The presentation of an HIV-1 neutralizing epitope on the surface of major structural protein VP7 of African horse sickness virus

deur

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Voorgelê ter vervulling van 'n deel van die vereistes vir die graad MSc in die Fakulteit Natuur- en Landbouwetenskappe

Universiteit van Pretoria

Pretoria

Augustus 2002

Dedicated to my parents,

Fred and Alice Meyer

ACKNOWLEDGEMENTS

I would like to express my most sincere gratitude and appreciation to the following people for their assistance and support throughout this study:

My supervisor, Prof. Henk Huismans for his insight and contribution to this study.

My family for their continued support and encouragement.

My best friend Sonia, for always being there.

The NRF for providing the necessary funding.

The biochemistry department for the use of their laboratories, in particular Sandra and Christine for their assistance and advice.

Mr. Mario Smuts at the Biomedical research institute for the care and handling of the test animals.

Also to Allan and Chris at the electron microscopy department for their assistance with the microscopic analysis.

The National Institute of Virology for providing the HIV viral lysates.

Everyone from the laboratory on the 8th floor for providing light entertainment along the way.

SUMMARY

THE PRESENTATION OF AN HIV-1 NEUTRALIZING EPITOPE ON THE SURFACE OF MAJOR STRUCTURAL PROTEIN VP7 OF AFRICAN HORSE SICKNESS VIRUS

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Two particulate structures based on AHSV major structural protein VP7, are under investigation as possible vectors for epitope display. Due to the extreme insolubility of the VP7 protein it aggregates into large hexagonal crystalline particles when expressed in an insect cell expression system (Chuma *et al*, 1992). To investigate its ability to present immunologically important epitopes to the immune system, VP7 mutants have been constructed that allow the presentation of foreign peptides on the surface these particles (Maree, 2000).

In part of this study AHSV core-like particles resulting from the co-expression of the two major structural proteins VP3 and VP7 were under investigation. Due to the low solubility of VP7, low core-like particle yields are obtained during co-expression (Maree, 2000). As a result not enough of the particles can be produced to make its use viable. To increase the core-like particle yield it is necessary to increase the solubility of VP7. Several amino acids have been implicated in the observed low solubility of VP7 (Monastyrskaya *et al*, 1997). One of these amino acids was targeted for site-specific mutation in an effort to increase protein solubility. Leucine 345 is located on the ninth C-terminal helix in the bottom domain of VP7 and was substituted to arginine, a polar positively charged residue. The substitution was made in wild type VP7 as well as in insertion mutants 177 and 200. The mutation was effected via PCR and the resulting mutant genes were expressed in the Bac-To-Bac expression system. The newly constructed mutant proteins were further investigated for an increase in solubility by differential and sucrose density centrifugation.

The site specific mutation in wild type VP7 and insertion mutant 200 resulted in a slight increase in solubility whereas the mutation in mutant 177 had no effect on solubility. As a result of failing to significantly increase the solubility of these mutants, no increase in the core-like particle yield was achieved.

In the second part of this study recombinant VP7 crystals were constructed which present a single and triple repeat of the HIV-1 transmembrane protein gp41 neutralizing antibody epitope, ELDKWA. To this end, oligonucleic acid adaptors coding for the epitope repeats were designed and cloned into VP7 insertion sites 144 and 177. The newly constructed VP7 mutant genes were expressed in the Bac-to-Bac expression system and the recombinant proteins were investigated for its solubility and crystal formation by sucrose density gradient centrifugation. All of the epitope presenting constructs were significantly less soluble than insertion mutants 144 and 177 and retained its ability to assemble into large hexagonal crystals. Gradient purified particles were injected into Balb/c mice and the epitope specific antibody response determined by dot and western blot analysis. Although a humoral immune response was induced against the VP7 constructs none were able to induce antibody responses specific to the ELDKWA epitope.

OPSOMMING

THE PRESENTATION OF AN HIV-1 NEUTRALIZING EPITOPE ON THE SURFACE OF MAJOR STRUCTURAL PROTEIN VP7 OF AFRICAN HORSE SICKNESS VIRUS

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Twee partikulêre strukture gebaseer op die APSV strukturele protiën VP7, word ondersoek as moontlike vektore vir die presentering van epitope aan die immuun sisteem. As gevolg van die swak oplosbare aard van VP7 lei dit daartoe dat die protiën aggregeer in groot heksagonale kristale tydens die uitdrukking van die geen in 'n insek sel geen uitdrukking sisteem (Chuma *et al*, 1992). Om die vermoë van die kritalle te toets om immunologies belangrike epitope te presenteer is daar drie VP7 invoegings mutante geskep naamlik, mutante 144, 177 en 200. Hierdie invoegings mutante laat die presentasie van eitope op die oppervlakte van die partikels toe (Maree, 2000).

In hierdie studie word Afrika perde siekte virus (APSV) kern partikels ondersoek. Die partikels vorm tydens die uitdrukking van die strukturele protiëne VP3 en VP7. As gevolg van die swak oplosbaarheid van VP7 is die opbrengs van die partikels geweldig laag, met die gevolg dat nie genoeg van die partikels gemaak kan word om die benadering sinvol te maak nie (Maree, 2000). 'n Aantal aminosure is geimpliseer by die waargenome swak oplosbaarheid van VP7 (Monastyrskaya *et al*, 1997). Een van die aminosure is geteiken vir setel-gerigte mutasie in 'n poging om VP7 se oplosbaarheid te verhoog. Die leusien in posisie 345 in die negende terminale C – heliks van VP7 is vervang met 'n polêr positief gelaaide residu nl, arginien. Die mutasie is deur middel van 'n polimerasie ketting reaksie (PKR) uitgevoer, en die nuwe VP7 mutante is uitgedruk in die Bac-To-Bac uitdrukkings sisteem. Die mutante protiëne is ondersoek na gelang van hul oplosbaarheid. 'n Effense toename in oplosbaarheid is waargeneem vir beide wildetipe VP7 sowel as vir die

invoegings mutant 200. Daar is egter geen teken van 'n toename in oplosbaarheid vir mutant 177 nie. As gevolg van die gebrek aan 'n noemenswaardige toename in die oplosbaarheid van die mutante word daar geen toename in die kern partikel opbrengs verwag nie.

Verder in die studie is daar drie rekombinante VP7 kristalle gekonstrueer wat of een of drie herhalings van 'n neutraliserende teenliggaam HIV-1 transmenbraan protiën gp41, epitoop (ELDKWA) presenteer. In die opsig is daar oligonukleiensuur koppel fragmente ontwerp wat kodeer vir die betrokke epitope. Die fragmente is in beide mutante 144 en 177 gekloneer. Die nuwe rekombinante gene is uitgedruk in die Bac-To-Bac uitdrukkings sisteem en die herwindende VP7 protiëne is ondersoek ten opsigte van hul oplosbaarheid sowel as hul vermoë om kristalle te vorm. Al die konstrukte het hul vermoë behou om groot heksagonale kristalle te vorm. Hierdie gesuiwerde partikels is gebruik om Balb/c muise te immuniseer om sodoende hul vermoë te bepaal om 'n epitoop spesifieke teenliggaam respons teen die ELDKWA epitoop uit te lok. Geen van die konstrukte het egter daarin geslaag nie.

ABBREVIATIONS

AHS	African horse sickness
AHSV	African horse sickness virus
AIDS	Acquired immunodeficiency syndrome
APSV	Afrika perde siekte virus
AP	Alkaline phosphatase
APC	Antigen presenting cells
BCG	*Bacilli* Calmette-Guèrin
bp	Base pairs
BSA	Bovine serum albumin
BTV	Bluetongue virus
°C	Degrees Celsius
ccc	Covalently closed circular
CLP	Core-like particle
CTL	Cytotoxic T lymphocyte
DNA	Deoxyribonucleic acid
ddH_2O	Deionized distilled water
EDTA	Ethylenediaminetetra-acetic acid
et al.	*et alia* (and others)
FCA	Freunds complete adjuvant
FCS	Fetal calf serum
FIA	Freunds incomplete adjuvant
FIV	Feline immunodeficiency virus
Fig.	Figure
FMD	Foot and mouth disease virus
g	Gram
HBV	Hepatitis B virus
HbsAg	Hepatitis B virus surface antigen
HSV	Herpes simplex virus
h	Hour
HIV	Human immunodeficiency virus
IPTG	Isopropyl-β-D-thiogalactopyranoside
ISCOMS	Immune-stimulating complexes

kDa	Kilodalton
kb	Kilobasepairs
l	Liter
LB	Luri Bertani
M	Molar
MCS	Multiple cloning site
mg	Milligram
MHC I	Major histocompatibility complex 1
MHC II	Major histocompatibility complex II
min	Minutes
ml	Milliliter
mM	Millimolar
MOI	Multiplicity of infection
M_r	Molecular weight
MVA	Modified vaccinia virus Ankara
ng	Nanogram
NS	Nonstructural
NVP	Nevirapine
OD	Optical density
OVA-MSs	Ovalbumin microspheres
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGLA	Poly-D-L-lactide-glycolide
PKR	Polimerase ketting reaksie
pmol	Picomol
polh	Polyhedrin
PSB	Protein solvent buffer
RNA	Ribonucleic acid
Rnase	Ribonuclease
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
sec	Second
Sf	Spodoptera frugiperda

SIV	Simian immunodeficiency virus
SMBV	Synthetic biomimetic supra molecular biovector
TC	Tissue culture
TEMED	N,N,N',N'-tetramethylethylendiamine
Tris	Tris-hydroxymethyl-aminomethane
µg	Microgram
µl	Microliter
UV	Ultraviolet
V	Volts
VLP	Virus like particle
VP	Viral protein
wcf	Whole cell fraction
w	Weight
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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CHAPTER 1

LITERATURE REVIEW

The science of vaccinology involves the identification and development of immunogenic agents that are capable of inducing long-term protection by the host immune defenses against infectious disease causing agents. Many different strategies are employed to develop vaccines against pathogens responsible for disease. To develop an effective vaccine it is necessary to understand what to present to the immune system and how to present it. More specifically the pathogenicity, epidemiology and the nature of protective immunity against the target pathogen should be elucidated. Technological advancement in the field of molecular biology provides new and exciting prospects for the development of safer and more effective vaccines. The main approaches to vaccine development are the use of i) live vaccines. ii) Inactivated vaccines. iii) Nucleic acid vaccines. iv) Subunit vaccines. In this chapter some of the different approaches to vaccine development are reviewed.

In this study a novel antigen delivery system based on one of the major structural proteins VP7, of African horse sickness virus is investigated as a means to display a well characterized human immunodeficiency virus type 1 (HIV –1) neutralizing antibody epitope (ELDKWA) to the immune system. The candidate antigen delivery system and the current status of HIV vaccine development are therefore also briefly reviewed in this chapter.

1.0 LIVE VACCINES

Live vaccines are capable of infecting target cells and replicating in the host. These microorganisms are attenuated, as a result their disease causing capability is reduced or inactivated. Several strategies exist to attenuate microorganisms i) biological attenuation can be accomplished by repeated passage of wild type virus through several different cell lines or repeated sub-culturing in the case of bacteria. In the process the virulent phenotype is lost or inactivated (Ellis, 2001). ii) Attenuation through chemical means proved to be useful for the production of a chemically mutated strain of *Salmonella typhi*, rendering it non-pathogenic (Levine *et al*, 1987). iii) A more innovative method that is also employed to attenuate microorganisms is based on the genetic manipulation of the microorganisms, where the genes responsible for toxicity or infection are deleted (Farrell *et al*, 1994).

The major advantage of the live vaccine approach is the elicitation of an immune response similar to that of the natural infection. In most cases this involves eliciting both humoral and cellular immunity. Antibodies serve to neutralize free viruses and toxins, and to remove these agents from the body fluids. Whereas the cellular immune response is responsible for identifying and destroying infected cells via cytotoxic T- lymphocytes, and plays an important role in protective immunity (Liljeqvist *et al*, 1999).

Safety is always a concern when vaccinating with attenuated live organisms. The possibility of causing disease due to incomplete attenuation or reversion through back mutation to the pathogenic phenotype can't be discounted. In the case of genetic attenuation the risk can however be reduced significantly. Despite safety concerns, the importance of live vaccines is reflected by the fact that of the seventeen viral vaccines licensed for use in the United States, ten are live (Hilleman, 2000).

There are several different strategies that are currently employed to develop live vaccines namely, i) live attenuated viruses and bacteria and, ii) recombinant viral and bacterial vectors. The different approaches are discussed by example in the following sections.

1.1 Attenuated viruses

1.1.1 Biological attenuation

The live attenuated virus approach has proven its effectiveness repeatedly (Hilleman, 2000). Its success is due mainly to the production of infectious virions capable of displaying mature viral proteins similar to that of the wild type organism and the induction of long lasting humoral and cell-mediated responses (Semple, 2000). The best known examples of this class of vaccine are those against childhood diseases like mumps (Hilleman, 1967) and measles (Enders *et al*, 1960).

Biological attenuation is an empirical process and the mechanism involved in reducing pathogenecity is not well understood (possibly by mutation or difference in the glycosylation patterns of different cell types). It is important that the viral vaccine phenotype still resembles the wild type organism to induce an immune response that will be effective against the wild type virus. It is well documented that altered glycosylation of viral envelope proteins occurs following passage and adaptation in cell lines. Different cell types or cell lines may show different glycosylation patterns (Goochee and Monica, 1990). The altered glycosylation of HIV-1

envelope proteins has been shown to influence both the virus infectivity and immunogenicity (Benjouad *et al*, 1992; Lee *et al*, 1992).

Dengue fever or dengue hemorrhagic fever is caused by four different serotypes of the dengue virus. The virus is transmitted by *Aedes* mosquitoes and annually results in the death of 35000 people in the tropical regions of the world (Vaughn *et al*, 1996). A live attenuated tetravalent vaccine has been developed and was shown to be safe, well tolerated and highly immunogenic. This vaccine was developed by passage in BSC-1 cells, LCC-MK_2 cells (x6) a rhesus monkey, mosquitoes (x2) and 53 times in a dog kidney cell line (Vaughn *et al*, 1996). This illustrates the impact the chosen cells have on effective attenuation.

1.1.2 Genetic attenuation

Attenuation by genetic manipulation i.e. the insertion of deletions or mutations in genes necessary for the pathogenicity of the virus can significantly reduce the risk of reversion to the pathogenic phenotype. Genetically attenuated viruses share much of the benefits of biologically attenuated viruses but are significantly safer. One such a candidate vaccine is that of Herpes simplex virus (HSV). In one of two approaches to develop a replication defective strain of HSV, a mutant virus was constructed carrying a deletion in the gene encoding a glycoprotein necessary for infection. As the virus is not capable of synthesizing this protein, it is supplied in trans by the cell line during *in vitro* cultivation. The viral progeny can initiate *in vivo* infection in the host, but is unable to replicate (Farrell *et al*, 1994). In another strategy two deletion mutations were inserted in two different genes both necessary for HSV DNA replication, resulting in a safer double deletion mutant that has been shown to induce protective immunity (Da Costa *et al*, 1999; Da Costa *et al*, 2000).

1.2 Recombinant viral vectors

The use of genetically engineered viruses to express foreign polypeptides to the host immune system is an innovative approach to vaccination. Genes or gene fragments of foreign epitopes are carried by the viral vector and is expressed in the host cell after infection or as part of the vectors viral envelope protein. This strategy attempts to mimic a natural infection, thereby providing an effective immune response. The expressed protein is either transported to the cell surface to stimulate antibody production or broken up and presented to elicit a cytotoxic T lymphocyte (CTL) response. The major advantage is the amplification of the immunogenic signal when the live vector replicates. There have been numerous studies undertaken to

develop viruses expressing a broad range of epitopes derived from bacterial, viral and parasitic pathogens (Roberts *et al*, 1998; Seong *et al*, 2001).

Vaccinia virus is considered the prototype viral vector and has been extensively studied since 1982 (Mackett *et al*, 1982; Ulaeto and Hruby, 1994). Concerns regarding its safety however restricted its use to animal vaccines (Smith *et al*, 1983; Fischetti *et al*, 1989; Mcmahon-Pratt *et al*, 1993). Fowlpox, canarypox as well as a highly attenuated strain of vaccinia virus namely modified vaccinia virus Ankara (MVA) have however since been developed for use in humans (Fries *et al*, 1996; Sutter and Moss, 1992).

1.3 Attenuated bacteria

Due to the limited success of attenuating bacteria *in vitro*, most live bacterial vaccines are based on the genetic manipulation of these microorganisms to remove genes or loci necessary for its virulence, colonization and survival. One of the few examples of non-recombinant attenuated bacterial vaccines is that of *Mycobacterium bovis* also known as *bacilli* Calmette-Guèrin (BCG). A strain of BCG has been used since 1920 for vaccination in humans. (Drabner and Guzman, 2001). The BCG vaccine strain was attenuated by *in vitro* sub-culturing over a thirteen year period (reviewed by Ellis, 2001).

The availability of molecular techniques to develop recombinant attenuated bacterial vaccines has made BCG the most commonly used bacterial vaccine worldwide (Medina and Guzman, 2001). A new approach involves the use of an attenuated strain of *Mycobacterium bovis,* carrying deletions in three distinct genomic regions (Mahairas *et al*, 1996). The mycobacterium mimics natural infection by entering and replicating in inactivated macrophages, after which it is killed by a strong Th1 helper response, IFN-γ production and cytotoxic lymphocytes (Drabner and Guzman. 2001). Despite its use some questions have been raised regarding its efficacy, with estimates ranging from 0-80% (Colditz *et al*, 1994).

1.4 Recombinant bacterial vectors

A major focus area for the development of live attenuated bacterial vectors has been the engineering of naturally occurring enteric bacteria to induce mucosal immune responses after oral administration. For some pathogens serum antibodies can provide effective protection, but generally pathogens enter the body at the mucosal surfaces.

Protection against disease in many cases therefore depends on the induction of mucosal immune responses at the site of infection. The use of bacterial vectors to present immunologically important epitopes to the immune system holds a number of advantages; these microorganisms are able to establish a limited infection, mimicking the natural disease that leads to the induction of both systemic and mucosal responses (Staats *et al*, 1994). Modest production and delivery costs facilitate its application for mass immunization programs. It makes this approach useful in non-industrialized countries. Typically it is also highly stable and provides long lasting protection even after a single dose. *Salmonella* is arguably the most widely exploited vaccine carrier system to date (reviewed by Liljeqvist *et al*, 1999; Georgiou *et al*, 1993). *Salmonella* has also been employed to produce virus like particles, by expressing Hepatitis B virus core antigen (Hopkins *et al*, 1995) and human Papiloma virus type 16 capsid protein (Nardelli-Haefliger *et al*, 1997). Both these particles were shown to induce immunity in the host.

Although this approach is largely successful, several problems associated with its use have been reported and should be noted (Medina and Guzman, 2001). i) The possibility of reversion back to the virulent phenotype ii) stability of the recombinant phenotype iii) horizontal gene transfer to the environment iv) pre-existing immunity to the carrier organism will lead to reduced efficacy and v) host genetic factors may also influence the efficacy of the vaccine.

2.0 INACTIVATED VACCINES

One of the oldest and most effective vaccination strategies rely on the inactivation of the whole virus and bacteria. Many inactivated vaccines have been developed and employed for veterinary use with great success, such as foot and mouth disease virus (FMD) (Samina *et al*, 1998; O'Donnell *et al*, 1997; Sugimura *et al*, 1996) and feline immunodeficiency virus (FIV) (Yamamoto *et al*, 1991). A popular method of inactivation is based on the use of formaldehyde, but concerns regarding its use to safely inactivate pathogens have been raised (Brown, 1993). The use of inactivated SIV vaccines have also been investigated (Cole *et al*, 1997; Hirsch *et al*, 1994; Carlson *et al*, 1990), however due to the obvious risks it seems to be an unlikely strategy for the development of an effective HIV-1 vaccine.

3.0 NUCLEIC ACID VACCINES

DNA and RNA have been investigated as a means to deliver antigens to the immune system, each with some success. Despite safety concerns DNA vaccines remain the most popular approach.

3.1 DNA vaccines

The use of DNA to elicit protective immune responses is relatively new to the field of vaccinology. The antigen of interest is placed under the control of a strong viral promoter that is recognized by mammalian cells, after DNA uptake it is expressed *in situ* and can be secreted or presented on the cell surface in a manner that triggers both humoral and cellular immune responses (Wolf *et al*, 1990). There are numerous advantages to this approach. i) The preparation of high concentrations of highly pure plasmid DNA carrying the immunologically important antigen is relatively easy. ii) Due to the high expression level large amounts of the antigen can be produced which usually results in a good immune response. iii) DNA vaccines are especially proficient at inducing cellular immunity via major histocompatibility complex I (MHC I) presentation of antigens, iv) the possibility of inducing mucosal immunity by oral or nasal route holds some promise. v) Enhancement of the immune response by co-delivery of immune stimulatory molecules coded for by the antigen expressing plasmid (Kim *et al*, 1997; Xiang and Ertl, 1995). vi) The ability of DNA to act as its own adjuvant. DNA motifs in which an unmethylated CpG dinucleotide is flanked by two 5' purines and two 3' pyrimidines dramatically increases B cell activation and reduces the amount of DNA required to induce a response (Krieg *et al*, 1995; Klinman *et al*, 1999; Sato *et al*, 1996; Davis *et al*, 1998). Two concerns regarding the use of DNA vaccines is the possibility of integration into the host genome causing cancer, and the production of anti DNA antibodies after immunization of plasmid DNA. Many studies has thus far been undertaken to test the efficacy of DNA vaccines to a wide range of pathogenic viruses some showing promising results, these include: Ebola virus (Xu *et al*, 1998), Hepatitis B, C, and E (Chow *et al*, 1997; Chen *et al*, 1995; He *et al*, 1997), Rabies (Xiang *et al*, 1994; Ray *et al*, 1997) and HIV (Wang *et al*, 1993; Boyer *et al*, 1997; Kim *et al*, 1997; Hinkula *et al*, 1997; Fuller *et al*, 1997).

