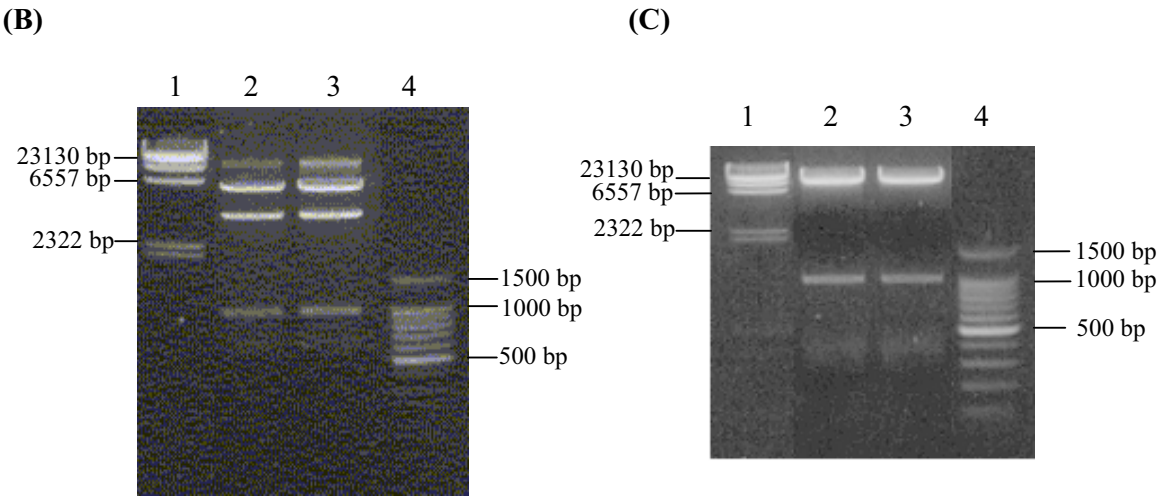


Fig. 3.2B,C Agarose gel electrophoretic analysis of pmutNAM/SAT2 plasmid DNA following digestion with (B) *Sac* I and (C) *Kpn* I. Lanes 1 and 4, DNA molecular weight markers; lanes 2 and 3, restriction endonuclease-digested recombinant plasmid DNA. The sizes of the molecular weight markers, phage λ DNA digested with *Hind* III (lanes 1) and a 100-bp ladder (lanes 4), are indicated to the left and right of the figures, respectively.

electrophoresis. Since the sizes of these fragments were in agreement with those predicted from a map of the mutant chimeric plasmid DNA, these results confirmed that the amplicon had been cloned successfully into the pSAT2 vector. Two chimeric clones, designated 1pmutNAM/SAT2 and 2pmutNAM/SAT2, were selected and characterized by nucleotide sequencing to verify the integrity of the desired mutation. Analysis of the nucleotide and deduced amino acid sequence indicated that the nucleotides encoding the RGD sequence in the G-H loop of capsid protein 1D of NAM/307/98, were replaced by nucleotides specifying a KGE sequence (Fig. 3.4). Both these clones were used in all subsequent experiments.

3.3.1.2 Construction of pBmutNAM/SAT2

An additional clone in which the KGE sequence in the mutated 1D-encoding region was reverted to the wild-type RGD sequence, was also constructed. Thus, should the cell-binding ability of the derived mutant viruses lacking a G-H loop RGD sequence be "rescued" through the re-introduction of the RGD sequence in capsid protein 1D of the derived viruses, it would provide strong supporting evidence as to the importance of the G-H loop RGD sequence, but not the second RGD sequence, to facilitate binding of the virus to susceptible cells.

The RGD-to-KGE mutation in the 1D-encoding region of the pmutNAM/SAT2 clones was substituted with the wild-type RGD sequence by a PCR-based site-directed mutagenesis approach similar to that described above, except that mutant chimeric clones 1pmutNAM/SAT2 and 2pmutNAM/SAT2 were used as templates in PCR reactions. The resultant amplicons (*ca.* 2.2 kb) were subsequently cloned into the corresponding pmutNAM/SAT2 chimeric clones, thus resulting in clones in which the mutant (KGE) external capsid-encoding region was replaced with that corresponding to the wild-type NAM/307/98 virus (RGD). After transformation of competent *E. coli* DH5α cells, plasmid DNA was extracted from ampicillin-resistant transformants and analyzed by digestion with *Sac* I and *Kpn* I, as described in the previous section.

Whereas digestion of the recombinant plasmid DNA with *Sac* I (Fig. 3.3A) yielded DNA fragments of 6.2, 3.8 and 1 kb, digestion of the recombinant plasmid DNA with *Kpn* I (Fig. 3.3B) yielded DNA fragments of 9.6, 1 and 0.3 kb. Since the sizes of the restriction fragments corresponded with the expected sizes following digestion of the recombinant