The use of bacterial vectors to deliver DNA vaccines is a strategy that holds a lot of promise, by exploiting the preference of intracellular bacteria to infect antigen presenting cells (APC) like macrophages. After phagocytosis these microorganisms can survive in the host by either preventing lysosomes to fuse with the phagosome, or by releasing itself into the host cell cytosol where it can release the DNA vaccine. The DNA enters the nucleus and the plasmid encoded antigen is expressed by the APC. The antigens can be presented by the APC together

with MHC class II and I molecules which results in both humoral and cellular responses (Dietrich *et al*, 2001). Some of the most commonly used organisms to deliver DNA vaccines are *S. flexneri* (Sizemore *et al*, 1997), *L. monocytogenes* (Dietrich *et al*, 1998), *S. typhimurium* (Darji *et al*, 1997).

3.2 RNA vaccines

Very little has been done on the use of RNA vaccine development. The single most important advantage RNA offers is its safety as there is no risk of cancer due to integration into the host genome. The use of RNA however presents several technical problems, its preparation and administration is somewhat troublesome due to the inherent low stability of RNA. Also RNA vaccines are relatively short lived once administered, as a result it is less effective at inducing an immune response. Despite its shortcomings there has been several cases were RNA vaccines have proved some measure of success. The induction of CTL as well as humoral immune responses to HIV-1 and Simian immunodeficiency virus (SIV) has been reported (Berglund *et al*, 1997; Mossman *et al*, 1996).

4.0 SUBUNIT VACCINES

The development of effective subunit based vaccines is made possible by the availability of powerful recombinant DNA technology. It is possible to isolate immunologically important epitopes form pathogens and present it to the immune system in a way that will confer protection against the disease causing agent. Subunit vaccines in most cases require the administration of several booster doses to obtain long-term protective immunity. The immunogenicity of the subunit vaccine also needs to be enhanced by co-administration with an adjuvant. Subunit vaccines predominantly stimulate humoral immune responses, although CTL responses have been induced with the aid of certain adjuvants and delivery systems. Subunit vaccines are generally safe provided that the extraction procedure or detoxifying method yields a pure product.

4.1 Natural and recombinant whole protein based vaccines

There are a number of effective approaches to the development of protein based vaccines. In the case of natural proteins, the direct purification of proteins from *B. pertussis* cultures has shown high levels of clinical efficacy (Greco *et al*, 1996). In most cases however the protein of interest needs to be inactivated before use. For chemical inactivation the bacterial toxins

responsible for pathogenicity are isolated and detoxified by incubation with formalin or gluteraldehyde. Toxins have been recovered and treated from organisms such as *C. diphteriae, C. tetani* and *B. pertussis.* Unfortunately there is a risk of altering the protective epitopes leading to a reduced immunogenicity. Also the risk of incomplete detoxification or reversion to the active toxin exists. A safer and more widely used method of inactivation is that of genetic inactivation where site-specific mutations are introduced into the toxin molecules to inactivate its toxic activity (Nencioni *et al*, 1990).

The basic principle for recombinant protein vaccines is that the gene encoding the antigen is isolated and transferred to a second non-pathogenic organism where it is produced and isolated for use in a vaccination strategy. The advantage of this approach is that the pathogen is entirely excluded from the production of the vaccine. There is therefore no risk of contamination with the pathogen, and also no risk of reversion to the virulent genotype or incomplete inactivation exists. The choice of the host to produce the recombinant vaccine should be carefully considered as each has its advantages and limitations. Commonly used hosts are bacteria (Makrides, 1996), yeast (Sudbery, 1996), insect cells (Possee, 1997) and mammalian cells (Geisse *et al*, 1995). A number of strategies exist to optimize the production of recombinant proteins. i) The use of appropriate promoters to increase the recombinant protein production level (Suarez *et al*, 1997). ii) The use of signal sequences to export proteins to the culture medium, which simplifies the recovery and purification process (Moks *et al*, 1987; Hansson *et al*, 1994). iii) And the development of strategies to counter proteolytic degradation (Murby *et al*, 1996).

An innovative application for the use of recombinant DNA technology in vaccine development is that of fusion protein construction. In principle a fusion is made to obtain chimeric antigens that are combined with a fusion partner that provides certain desirable properties (for review see LaVallie and McCoy, 1995). Examples of such properties are i) the use of affinity fusion partners enabling recovery of the product via affinity chromatography (Nygren *et al*, 1994; Nilsson *et al*, 1997). The affinity handles can then be removed after purification by site- specific enzymatic cleavage (Jonasson *et al*, 1998). ii) Fusion of the expressed protein to a secretion signal (Hansson *et al*, 1994). iii) Fusion to a highly soluble partner to increase solubility that will ultimately simplify recovery of the product (Samuelsson *et al*, 1994; Murby *et al*, 1995). iv) Targeting antigens to immunoreactive sites by combining the antigen with a molecule that specifically binds to a eukaryotic cell receptor (Hajishengallis *et al*, 1995). v) The creation of chimeric composite immunogens, by fusing several different antigens (Lebens *et al*, 1996; Power *et al*, 1997). vi) The use of fusion proteins to improve immunogenicity (Libon *et al*, 1999).

4.2 Peptide based vaccines

Peptide based vaccines are made possible by the identification of immunologically important peptides through epitope mapping. After identification, the epitope can be synthesized *in vitro*, purified and administered to test animals, to evaluate its immunogenicity. In most cases the synthesized peptides are conjugated to a carrier protein like bovine serum albumin (BSA) (Nyambi *et al*, 1998; Earl *et al*, 1997). As with other subunit vaccines, peptides also require the co-administration of an adjuvant to induce an immune response. Since the availability of safe adjuvants suitable for human use is limited, its effective use in humans is also somewhat restricted.

The most important obstacle for the development of effective peptide vaccines is the structural conformation of the epitope. Chemically synthesized peptides are generally conformationally heterogeneous in an aqueous solution, thus differing form the structure the cognate sequence adapts in the native protein. As a result there is a reduced affinity by the peptide for antibodies generated by the native protein (Jemmerson, 1987). Inversely, antibodies elicited by a heterogeneous pool of peptides show a lower antigenicity towards the native protein. They are mostly incompatible with the native protein surfaces (Stanfield *et al*, 1990). It has been shown however that the immunological properties of peptides can be improved by constraining them to mimic protein substructures (Kaumaya *et al*, 1992). Recently a method to chemically stabilize the structure of synthetic peptides in aqueous solutions, resulting in improved immunogenicity was reported (Cabezas *et al*, 2000).

4.3 Particulate vaccine delivery systems

There is considerable interest in developing protein based as well as synthetic particulate vaccine delivery systems. Some protein based systems are aimed at generating non-infectious particles that can present foreign epitopes to the immune system. Generally a constituent structural protein is mutated to allow the expression of the epitope on the surface of the particle. The recombinant particles spontaneously assemble after the expression of one or more viral structural proteins in an expression system. The relative ease with which these particles can be constructed and its complete safety makes this a worthwhile strategy to explore. There is however a size limitation for the epitope that can be displayed and as with other subunit vaccines these particles generally also require the co-administration with an adjuvant (El Mir and Triebel, 2000).

The expression of hepatitis B virus surface antigen (HBsAg) in *S. cerevisiae* resulted in the formation of virus like particles similar to that of the hepatitis b virus (HBV) virion (Valenzuela *et al*, 1982). Since then hepatitis particles have been exploited to successfully deliver numerous epitopes, probably making it the most widely used particulate delivery system (Murray and Shiou, 1999; Borisova *et al*, 1999). It has been shown that fragments as large as 238 amino acids can be presented successfully in the hepatitis core antigen (Beterams *et al*, 2000, Pumpens and Grens, 1999). Apart from its remarkable stability the particle generally succeeds in eliciting both mucosal and systemic humoral and cellular immune responses when administered with the appropriate adjuvant (El Mir and Tiebel, 2000; Hui *et al*, 1999; Paoletti *et al*, 2001; Milich *et al*, 2001). Many other particulate vectors derived from viral structural proteins have also been developed and shown to hold promise, for example bluetongue virus (BTV) core-like particles (Adler *et al*, 1998) and parvovirus virus-like particles (Casal *et al*, 1999). Apart from presenting foreign epitopes to the immune system some virus-like particles are also developed as candidate vaccines against the virus self. Candidate vaccines for hepatitis (Hui *et al*, 1999), bursal disease virus (Wang *et al*, 2000) and Japanese encephalitis virus (Konishi *et al*, 1997) have proven to be potentially useful.

Synthetic particulate delivery systems are also in development. Most of the effort is focused on developing systems that require only one administration of the vaccine in the hope of cutting down on production and supply costs. Delivery systems like these are especially useful for vaccination programs in poor unindustrialized countries. Some of the approaches taken are i) synthetic micro-particles. ii) Lipid-based delivery systems.

There are numerous examples of different microspheres in development, some pointing to the possibility of obtaining optimal and long lasting immune responses by single administration. i) Ovalbumin microspheres (OVA-MSs) (Puri *et al*, 2000). ii) Fluorescent latex microspheres (Puri *et al*, 2000). iii) Collagen mini pellets (Higaki *et al*, 2001). iv) Immunostimulating complexes (ISCOMS) (Hu *et al*, 2001). v) Non-ionic surfactant vesicles (NISV) (Mohamedi *et al*, 2000). vi) Cationic polysaccharide (Chitosan) (Illum *et al*, 2001). vii) Synthetic biomimetic supra molecular biovector™ (SMBV™) (von Hoegen, 2001).

One microparticle in particular is showing a lot of promise, PGLA (poly-D-L-lactide-glycolide). Oral administration of these microparticles containing experimental antigens demonstrated the induction of systemic and mucosal, humoral and cellular immune responses in animal models (Challocombe *et al*, 1992). Some advantages of PGLA microparticles are, excellent tissue compatibility, non-toxicity, and the fact that it has been used extensively for medical implants and drug delivery. Also it can deliver both DNA and protein vaccines and dramatically reduces

the number of inoculations and the amount of immunogen needed to generate a good immune response (Rosas *et al*, 2001).

Liposomes has been shown to effectively transport protein, peptide and DNA based vaccines and has also proved effective in inducing both humoral and cellular immunity (Rao and Alving, 2000). The ease with which different sized particles can be formulated at a high yield has made this a very promising strategy (Gregoriadis *et al*, 1999). Numerous studies are under way investigating the use of lipid-based vaccine delivery systems (Owais *et al*, 2001; Conacher *et al*, 2001; Lian *et al*, 2001).

In this study we are interested in developing two protein based antigen delivery systems for the presentation of linear epitopes to the immune system. Both of the vectors are based on structures formed during the expression of two major structural proteins of African horse sickness virus. The co-expression of structural proteins VP3 and VP7 results in the formation of core-like particles. One strategy is aimed at developing these hollow spheres for epitope display. In a second approach the expression of VP7 alone results in the assembly of large hexagonal crystals. These crystals are also under investigation for its use in epitope display. The following sections review the origin of these structures and its subsequent development as presentation systems.

4.3.1 AHSV structural protein based antigen delivery systems

4.3.1.1 The AHSV Virion

African horse sickness (AHS) is a gnat (*Cullicoides*) transmitted disease of equines. The etiological agent, African horse sickness virus (AHSV) is a member of the Orbivirus genus within the Reoviridae family (House, 1993). The morphological and molecular characteristic of AHSV is similar to that of BTV, the prototype virus of the orbivirus genus. The viral genome consists of 10 dsRNA segments varying in size, namely L1-L3 (large), M4-M6 (medium) and S7-S10 (small) and codes for seven structural and four non-structural proteins. The virion is constructed from seven structural proteins (VP1 -7) that are organized into a double layered non-enveloped capsid (Roy *et al*, 1994). The outer capsid consists of VP5 and VP2 (the serotype specific antigen) where as the inner core is constructed from the two major structural proteins, VP3 and VP7. The minor structural proteins VP1, VP4 and VP6 are enclosed within the core, and are believed to have enzymatic functions.

4.3.1.2 Major core protein VP7

VP7 is 349 amino acids long and has a calculated weight of 38 KDa (Roy *et al*, 1991). A high degree of similarity (70%) to BTV VP7 has been reported (Roy *et al*, 1991), implying a similarity in protein structure and function. The crystal structure for BTV VP7 has been completely resolved (Basak *et al*, 1992); where as the x-ray structure for AHSV VP7 has only partially been solved (Basak *et al*, 1996). The results however confirm its predicted similarity in structure.

The VP7 monomer is characterized by its division into an upper domain (aa 121-249) that is folded into antiparralel β-strands connected by β-turns and a lower domain (aa 1 -120, 250-349), which forms a complex of 9 α-helices (Grimes *et al*, 1995). The VP7 monomers spontaneously organize into trimers after production, to produce subunits consisting of three non-covalently linked monomers. The domains of the monomers are twisted anticlockwise in the trimer, allowing the top domain of one monomer to rest on the C-terminal region of the threefold related subunit (Grimes *et al*, 1995, Monastyrskaya *et al*, 1997).

There is a distinctive difference in solubility between the VP7 of BTV and AHSV. AHSV VP7 is intrinsically much less soluble and has been reported to form hexagonal crystals up to 6μm in size when expressed in insect cells or during AHSV infection of BHK1 cells (Chuma *et al*, 1992, Burroughs *et al*, 1994). BTV VP7 however is completely soluble (Oldfield *et al*, 1990). Due to the extreme hydrophobicity of AHSV VP7 it is reasonable to assume that hydrophobic interaction between the trimers play a large role in the aggregation of the protein into these particulate structures.

4.3.1.3 VP7 insertion mutants

The ease with which these structures can be synthesized and purified compounded by its group specific antigenicity led workers to investigate its use as a subunit vaccine against AHSV (Wade-Evans *et al*, 1998), they concluded that the protection obtained was primarily cellular in nature. In our laboratory the use of this particulate structure as a vehicle for the expression of foreign epitopes to elicit humoral response is under investigation. Three hydrophilic regions within the top domain (aa positions 144, 177 and 200) of VP7 have been identified. These sites were targeted to construct three insertion mutants each having a cloning site of 18 nucleotides in length for the insertion of foreign DNA (Maree, 2000, Maree *et al*, submitted for publication). By inserting foreign DNA encoding an epiptope into such a hydrophilic patch the aim is to present the epitope on the surface of the trimer and ultimately the crystalline structure. The ability of the crystalline structures to present a linear HIV neutralizing antibody epitope

(ELDKWA) in order to induce a humoral immune response is the subject of investigation in chapter 3.

4.3.1.4 The AHSV core-like particle

The atomic structure of the BTV inner core has been resolved at a resolution approaching 3.5 Å (Grimes *et al*, 1998). It has a diameter of 70nm and is composed of two major structural proteins VP3 and VP7. The core has an icosahedral shape and is comprised of 780 copies of the VP7 monomer organized as 260 trimers on a T=13 quasi-equivalent lattice (Grimes *et al*, 1998). The three fold related axes of each trimer is perpendicular to the core surface, and interacts via a broad hydrophobic base with an inner VP3 scaffold. Which is arranged in a T=2 icosahedral symmetry and consists of 120 copies.

It is reasonable to assume a similar structure for AHSV core particles. The co-expression of the major core proteins VP3 and VP7, in a eukaryotic expression system reported the production of core-like particles similar in morphology to that described for BTV (Maree *et al*, 1998, Burroughs *et al*, 1994, Le Blois and Roy, 1993).

In our laboratory we are interested in the development of antigen delivery systems based on AHSV VP7 insertion mutants. In chapter 2 the possible use of AHSV core-like particles acting as a vector for epitope presentation is discussed. The principle is essentially the same as in the case of VP7 crystals. DNA coding for an immunologically important epitope is cloned into an insertion site. The mutant VP7 is then co-expressed however with VP3 resulting in the formation recombinant core-like particles presenting the foreign epitope. Such constructs have already been made although not tested for its immunogenicity (Maree, 2000). A similar approach based on BTV core-like particles has been investigated (Adler *et al*, 1998).

The extreme insolubility of AHSV structural protein VP7 poses a problem for the development of core-like particle vectors as a means to present foreign epitopes. Due to its insolubility the majority of the VP7 trimers are assembled into crystals when co-expressed with VP3. This results in a low core-like particle yield (Maree, 2000). It is therefore necessary to significantly increase the solubility of VP7. In this regard a non-polar uncharged residue present in the bottom domain of VP7, namely Leu 345 was identified as possibly contributing to the proteins insolubility (Monastyrskaya *et al*, 1997). The effect on solubility of this single amino acid substitution is the subject of investigation in chapter 2.

5.0 HIV VACCINE DEVELOPMENT

5.1 The human immunodeficiency virus

5.1.1 Virion structure

The human immunodeficiency virus is a complex retrovirus that belongs to the genus *Lentivirinae* (Narayan and Clements, 1990). It is similar in structure to other retroviruses with infectious particles approximately 100nm in size (Fig. 1.1). A dense cylindrical shaped protein core made up of 2000 copies of the viral protein p24 characterizes the virion. The core encases the viral RNA genome as well as several enzymes that play an important role in the life cycle of the virus (reverse transcriptase, integrase and protease). A single stranded RNA genome encodes the proteins necessary for infection and the production of new virus particles, namely gag, pol, env, tat, rev, nef, vif, vpr and vpu (Wong-Staal, 1990). A protein matrix and a membrane envelope surround the virus core. The viral envelope is composed of a lipid bilayer and is derived from the host cell after budding of the virus had occurred. Apart from the host proteins embedded in the membrane there are also 72 knob-like protrusions or "spikes" each formed by a glycoprotein trimer (Wong-Staal, 1990). Each envelope glycoprotein is made up of two non-covalently linked subunits, an extra cellular protein, gp120 and a transmembrane protein gp41 that is generated by site-specific cleavage of a precursor polypeptide gp160 (Freed and Martin, 1995). GP120 has been shown to determine viral tropism by binding to target cell receptors, whereas gp41 is responsible for mediating fusion between the viral and cellular membranes (Chan *et al*, 1997).

5.1.2 Routes of transmission

HIV was identified as the etiological agent responsible for the onset of acquired immunodeficiency syndrome (AIDS) (Barre-Sinoussi *et al*, 1983; Gallo *et al*, 1984; Wong-Staal and Gallo, 1985). The human immunodeficiency virus is divided into two types, type 1 and 2. Type-1 HIV is implicated in the worldwide AIDS epidemic whereas HIV-2 is found predominantly in West Africa (Kanki *et al*, 1986). Both of these viruses are responsible for immunodeficiency in humans, although HIV-2 may be less virulent (Wong-Staal, 1990).

A central African origin for HIV-1 has been suggested and confirmed with the identification of its primary reservoir (Zhu *et al*, 1998; Gao *et al*, 1999). The common chimpanzee *Pan troglodytes* is believed to be the source of at least three independent introductions of SIVcpz to the human population (Gao *et al*, 1999) leading to the establishment of the three recognized groups within HIV-1 namely group M (main), O (outlier) and N (non-M/non-O) (Montavon *et al*, 1999; Simon *et*

al, 1998). Group M is further divided into several subtypes (A-D, F1, F2, G, H, J, K) present in different geographic regions of the world and is the main driving force behind the AIDS epidemic (Fig. 1.2).

The virus is most commonly transmitted through unprotected sexual contact with an infected individual. In the early 1980's when little was known about the virus many hemophiliacs were inadvertently infected after receiving contaminated blood or blood products. Since then a lot has been done to ensure the safety of these products, cutting down on the risk of infection. The virus can however still be transmitted through blood amongst intravenous drug abusers who are infected and do share needles. The virus can also be transmitted from an infected mother to her child either during pregnancy, birth or breast feeding (Hirsch and Curren, 1990). Recently however it has been shown that the antiretroviral drug nevirapine (NVP) given to an HIV-infected woman in labor and to her baby within three days of birth could significantly reduce the risk of transmission to the infant (Guay *et al*, 1999).

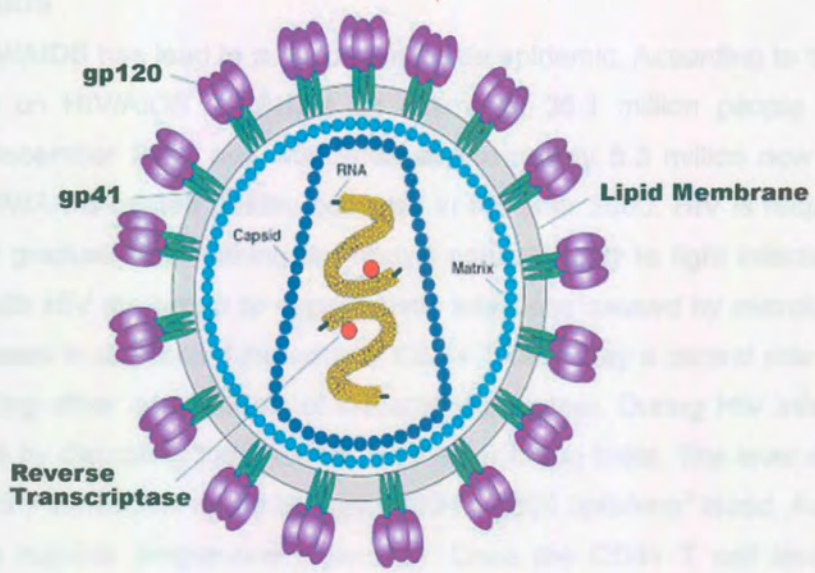

Fig. 1.1 The organization of the HIV virion.
Illustration obtained from the NIAID website.

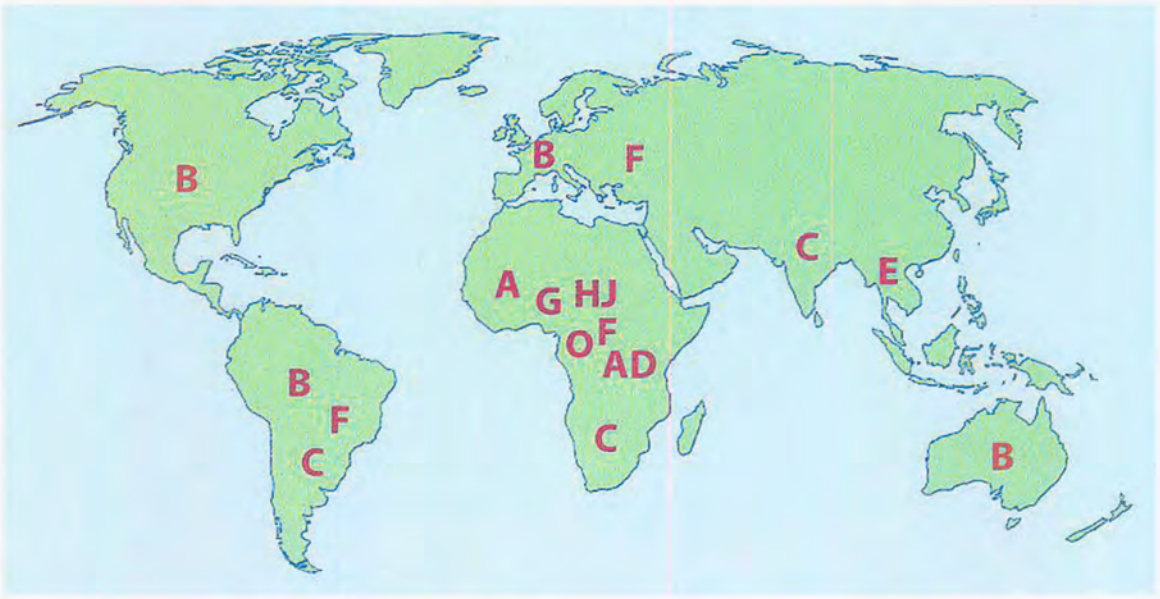

Fig. 1.2 The worldwide distribution of HIV-1 subtypes.
Illustration obtained from the NIAID website.

5.1.3 HIV and AIDS

The spread of HIV/AIDS has lead to a major worldwide epidemic. According to the Joint United Nations Program on HIV/AIDS (UNAIDS) an estimated 36.1 million people are living with HIV/AIDS from December 2000 and worldwide approximately 5.3 million new HIV infections and 3.0 million HIV/AIDS-related deaths occurred in the year 2000. HIV is responsible for the onset of AIDS by gradually diminishing the body's natural ability to fight infection. As a result people infected with HIV are prone to opportunistic infections caused by microbes that usually do not cause disease in uninfected individuals. CD4+ T cells play a central role in the immune response, regulating other components of the immune system. During HIV infection the virus targets these cells by disrupting their normal function or killing them. The level of CD4+ T cells in uninfected healthy individuals range between 800-to 1,200 cells/mm^3 blood. As a result of the HIV infection this number progressively declines. Once the CD4+ T cell level drops below 200/mm^3 the individual becomes particularly susceptible to the opportunistic infections that can ultimately result in death (Sterling *et al*, 2001). There is a wide spectrum of diseases associated with people living with AIDS, some of the general afflictions are infections of the lungs, intestinal tract, brain, eyes and other organs, as well as weight loss, diarrhea, neurological conditions and cancers such as Kaposi's sarcoma and certain types of lymphomas.

5.2 HIV vaccines

There exists an obvious need for the development of an effective prophylactic vaccine against HIV. Up till now the only recourse against HIV/AIDS has been the use of antiretroviral therapy to reduce the risk of infection and to control disease progression. These drugs are not capable of clearing the virus from the body and generally result in other health complications. It is also extremely expensive making it unaffordable for developing countries where the epidemic is raging out of control. The use of a prophylactic vaccine in conjunction with risk behavior education will certainly be the most effective approach to fight the epidemic.

The exact nature of the protective immunity required against HIV is one of the difficulties facing scientists tasked with developing a vaccine. It is generally accepted that a successful immunization strategy against HIV must be able to induce systemic as well as mucosal immunity including both humoral and cell-mediated responses against the virus (Morris *et al*, 2000). It has been reported that the induction of anti HIV-1 CD8+ CTL and Th1 cytokine responses against HIV-1 is most desirable (Gorse *et al*, 2001). CD8+ CTL activity during early HIV-1 infection has been associated with viral clearance and long-term non-progression of HIV infection (Borrow *et al*, 1994) whereas cytokine response to HIV-1 antigens may influence resistance to HIV-1 infection (Clerici *et al*, 1994).

5.2.1 The current status of HIV vaccine development

Many of the vaccine strategies discussed previously are employed for the development of a HIV vaccine. Much has been learned so far from HIV vaccine research. i) Neutralizing antibodies protect chimpanzees against laboratory adapted HIV strains (reviewed by Klein, 1999). ii) Strong T-cell responses are detected in long-term non-progressors and highly exposed seronegative individuals (Brander and Walker, 1999). iii) There seem to be some evidence pointing to the protective role of mucosal immunity (Mazzoli *et al*, 1997). So far more than sixty phase i and ii trials of thirty candidate vaccines has been initiated. Some of the candidate vaccines tested has proven to be safe, well tolerated and immunogenic (for review see Esparza and Bhamarapravati, 2000; Klein 2001). There is some disagreement amongst scientists regarding phase iii HIV trials. It is uncertain what type and level of immune response the candidate vaccine should induce (Desai *et al*, 1999). Never the less two phase iii trials were launched in the USA in 1998 (Francis *et al*, 1998) and in Thailand in 1999 (Berman *et al*, 1999). The candidate vaccines (AIDSVAX) are based on different envelope protein antigens in alum formulations and is derived from the gp120 envelope proteins of HIV-1 subtypes B and E. The vaccines were tested in chimpanzees and shown to be safe as well as effective in inducing strong neutralizing antibody and memory responses (Francis *et al*, 1998). Both of the trials aim

to test the efficacy of the vaccine in inducing protection against infection and disease. The vaccines are tested in populations with a high HIV incidence involving several thousand volunteers. For ethical reasons these trials are conducted as double blind placebo controlled trials where the volunteers also benefit from risk reduction interventions (Francis *et al*, 1998). Preliminary results indicating the efficacy of the candidate vaccines should be available soon (Migasena *et al*, 2000).

Despite best efforts there remains a need for the development of new HIV vaccine strategies. Most of the effort is aimed at developing a vaccine against HIV-1 subtype B. If one considers that 55% of all HIV-1 infections are due to in subtype C it becomes evident that a lot remains to be done, particularly as it is uncertain to what extend cross protection may be afforded by the candidate vaccines currently tested at phase iii (Esparza and Bhamarapravati, 2000). The following sections briefly review some examples of HIV vaccine strategies and current approaches to HIV vaccine development.

5.2.2 Subunit based vaccines

The first generation of HIV subunit vaccines was based on the use of monomeric gp120 and gp160 envelope proteins (Schwartz *et al*, 1993; Belshe *et al*, 1993). Studies showed that this strategy was not able to induce protective immunity as vaccine trials demonstrated that the envelope proteins alone could not confer protection, despite the induction of in vitro neutralizing antibodies (Kahn *et al*, 1995; McElrath *et al*, 1996). The use of oligomeric envelope proteins was also investigated. It was shown that the modification of oligomeric envelope vaccines to increase the exposure of conserved neutralizing epitopes might however be necessary for it to be an effective approach (Barnett *et al*, 2001). An oligomeric HIV-1 IIIB ENV protein gp140 vaccine although inducing strong neutralizing antibody responses only lead to modest neutralization of laboratory adapted isolates (Earl *et al*, 2001). These results casts some doubt on the efficacy of envelope based vaccines.

Complex peptides or multi-epitope vaccines are showing a lot of promise for the development of powerful subunit based vaccines. In this approach long peptides are synthesized that contain multiple copies of one or more epitopes. With this strategy a remarkable increase in the immune response is obtained, there are numerous putative HIV vaccines in development based on this principle (Lu *et al*, 2000; Chen *et al*, 1999; Liao *et al*, 2000). The multi-epitope vaccine approach is discussed in more detail as part of the introduction in Chapter 3. Many other HIV peptide vaccines are also under investigation, ranging in different target epitopes, in an effort to

induce both mucosal and systemic, humoral (Raya *et al*, 1999; Boykins *et al*, 2000; Kato *et al*, 2000; Toledo *et al*, 2001; Wang *et al*, 1991) and cell-mediated immune responses (Morris *et al*, 2000; Elliot *et al*, 1999; Peter *et al*, 2001; Sarin *et al*, 1999).

The development of virus-like particle vaccine strategies was discussed previously. Several studies have aimed at employing some of these particles to present HIV-1 epitopes. Hepatitis B core particles expressing HIV-1 gp41 and p34 pol antigens were successful in inducing a T-cell response (Isaguliants *et al*, 1996). The yeast retrotransposon protein Ty also form virus-like particles and was able to induce both humoral and cellular immune responses in humans to p24 (Kingsman *et al*, 1995). Another particulate vector strategy is aimed at developing non-infectious SIV or HIV virus-like particles as possible vaccines. SIV (gag) virus-like particles presenting the complete HIV-1 gp120 envelope protein induced neutralizing antibodies and cell mediated immunity, resulting in viral clearance in rhesus macaques (Notka *et al*, 1999). There are several other studies using similar approaches reporting on the promising potential of VLP vaccines (Tobin *et al*, 1997; Klavinskis *et al*, 1996; Schrimbeck *et al*, 1995).

5.2.3 Viral based vaccines

The HIV NEF protein has been implicated in several viral functions. i) It is responsible for the high replication rate of HIV by enhancing reverse transcription (Harris, 1994). ii) The expression of the NEF gene also enhances HIV pathogenicity (Harris, 1999) and iii) it also down-regulates the cell surface expression of $CD4^+$ receptors (Ross *et al*, 1999). High levels of $CD4^+$ may interfere with the production and budding of new infectious particles. There has been evidence to suggest that HIV strains lacking the NEF gene appear to delay the onset of AIDS (Desrosiers, 1992; Kestler *et al*, 1991). As a result these viruses were tested as a possible vaccine and proved promising in adult Rhesus monkeys (Daniel *et al*, 1992). However further studies undertaken on NEF deficient HIV strains all suggest that it is unsafe and not suitable for vaccination as oral immunization of new born macaques proved to be pathogenic (Baba *et al*, 1995) and several humans accidentally infected with a NEF deficient strain also developed AIDS related symptoms fifteen years later (Greenough *et al*, 1999).

Recombinant viral vectors delivering HIV-1 epitopes to the immune system is an approach that holds a lot of promise. A candidate HIV vaccine based on the use of recombinant vaccinia virus expressing a chimeric HIV-1 glycoprotein on its surface has demonstrated the usefulness of this approach by proving to be highly immunogenic (Katz *et al*, 1997). Several other vectors presenting HIV-1 epitopes have since been developed; most focused on eliciting CTL

responses (McInerney *et al*, 1999; De Berardinis *et al*, 1999; Letvin *et al*, 2001). Some of the typical vectors used are canarypox virus, Sinbis, Semliki forest virus and Venezuelan equine encephalitis (Frolov *et al*, 1996; Berglund *et al*, 1998; Calley *et al*, 1999).

5.2.4 Prime boost strategies

Combining different vaccine strategies will likely be the most effective approach for the development of an effective HIV vaccination strategy. A prime boost strategy using recombinant canarypox virus, expressing several HIV-1 gene products in combination with recombinant gp120 subunit vaccine as a booster showed promising results (Gorse *et al*, 2001). Combination of the two vaccines produced higher levels of cytokine response to the HIV-1 antigens than the canarypox vaccine alone. Phase i and ii trials of the prime boost strategy using recombinant canarypox virus has shown to be safe well tolerated and capable of inducing humoral and cellular immune responses in a high percentage of volunteers (Tartaglia *et al*, 1998; Verrier *et al*, 2000). Other prime boost strategies rely on combining DNA and peptide vaccines and also show some promise (Yoshizawa *et al*, 2001; Sandberg *et al*, 2000; Calarota *et al*, 2001).

5.2.5 HIV and mucosal immunity

HIV is sexually transmitted at the mucosal sites. The realization of the importance of mucosal immunity against HIV infection has driven a lot of research on the induction of mucosal immunity. The problem with subunit vaccines applied to mucosal surfaces, is that it is poorly immunogenic. Naturally the use of adjuvants is investigated as a means to overcome this problem, with mixed results (Morris *et al*, 2000; McCormack *et al*, 2000). A promising approach is the use of genetically detoxified heat-labile enterotoxin produced by some enterotoxigenic strains of *E.coli* as a mucosal adjuvant (Clements *et al*, 1988; Dickinson *et al*, 1995). The use of this adjuvant greatly increased systemic and mucosal antibody and CTL responses to an oligomeric gp160 vaccine (Morris *et al*, 2000). Several strategies has been employed in an attempt to induce mucosal immunity, for instance the use of attenuated bacteria like *Shigella* and *Salmonella* as HIV-1 antigen delivery systems (Sizemore *et al*, 1997; Darj *et al*, 1997), pox based viruses (Belyakov *et al*, 1999), encapsulated DNA (Kaneko *et al*, 2000) and multi-component peptide vaccines (Bukawa *et al*, 2000).

AIMS AND STRATEGY OF THIS STUDY

- To investigate the effect on solubility and particle formation of a specific amino acid substitution in the bottom domain of AHSV9 structural protein VP7.

    - Introduction of a site specific mutation into VP7 and insertion mutants via PCR
    - Expression of newly constructed mutant genes in baculovirus expression system
    - Solubility and electron microscopic analysis of expressed proteins

- To determine the ability of VP7 insertion mutant crystalline particles to present a linear HIV neutralizing epitope to the murine immune system in a manner that will elicit an epitope specific antibody response

    - Cloning of DNA adapters coding for the linear epitope into insertion mutants 144 and 177
    - Expression of newly constructed mutant genes in baculovirus expression system
    - Solubility and electron microscopic analysis of expressed proteins
    - Immunoblot analysis of serum obtained after inoculation of mice with the epitope expressing VP7 particles

CHAPTER 2

EFFECT OF SITE DIRECTED MUTATIONS IN THE MAJOR CORE PROTEIN VP7 OF AFRICAN HORSE SICKNESS VIRUS, ON SOLUBILITY AND PARTICLE FORMATION

2.1 INTRODUCTION

The usefulness of AHSV core-like particles as a vector for epitope presentation depends on the ability to produce large amounts of these particles when VP7 is co-expressed with VP3. In this regard the extreme insolubility of VP7 poses a problem for the development of core-like particle vectors. The majority of the VP7 trimers aggregate into crystals when co-expressed with VP3, resulting in a low core-like particle yield (Maree, 2000). To overcome this obstacle it is necessary to significantly increase the solubility of VP7 in an effort to increase the yield. It has been suggested that the concentration of VP7 trimers in solution is the driving force behind core-like particle formation (Le Blois and Roy, 1993).

The VP7 protein of bluetongue virus is completely soluble. Experiments involving domain switching between the VP7 proteins of BTV and AHSV led to the identification of several amino acids possibly involved in the observed difference in solubility between the proteins (Monastyrskaya *et al*, 1997). A non-polar uncharged residue present in the ninth C-terminal helix of the bottom domain, namely Leu 345 was one of the amino acids identified as possibly contributing to the insolubility of AHSV VP7.

Single amino acid substitutions were shown to have a drastic effect on protein solubility (Limn *et al*, 2000, Zhao and Somerville, 1992). In order to investigate the effect of Leu 345 on solubility of VP7 the amino acid was substituted by a polar positively charged arginine residue, the BTV equivalent at position 345. As we are interested in developing the core-like particles as vectors, the substitutions were made not only in the wild type VP7 but also in insertion mutants 177 and 200. The aims of this chapter were to, i) introduce the site-specific amino acid substitution in the target genes via a PCR based strategy. ii) To determine what effect the mutation had on the solubility of the expressed VP7 mutant proteins.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Restriction endonucleases, Rnase, T4 DNA ligase, Klenow polymerase, dNTPs, DNA molecular weight marker II (MW II) and the High Pure™ DNA Plasmid and PCR product purification kits were purchased from Boehringer Mannheim. *Hae*III digested ϕX 174 DNA were obtained from Promega. The Geneclean™ DNA purification kit was purchased from Bio 101 Inc. Rainbow™ protein molecular weight marker was bought from Amersham. Cellfectin™, oligonucleotide primers, TaKaRa Ex Taq™ polymerase and the BAC-TO-BAC™ baculovirus expression system were obtained from Gibco BRL (Life technologies). Grace's insect medium and fetal calf serum were obtained from Highveld Biological. Amplitaq^R DNA polymerase FS was supplied in the ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer). All other chemicals were purchased from Merck and Sigma. The NERC Institute of Virology and Environmental Microbiology, Oxford, UK supplied Sf9 insect cells. The pBS-S7, pFB-S7mt177 and pFB-S7mt200 plasmid vectors were obtained from Dr. F.F. Maree (UP).

2.2.2 Restriction endonuclease digestions

Restriction endonuclease digestions were performed for the preparation of DNA fragments during cloning procedures or as a method to characterize newly constructed recombinant DNA clones, the manufacturers (Boehringer Mannheim) specifications were followed for the enzymes concerned. Digestion products were analyzed on 0.7 – 1 % agarose gels, with the applicable controls and molecular weight size markers.

2.2.3 Klenow reaction

In some instances for the purpose of the cloning strategy, sticky ends generated by restriction enzyme digestion were filled in a Klenow reaction. Klenow polymerase (2U/1µl) and 4µl of a 0.5mM dNTP mix was added to a 30µl restriction reaction, the volume was adjusted to 40µl with UHQ and incubated for 40 minutes at 37°C.

2.2.4 Recovery of DNA fragments from agarose

During all cloning procedures, DNA fragments of interest were recovered from agarose gels via the Geneclean™ II kit (Bio101). Excised gel slices containing DNA were melted at 45-55°C in 2.5 volumes 3M NaI. After the addition of 5µl ice cold glasmilk suspension, the DNA was left to bind to the glasmilk by gentle agitation for 15min. The mix was then incubated on ice for a further 3min. After pelleting the glasmilk and bound DNA, the pellet was washed three times with 500µl New Wash (NaCl, Tris, EDTA, Ethanol, water). The DNA was eluted twice in UHQ at 45-55°C for 8min to a final volume of 16µl.

2.2.5 Ligation

Ligation reactions were set up according to the specifications provided by the manufacturer (Boehringer Mannheim). Typically reactions were done in 15µl with 1 unit T4 DNA ligase in the appropriate ligation buffer, at 16°C for sticky end or 22°C for blunt end ligations.

2.2.6 Preparation of competent *E.coli* cells

To prepare competent *E.coli* cells the standard calcium chloride method described by Cohen *et al.* (1972) was used. *E.coli* XI1Blue cells were used for all transformations of pFastbac and pBS vectors. An overnight *E.coli* cell culture was used to inoculate 50ml Lauria-Bertani (LB) medium (1% tryptone (w/v), 0.5% yeast (w/v), 1% NaCl (w/v)) and the cells were grown to logarithmic (log) phase (OD_{550} = 0.5) by incubation at 37°C with shaking. Cells were collected by centrifugation (5000rpm, 5min, 4°C), and gently resuspended in half the original volume of ice cold freshly prepared 50mM $CaCl_2$, again collected by centrifugation and resuspended in $^1/_{20}$ of the original volume of 50mM $CaCl_2$. The cells were kept on ice for 1h before use, or immediately frozen away at -70°C after addition of sterile glycerol (15%v/v).

2.2.7 Transformation of competent cells

Plasmid DNA was added to 100µl competent cells and allowed to adsorb for 30min on ice. The cells were then subjected to a heat shock at 42°C for 90s and cooled on ice for 2min, before adding 900µl LB and incubating at 37°C with shaking for 1h. Aliquots of 100µl were plated out with 50µl 2% 5-bromo-4chloro-3indolyl-β-D-galactopyranoside (X-gal) in dimethylformamide and 10µl 100mM isopropyl-β-D-thiogalactopyranoside (IPTG) when relevant on LB agar plates (1.2% agar in LB medium) containing the appropriate antibiotics (12.5µg/ml tetracycline hydrochloride (tet) or 100µg/ml ampicillin (amp)). Plates were incubated overnight at 37°C. Colonies of the desired phenotype were picked and grown overnight in 3ml LB medium with the appropriate antibiotics.