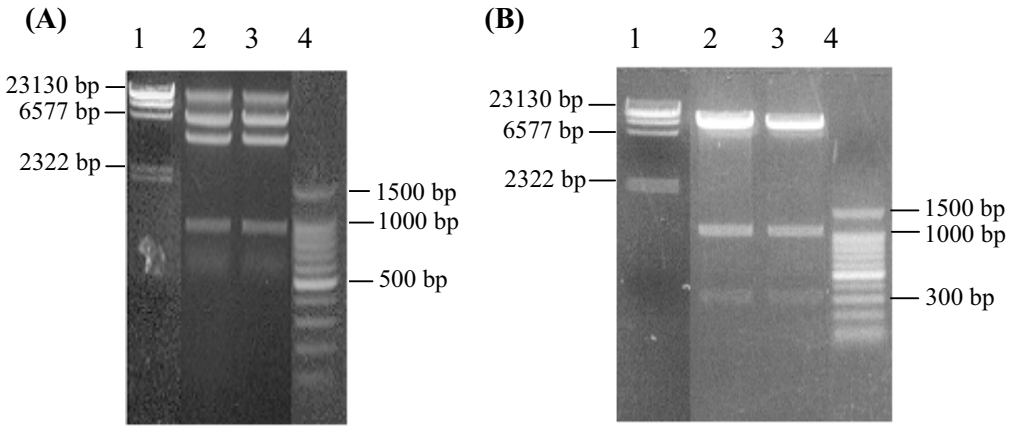


Fig. 3.3A,B Agarose gel electrophoretic analysis of pBmutNAM/SAT2 plasmid DNA following digestion with (A) *Sac* I and (B) *Kpn* I. Lanes 1 and 4, DNA molecular weight markers; Lanes 2 and 3, restriction endonuclease-digested recombinant plasmid DNA. The sizes of the molecular weight markers, phage λ DNA digested with *Hind* III (lanes 1) and a 100-bp DNA ladder (lanes 4), are indicated to the left and right of the figures, respectively.

Fig. 3.4 Nucleotide sequence alignment of the external capsid-encoding region of NAM/307/98, the chimeric pNAM/SAT2 clone, the mutant pmutNAM/SAT2 clones and the back-mutated pBmutNAM/SAT2 clones. Partial nucleotide sequences are indicated; the pSAT2 sequence is indicated in blue and the P1 sequence of NAM/307/98 in red. The *Xma* I cloning site is indicated above the cloning junction. Nucleotide sequences encoding for the KGE mutation are indicated in black, whilst those encoding for the RGD sequence are indicated in green. The nucleotide sequences encoding for the second non-conserved RGD motif are indicated in cyan. The region encoding the G-H loop is indicated by *, corresponding to amino acid residues 661-687 (residues 136-162 of 1D).

plasmid DNA with the respective restriction endonucleases, it was concluded that the amplicon had indeed been successfully cloned. The recombinant clones were designated 1pBmutNAM/SAT2 and 2pBmutNAM/SAT2, where 1 and 2 denotes that the clones were derived using 1pmutNAM/SAT2 and 2pmutNAM/SAT2 as the genetic backbone, respectively. The integrity of the desired mutation was verified by nucleotide sequencing through the cloning sites in the respective pBmutNAM/SAT2 clones. Analysis of the sequence obtained indicated that the nucleotides encoding the KGE sequence were substituted by nucleotides specifying the wild-type RGD sequence (Fig. 3.4). Both these clones were used in all subsequent experiments.

3.3.2 Transfection of BHK-21 cells with *in vitro*-synthesized RNA derived from the pmutNAM/SAT2 and pBmutNAM/SAT2 chimeric clones

To determine whether the second, non-G-H loop RGD motif plays a role in the ability of NAM/307/98 to bind to BHK-21 cells, *in vitro*-synthesized RNA generated from the respective pmutNAM/SAT2 and pBmutNAM/SAT2 clones were transfected into BHK-21 cells using lipofectamine reagent. *In vitro*-transcribed RNA derived from pSAT2 and pNAM/SAT2 were also included in these assays as controls, since the derived viruses have been shown previously to replicate in BHK-21 cells (Chapter 2). The supernatants obtained from the transfected cells were used to infect BHK-21 cells and then monitored for signs of CPE by comparison to mock-infected cells. The results obtained following repeated passaging in BHK-21 cells are shown in Table 3.2.

The BHK cells transfected with RNA derived from pSAT2 displayed 100% CPE after 48 h of incubation upon the first round of passage. Although no CPE was initially observed for BHK cells transfected with RNA derived from the chimeric pNAM/SAT2 clone, the amount of CPE increased upon subsequent passaging and CPE of 40-60% was observed for vNAM/SAT2 after 48 h of incubation on the fourth passage. In contrast, BHK cells transfected with RNA derived from the mutant pmutNAM/SAT2 clones did not display CPE, despite being passaged five times. Rather, the cells resembled the control mock-infected cells. Notably, the results obtained for BHK cells transfected with RNA derived from the corresponding pBmutNAM/SAT2 clones resembled those obtained for vNAM/SAT2. After an initial lack of CPE, continued passaging of the derived viruses resulted in 40-60% CPE being observed after 48 h of incubation on the fifth passage.