2.2.8 DNA isolation and purification

The alkaline lysis method (Sambrook *et al,* 1989), first described by Birnboim and Doly (1979), was used to extract plasmid DNA. Small scale extractions were performed using small cultures. Selected colonies were used to inoculate 3ml LB broth supplemented with the appropriate antibiotics and grown overnight. Cells were harvested by centrifugation at 10000g for 1 minute. The pellets were resuspended in 100µl of solution 1(50mM glucose, 10mM EDTA, 25mM Tris (pH 8.0) and incubated at room temperature for 5 min. Complete lysis of the cells and denaturation of DNA was achieved by adding 200µl 0.2M NaOH, 1% SDS and placing the

tubes on ice for 5 min. Genomic DNA, high molecular weight RNA and protein were precipitated by the addition of 150µl 3M NaAc (pH 4.8). After 10 min on ice, cell debris and precipitates were pelleted by centrifugation at 15000rpm (10min, 4°C). The DNA was precipitated from the supernatant in two volumes 96% ethanol for 30min. After the final centrifugation and 80% ethanol wash step the DNA was resuspended in UHQ.

2.2.9 Polymerase chain reaction

The site-specific mutation was introduced into the gene of interest with the use of a polymerase chain reaction. The reactions were set up to a final volume of 50µl; 10ng DNA template, 1µl (500ng) of each primer (table 2.1), 5µl 10 x TaKaRa Ex Taq™ polymerase buffer (20mM $MgCl_2$, 500mM KCl, 250mM TAPS pH 9.3, 10mM 2-mercaptoethanol), 5µl 2,5mM dNTP mix, 0.5µl (5U) TaKaRa Ex Taq™ polymerase (Life technologies). The reactions were performed in an ABI thermal cycler 9600 PCR machine. The conditions were as follows, 1 cycle 94°C, 3min. 25 cycles; 93°C, 45sec, 58°C, 2min, 72°C, 2min; 1 cycle 72°C, 10min.

The amplified PCR products were recovered from the respective reaction mixtures using the High pure™ PCR product purification kit (Boehringer Mannheim). The purified products were used for the subsequent cloning procedures.

Table 2.1. A summary of primers used in the construction of the site-specific mutants.

FUNCTION	POSITON AND POLARITY	OLIGONUCLEOTIDE SEQUENCE
Insertion of site specific mutation	1047-1052 [-], point mutation	5'-CAG**CCCGGG**CAGCCTACCACTAGTGGC-3'
	1047-1052 [+], point mutation	5'- CAG**CCCGGG**CAAGAGCTCTGTTGATCT-3'
Sequencing (External)	[+], VP7, 5' end-specific	5'-CACAGATCTTTCGGTTAGGATGGACGCG-3'
	[-], VP7, 3' end-specific	5'-CACAGATCTGTAAGTGTATTCGGTATTGAC-3'
(Internal)	[+], VP7, nt 548-549	5'-GCTCTAGAGTCGACAGGGGGGACGCAGTCATG-3'

2.2.10 Nucleotide sequence determination

All DNA templates sequenced were purified with the High pure™ plasmid purification kit (Boehringer Mannheim) according to the manufacturers instructions. DNA Concentrations were calculated from absorbency readings obtained with a Beckman DU®64 spectrophotometer at 260nm, and factoring in the extinction coefficient for dsDNA ($1A_{260}$ = 50µg/µl).

All sequence reactions were done using the ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) in an ABI thermal cycler 9600. Briefly, half reactions (10µl) were set up as follows, 1µl (250ng) DNA template, 2µl (3.2pmol) primer of interest, 4µl Terminator Ready Reaction Mix and 3µl UHQ. PCR conditions, (x25) 96°C, 10 sec, 50°C, 45sec, 60°C, 4 min.

Unincorporated dye terminators were removed by ethanol precipitation. 1µl 3M NaAc, 25µl 98% ethanol was added to the 10µl reaction and left on ice for 10 min. The reaction was then centrifuged for 30 min at 13000 rpm on a standard tabletop centrifuge. The pellet was washed in 100µl 80% ethanol, recentrifuged for 20 min at 13000 rpm and dried in a vacuum dryer. The sequencing reactions were analyzed using an ABI PRISM 377 sequencer. Dried samples were resuspended in 3µl sequencing loading buffer (5:1 deionised formamide: 25mM EDTA pH 8 containing 50mg/ml dextran blue) prior to loading. Samples were denatured at 95°C for 2 min, 1.5µl was loaded onto a 4% denaturing polyacrylamide gel and run for 7h at 1.6kV. Sequences were analyzed using the ABI PRISM sequencing analysis™ program as well as the ABI PRISM Navigator™ program.

2.2.11 Cells and Media

Wild type and recombinant baculoviruses were propagated in *Spodoptera frugiperda* (Sf9) cells grown in suspension culture at 27°C in Grace's insect medium supplemented with 10% fetal calf serum and antibiotics (penicillin, streptomycin, fungizone).

2.2.12 Transposition

Recombinant pFB DNA was added, to 100µl competent DH10Bac cells. DH10Bac is an *E.coli* strain in which the baculovirus genome is propagated as a 135Kb plasmid. The mixture was left on ice for 30 minutes where after it was subjected to a 45 sec heat shock at 42°C. The mixture was left on ice for 2 minutes before adding 900µl pre-warmed LB medium without antibiotics. The samples were incubated for 4h at 37°C. After incubation 100µl of the mixture was plated out on agar plates containing Kanamycin (50µg/ml), Gentamycin (7µg/ml), Tetracycline

(10µg/ml), IPTG (40µg/ml) and X-gal (300µg/ml). The plates were then incubated for 48h at 37°C. White colonies were picked and used to inoculate 3 ml of LB medium with appropriate antibiotics. The mini cultures were incubated overnight at 37°C. The recombinant bacmid DNA was isolated from the DH10Bac cells with an alkaline lysis method that have been adapted for the isolation of large plasmid molecules (Amemiya *et al*, 1994).

2.2.13 Transfection

A six well TC plate was seeded with 1 x 10^6 Sf9 cells/ well in 2ml graces medium. The cells were allowed to attach to the bottom of the plate for an hour. Two solutions were prepared, one containing 6µl bacmid DNA and the other 6µl CELLFECTIN™, both in 100µl Grace's medium without antibiotics or serum. The two solutions were combined and left at room temperature for 45 minutes. The seeded cells were washed twice with 2ml Grace's medium without serum or antibiotics. Clean Grace's medium (800µl) was added to the lipid-DNA complexes. The wash media was removed from the cells and the DNA-lipid solutions were added to the cells. The transfection samples were incubated in a Tupperware™ container for 5h at 27°C, where after the transfection mixtures were replaced with 2ml complete Grace's medium containing serum and antibiotics. The samples were left to incubate for a further 96h. The medium containing the recombinant viruses were removed and stored at 4°C for further use.

2.2.14 Plaque purification and preparation of viral stock

Viral stocks of recombinant baculoviruses were prepared by inoculating 75cm^3 tissue cultures flasks (1 x 10^7 cells per flask) with 200µl of the transfection supernatant. After 3 days the supernatant was harvested and filter sterilized. To determine the viral titer of the recombinant baculoviruses, a six well TC plate was seeded with Sf9 cells at 1.4x10^6 cells/well and left on the workbench for one hour to allow the cells to attach. A dilution series of the recombinant viruses were prepared, ranging from 10^{-1} to10^{-9}. The medium was removed one well at a time and replaced by virus dilutions (10^{-4} to10^{-9}), dishes were left for 2h at room temperature. After two hours the viral dilutions were removed and 2ml cooled (37°C) agarose (2%)/ Grace's medium was carefully layered over the cells. Cells were left to incubate for 4 days at 28°C. In order to see the plaques, 1ml neutral red (1mg/ml in ddH_2O, diluted 10x with Grace's medium) was added to each well and left to incubate for 5 hours at 28°C. Where after the neutral red was removed and incubation continued over night. Plaques were visible as light red patches in a darker red background. The viral titer was calculated from the number of plaques at a given dilution.

2.2.15 Large scale expression and isolation of recombinant proteins

S. frugiperda cells in 75cm^3 tissue cultures flasks (1 x 10^7 cells per flask) were infected with recombinant baculoviruses at a MOI of 5 to 10 pfu/cell. The expressed protein was harvested 72 hours post infection, essentially as described by Basak *et al.* (1996). Briefly, the infected cells were harvested by low speed centrifugation (3000 rpm for 5 min) and the pellet washed in 1xPBS. The cells were resuspended in TNN lysis buffer (50mM Tris-HCL pH 8.0, 150mM NaCl, 0.5% Nonidet P-40) at 2 x 10^7 cells per ml and incubated on ice for 15 min. The expressed protein was recovered from the whole cell fraction by centrifugation on a sucrose density gradient, section 2.2.18.

2.2.16 SDS polyacrylamide gel electrophoresis

Prior to electrophoresis, protein samples were diluted 1:1 with 2 x protein solvent buffer (0.125M Tris-HCL pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol), boiled at 100°C for 10 min. Samples were then separated by 10 or 12% SDS-PAGE as described by Laemmli (1970). 0.75mm thick separating gels containing 0.375M Tris-HCL pH 8.8 and 0.1% SDS and 5% stacking gels containing 0.125M Tris-HCL pH 6.8 and 0.1% SDS were prepared from a stock of 30% acrylamide, 0.8% bisacrylamide. The gels were polymerized by the addition of 0.008% (v/v) tetra-methyl-ethylene-diamine (TEMED) and 0.08% (w/v) ammonium peroxysulfate. Electrophoresis was carried out in TGS buffer (0.025M Tris-HCL pH 8.3, 0.192M glycine, 0.1% SDS) in a Mighty Small II SE250 unit (Hoefer Scientific Instruments) for 2 hours at 120V.

2.2.17 Coomassie brilliant blue staining

Gels were stained in 0.125% coomassie brilliant blue and 50% methanol, after 20 min the gels were destained for as long as necessary to the visualize protein bands, in destaining solution (5% acetic acid, 5% methanol).

2.2.18 Solubility studies

For protein purification and analysis of solubility two different experiments were performed.

2.2.18.1 Ultracentrifugation

A whole cell fraction of the expressed protein was loaded onto a 40-70% (w/v) discontinuous sucrose gradient in 50mM Tris-HCL pH 8.0, 150mM NaCl. The sample was centrifuged at 40000 rpm for 20 hours at 4°C in a SW 50.1 Beckman rotor and fractions were taken from the bottom of the gradient. A sample of each fraction was taken for SDS PAGE analysis. The amount of expressed protein in each fraction was quantified from the polyacryl amide gel using

the Sigma Gel™ software package. Fractions in which the expressed protein was found was diluted (6:1) with 50mM Tris-HCL pH 8.0, 150mM NaCl, and recovered by centrifugation for 45min at 5000 rpm for electron microscopic analysis.

2.2.18.2 Differential centrifugation

Whole cell fractions were separated into a soluble and particulate fraction by differential centrifugation (5000rpm, 30min). The recovered particulate fraction was resuspended in 300µl (50mM Tris-HCL pH 8.0, 150mM NaCl). To recover the soluble protein from the supernatant 20%(v/v) of a saturated ammonium sulphate solution was added to the supernatant and left to incubate for 20min. The precipitated protein was recovered by centrifugation at 5000rpm for 45min. The pellet was also resuspended to a final volume of 300µl. Equal volumes of both fractions were loaded onto a polyacrylamide gel for analysis. Again the Sigma Gel™ software package was used to determine the relative amount of the protein of interest.

2.2.19 Electron microscopy

Pure VP7 particles were recovered from the sucrose fractions after density gradient centrifugation. These samples were fixed for 30 minutes in phosphate buffered 2.5% formaldehyde/0.1% gluteraldehyde at room temperature. After fixation the samples were filtered through a 0.22 µm nylon filter and washed three times with 0.075M Na_2HPO_4, waiting 10 minutes between each wash. The samples were then dehydrated with successive treatments of 50%, 70%, 90% and 100% (x3) ethanol. The filters were dried in a critical point dryer with liquid CO_2 and mounted onto an aluminum stub before being spatter coated with gold-beladium. The samples were viewed at 5kV in a JEOL 840 scanning electron microscope.

2.2.20 Light microscopy

Sf9 cells were grown on Esco™ cover slips that had previously been rinsed in 70% ETOH, flamed and placed in 35mm six well tissue culture plates (Nunclon™). After overnight subjection to UV-irradiation, each well was seeded with 1 x 10^6 cells. Cells were infected with recombinant baculoviruses expressing the different VP7 mutants at a MOI of 5pfu/cell. Uninfected Sf9 as well as wild type baculovirus infected cells were included as controls. The cover slips were removed 48hpi and placed on slides where after they were viewed on a Nikon light microscope.

2.3 RESULTS

The amino acid Leu 345 is located in the ninth C-terminal helix of VP7 and has been implicated in the observed insolubility of the protein (Monastyrskaya *et al*, 1997). To investigate what effect this amino acid has on the solubility of VP7 it was targeted for site-specific mutation. The bluetongue virus VP7 equivalent at this position is arginine, a polar positively charged residue. As a result of this charged amino acid, the C-terminal region of BTV VP7 is more hydrophilic than that of AHSV VP7 and this ultimately contributes to its complete solubility. The substitution of leucine to arginine at position 345 was made in wild type VP7 as well as in the insertion mutants 177 and 200. The mutants were included to determine if the substitution could further increase its solubility since an increase in solubility was previously reported for mutant 177 (Maree, 2000).

The site-specific mutation was introduced via a polymerase chain reaction with the use of two primers specifically designed to effect the change. The primers annealed with its 5' ends overlapping, and amplified the entire plasmid. The linear PCR product was subsequently cleaved with the appropriate restriction enzyme and then self ligated to reconstruct the gene with the site-specific mutation in place. To avoid problems associated with amplifying large amplicons, a strategy was devised whereby the target genes for mutation were first sub-cloned into a smaller vector before amplification (pBS 3.2kb). As another worker in our laboratory had already previously cloned VP7 into pBS and mutants 177 and 200 into pFB, only mutants 177 and 200 had to be sub-cloned into pBS (Maree, 2000). However due to the strategy employed to construct the pFB vectors it was not possible to recover the complete mutant VP7 genes, as the 5' ends were cloned into BglII sites with BamHI overhangs thus making recovery with either of these enzymes impossible (Maree, 2000). A different strategy had to be employed where an internal fragment of the VP7 mutant gene was cloned into a pBS vector already carrying the remainder of the VP7 gene, and thus reconstructing the complete gene.

2.3.1 Recloning VP7 mutants 177 and 200 into pBS

The pBS vector carrying VP7 was designated pBS-S7 (4.3kb) and used to obtain a vector fragment for the sub-cloning procedure (Fig. 2.0a). pBS-S7 was subjected to complete BamHI and HindIII digestion, producing two DNA fragments of 3.3 and 1kb in size (Fig. 2.1a). The BamHI and HindIII sites are respectively located 100bp downstream from the 5' end in the VP7 gene and several bases downstream from the 3' end in the MCS of the vector. The two pFB vectors were designated pFB-S7mt177 and pFB-S7mt200 and used as templates for the recovery of the mutant gene fragments (Fig. 2.0b). As both insertion mutants have an internal cloning site (HindIII/XbaI/SaI, Maree, 2000) it was necessary to recover these gene fragments

through partial HindIII digestion. Both these vectors were subjected to complete BamHI and partial HindIII digestion, producing an array of different sized fragments most notably the gene fragment of interest (Fig. 2.1b). The 1kb DNA fragments were recovered from the agarose gel and cloned into the pBS vector fragment to reconstruct both mutants and thereby creating two new constructs designated pBS-S7mt177 and pBS-S7mt200. The integrity of the newly constructed clones was verified by restriction enzyme analysis (results not shown).

The strategy that was followed to introduce the site specific mutation required the design of two overlapping primers each with a SmaI recognition site that was used to reconstruct the gene after PCR. Since the pBS vectors already contained a SmaI site in its MCS (Fig. 2.0a) it was necessary to remove this site before PCR amplification. By digesting each of the pBS constructs with EcoRI and SmaI, a 4.3kb linear fragment was produced of which the sticky ends were filled in a klenow reaction followed by self ligation, this resulted in the loss of the entire MCS. New templates lacking the SmaI site were selected by digestion with SmaI and EcoRI and used as templates in the polymerase chain reaction (results not shown).

2.3.2 Introduction of the site specific mutation via PCR

To mutate codon 345 in the target genes, two 27 nt primers were designed that annealed to the templates with its 5' ends overlapping the target site. A part of the overlapping region of each primer coded for the recognition sequence of SmaI (5'- CCCGGG -3'). By inserting the SmaI site at nucleotide positions 1047 to 1052, the site-specific mutation was effected as codon 345 was now mutated (5'-CTA-3' $\rightarrow$ 5'-CCCGGG-3'). This change resulted in the substitution of Leu to Arg. Codon 344 was also subsequently mutated from Val to Ala as a result of the nucleotide substitution. The chemical characteristics of these residues are quite similar, consequently the effect of this mutation was considered to be negligible. The insertion of the SmaI site simplified the reconstruction of the mutated genes, as the amplified PCR products were merely digested with SmaI and then self ligated. Due to the size of the plasmid templates a high fidelity Taq polymerase was used to avoid the introduction of any non-specific mutations during the PCR. In each case a 4.3kb amplified product was produced (Fig. 2.2). The PCR products were purified before and after digestion with SmaI and then self ligated. The constructs that contained the SmaI insertion were designated pBS-S7L345R, pBS-S7mt177L345R and pBS-S7mt200L345R and sequenced to confirm that no unwanted changes were introduced.

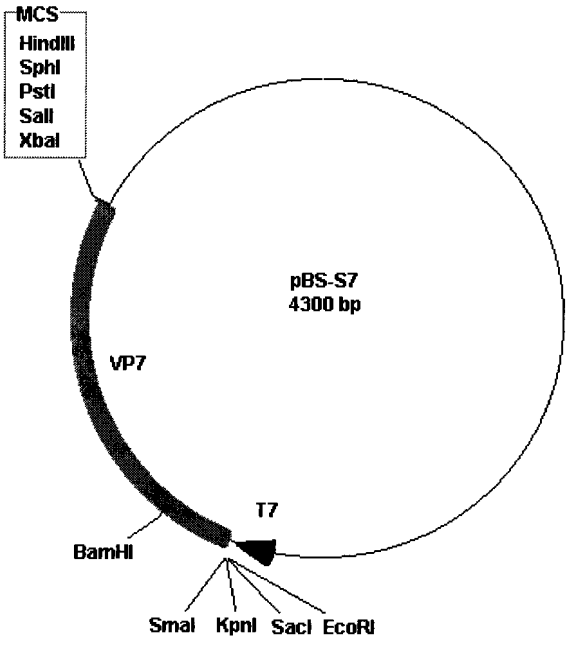

Fig. 2.0a A plasmid map of the pBS-S7 vector used in the recloning strategy of mutants 177 and 200, pBS-S7 was subjected to complete BamHI and HindIII digestion.

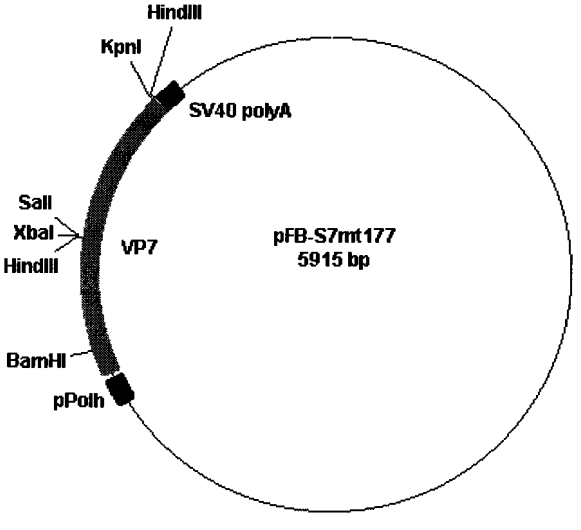

Fig. 2.0b In order to obtain the gene fragment from pFB-S7mt177, the plasmid was subjected to complete BamHI and partial HindIII digestion. The vector and insert fragments were ligated overnight to produce pBS-S7mt177. The same procedure was followed for the construction of pBS-S7mt200.

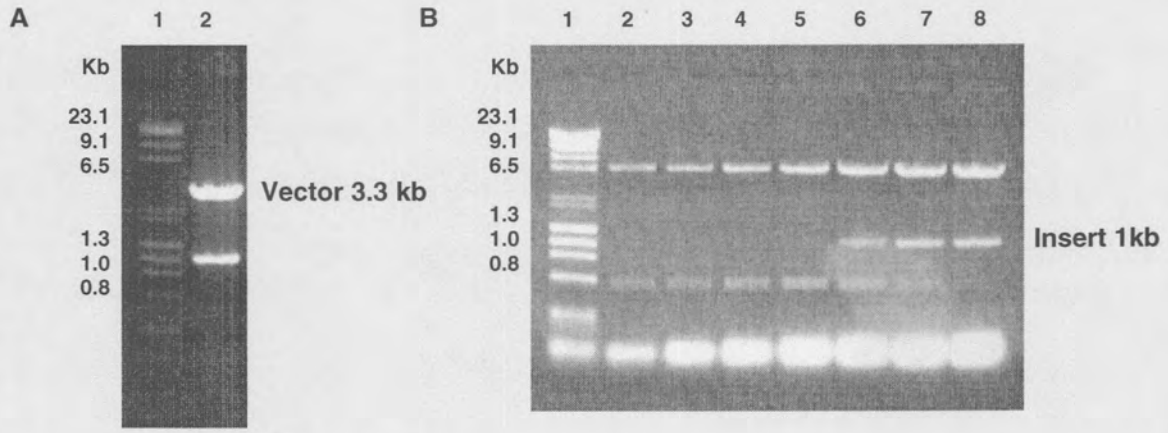

Fig 2.1 a) 1) Molecular weight size marker. 2) pBS-S7 digested with BamHI and HindIII. b) 1) Molecular weight size marker. 2-8) Complete BamHI, partial HindIII digestion of pFB-S7mt177. 0.5 U HindIII was serially diluted over seven digestion reactions and the target DNA digested for 1 hour.

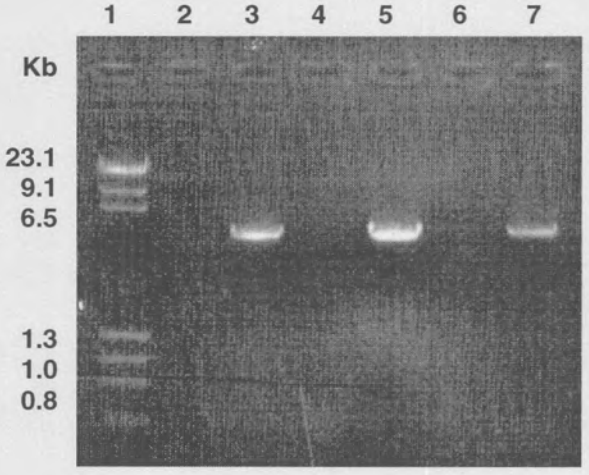

Fig 2.2 A 1% agarose gel showing the results of the polymerase chain reaction. 4.3Kb DNA fragments were amplified. 1) Molecular weight size marker. 2) Empty. 3) S7L345R-PCR. 4) Empty. 5) S7mt177L345R-PCR. 6) Empty. 7) S7mt200L345R-PCR

2.3.3 Nucleotide sequence determination

The nucleotide sequence was determined for each of the newly constructed mutant genes. This was done to verify the substitution of the target nucleotides. To this end an external forward primer binding to the 5' end of VP7 as well as an internal VP7 primer binding at nt position 548 was used (Table 2.1). From this, two overlapping sequences could be obtained for each mutant. As expected the leucine residue at position 345 was substituted to arginine, and valine at position 344 to an alanine. The complete nucleotide and deduced amino acid sequences of the open reading frame of each mutant is presented in figures 2.3 (a to d).

2.3.4 The baculovirus expression system

Bac-to-Bac™ is a eukaryotic expression system that utilises recombinant baculoviruses for heterologues gene expression. Recombinant baculoviruses are generated by incorporating the gene of interest under the control of a strong viral promoter. The gene is initially cloned into a transfer vector (pFastbac1, Fig 2.4), under the control of the polyhedrin promoter (section 2.3.5). DH10BAC™ cells are modified *E. coli* organisms that carry the viral genome as an extra chromosomal plasmid. The recombinant transfer vector is used in a transposition reaction where the gene and promoter is inserted into the 135Kb baculovirus genome through homologues recombination via the Tn7L and Tn7R regions present in both the vector and genome. The recombinant viral genome (Bacmid) is subsequently used for the generation of recombinant baculoviruses that can express the gene (sections 2.2.11 to 2.2.15).

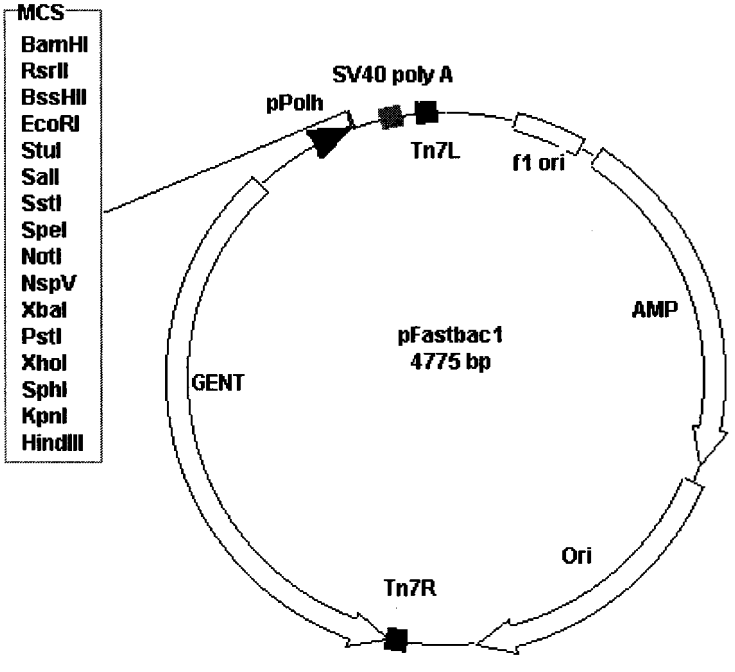

Fig 2.4 A map of the transfer vector pFastbac1 that was used for the expression of the L345R mutant genes. The plasmid map illustrates some of the important features of the vector.

```
   1 ATGGACGCGATACGAGCAAGAGCCTTGTCCGTTGTACGGGCATGTGTCACAGTGACAGAT   60
   1 M  D  A  I  R  A  R  A  L  S  V  V  R  A  C  V  T  V  T  D    20

  61 GCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGATTGCAATCAATAGGTAT  120
  21 A  R  V  S  L  D  P  G  V  M  E  T  L  G  I  A  I  N  R  Y    40

 121 AATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACCCAAGCAGAACGAAATGAA  180
  41 N  G  L  T  N  H  S  V  S  M  R  P  Q  T  Q  A  E  R  N  E    60

 181 ATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCGCTGAACGTCCAAATTGGGAATATT  240
  61 M  F  F  M  C  T  D  M  V  L  A  A  L  N  V  Q  I  G  N  I    80

 241 TCACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTCGCAACGACTGAAATTCCA  300
  81 S  P  D  Y  D  Q  A  L  A  T  V  G  A  L  A  T  T  E  I  P   100

 301 TATAATGTTCAGGCCATGAATGACATCGTTAGAATAACGGGTCAGATGCAAACATTCGGA  360
 101 Y  N  V  Q  A  M  N  D  I  V  R  I  T  G  Q  M  Q  T  F  G   120

 361 CCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAGGTGCAACAATCTGGCAGA  420
 121 P  S  K  V  Q  T  G  P  Y  A  G  A  V  E  V  Q  Q  S  G  R   140

 421 TATTACGTACCGCAAGGTCGAACGCGTGGTGGGTACATCAATTCAAATATTGCAGAAGTG  480
 141 Y  Y  V  P  Q  G  R  T  R  G  G  Y  I  N  S  N  I  A  E  V   160

 481 TGTATGGATGCAGGTGCTGCGGGACAGGTCAATGCGCTGCTAGCCCCAAGGAGGGGGGAC  540
 161 C  M  D  A  G  A  A  G  Q  V  N  A  L  L  A  P  R  R  G  D   180

 541 GCAGTCATGATCTATTTCGTTTGGAGACCGTTGCGTATATTTTGTGATCCTCAAGGTGCG  600
 181 A  V  M  I  Y  F  V  W  R  P  L  R  I  F  C  D  P  Q  G  A   200

 601 TCACTTGAGAGCGCTCCAGGAACTTTTGTCACCGTTGATGGAGTAAATGTTGCAGCTGGA  660
 201 S  L  E  S  A  P  G  T  F  V  T  V  D  G  V  N  V  A  A  G   220

 661 GATGTCGTCGCATGGAATACTATTGCACCAGTGAATGTTGGAAATCCTGGGGCACGCAGA  720
 221 D  V  V  A  W  N  T  I  A  P  V  N  V  G  N  P  G  A  R  R   240

 721 TCAATTTTACAGTTTGAAGTGTTATGGTATACGTCCTTGGATAGATCGCTAGACACGGTT  780
 241 S  I  L  Q  F  E  V  L  W  Y  T  S  L  D  R  S  L  D  T  V   260

 781 CCGGAATTGGCTCCAACGCTCACAAGATGTTATGCGTATGTCTCTCCCACTTGGCACGCA  840
 261 P  E  L  A  P  T  L  T  R  C  Y  A  Y  V  S  P  T  W  H  A   280

 841 TTACGCGCTGTCATTTTTCAGCAGATGAATATGCAGCCTATTAATCCGCCGATTTTTCCA  900
 281 L  R  A  V  I  F  Q  Q  M  N  M  Q  P  I  N  P  P  I  F  P   300

 901 CCGACTGAAAGGAATGAAATTGTTGCGTATCTATTAGTAGCTTCTTTAGCTGATGTGTAT  960
 301 P  T  E  R  N  E  I  V  A  Y  L  L  V  A  S  L  A  D  V  Y   320

 961 GCGGCTTTGAGACCAGATTTCAGAATGAATGGTGTTGTCGCGCCAGTAGGCCAGATTAAC 1020
 321 A  A  L  R  P  D  F  R  M  N  G  V  V  A  P  V  G  Q  I  N   340

1021 AGAGCTCTTGTGCTAGCAGCCTACCACTAG                                1050
 341 R  A  L  V  L  A  A  Y  H  *                                  350
```

Fig. 2.3a Nucleotide and amino acid sequence of wild type S7 (VP7).

```
   1 ATGGACGCGATACGAGCAAGAGCCTTGTCCGTTGTACGGGCATGTGTCACAGTGACAGAT    60
   1 M  D  A  I  R  A  R  A  L  S  V  V  R  A  C  V  T  V  T  D     20

  61 GCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGATTGCAATCAATAGGTAT   120
  21 A  R  V  S  L  D  P  G  V  M  E  T  L  G  I  A  I  N  R  Y     40

 121 AATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACCCAAGCAGAACGAAATGAA   180
  41 N  G  L  T  N  H  S  V  S  M  R  P  Q  T  Q  A  E  R  N  E     60

 181 ATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCGCTGAACGTCCAAATTGGGAATATT   240
  61 M  F  F  M  C  T  D  M  V  L  A  A  L  N  V  Q  I  G  N  I     80

 241 TCACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTCGCAACGACTGAAATTCCA   300
  81 S  P  D  Y  D  Q  A  L  A  T  V  G  A  L  A  T  T  E  I  P    100

 301 TATAATGTTCAGGCCATGAATGACATCGTTAGAATAACGGGTCAGATGCAAACATTCGGA   360
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 361 CCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAGGTGCAACAATCTGGCAGA   420
 121 P  S  K  V  Q  T  G  P  Y  A  G  A  V  E  V  Q  Q  S  G  R    140

 421 TATTACGTACCGCAAGGTCGAACGCGTGGTGGGTACATCAATTCAAATATTGCAGAAGTG   480
 141 Y  Y  V  P  Q  G  R  T  R  G  G  Y  I  N  S  N  I  A  E  V    160

 481 TGTATGGATGCAGGTGCTGCGGGACAGGTCAATGCGCTGCTAGCCCCAAGGAGGGGGGAC   540
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 541 GCAGTCATGATCTATTTCGTTTGGAGACCGTTGCGTATATTTTGTGATCCTCAAGGTGCG   600
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 601 TCACTTGAGAGCGCTCCAGGAACTTTTGTCACCGTTGATGGAGTAAATGTTGCAGCTGGA   660
 201 S  L  E  S  A  P  G  T  F  V  T  V  D  G  V  N  V  A  A  G    220

 661 GATGTCGTCGCATGGAATACTATTGCACCAGTGAATGTTGGAAATCCTGGGGCACGCAGA   720
 221 D  V  V  A  W  N  T  I  A  P  V  N  V  G  N  P  G  A  R  R    240

 721 TCAATTTTACAGTTTGAAGTGTTATGGTATACGTCCTTGGATAGATCGCTAGACACGGTT   780
 241 S  I  L  Q  F  E  V  L  W  Y  T  S  L  D  R  S  L  D  T  V    260

 781 CCGGAATTGGCTCCAACGCTCACAAGATGTTATGCGTATGTCTCTCCCACTTGGCACGCA   840
 261 P  E  L  A  P  T  L  T  R  C  Y  A  Y  V  S  P  T  W  H  A    280

 841 TTACGCGCTGTCATTTTTCAGCAGATGAATATGCAGCCTATTAATCCGCCGATTTTTCCA   900
 281 L  R  A  V  I  F  Q  Q  M  N  M  Q  P  I  N  P  P  I  F  P    300

 901 CCGACTGAAAGGAATGAAATTGTTGCGTATCTATTAGTAGCTTCTTTAGCTGATGTGTAT   960
 301 P  T  E  R  N  E  I  V  A  Y  L  L  V  A  S  L  A  D  V  Y    320

 961 GCGGCTTTGAGACCAGATTTCAGAATGAATGGTGTTGTCGCGCCAGTAGGCCAGATTAAC  1020
 321 A  A  L  R  P  D  F  R  M  N  G  V  V  A  P  V  G  Q  I  N    340

1021 AGAGCTCTTGCCCGGGCAGCCTACCACTAG                                  1050
 341 R  A  L  A  R  A  A  Y  H  *                                    350
```

Fig. 2.3b Nucleotide and amino acid sequence of S7-L345R.

```
   1 ATGGACGCGATACGAGCAAGAGCCTTGTCCGTTGTACGGGCATGTGTCACAGTGACAGAT   60
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  61 GCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGATTGCAATCAATAGGTAT  120
  21 A  R  V  S  L  D  P  G  V  M  E  T  L  G  I  A  I  N  R  Y    40

 121 AATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACCCAAGCAGAACGAAATGAA  180
  41 N  G  L  T  N  H  S  V  S  M  R  P  Q  T  Q  A  E  R  N  E    60

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 361 CCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAGGTGCAACAATCTGGCAGA  420
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 421 TATTACGTACCGCAAGGTCGAACGCGTGGTGGGTACATCAATTCAAATATTGCAGAAGTG  480
 141 Y  Y  V  P  Q  G  R  T  R  G  G  Y  I  N  S  N  I  A  E  V   160

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 181 R  V  D  R  G  D  A  V  M  I  Y  F  V  W  R  P  L  R  I  F   200

 601 TGTGATCCTCAAGGTGCGTCACTTGAGAGCGCTCCAGGAACTTTTGTCACCGTTGATGGA  660
 201 C  D  P  Q  G  A  S  L  E  S  A  P  G  T  F  V  T  V  D  G   220

 661 GTAAATGTTGCAGCTGGAGATGTCGTCGCATGGAATACTATTGCACCAGTGAATGTTGGA  720
 221 V  N  V  A  A  G  D  V  V  A  W  N  T  I  A  P  V  N  V  G   240

 721 AATCCTGGGGCACGCAGATCAATTTTACAGTTTGAAGTGTTATGGTATACGTCCTTGGAT  780
 241 N  P  G  A  R  R  S  I  L  Q  F  E  V  L  W  Y  T  S  L  D   260

 781 AGATCGCTAGACACGGTTCCGGAATTGGCTCCAACGCTCACAAGATGTTATGCGTATGTC  840
 261 R  S  L  D  T  V  P  E  L  A  P  T  L  T  R  C  Y  A  Y  V   280

 841 TCTCCCACTTGGCACGCATTACGCGCTGTCATTTTTCAGCAGATGAATATGCAGCCTATT  900
 281 S  P  T  W  H  A  L  R  A  V  I  F  Q  Q  M  N  M  Q  P  I   300

 901 AATCCGCCGATTTTTCCACCGACTGAAAGGAATGAAATTGTTGCGTATCTATTAGTAGCT  960
 301 N  P  P  I  F  P  P  T  E  R  N  E  I  V  A  Y  L  L  V  A   320

 961 TCTTTAGCTGATGTGTATGCGGCTTTGAGACCAGATTTCAGAATGAATGGTGTTGTCGCG 1020
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1021 CCAGTAGGCCAGATTAACAGAGCTCTTGCCCGGGCAGCCTACCACTAG              1068
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```

Fig. 2.3c Nucleotide and amino acid sequence of S7mt177L345R. Amino acids coding for the insertion site 177 inserted previously are underlined.

```
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  61 GCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGATTGCAATCAATAGGTAT  120
  21 A  R  V  S  L  D  P  G  V  M  E  T  L  G  I  A  I  N  R  Y    40

 121 AATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACCCAAGCAGAACGAAATGAA  180
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 181 ATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCGCTGAACGTCCAAATTGGGAATATT  240
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 241 TCACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTCGCAACGACTGAAATTCCA  300
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 101 Y  N  V  Q  A  M  N  D  I  V  R  I  T  G  Q  M  Q  T  F  G   120

 361 CCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAGGTGCAACAATCTGGCAGA  420
 121 P  S  K  V  Q  T  G  P  Y  A  G  A  V  E  V  Q  Q  S  G  R   140

 421 TATTACGTACCGCAAGGTCGAACGCGTGGTGGGTACATCAATTCAAATATTGCAGAAGTG  480
 141 Y  Y  V  P  Q  G  R  T  R  G  G  Y  I  N  S  N  I  A  E  V   160

 481 TGTATGGATGCAGGTGCTGCGGGACAGGTCAATGCGCTGCTAGCCCCAAGGAGGGGGGAC  540
 161 C  M  D  A  G  A  A  G  Q  V  N  A  L  L  A  P  R  R  G  D   180

 541 GCAGTCATGATCTATTTCGTTTGGAGACCGTTGCGTATATTTTGTGATCCTCAAGGTGCG  600
 181 A  V  M  I  Y  F  V  W  R  P  L  R  I  F  C  D  P  Q  G  A   200

 601 AAGCTTTCTAGAGTCGACTCACTTGAGAGCGCTCCAGGAACTTTTGTCACCGTTGATGGA  660
 201 K  L  S  R  V  D  S  L  E  S  A  P  G  T  F  V  T  V  D  G   220

 661 GTAAATGTTGCAGCTGGAGATGTCGTCGCATGGAATACTATTGCACCAGTGAATGTTGGA  720
 221 V  N  V  A  A  G  D  V  V  A  W  N  T  I  A  P  V  N  V  G   240

 721 AATCCTGGGGCACGCAGATCAATTTTACAGTTTGAAGTGTTATGGTATACGTCCTTGGAT  780
 241 N  P  G  A  R  R  S  I  L  Q  F  E  V  L  W  Y  T  S  L  D   260

 781 AGATCGCTAGACACGGTTCCGGAATTGGCTCCAACGCTCACAAGATGTTATGCGTATGTC  840
 261 R  S  L  D  T  V  P  E  L  A  P  T  L  T  R  C  Y  A  Y  V   280

 841 TCTCCCACTTGGCACGCATTACGCGCTGTCATTTTTCAGCAGATGAATATGCAGCCTATT  900
 281 S  P  T  W  H  A  L  R  A  V  I  F  Q  Q  M  N  M  Q  P  I   300

 901 AATCCGCCGATTTTTCCACCGACTGAAAGGAATGAAATTGTTGCGTATCTATTAGTAGCT  960
 301 N  P  P  I  F  P  P  T  E  R  N  E  I  V  A  Y  L  L  V  A   320

 961 TCTTTAGCTGATGTGTATGCGGCTTTGAGACCAGATTTCAGAATGAATGGTGTTGTCGCG 1020
 321 S  L  A  D  V  Y  A  A  L  R  P  D  F  R  M  N  G  V  V  A   340