Table 3.2: Cytopathic effect (CPE) observed during serial passage of the vSAT2, vNAM/SAT2, as well as vmutNAM/SAT2 and vBmutNAM/SAT2 viruses in BHK-21 cells

Passage	***CPE Observed**						
	Mock-infection	**vSAT2**	**vNAM/SAT2**	**1vmutNAM/SAT2**	**1vBmutNAM/SAT2**	**2vmutNAM/SAT2**	**2vBmutNAM/SAT2**
BHK p1 - 48 h	-	++++	-	-	-	-	-
BHK p2 - 48 h	-	++++	-	-	-	-	-
BHK p3 - 48 h	-	++++	+	-	+	-	-
BHK p4 - 48 h	-	++++	++	-	++	-	+
BHK p5 - 48 h	-	++++	++	-	++	-	++

* - = No CPE observed

+ = 10-30% CPE; ++ = 40-60% CPE; +++ = 65-90% CPE; ++++ = 100% CPE

Since neither of the pmutNAM/SAT2 constructs gave rise to measurable amounts of recovered viruses, as evidenced by the lack of CPE, the results suggest that substitution of the conserved G-H loop RGD motif by an KGE tripeptide sequence apparently abolished the infectivity of these chimeric clones. This is furthermore supported by the observation that chimeric viruses derived from the corresponding pBmutNAM/SAT2 clones, which contained a KGE-for-RGD substitution and thus resembled the pNAM/SAT2 clone, led to productive infection accompanied by visible CPE. It would therefore appear unlikely that the upstream non-G-H loop RGD motif in capsid protein 1D of NAM/307/98 serves as a ligand for integrin receptor-binding in BHK-21 cells.

3.3.3 Determination of viral replication in BHK-21 cells

Although the lack of visible CPE for cells infected with mutant vmutNAM/SAT2 viruses could have been due to the KGE-for-RGD substitution, thereby abolishing the infectiousness of the viruses, it is, however, also possible that these constructs might not have encoded for infectious RNA. Therefore, an experiment was designed to distinguish between these two possibilities. To investigate, viral RNA was extracted from RNase-treated samples prepared from cell-free tissue culture supernatants and from cell monolayers used during transfection and the first passage, as well as from lysates (supernatants and cells) of cell monolayers used in subsequent passaging. The extracted viral RNA was reverse transcribed using a virus-specific oligonucleotide, and oligonucleotides WDA and VP1UB were then used to PCR-amplify an *ca.* 500-bp amplicon comprising the region flanking the *Xma* I cloning site at the 3' end of the cloned external capsid-encoding regions.

The results that were obtained for cells transfected with *in vitro*-synthesized RNA indicated that an amplicon of the expected size was amplified from the samples prepared from both the tissue culture supernatant (Fig. 3.5A) and the cellular fractions (Fig. 3.5B) of cells transfected with RNA derived from the various plasmid constructs. Analysis of the results obtained for samples prepared from the tissue culture supernatant (Fig. 3.5C) and the cellular fractions (Fig. 3.5D) following the first passage on BHK cells yielded similar results, but with the notable exception that no amplicon could be detected in instances where the viral RNA was extracted from cells infected with vmutNAM/SAT2 (Fig. 3.5D, lanes 5 and 6). Analysis of samples prepared from the lysate of BHK cells used in subsequent virus passaging (BHKp2, BHKp3 and BHKp4) yielded an amplicon of the expected size for cells infected with the

control vSAT2 virus, the chimeric vNAM/SAT2 virus and back-mutated vBmutNAM/SAT2 chimeric viruses. In contrast, no amplicon was obtained from samples prepared from vmutNAM/SAT2-infected BHK cells (Fig. 3.5E, F and G). At no stage during these experiments were amplicons produced from samples prepared from mock-infected cells, thus confirming the specificity of the RT-PCR assay for viral RNA only.

To characterize the recovered viruses, the nucleotide sequence of the amplicons obtained from samples prepared from the tissue culture supernatant of transfected cells and of the lysates prepared from the highest passaged cells was determined. Analysis of the deduced amino acid sequence (Fig. 3.6) of the partially sequenced capsid-encoding regions confirmed the presence and stable maintenance of the two RGD motifs located in the capsid protein 1D of the chimeric vNAM/SAT2 virus and back-mutated vBmutNAM/SAT2 chimeric viruses. Furthermore, sequence analysis of the amplicon derived from samples prepared from the tissue culture supernatant of transfected cells confirmed the presence of an KGE-for-RGD replacement in the capsid protein 1D of the mutant vmutNAM/SAT2 chimeric viruses.

Assuming that RNase treatment of the tissue culture samples was effective in degrading intracellular and unpackaged (extracellular) viral RNA, these results indicate that the amplicons were derived from packaged viral RNA following their extraction from the virus particles with Trizol. Thus, viral particles could have been produced following transfection of the BHK-21 cells, but unlike vmutNAM/SAT2 viruses, which were unable to produce CPE in cell culture, the vNAM/SAT2 and vBmutNAM/SAT2 viruses were infectious as evidenced by increasingly more severe CPE upon further passaging (Table 3.2, Fig. 3.5). The ability of the latter chimeric viruses to infect BHK-21 cells can therefore be ascribed to the presence of a functional RGD motif in the G-H loop of the capsid protein 1D of NAM/307/98. Since mutation of this tripeptide sequence abolished the infectiousness of the derived vmutNAM/SAT2 viruses, it is apparent that the non-conserved RGD sequence in 1D of NAM/307/98 does not play a role in the ability of the virus to infect BHK-21 cells.