1021 CCAGTAGGCCAGATTAACAGAGCTCTTGCCCGGGCAGCCTACCACTAG              1068
 341 P  V  G  Q  I  N  R  A  L  A  R  A  A  Y  H  *                360
```

Fig. 2.3d Nucleotide and amino acid sequence of S7mt200L345R. Amino acids coding for the insertion site 200 inserted previously are underlined.

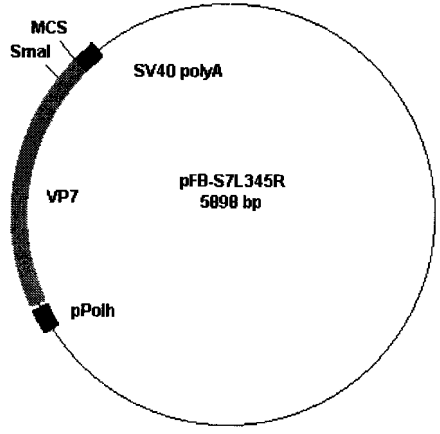
2.3.5 The construction of L345R mutant pFastbac1 transfer vectors

In order to express the L345R mutants in the baculovirus expression system, it was necessary to insert each of the genes into a pFastbac1 expression vector. The strategy used was similar to that previously described for the construction of pBS-S7-mt177. Shortly, a 4.9kb vector fragment was produced by complete digestion of a pFB-S7 template (5.9kb) with BamHI and HindIII. The 1kb gene fragment was recovered from pBS-S7L345R via complete BamHI and HindIII digestion, where as the pBS-S7mt177L345R and pBS-S7mt200L345R fragments were recovered by complete BamHI and partial HindIII digestion. The gene fragments were cloned into the vector fragment to produce three new constructs, namely pFB-S7L345R, pFB-S7mt177L345R and pFB-S7mt200L345R (Fig. 2.5). The integrity of the new constructs was verified via SmaI digestion (Fig. 2.6).

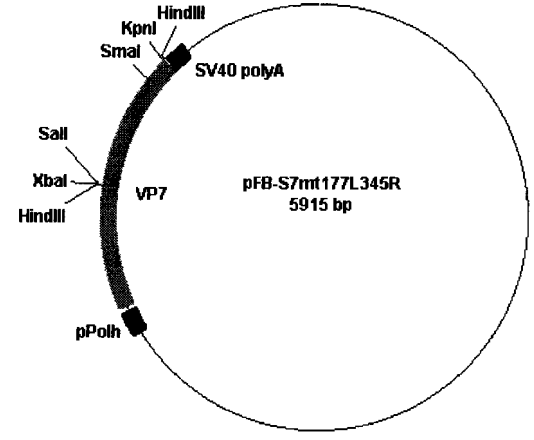
2.3.6 Baculovirus expression of VP7-L345R mutants

DH10BAC™ cells were transformed with transfer vectors pFB-S7L345R, pFB-S7mt177L345R and pFB-S7mt200L345R. After transformation the cells were plated out on agar plates enriched with X-gal and IPTG, blue/white selection was used for the identification of recombinant colonies. White colonies were picked and re-plated on agar plates to verify its true white nature. The colonies were picked and used to inoculate a mini culture, from which the recombinant baculovirus genome (Bacmid DNA) was isolated with the modified plasmid purification procedure (section 2.2.12). The viral DNA was transfected into Sf9 insect cells for the generation of recombinant baculoviruses. The recombinant viruses were harvested and used to prepare a viral stock to express S7L345R, S7mt177L345R and S7mt200L345R. Sf9 monolayers were infected with the respective recombinant viral stocks at a MOI of 5-10 pfu/cell. Expression of the mutants was verified by polyacrylamide gel electrophoresis (Fig. 2.7). As expected the substitution of the target amino acids had no effect on the size of the respective proteins, S7L345R (38KDa), S7mt177L345R (39KDa) and S7mt200L345R (39KDa), the sizes correspond to that previously published for VP7 and the insertion mutants 177 and 200 (Maree, 2000).

(a)



(b)



(c)

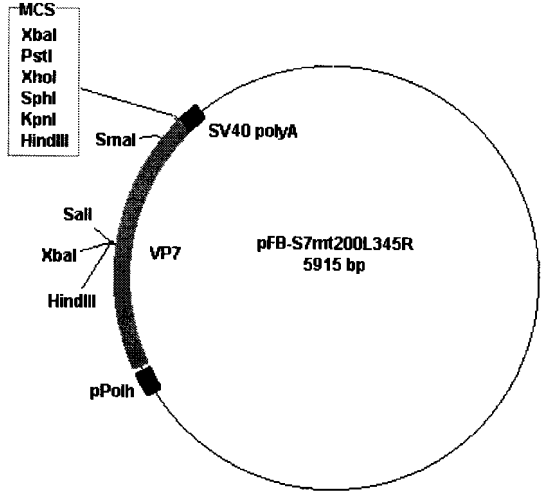


Fig. 2.5 a,b and c respectively show the plasmid maps for pFB- S7L345R, pFB-S7mt177L345R and pFB-S7mt200L345R, illustrating the insertion of the SmaI site in position 1047 to 1052. To verify the correct reconstruction of the target genes and thus the insertion of the site-specific mutation, pFB clones were digested with SmaI. Constructs that linearized after digestion were viewed as positive for the substitution.

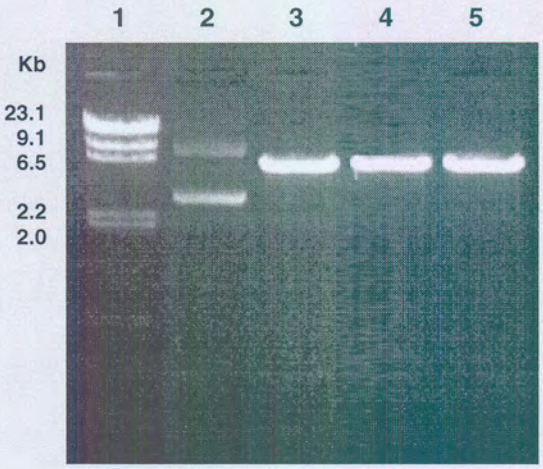


Fig 2.6 The reconstructed pFastbac1 vectors carrying the L345R mutant genes were digested with SmaI and the reactions were analysed on a 1% agarose gel. Lane 1) Molecular Weight marker II. 2) pFB-S7 (SmaI neg. control. 3) pFB-S7L345R. 4) pFB-S7mt177L345R. 5) pFB-S7mt200L345R.

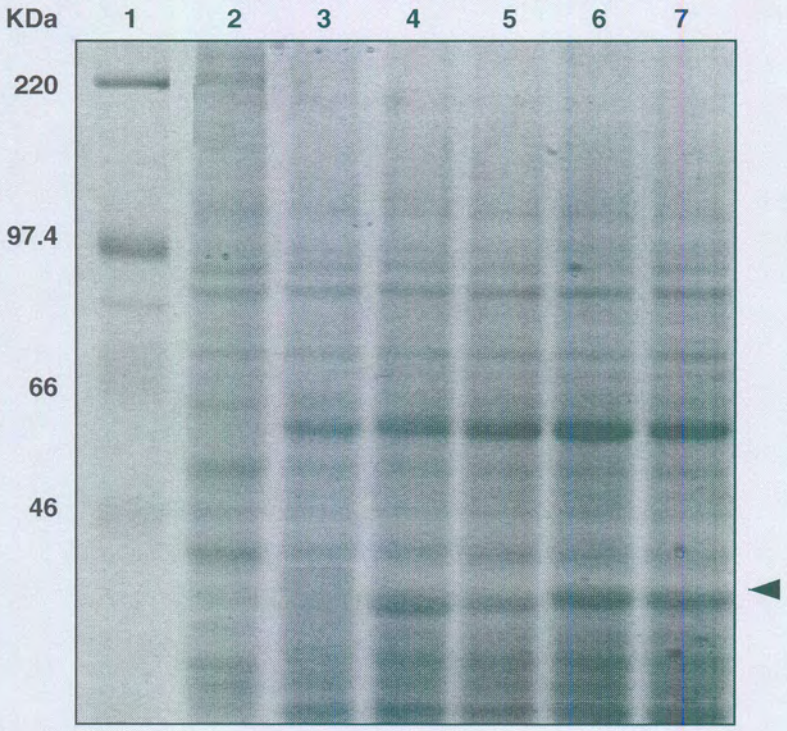


Fig. 2.7 A 10% Polyacrylamide gel showing the expression of L345R mutants in Sf9 insect cells via the baculovirus expression system. Lane 1) Molecular weight marker. Lane 2) Uninfected Sf9 cells. Lane 3) Sf9 cells infected with wild type baculovirus. Lane 4) S7, 38kDa. Lane 5) S7L345R, 38kDa. 6) S7mt177L345R, 39kDa. 7) S7mt200L345R, 39kDa. The arrow indicates the position of VP7.

2.3.7 Solubility studies

A two-pronged approach was used to study protein solubility. Firstly, Sf9 cells were infected with recombinant viruses expressing S7, S7L345R, S7mt177L345R and S7mt200L345R. The cells were harvested 72 hours post infection. Care was taken to ensure that infected cells were still intact before harvesting. This is important because as the cells are harvested by low speed centrifugation, some soluble protein can be lost due to cell lyses thus compromising the integrity of the assay. The whole cell fractions were subjected to low speed differential centrifugation, which separated the VP7 particulate faction from the VP7 trimers, which remained in the soluble fraction. The VP7 trimers were recovered from the soluble fractions by ammonium sulphate precipitation. The soluble and particulate fractions were resuspended in equal volumes, and a fraction of each analysed by PAGE (Fig. 2.8). The amount of VP7 protein present in the soluble faction of each mutant was quantified from the polyacrylamide gel using the Sigma Gel™ software package. The difference in amount of protein present in the soluble fractions of wild type VP7 (S7) and mutants S7L345R, S7mt177L345R and S7mt200L345R is visibly significant (Fig. 2.8). This experiment was repeated several times and a quantification of the results is presented in Fig. 2.10 in which the solubility results are combined with a second approach to study solubility.

In this approach whole cell fractions were prepared as before, taking the same factors into consideration. The whole cell fraction was loaded onto a 40-70% discontinuous sucrose gradient and centrifuged as described in the methods section. The gradients were fractionated and a sample of each fraction analysed by 10% PAGE. Under the specific centrifugation conditions it was possible to separate the particulate VP7 (fractions 1-6) from the soluble trimers (fractions 7-11) (Fig. 2.9), the Sigma Gel™ software package was again employed to quantify the amount of soluble protein.

Fig. 2.9 shows the amount of soluble protein present in fractions 7 to 11, of S7L345R, S7mt177L345R and S7mt200L345R compared to its particulate fractions (1-6, and pellet) is comparatively more then that present in wild type VP7 (S7). The data obtained from both methods used to determine the amount of soluble VP7, is represented in Fig. 2.10. The results show that only 17% (SD±11%) of wild type VP7 is present in trimeric form, as the majority assembles into particulate structures. S7L345R shows an increase to 47% (SD±6%), S7mt177L345R to 37% (SD±6%) and S7mt200L345R to 38% (SD±3%). The standard deviation of the mean values is large, reflecting the crude nature of the quantification method. The data obtained in this assay should therefore be seen as a rough estimation of the amount of soluble protein.

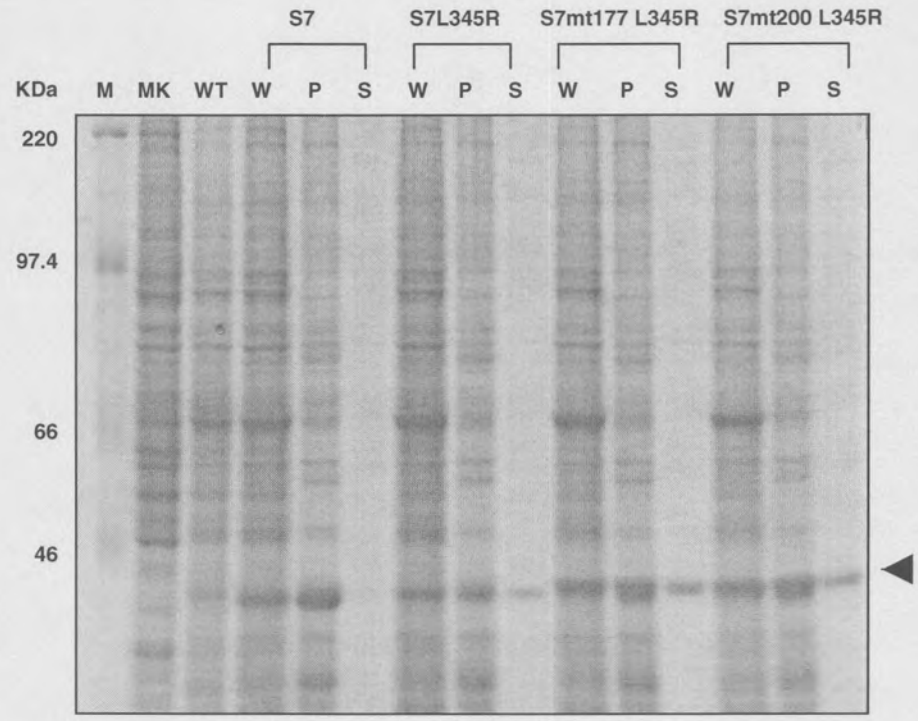


Fig. 2.8 Proportional fractions of the whole cell (W), particulate (P) and soluble (S) fractions of each mutant was analysed by 10% PAGE. M) Molecular weight marker. MK) Uninfected Sf9 cells (MK). WT) Sf9 cells infected with wild type baculovirus. The black arrow indicates the position of VP7.

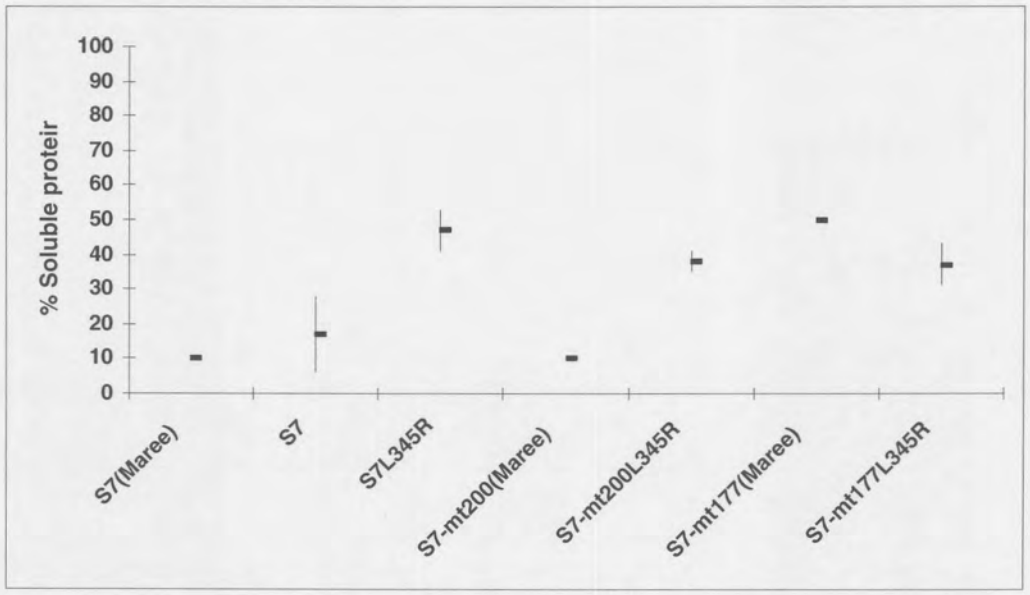


Fig. 2.10 The mean value of % soluble protein for each mutant is presented showing the calculated standard deviation. The data shows an increase in solubility for the L345R mutants compared to wild type VP7and S7mt177 (50%), S7mt200 (10%) and S7 (10%) as determined by Maree (2000). S7 (17±11%), S7L345R (47±6%), S7mt177L345R (37±6%) and S7mt200L345R (38±3%).

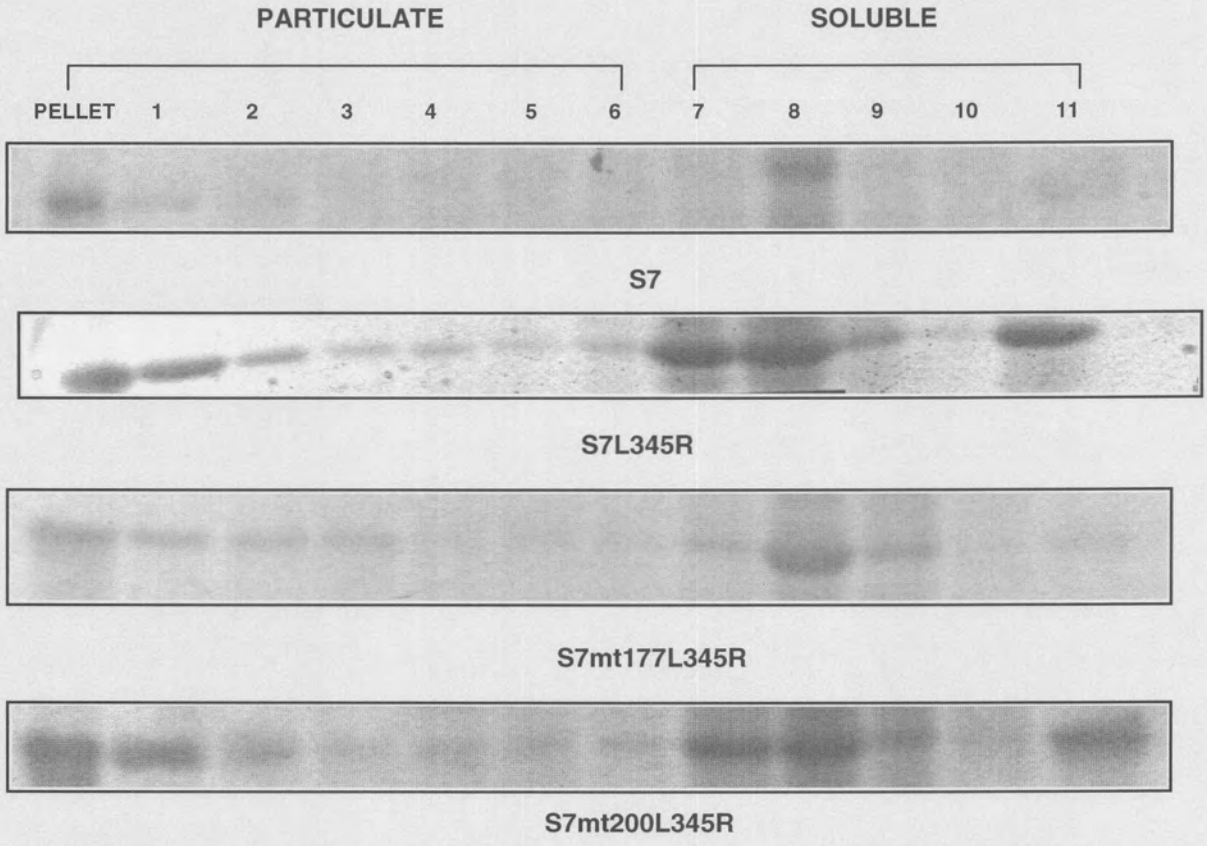


Fig. 2.9 Whole cell fractions of S7, S7L345R, S7mt177L345R and S7mt200L345R were prepared and loaded onto a 40-70% discontinuous sucrose gradient. After fractionation, a sample of each fraction was resolved on a 10% polycrylamide gel. The relative amount of VP7 in each band was determined using the Sigma Gel™ software package.

2.3.8 Trimerization assay

The important role that the C-terminal residues of VP7 play in trimer stability has been clearly shown (Grimes *et al*, 1998, LeBlois and Roy, 1993). As an obvious cause for the increased solubility of the L345R mutants is trimer destabilization, a trimerization assay was performed similar to that described by Limn *et al* (2000). Boiled as well as unboiled samples taken from the soluble fraction of each mutant was subjected to 10% SDS-PAGE. Figure 2.11 shows the presence of trimers (111KDa) for each of the mutants, suggesting that the specific amino acid substitutions made in the 9th C-terminal helix had no effect on the ability of the mutants to trimerize. The lack of any sign of dimers confirms the stability of monomer interaction in forming the trimers, this consequently rules out trimer destabilization as the mechanism for the increased solubility.

2.3.9 Microscopic analysis

Cells infected with recombinant baculoviruses expressing S7, S7L345R, S7mt177L345R and S7mt200L345R were viewed under 40x magnification with a Nikon light microscope (Fig. 2.12). Only in cells in which wild type VP7 is expressed (Fig. 2.12c), are the characteristic large crystals reported by Chuma *et al* (1992) observed. The absence of these large crystals in the L345R infections could perhaps best be explained by an increase in solubility since the expression levels of all the mutants are relatively similar to that of the wild type VP7 (Fig. 2.7). The difference can therefore not be explained by poor expression of one of the mutant VP7 proteins. Both the mock and wild type baculovirus infections included as negative controls show the lack of any crystalline particles, as expected. It is evident from the sucrose gradient results reported in Fig. 2.9 that the L345R mutants still assemble into particulate structures, as the separation of different size particles can be seen on the gradient (fraction 1-6). To verify particulate structure formation these structures were recovered from the sucrose and viewed by electron microscopy and particle assembly was confirmed (Fig. 2.13).

Figure 2.14 shows the size separation of the VP7 particles. Whole cell fractions were loaded onto a 40-70% sucrose gradient and centrifuged under the conditions described (2.2.18.1). The bar graph indicates that a large percentage of the particulate structures migrated to the pellet under the centrifugation conditions. This fraction represents the largest of the assembled VP7 particles, while the smaller particulate fragments remain in the gradient. The top millilitre of the gradient represents the soluble fraction. It is evident that there is less soluble S7 present compared to the L345R mutants.

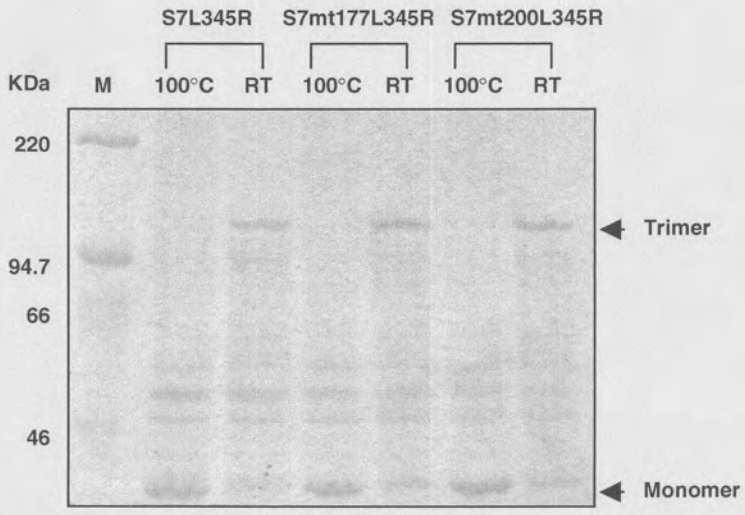


Fig. 2.11 Boiled as well as unboiled samples of the soluble fractions for mutants S7L3454R. S7mt177L345R and S7mt200L345R were loaded onto a 10% polyacrylamide gel and run for 2 hours at 110kV. The first lane shows molecular weight size marker. The positions of the trimers and monomers are indicated.

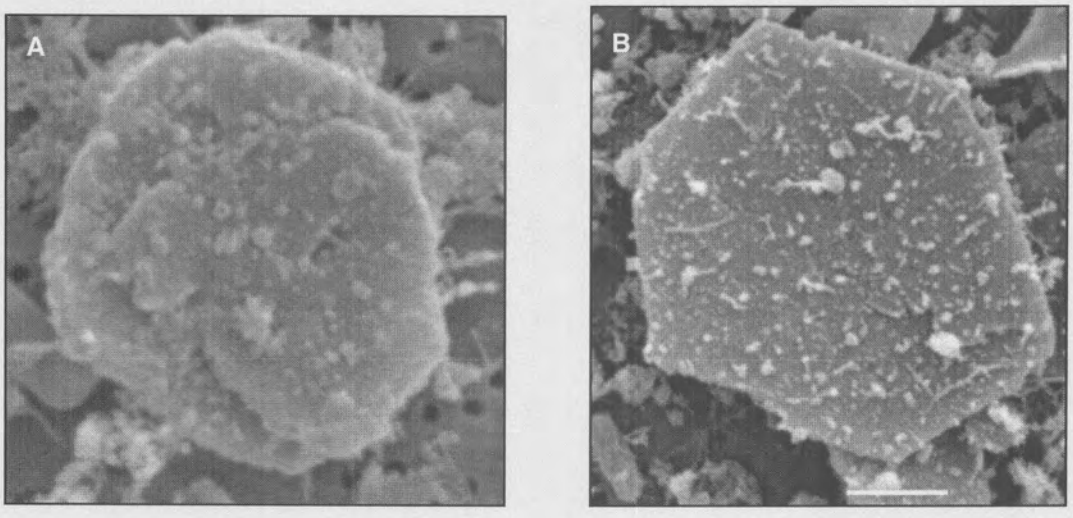


Fig. 2.13 Electron microscopic photos of L345R particulate structures. a) S7mt177L345R and b) S7mt200L345R

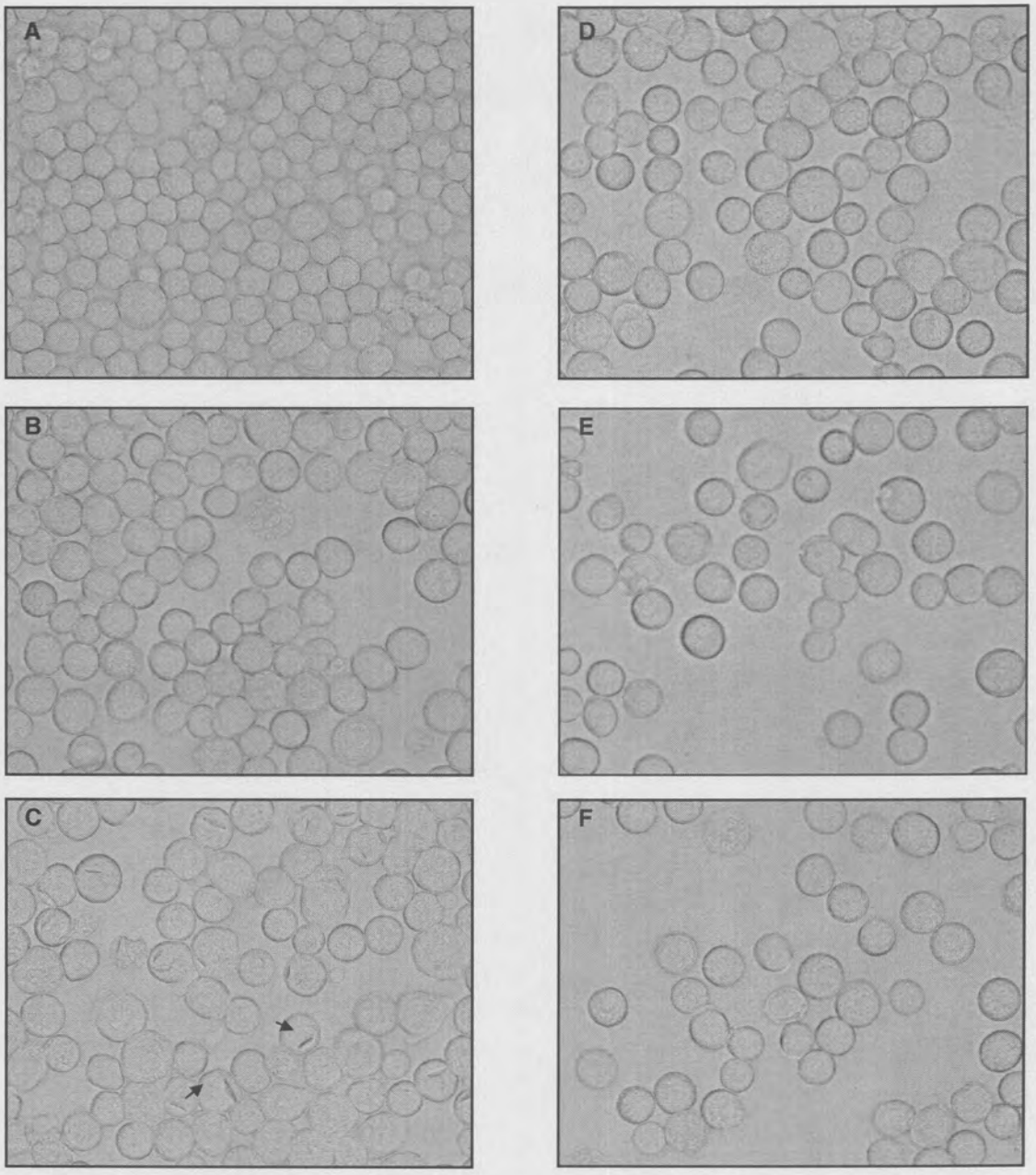


Fig. 2.12 Light microscopy photos of Sf9 cells infected with recombinant baculoviruses. A) Uninfected Sf9 cells. B) Sf9 cells infected with wild type baculovirus. C) S7 (arrows indicate presence of crystals). D) S7L345R. E) S7mt177L345R. F) S7mt200L345R. Only wild type VP7 (C) shows the presence of the characteristic crystals (Chuma *et al*, 1992). A and B were included as negative controls.

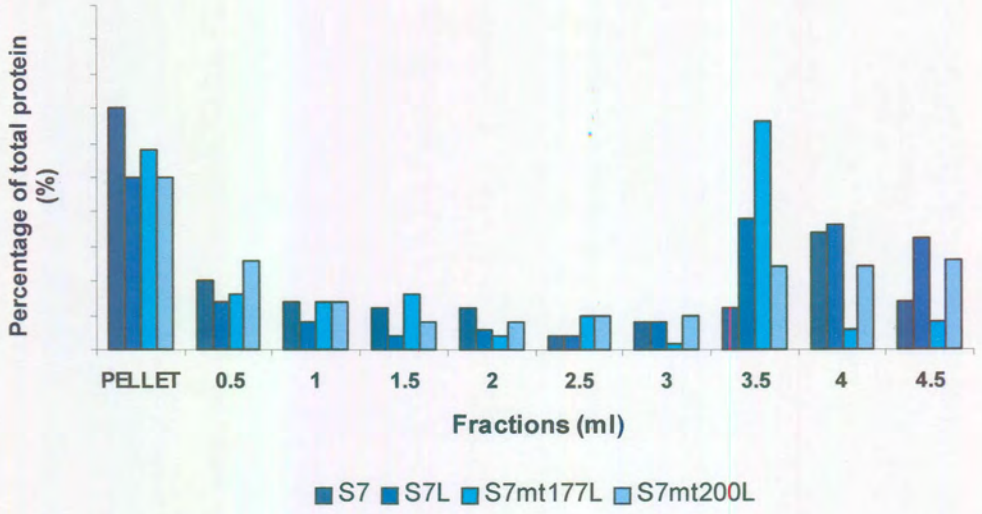


Fig. 2.14 The graph shows the separation of the S7, S7L345R, S7mt177L345R and S7mt200L345R whole cell fractions on a 40-70% discontinuous sucrose gradient. The bottom fractions (up till 3ml) show the separation of the particulate structures. The top 1ml of the gradient represents the soluble protein.

2.4 DISCUSSION

The extreme insolubility of VP7 poses a problem for the development of AHSV core-like particles as a vector for epitope display. The low core-like particle yield observed in co-expression experiments with VP3 and VP7, can be attributed to the inherent insolubility of VP7 (Maree, 2000). The hydrophobic interaction between the VP7 trimers seems to favour its aggregation into crystalline structures that are characteristic of AHSV VP7 (Chuma *et al*, 1992; Burroughs *et al*, 1994).

The production of recombinant core-like particles, derived from the co-expression of VP3 and VP7 insertion mutants, showed that by increasing the solubility of VP7 it was also possible to increase the core-like particle yield. In this instance the insertion of 6 amino acids at position 177 significantly altered the solubility of VP7, increasing it by an estimated 40% (Maree, 2000). The obtained increase in core-like particle yield was however still not enough to make its use practical.

Two amino acids located in the top domain of VP7 (A167 and F209) were implicated and investigated for its role in the observed insolubility of the protein. Mutation of these sites in wild type VP7 (A167→ R and F209→ T) resulted in no increase in solubility, but pointed towards an amino acid (Leu345) located in the 9th C-terminal helix as possibly contributing to the observed insolubility (Monastyrskaya *et al*, 1997). There have been several reports describing the drastic effect of a single amino acid substitution on protein solubility (Limn *et al*, 2000, Zhao and Somerville, 1992; Dyda *et al*, 1994). In order to investigate the significance of the proposed amino acid (Leu345), it was targeted for site directed mutagenesis in wild type VP7 as well as insertion mutants 177 and 200. Sequence alignment of AHSV and BTV VP7, revealed the presence of an arginine at position 345 in the soluble BTV VP7 contributing to the surrounding region being more hydrophilic than that of AHSV VP7. It was subsequently decided to substitute Leu 345 to Arg in AHSV VP7.

The newly constructed VP7 L345R mutants were expressed in the baculovirus expression system. Each of the mutant proteins were subsequently investigated for the effect on solubility. To this end two different solubility assays were performed. Both assays however relied on obtaining a rough estimation of the amount of VP7 trimers that remained in a soluble form. The ratio of the amount of soluble trimers to that assembled in particulate form was therefore used as the basis for judging protein solubility. This approach is inherently subject to experimental error, the assays were therefore repeated several times and an average value was calculated for each of the proteins under investigation. The results obtained for the single substitution in

wild type VP7 and mutant 200 indicates an increase from 10% (Maree, 2000) to 47% and 38% respectively. It should be noted that the significance of these calculated values should not be over estimated as the margin of error is quite considerable for this method of quantification. The data does however show some increase in solubility. Previously Maree (2000) reported that VP7 mutant 177 was significantly more soluble than both wild type VP7 and mutant 200. This presented an opportunity to investigate if the single amino acid substitution could have an additive effect on solubility. As a control in this study the solubility of VP7 mutant 177 was also determined and was found to agree with that reported by Maree (2000), (45-50%). The solubility of mutant S7mt177L345R was determined to be 37±6% and no evidence of any additional increase in solubility was found.