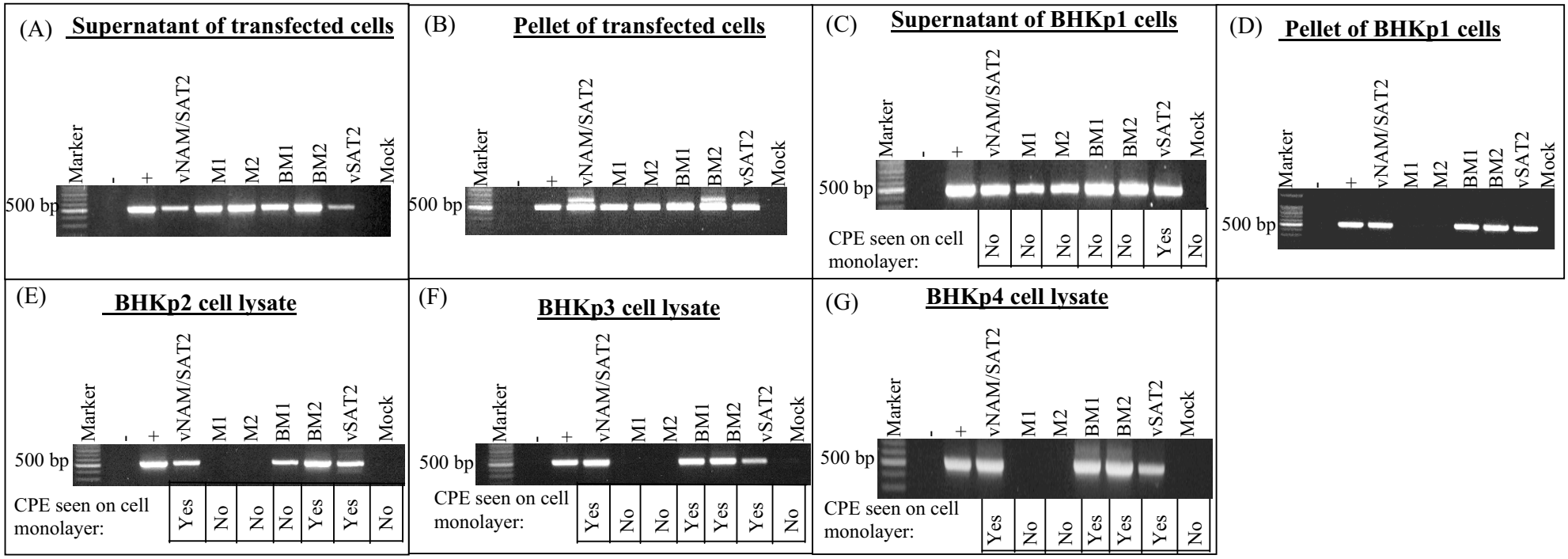
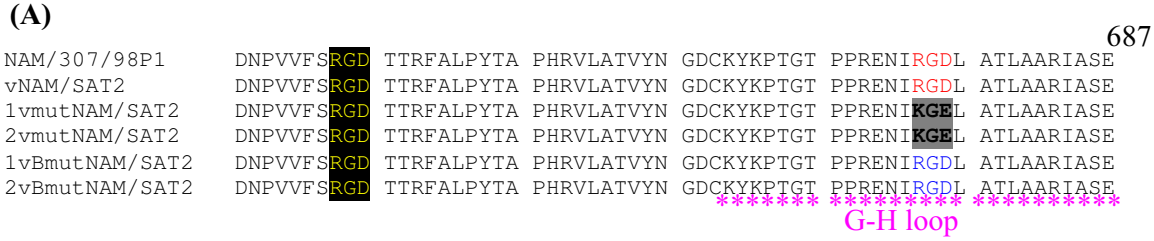


Fig. 3.5 Agarose gel electrophoretic analysis of the amplicons obtained following RT-PCR amplification of the partial 1D-encoding region of the chimeric virus vNAM/SAT2, mutant viruses 1vmutNAM/SAT2 and 2vmutNAM/SAT2, back-mutated viruses 1vBmutNAM/SAT2 and 2vBmutNAM/SAT2 and the control vSAT2 virus. Following transfection of the BHK-21 cells with RNA derived from the respective clones, the supernatant (A) and cell pellet (B) of transfected cells, the supernatant (C) and cell pellet (D) of BHK passage 1, as well as the cell lysate of BHKp2 (E), BHKp3 (F) and BHKp4 (G) monolayers were recovered and treated with RNases. Viral RNA was then extracted, reverse-transcribed and the partial 1D-encoding region of the respective viruses amplified. For each of the agarose gel images: lane 1 contains a 100-bp DNA molecular weight ladder; lane 2, negative control PCR reaction lacking template DNA; and lane 3, PCR reaction containing pNAM/SAT2 as template DNA. The amplicons obtained for the derived viruses are indicated as follows: lane 4, chimeric vNAM/SAT2 virus; lanes 5 and 6, mutant vNAM/SAT2 viruses (indicated by M1 and M2, respectively); lanes 7 and 8, back-mutated vBmutNAM/SAT2 viruses (indicated by BM1 and BM2, respectively); lane 9, control vSAT2 virus. Mock-infected cells (lane 10) were treated identically and included as a control in these assays. The cytopathic effect (CPE) observed on the BHK cell monolayers following each round of passage is indicted below the corresponding agarose gel images.

(A)



(B)

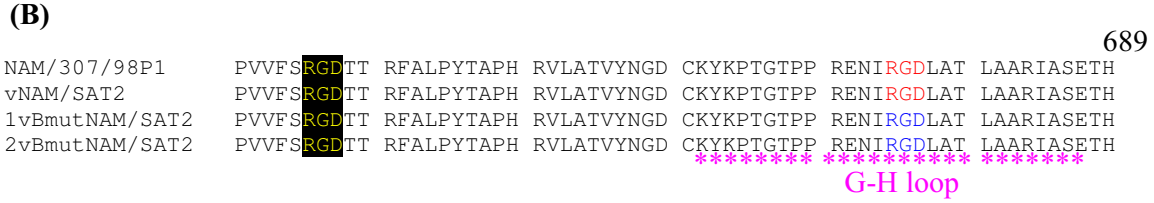


Fig. 3.6 Amino acid sequence alignment of the partial 1D protein of NAM/307/98, chimeric vNAM/SAT2, vmutNAM/SAT2 and vBmutNAM/SAT2 viruses. The deduced amino acid sequence of the partially sequenced 1D capsid-encoding regions from amplicons derived from samples prepared from (A) the supernatant of transfected BHK-21 cells and (B) lysates prepared from the highest passage cells (BHKp4) is presented. The G-H loop RGD sequence is indicated in red, whilst a second, upstream RGD motif is highlighted in black. Substitution of the G-H loop RGD sequence with an KGE sequence is highlighted in grey, whilst substitution of the KGE sequence with an RGD sequence is indicated in blue. The G-H loop is indicated by *, corresponding to amino acid residues 661-687 (residues 136-162 of 1D).

3.4 DISCUSSION

Crystal structures of viruses representative of several FMDV serotypes have been determined, and a major feature of the outer capsid surface is a long, conformationally flexible loop (Acharya *et al*., 1989; Logan *et al*., 1993; Fry *et al*., 1999). This loop, the G-H loop of capsid protein 1D, contains the major immunodominant epitopes of the virion (Baxt and Becker, 1990; Jackson *et al*., 1997) and includes at its apex a highly conserved RGD sequence. Subsequent studies have reported that FMDV uses RGD-dependent integrins as receptors through an interaction mediated by the RGD motif in the G-H loop of capsid protein 1D (Fox *et al*., 1989; Lea *et al*., 1994; Mason *et al*., 1994; Curry *et al*., 1996). Previously, sequence analysis of the 1D-encoding region of a SAT1 FMDV field isolate, NAM/307/98, indicated the presence of a second RGD motif located upstream of the conserved RGD sequence in the G-H loop (Bastos *et al*., 2001). In this part of the study, the ability of this second RGD sequence to mediate attachment of NAM/307/98 to BHK-21 cells was investigated.

To investigate, an infectious cDNA clone of the SAT2 FMDV ZIM/7/83 (Van Rensburg *et al.*, 2004) was used to engineer chimeric viruses of which the conserved RGD sequence in the G-H loop of capsid protein 1D of NAM/307/98 was mutated to KGE. The KGE-for-RGD substitution was specifically chosen as it maintained the "positive-glycine-negative" charge motif of the wild-type RGD sequence. In addition, the introduction of mutations within the RGD sequence, amongst them a KGE-for-RGD substitution, have been reported to yield FMD mutant viruses that were able to bind to a panel of monoclonal antibodies (Mason *et al*., 1994). Therefore, this mutation was not expected to significantly alter the antigenic structure of the G-H loop or to induce extensive rearrangements in the capsid protein 1D. Hence, it was expected that the function of the NAM/307/98 1D protein, except that of the RGD motif itself, would be maintained.

BHK-21 cells transfected with the *in vitro*-synthesized mutant RNA transcripts showed CPE, but no cell lysis could be observed upon further passage, indicating that the mutant vmutNAM/SAT2 virus was unable to produce cytopathic effect in cell culture. Therefore, the loss of infectivity of the chimeric vmutNAM/SAT2 viruses might have been a consequence of the altered G-H loop RGD sequence. This is also supported by the finding that substitution of the KGE sequence by an RGD sequence in capsid protein 1D of these mutant chimeric

viruses yielded viruses that regained their ability to bind to and initiate infection of BHK-21 cells (Table 3.2). To exclude the possibility that BHK-21 cells are not susceptible for a second pathway that may utilize the non-G-H loop RGD sequence, IB-RS-2 cells were also tested in further experiments. The results appear to be in agreement with those obtained for BHK-21 cells. Cells transfected with RNA transcripts derived from the control non-mutant pNAM/SAT2 chimeric clone yielded viruses capable of infecting IB-RS-2 cells, whereas cells transfected with transcripts derived from the mutant pmutNAM/SAT2 clones yielded viruses that were unable to produce CPE in cell culture (results not shown). The above results are therefore not only in agreement with previous reports indicating that the G-H loop RGD sequence is essential for binding FMDV to susceptible cells (Fox *et al*., 1989; Logan *et al*., 1993; Lea *et al*., 1994; Mason *et al*., 1994; Curry *et al.*, 1996), but it also suggested that a second upstream RGD motif located in capsid protein 1D of NAM/307/98 does not mediate attachment of the virus to either BHK-21 or IB-RS-2 cells.