To understand the mechanism responsible for the observed increase in solubility a trimerization assay was performed for each of the mutants. The interaction between VP7 monomers within a trimer consist of 13 contact points and involves both the lower domains. The interactions between the lower domains mostly involve helices 5 and 6. The large cavities at the centre of each trimer (along the three fold related axes) are surrounded by mostly uncharged residues (Grimes *et al*, 1998), which probably result in the stabilization of the trimers through hydrophobic interaction. There are a number of hydrophobic regions within the last 50 amino acids of the C-terminus. That the involvement of these domains (in both intra or intermolecular interactions) is important for protein function has been suggested by (Le Blois and Roy, 1993). Therefore due to the location where the substitution was made (the 9th C-terminal helix), it had to be determined if this mutation prevented VP7 monomers from interacting to form stable trimers. The results presented in figure 2.11 however suggest stable trimer formation, ruling out the possibility that a lack of trimer formation caused the increase in solubility. From later electron microscopic analysis, particulate structures were observed which formation rely solely on the presence of trimers, also confirming trimer stability (Fig. 2.13). A second possible explanation for the increase in solubility is a weakening in trimer-trimer interaction by decreasing the hydrophobic effect that stabilizes the interaction and causes particle formation. This will necessarily result in more trimers being present in solution, as the trimers are less likely to assemble into particles. Previous deletion experiments identified residues 334 to 349 as essential for trimer interaction (Le Blois and Roy, 1993). The interaction between different trimers is non-specific involving a set of hydrophobic residues believed to form a thin hydrophobic band around the lower domain. There is very limited contact area at the three fold interfaces indicating that the trimer-trimer interaction is relatively weak (Grimes *et al*, 1998, Limn *et al*, 2000). Hydrophilicity plots drawn for each mutant based on the algorithm of Hopp and Woods (1981) with a window width of seven amino acids predicts a decrease in

hydrophobicity in the C-terminal region for each of the mutants (Fig. 2.15). Therefore it seems reasonable to assume that the insertion of the hydrophilic arginine residue lead to a decrease in the local hydrophobicity of that region, resulting in the trimers being more hydrophilic and consequently more soluble.

From the microscopic data the effect of the increase in solubility can be seen as none of the L345R mutants aggregate into crystalline structures large enough to be visible within the insect cells, it is expressed in (Fig. 2.12). This probably reflects the increase in solubility because the expression level for the mutants are comparable to that of wild type VP7, which aggregates into large crystals. The mutants do however still assemble into particles, showing some sign of the characteristic hexagonal shape (Fig. 2.13). The bar graph in figure 2.14 confirms the fact that the structures formed by the L345R mutants seem to be generally smaller, as the majority of the particles still remain in the gradient even under tremendously high centrifugation conditions (section 2.2.18.1), while most of the particulate wild type VP7 sediments in the pellet.

Although the L345R mutants were shown to form trimers, its ability to assemble into stable core-like particles remains undetermined. It has been shown that trimerization alone does not imply core-like particle formation (Monastyrskaya *et al*, 1997). The trimers are believed to interact with the VP3 scaffold via its flat hydrophobic bottom surface. Since none of the residues in the C-terminal part where the substitution is located contribute to the base of the trimer they do not interact with VP3. It seems therefore unlikely that the mutation would abrogate core-like particle formation. Even though there is some increase in solubility the increase may be to small to result in a drastic core-like particle yield. It is probably unlikely that the solubility of VP7 or its insertion mutants could be increased to a level where the core-like particle yield would be significant enough to make its use practical. It was decided to rather focus on the development of the crystalline particles as a vector for epitope presentation. This is the subject of investigation in chapter 3.

A comparison of the Hydrophilicity profiles of S7/insertion mutants and L345R mutants

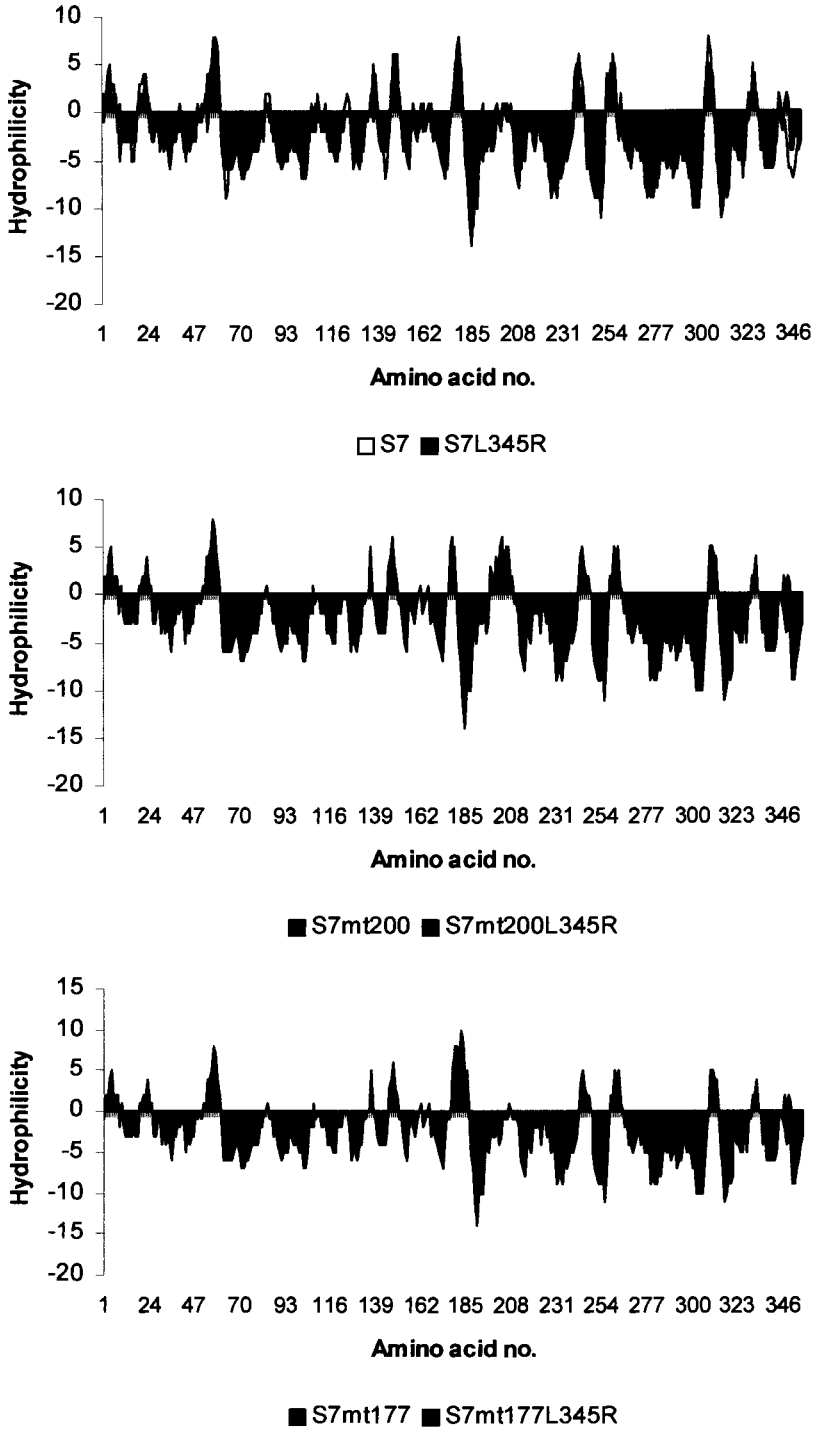


Fig. 2.15 Comparison of the hydrophilicity plots of VP7 and its insertion mutants before and after the single amino acid substitution according to the algorithm of Hopp and Woods (1981).

CHAPTER 3

EXPRESSION OF HIV-1 NEUTRALIZING ANTIBODY EPITOPE ELDKWA IN AHSV VP7 INSERTION MUTANTS AND ITS EFFECT ON CRYSTAL FORMATION AND IMMUNOGENICITY

3.1 INTRODUCTION

Although the majority of the HIV-1 gp41 protein is not accessible to the immune system, an ectodomain region located near the membranous region of gp41 were shown to be recognized by sera obtained from infected individuals (Xu *et al*, 1991). An amino acid sequence within this region, ELDKWA (aa position 671-676) was subsequently shown to elicit a neutralizing monoclonal antibody (2F5) that could neutralize not only laboratory adapted HIV strains but also several clinical isolates (Muster *et al*, 1993; Broliden *et al*, 1992).

The ELDKWA epitope is conserved in 72% of HIV-1 isolates (Muster *et al*, 1993) and has become the subject of extensive investigation for its possible application in HIV vaccine development. The likelihood of the epitope reflecting a specific conformation of the contributing amino acids that is important for the induction of a neutralizing antibody response has been documented (Eckhart *et al*, 1996; Muster *et al*, 1994; Coeffier *et al*, 2001).

The molecular context in which an epitope is presented to the immune system plays an important role in the ability of the epitope to induce a suitable immune response. The expression of a multi-epitope ELDKWA peptide in the *E. coli* MalE protein could not induce anti-HIV neutralizing antibodies even though the epitope has repeatedly shown to be highly immunogenic and capable of inducing neutralizing immune responses (Coeffier *et al*, 2001; Martineau *et al*, 1992). This points to the importance of the molecular context in which the epitope is presented.

Consequently the ability of VP7 insertion mutants to present the ELDKWA epitope to the immune system in a conformation that can induce an effective immune response was investigated. A single repeat of the ELDKWA epitope was subsequently expressed on the surface of insertion mutant 144 and 177 crystalline particles and investigated for its ability to induce an immune response in mice. As the aim of this chapter is to utilize the insoluble crystalline particles as a vector, the original unmodified mutant 177 insertion mutant was

chosen to continue the study. Also insertion mutant 144 was chosen as other studies investigating mutant 200 was already underway.

In light of the fact that multi-epitope vaccines expressing several repeats of neutralizing epitopes has been shown to induce even higher levels of epitope specific antibody responses (Lu *et al*, 2000; Liao *et al*, 2001; Chen *et al*, 1999), that are considered to confer long lasting protection it was decided to test the ability of such a multi-epitope approach on VP7 crystals. Three repeats of the ELDKWA epitope was expressed on the surface of insertion mutant 177 crystals and investigated for its immunogenicity.

In short the aims of this chapter were i) to construct recombinant VP7 proteins presenting either a single or triple repeat of the neutralizing antibody epitope ELDKWA. ii) To investigate the effect of such an insertion on VP7 crystal formation. iii) And to determine the ability of the recombinant crystals to induce an epitope specific antibody response in mice.

3.2 MATERIALS AND METHODS

3.2.1 Adaptor reconstruction

To reconstruct the adapters coding for single and triple repeat copies of the epitope, the commercially synthesized oligonucleotide primers (Life technologies) were resuspended in 15% acetonitryl to a final concentration of 100pmol/µl. An annealing reaction was set up for each adaptor as follows, 200pmol primer [+], 200pmol primer [-] and 10X annealing buffer (100nM Tris-HCl pH 7.5, 1M NaCl, 10mM EDTA in DEPC treated water), reactions were made up to a final volume of 20µl. The reaction mix was denatured at 92°C for 10min followed an annealing step, 1hour at 89°C. The annealed adaptors were frozen away at -20°C until further use.

Table 3.1 Adapters used to insert single and triple repeat copies of the ELDKWA epitope.

NO. REPEATS	SITE AND POLARITY	OLIGONUCLEOTIDE SEQUENCE
Single	144 [+]	5'-AATTCGAACTGCTAGAGCTCGACAAGTGGGCAAGCTTGTGGC-3'
	177 [+]	5'-AGCTTGAACTGCTAGAGCTCGACAAGTGGGCAAGCTTGTGGC-3'
	144/177 [-]	5'- TCGAGCCACAAGCTTGCCCACTTGTCGAGCTCTAGCAGTTCA-3'
Triple	177 [+]	5'-AGCTTGAATTAGATAAATGGGCAGAGCTCGACAAGTGGGCAGAGCTAGATAAATGGGCAC-3'
	177 [-]	5'-TCGAGTGCCCATTTATCTAGCTCTGCCCACTTGTCGAGCTCTGCCCATTTATCTAATTCA-3'

3.2.2 Induction of immune response against recombinant VP7 constructs

A group consisting of five 6-week old Balb/c mice were injected subcutaneously with approximately 10µg of antigen per mouse. For the initial injection (day #0) the antigen was made up to a final volume of 50µl (1:1 dilution in Freunds complete adjuvant (FCA)). Each animal received two booster shots (±10µg) on days 14 and 28, the FCA was substituted

however with Freunds incomplete adjuvant (FIA). On day 42, the animals were bled by cardiac puncture and approximately 1ml of blood was recovered from each animal. The serum was recovered as described by (Johnson and Thorpe, 1990) and frozen away at –70°C until further use.

3.2.3 Chemilumanesence blot

A PVDF membrane was soaked in 100% methanol and then transferred to 10mM CAPS pH > 9. The proteins of interest were spotted onto the membrane and the background blocked in TBSTT pH 7.4 (1% skimmed milk, 10mM Tris, 150mM NaCl, 0.1% Tween 20, 0.1% TritonX100) for one hour at room temperature. The primary antibody was prepared in antibody buffer (100mM Tris, 0.9% NaCl, 1% skimmed milk, pH 7.4) to final dilutions of 1/1000 and 1/10000. The membrane and antibody solution was incubated for one hour at RT. After the incubation the membrane was washed three times in TBSTT. The primary antibodies were detected by the anti-mouse goat immunoglobulin conjugate provided by the Super signal ^{R} kit (obtained from Pierce). All the manufactures specifications were followed for the detection.

3.2.4 Western blot analysis

The proteins of interest were separated by 12% PAGE for 90 minutes at 120V and then blotted onto a nitrocellulose membrane (Hybond C) for 90 minutes. The membrane was washed in wash buffer (0.005% Tween 80, 1 X PBS pH7.4) for 5 min before being blocked to prevent non-specific binding of the primary antibodies, (incubation in blocking buffer 1% skimmed milk powder, 1 x PBS pH 7.4 for 30 minutes at RT). A primary antibody solution was prepared in blocking buffer to a final volume of 10ml (1 in a 1000 dilution). The membrane was sealed in a plastic bag and incubated overnight at 4°C while shaking. After incubation the membrane was washed thoroughly (3x15min) in TBSTT pH 7.4 (1% skimmed milk, 10mM Tris, 150mM NaCl, 0.1% Tween 20, 0.1% TritonX100). The secondary antibody (1 in a 1000 dilution) was prepared in antibody buffer (100mM Tris, 0.9% NaCl, 1% skimmed milk, pH 7.4) to a final volume of 25ml and the membrane was incubated at RT for 30min. The excess secondary antibodies were removed by three 15 min washes in TBSTT. The Super signal ^{R} kit obtained from Pierce was subsequently used for the detection of bound primary antibodies. All the manufactures specifications were followed.

3.3 RESULTS

To clone the small linear neutralizing HIV epitope ELDKWA into the VP7 insertion mutants, several nucleotide primers that could be annealed to form adaptors that encode the epitope were designed (Table 3.1). The primers were designed such, that after annealing 5' and 3' overhangs were created. The adaptors inserted into mutants 177 and 144 required 5'HindIII and SalI3', 5'EcoRI and XhoI3' overhangs respectively. Adaptors were designed for the expression of a single, as well as a triple repeat of the ELDKWA epitope. The single repeat (ELLELDKWASLW) was inserted into both mutants 144 and 177. The additional amino acids flanking the epitope correspond to that in the transmembrane protein gp41 for HIV-1 subtype B (Conley *et al*, 1994). To investigate the efficacy of presenting multiple copies of the epitope on the VP7 display system a second 60nt adaptor coding for three repeats of ELDKWA was designed (ELDKWAELDKWAELDKWA); this epitope was introduced into mutant 177. To simplify the selection of newly constructed recombinants a marker restriction enzyme site (SacI 5' GAGCTC 3') was inserted into each adaptor.

3.3.1 Construction of mutant 144 and 177 ELDKWA transfer vectors

Vectors were prepared by digesting pFB-S7-144 and pFB-S7-177 with EcoRI/XhoI and HindIII/SalI respectively. The pFB-S7-177 construct used, had previously been modified to remove the HindIII site from the MCS of the vector in order to simplify the cloning strategy when cloning into the HindIII site at position 177 (Maree, 2000). In each case a linear 5.9kb fragment was produced and recovered from an agarose gel. The adaptors were reconstructed as described in section 3.2.1, and cloned into the prepared vector fragments. Recombinant clones were selected by digestion with SacI. Clones in which the adaptors were inserted successfully produced three DNA fragments, S7-144 produced fragments 5100, 605 and 137bp in size, where as both the single and triple repeat mutant 177 constructs produced three fragments approximately 5100, 508 and 234bp in size. The constructs were designated pFB-S7-144Mon-E, pFB-S7-mt177Mon-E and pFB-S7-177Tri-E (Fig. 3.1). Plasmid maps of the newly constructed vectors are shown in Fig. 3.2.

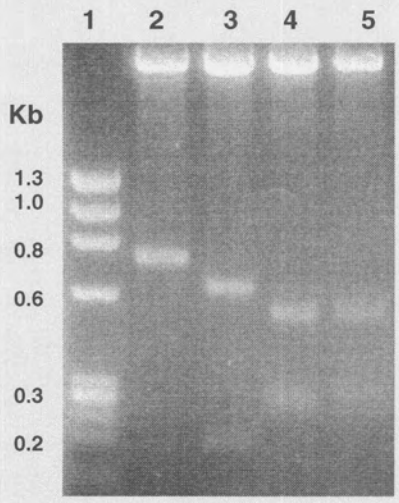


Fig. 3.1 A 2% agarose gel of the obtained DNA fragments after SacI digestion. Lane 1) ϕX molecular weight size marker. 2) pFB-S7. 3) pFB-S7-144Mon-E. 4) pFB-S7-177Mon-E. 5) pFB-S7-177Tri-E.

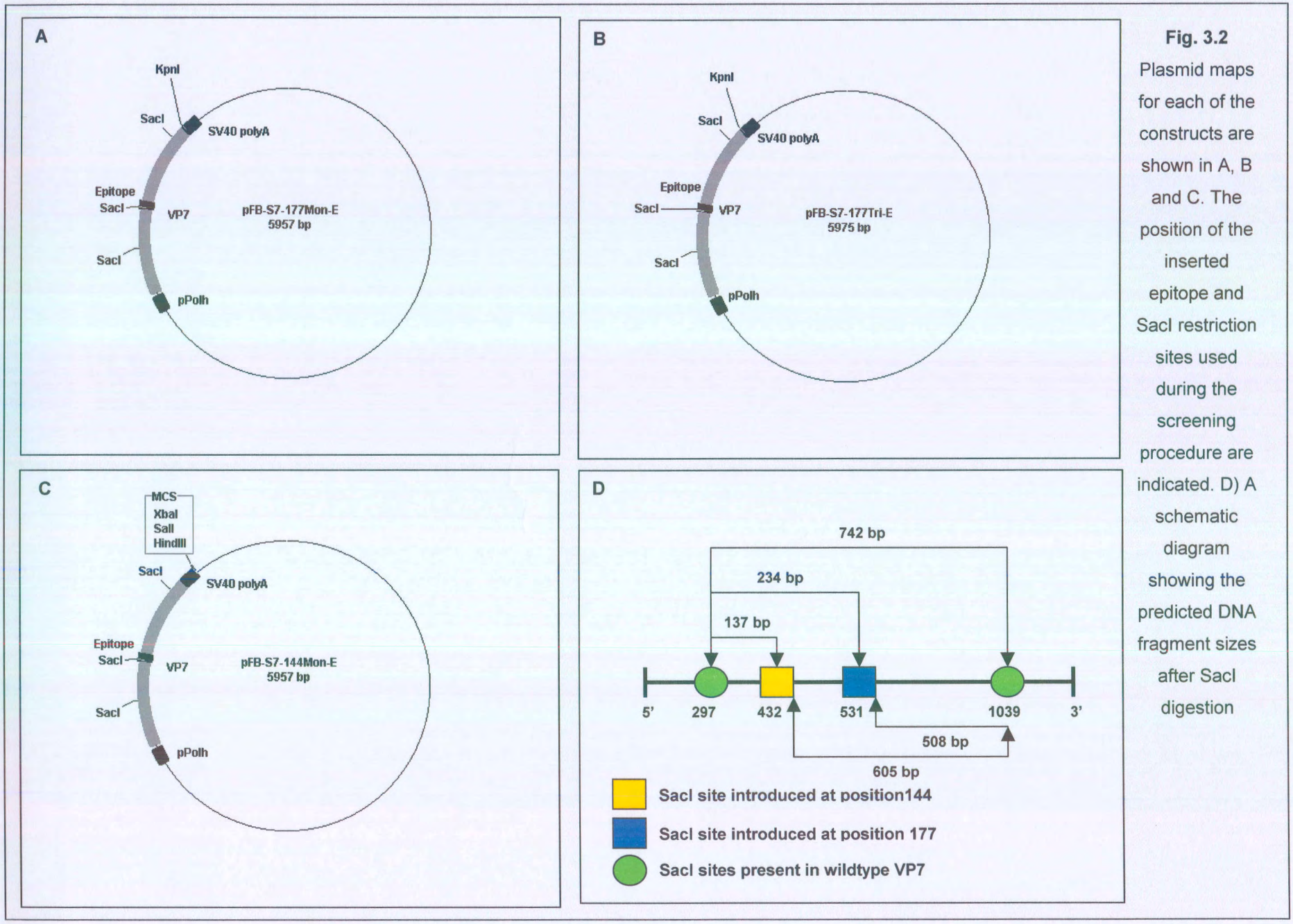


Fig. 3.2 Plasmid maps for each of the constructs are shown in A, B and C. The position of the inserted epitope and SacI restriction sites used during the screening procedure are indicated. D) A schematic diagram showing the predicted DNA fragment sizes after SacI digestion

3.3.2 Nucleotide sequence determination

The external primers listed in table 2.1, section 2.2.8 were used to determine the nucleotide sequence of the insertion mutants. Two overlapping sequences were generated from which the complete gene sequence could be deduced. The mutant gene sequences were translated using the ABI PRISM Navigator™ program to obtain an amino acid sequence for each mutant. This was done to verify the integrity of the newly constructed mutant genes. The sequences determined for S7-144Mon-E and S7-177Mon-E showed the insertion of 12 amino acids (ELLELDKWASLW) at the respective insertion sites. S7-144Mon-E increased in size to 367 amino acids, where as S7-177Mon-E only increased to 365 amino acids. The difference in size is due to the loss of two codons during the cloning procedure. As the insertion site at position 177 has recognition sequences for 5'-HindIII, XbaI and SalI-3' and the adaptor was cloned between the HindIII and SalI sites, this resulted in the loss of the XbaI site and thus a decrease in two amino acids. The deduced amino acid sequence of S7-177Tri-E showed the insertion of the 18 amino acid triple repeat (ELDKWAELDKWAELDKWA) and a total size of 371 amino acids. The translated open reading frames of the insertion mutants are given in Fig. 3.3. The obtained sequences verify the suitability of the genes for subsequent expression.

3.3.3 Baculovirus expression of insertion mutants

Baculovirus recombinants were constructed for the expression of S7-144Mon-E, S7-177Mon-E and S7-177Tri-E as previously described in sections 2.2.11 to 2.2.17. The constructs were expressed on a large scale, and a sample of each whole cell fraction was analysed by SDS-PAGE (Fig. 3.4). The increase in the size of the insertion mutants are visible and as follows, S7-144Mon-E (40KDa), S7 177Mon-E (40KDa) and S7-177Tri-E (41KDa). All of the VP7 mutants showed an expression level similar to that of wild type VP7. A high expression level is important for the production of particulate structures, since large amounts of these particles are needed for further structural and immunogenic analysis.