Due to a lack of appropriate microscopy facilities at the Exotic Diseases Division (EDD), it was not possible to view virus particles directly using transmission electron microscopy (TEM) nor was it possible to verify viral protein synthesis by immunofluorescence microscopy using an appropriate FMDV-specific monoclonal antibody directed against the 1D protein and/or other viral proteins. Therefore, an experiment was designed whereby it could be investigated whether RNA transfected into BHK-21 cells yielded viruses which are capable of replication, but which cannot initiate further cycles of infection since they cannot interact with the cell receptor. For this purpose, *in vitro*-synthesized RNA derived from the mutant clones, as well as from control infectious non-mutant and back-mutated chimeric clones was transfected into BHK-21 cells and the derived chimeric viruses were subsequently passaged four times on BHK-21 cells. Samples of the clarified tissue culture supernatants and lysed cell monolayers were treated with RNases after which viral RNA was extracted, followed by reverse transcription using a virus strand-specific oligonucleotide, and the partial 1D-encoding regions were amplified by PCR and then sequenced.

By using the above approach, RNA replication could be demonstrated with the resultant chimeric viruses (Fig. 3.5). Amplicons corresponding to the 1D-encoding regions of both the non-mutant vNAM/SAT2 and back-mutant vBmutNAM/SAT2 chimeric viruses were obtained from all the samples tested in this assay, demonstrating that the virus particles produced following transfection were viable and capable of adsorbing to BHK-21 cells. In

contrast, an amplicon corresponding to the RGD→KGE mutated 1D-encoding region of vmutNAM/SAT2 chimeric viruses was only produced when samples of the transfected cells and the tissue culture supernatant of cells used in the first passage were used in the assay. No amplicon, however, was obtained from samples prepared of the cells used in the first passage, which had been rinsed with a low pH buffer to aid in the disassembly of the extracellular cell-associated virus particles prior to cell lysis and RNase treatment. The inability to produce an amplicon from the latter samples may therefore indicate that although virus particles were present in the tissue culture supernatant, they were unable to bind to the cells or, alternatively, that they were bound to the cells but not internalized. Although the experimental design does not allow a clear distinction between these two possibilities, it is likely that the virus did not bind to the cells, since Mason and co-workers (1994), using virus-binding studies, have reported that an A_{12} FMDV containing a mutation identical to that introduced into the 1D region of NAM/307/98 was unable to bind to BHK-21 cells. Based on these results, it was therefore concluded that the second RGD motif situated upstream of the G-H loop RGD sequence in capsid protein 1D of NAM/307/98 does not function as a receptor-binding site for infection of BHK-21 cells.

In conclusion, a double mutation introduced into the RGD sequence in the G-H loop of capsid protein 1D of NAM/307/98 via an infectious cDNA clone from ZIM/7/83, a SAT2 type FMDV, resulted in the production of non-infectious chimeric viral particles that could not interact with cellular receptors, despite the presence of a second, intact RGD motif. The inability of the second RGD motif to substitute as a cell receptor ligand may be related to its location in the capsid protein 1D of NAM/307/98. Although this second RGD sequence is situated on the surface-exposed βF and βG1 loop, the loop is shorter compared to the G-H loop and is also more rigid compared to the flexible G-H loop (Chapter 2, Fig. 2.5). Since the protruding Arg and Asp side chains of the G-H loop RGD motif have been shown to directly interact with residues in the concave paratope of an anti-virus neutralizing antibody (Verdaguer *et al*., 1995; Mateu *et al*., 1996; Domingo *et al*., 1999), it has been proposed that they may similarly interact with the cell receptor (D'Souza *et al*., 1991; Mateu *et al*., 1996; Domingo *et al*., 1999). Therefore, it might be that the short, rigid F-G loop does not present the second RGD motif in a conformation or orientation that is favourable for direct interaction with the RGD-dependent cell receptors.

CHAPTER 4

CONCLUDING REMARKS

This investigation was initiated following the observation that the amino acid sequence deduced from the 1D-encoding region of a SAT1 type FMD virus, NAM/307/98, isolated from an African buffalo, contained two RGD sequences. An additional RGD motif was identified upstream of the surface-exposed G-H loop on another surface-exposed loop, the short F-G loop in the 1D capsid protein (Bastos *et al*., 2001). Based on the important role that the RGD sequence plays in binding naturally-occurring isolates of FMDV to one or more RGD-dependent integrin receptors (Berinstein *et al*., 1995; Jackson *et al*., 1997; 2000b; 2002; 2004; Neff *et al*., 1998; 2002), the presence of the second surface-localized RGD sequence warranted further investigation. Thus, the primary aim of this investigation was to determine whether the second non-G-H loop RGD sequence in capsid protein 1D of NAM/307/98 could be utilized as an alternative ligand for binding the virus to susceptible host cells.

A first step in this investigation was to determine whether NAM/307/98 would indeed be capable of using integrin receptors for cell binding and whether this virus utilizes alternative receptors for cell binding. The ability of NAM/307/98 to utilize integrin receptors was established by plaque assays on different tissue culture cell lines (Chapter 2). The virus was able to replicate and form plaques on both BHK-21 and IB-RS-2 cells, which is in agreement with previous studies indicating that both these cell lines are permissive for field strains of FMDV (de Castro, 1964; de la Torre *et al*., 1985; 1988; Dunn and Donaldson, 1997). However, NAM/307/98 was unable to form plaques on CHO-K1 cells, which have been reported to be non-permissive for field strains of FMDV due to a lack of appropriate integrin receptors at its cell surface for binding FMDV (Mason *et al*., 1993; Jackson *et al*., 1997; Neff *et al.,* 1998; You *et al*., 2001; Jackson *et al*., 2002). These results also indicated that NAM/307/98 does not use heparan sulfate proteoglycans for binding of the host cell. Consequently, BHK-21 cells were used throughout this investigation.

Various different approaches have been described whereby it was shown that the RGD sequence in the G-H loop of capsid protein 1D plays an important role in binding FMDV to susceptible cells (Mason *et al*., 1994; Berinstein *et al*., 1995; McKenna *et al*., 1995; Mateu *et*

al., 1996; Jackson *et al*., 1997; 2000b; 2002; 2004). Although powerful in its application, the use of synthetic RGD-containing peptides (Berinstein *et al*., 1995; Mateu *et al*., 1996; Neff and Baxt, 2001; Jackson *et al*., 1997; 2000b; 2002; 2004) or function blocking antibodies (Berinstein *et al*., 1995; Jackson *et al*., 2000b; 2002; 2004) was not considered for use in this investigation, as it would not be possible to ascribe changes in virus-cell binding to a specific RGD sequence. Therefore, the RGD sequence situated in the G-H loop of capsid protein 1D of NAM/307/98 was rather targeted for inactivation through the introduction of a double mutation by site-directed mutagenesis procedures, thus leaving the second, upstream RGD sequence intact. The RGD sequence was subsequently replaced with a KGE sequence, since previous studies have shown that the KGE sequence in the context of either a serotype A_{12} virus (Mason *et al*., 1994; McKenna *et al*., 1995) or a type O_1 virus (Neff *et al*., 1998), was incapable of substituting for RGD in allowing the virus to replicate in culture. The introduction of such a mutation was also not expected to alter the structure or function of the NAM/307/98 1D protein, except that of the RGD motif itself. Consequently, a genome-length chimeric cDNA clone was constructed between NAM/307/98 and pSAT2 as genetic backbone, which is a stable infectious genome-length cDNA clone engineered from the SAT2 type virus ZIM/7/83 (Van Rensburg *et al*., 2004). The genome-length cDNA clones generated in this study comprised of pNAM/SAT2, a pSAT2 derivative that contained a cloned cDNA copy of the wild-type NAM/307/98 capsid-encoding region (Chapter 2), pmutNAM/SAT2, which contained a mutated copy of the NAM/307/98 capsid-encoding region (Chapter 3), and pBmutNAM/SAT2, which contained a cloned copy of the capsid-encoding region in which the KGE sequence was replaced with the wild-type RGD sequence in the 1D-encoding region (Chapter 3).

To determine whether the non-G-H loop RGD sequence present in capsid protein 1D of NAM/307/98 is utilized as a ligand for integrin receptors, two separate experiments were performed (Chapter 3). In the first experiment, BHK-21 cell monolayers transfected with *in vitro*-synthesized RNA transcripts derived from the respective clones, as well as cell monolayers used in passaging of the resultant viruses were monitored for signs of productive infection, as indicated by the appearance of CPE. The results indicated that infectious viruses could be derived from all but the pmutNAM/SAT2 clones. However, despite having used the same genetic backbone for all the plasmid constructions, the possibility that the pmutNAM/SAT2 clones yielded non-infectious RNA could not be excluded by the above experiments. Therefore, a second experiment was performed aimed at detecting viral RNA

replication in both transfected and infected BHK-21 cells. The results obtained indicated that for both mutant and non-mutant RNA transcripts transfected into BHK-21 cells, replication occurred and virus particles could be produced. Although the existence of virus particles in the tissue culture supernatants was not directly proven, several lines of reasoning would argue that infectious virus particles were derived from both the pNAM/SAT2 and pBmutNAM/SAT2 clones and that non-infectious virus particles were derived from pmutNAM/SAT2. Firstly, cells transfected with mutant RNA showed cell lysis, but no CPE upon subsequent passaging, whereas increasingly more severe CPE could be observed in cell monolayers used for passaging of the viruses derived from non-mutated and back-mutated viral RNA. Secondly, due to the extensive RNase treatment of the cell-free tissue culture supernatants and lysed cellular fractions prior to extraction and reverse transcription of viral RNA, it is unlikely that the virus-specific cDNA subsequently synthesized might have originated from residual synthetic RNA transcripts. Thirdly, carry-over of RNA transcripts or lipofectamine-RNA complexes upon subsequent passaging of the derived viruses would result in them being diluted from the samples, and consequently should not be detectable in the assay. Therefore, the most likely source of RNA available for virus-specific cDNA synthesis would be that of packaged RNA (and therefore protected from degradation by the RNase treatment) in progeny viral particles. More direct proof as to the existence of virus particles could be obtained through analysis of the culture supernatants by electron microscopy (Leippert *et al*., 1997), but the facilities for such an analysis are not available at the EDD. Alternatively, sucrose gradient analysis of culture supernatants may be used to verify the presence of virions (140S particles) (Mason *et al*., 1994), but as a consequence of the small scale in which the experiments were performed, it would be insufficient for 146S determination.

Based on the above results indicating that mutation of the G-H loop RGD sequence led to adsorption-deficient virus mutants, the most likely conclusion is that the second RGD sequence in capsid protein 1D of NAM/307/98 is not able to mediate attachment of the virus to cell receptors. This may be as a consequence of its location on the virion surface. The long, mobile G-H loop may present the RGD motif, located at its apex, in a conformation appropriate for direct interaction of the oppositely orientated Arg (R) and Asp (D) side chains with the cell receptors (D'Souza *et al*., 1991; Mateu *et al*., 1996; Domingo *et al*., 1999). In contrast, the second RGD sequence is located in the much shorter and more rigid F-G loop that might therefore not possess the flexibility required to present the RGD motif in such a

way as to allow for interaction with the cellular receptor. Alternatively, based on reports indicating that FMDV can use more than one RGD-binding integrin as cell surface receptors (Berinstein *et al*., 1995; Jackson *et al*., 2000b; 2002) together with the lack of information regarding the type and nature of integrins or other receptors present on the cell surface of BHK-21 cells, it may be that BHK cells lacked a receptor that may be utilized by the second non-G-H loop RGD sequence. The inability of the virus mutants lacking a G-H loop RGD sequence, but containing an intact F-G loop RGD sequence, to attach to BHK-21 cells may then be due to factors such as lower abundance or lower affinity of the virus for the receptors present on the BHK cells. However, this appears unlikely as results similar to those described above for BHK-21 cells were obtained following transfection of IB-RS-2 cells with RNA transcripts derived from the pNAM/SAT2 and pmutNAM/SAT2 clones. The mutant virus also lysed transfected cells, but, in contrast to the non-mutant virus, could not replicate in cell culture.

The results obtained in this investigation could also suggest that another region of the NAM/307/98 capsid, which may include the second RGD sequence, cannot bring about binding to the appropriate cell receptor in the absence of the G-H loop RGD sequence. This is based on evidence indicating that the C-terminus of capsid protein 1D is involved in receptor interactions, since virus-cell binding can be blocked by antibodies to this region and removal of this region resulted in virus particles with greatly reduced infectivity (Fox *et al*., 1989; Parry *et al*., 1989). Furthermore, as it is common for ligand-integrin interactions to include more than one site for adhesion (Hynes, 1992), it might therefore not be surprising that more than one region of the viral capsid should participate in virus-receptor interaction. Although the second RGD sequence is present in a region of capsid protein 1D that appears to be subject to extensive genetic variation (Bastos *et al*., 2001) and mutation (Botha, 2002) in SAT1 type viruses, it is nevertheless an intriguing possibility. This could in future be investigated by comparing differences in the cell-binding ability of the non-mutant virus and virus mutants containing mutated RGD sequences in the G-H loop and F-G loop, respectively. For this purpose, virus-binding assays similar to that described by Baxt and co-workers (1984) could be used.

In conclusion, based on the results obtained in this investigation it is unlikely that the second non-G-H loop RGD sequence plays a role in mediating attachment of NAM/307/98 to its target cells. Similarly, hepatitis A virus and pertactin each contain an RGD motif, but it does

not play a role in binding them to their target cells (Stapleton *et al*., 1991; Everest *et al*., 1996). Therefore, the presence of an RGD sequence does not always correlate with a functional role in cell adhesion, but it does not exclude the possibility that the second RGD sequence may have some other, as yet, unknown function.

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CONGRESS CONTRIBUTIONS

STOREY, P., Van Rensburg, H.G. and Theron, J. Investigating a second RGD triplet in SAT1 Foot-and-Mouth Disease Virus. 18th Congress of the South African Genetics Society/South African Microbiology Society, Cape Town, 4-7 April 2004. (Poster)

Maree, F.F., Van Rensburg, H.G., Lekoana, L.T., Böhmer, B., **STOREY, P** and Vosloo, W. Phylogenetic relationships for the capsid coding regions of the South African Territories (SAT) types Foot-and-Mouth Disease Virus. 3rd Annual Congress of the South African Society for Veterinary Epidemiology and Preventative Medicine, Pretoria, 21-22 August 2003. (Poster)

STOREY, P., Van Rensburg, H.G. and Theron, J. Analysing the functional role of a second RGD triplet in a Namibian SAT1 Foot-and-Mouth Disease Virus. 18th Congress of the South African Society of Biochemistry and Molecular Biology, Pretoria, 6-9 July 2003. (Poster)

APPENDIX

- L6 Buffer

  8.3 M GuSCN, 80 mM Tris-HCl (pH 6.4), 36 mM EDTA, 33 mM TritonX-100

- L2 Wash Buffer

  10.2 M GuSCN, 0.1 M Tris-HCl (pH 6.4)

- Methylene Blue Stain

  1% (w/v) methylene blue dissolved in absolute EtOH to an equal volume of formaldehyde prepared with phosphate-buffered saline; pH 7.4

- STET buffer

  0.23 M sucrose, 77.3 mM TritonX-100, 50 mM EDTA (pH 8), 50 mM Tris (pH 8)

- Tragacanth overlay Media

  2 × MEM, 1.2% gum Tragacanth, 1% FCS