```
   1 ATGGACGCGATACGAGCAACAGCCTTGTCCGTTGTACGGGCATGTGTCACAGTGACAGAT   60
   1 M  D  A  I  R  A  T  A  L  S  V  V  R  A  C  V  T  V  T  D    20

  61 GCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGATTGCAATCAATAGGTAT  120
  21 A  R  V  S  L  D  P  G  V  M  E  T  L  G  I  A  I  N  R  Y    40

 121 AATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACCCAAGCAGAACGAAATGAA  180
  41 N  G  L  T  N  H  S  V  S  M  R  P  Q  T  Q  A  E  R  N  E    60

 181 ATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCGCTGAACGTCCAAATTGGGAATATT  240
  61 M  F  F  M  C  T  D  M  V  L  A  A  L  N  V  Q  I  G  N  I    80

 241 TCACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTCGCAACGACTGAAATTCCA  300
  81 S  P  D  Y  D  Q  A  L  A  T  V  G  A  L  A  T  T  E  I  P   100

 301 TATAATGTTCAGGCCATGAATGACATCGTTAGAATAACGGGTCAGATGCAAACATTCGGA  360
 101 Y  N  V  Q  A  M  N  D  I  V  R  I  T  G  Q  M  Q  T  F  G   120

 361 CCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAGGTGCAACAATCTGGCAGA  420
 121 P  S  K  V  Q  T  G  P  Y  A  G  A  V  E  V  Q  Q  S  G  R   140

 421 TATTACGTACCGCCCGGGGAATTTGAACTGCTAGAGCTCGACAAGTGGGCAAGCTTGTGG  480
 141 Y  Y  V  P  P  G  E  F  E  L  L  E  L  D  K  W  A  S  L  W   160

 481 CTCGAGCAAGGTCGAACGCGTGGCGGGTACATCAATTCAAATATTGCAGAAGTGTGTATG  540
 161 L  E  Q  G  R  T  R  G  G  Y  I  N  S  N  I  A  E  V  C  M   180

 541 GATGCAGGTGCTGCGGGACAGGTCAATGCGCTGCTAGCCCCAAGGAGGGGGGGACGCAGTC  600
 181 D  A  G  A  A  G  Q  V  N  A  L  L  A  P  R  R  G  D  A  V   200

 601 ATGATCTATTTCGTTTGGAGACCGTTGCGTATATTTTGTGATCCTCAAGGTGCGTCACTT  660
 201 M  I  Y  F  V  W  R  P  L  R  I  F  C  D  P  Q  G  A  S  L   220

 661 GAGAGCGCTCCAGGGACTTTTGTCACCGTTGATGGAGTAAATGTTGCAGCTGGAGATGTC  720
 221 E  S  A  P  G  T  F  V  T  V  D  G  V  N  V  A  A  G  D  V   240

 721 GTCGCATGGAATACTATTGCACCAGTGAATGTTGGAAATCCTGGGGCACGCAGATCAATT  780
 241 V  A  W  N  T  I  A  P  V  N  V  G  N  P  G  A  R  R  S  I   260

 781 TTACAGTTTGAAGTGTTATGGTATACGTCCTTGGATAGATCGCTAGACACGGTTCCGGAA  840
 261 L  Q  F  E  V  L  W  Y  T  S  L  D  R  S  L  D  T  V  P  E   280

 841 TTGGCTCCAACGCTCACAAGATGTTATGCGTATGTCTCTCCCACTTGGCACGCATTACGC  900
 281 L  A  P  T  L  T  R  C  Y  A  Y  V  S  P  T  W  H  A  L  R   300

 901 GCTGTCATTTTTCAGCAGATGAATATGCAGCCTATTAATCCGCCGATTTTTCCACCGACT  960
 301 A  V  I  F  Q  Q  M  N  M  Q  P  I  N  P  P  I  F  P  P  T   320

 961 GAAAGGAATGAAATTGTTGCGTATCTATTAGTAGCTTCTTTAGCTGATGTGTATGCGGCT 1020
 321 E  R  N  E  I  V  A  Y  L  L  V  A  S  L  A  D  V  Y  A  A   340

1021 TTGAGACCAGATTTCAGAATGAATGGTGTTGTCGCGCCAGTAGGCCAGATTAACAGAGCT 1080
 341 L  R  P  D  F  R  M  N  G  V  V  A  P  V  G  Q  I  N  R  A   360

1081 CTTGTGCTAGCAGCCTACCACTAG                                      1104
 361 L  V  L  A  A  Y  H  *                                        368
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Fig. 3.3 (a) Nucleotide and amino acid sequence of S7-144Mon-E

```
   1 ATGGACGCGATACGAGCAAGAGCCTTGTCCGTTGTACGGGCATGTGTCACAGTGACAGAT   60
   1 M  D  A  I  R  A  R  A  L  S  V  V  R  A  C  V  T  V  T  D    20

  61 GCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGATTGCAATCAATAGGTAT  120
  21 A  R  V  S  L  D  P  G  V  M  E  T  L  G  I  A  I  N  R  Y    40

 121 AATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACCCAAGCAGAACGAAATGAA  180
  41 N  G  L  T  N  H  S  V  S  M  R  P  Q  T  Q  A  E  R  N  E    60

 181 ATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCGCTGAACGTCCAAATTGGGAATATT  240
  61 M  F  F  M  C  T  D  M  V  L  A  A  L  N  V  Q  I  G  N  I    80

 241 TCACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTCGCAACGACTGAAATTCCA  300
  81 S  P  D  Y  D  Q  A  L  A  T  V  G  A  L  A  T  T  E  I  P   100

 301 TATAATGTTCAGGCCATGAATGACATCGTTAGAATAACGGGTCAGATGCAAACATTCGGA  360
 101 Y  N  V  Q  A  M  N  D  I  V  R  I  T  G  Q  M  Q  T  F  G   120

 361 CCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAGGTGCAACAATCTGGCAGA  420
 121 P  S  K  V  Q  T  G  P  Y  A  G  A  V  E  V  Q  Q  S  G  R   140

 421 TATTACGTACCGCAAGGTCGAACGCGTGGTGGGTACATCAATTCAAATATTGCAGAAGTG  480
 141 Y  Y  V  P  Q  G  R  T  R  G  G  Y  I  N  S  N  I  A  E  V   160

 481 TGTATGGATGCAGGTGCTGCGGGACAGGTCAATGCGCTGCTAGCCCCAAGGAAGCTTGAA  540
 161 C  M  D  A  G  A  A  G  Q  V  N  A  L  L  A  P  R  K  L  E   180

 541 CTGCTAGAGCTCGACAAGTGGGCAAGCTTGTGGCTCGACAGGGGGGGACGCAGTCATGATC  600
 181 L  L  E  L  D  K  W  A  S  L  W  L  D  R  G  D  A  V  M  I   200

 601 TATTTCGTTTGGAGACCGTTGCGTATATTTTGTGATCCTCAAGGTGCGTCACTTGAGAGC  660
 201 Y  F  V  W  R  P  L  R  I  F  C  D  P  Q  G  A  S  L  E  S   220

 661 GCTCCAGGAACTTTTGTCACCGTTGATGGAGTAAATGTTGCAGCTGGAGATGTCGTCGCA  720
 221 A  P  G  T  F  V  T  V  D  G  V  N  V  A  A  G  D  V  V  A   240

 721 TGGAATACTATTGCACCAGTGAATGTTGGAAATCCTGGGGCACGCAGATCAATTTTACAG  780
 241 W  N  T  I  A  P  V  N  V  G  N  P  G  A  R  R  S  I  L  Q   260

 781 TTTGAAGTGTTATGGTATACGTCCTTGGATAGATCGCTAGACACGGTTCCGGAATTGGCT  840
 261 F  E  V  L  W  Y  T  S  L  D  R  S  L  D  T  V  P  E  L  A   280

 841 CCAACGCTCACAAGATGTTATGCGTATGTCTCTCCCACTTGGCACGCATTACGCGCTGTC  900
 281 P  T  L  T  R  C  Y  A  Y  V  S  P  T  W  H  A  L  R  A  V   300

 901 ATTTTTCAGCAGATGAATATGCAGCCTATTAATCCGCCGATTTTTCCACCGACTGAAAGG  960
 301 I  F  Q  Q  M  N  M  Q  P  I  N  P  P  I  F  P  P  T  E  R   320

 961 AATGAAATTGTTGCGTATCTATTAGTAGCTTCTTTAGCTGATGTGTATGCGGCTTTGAGA 1020
 321 N  E  I  V  A  Y  L  L  V  A  S  L  A  D  V  Y  A  A  L  R   340

1021 CCAGATTTCAGAATGAATGGTGTTGTCGCGCCAGTAGGCCAGATTAACAGAGCTCTTGTG 1080
 341 P  D  F  R  M  N  G  V  V  A  P  V  G  Q  I  N  R  A  L  V   360

1081 CTAGCAGCCTACCACTAG                                          1098
 361 L  A  A  Y  H  *                                             366
```

Fig. 3.3(b) Nucleotide and amino acid sequence of S7-177Mon-E.

```
   1 ATGGACGCGATACGAGCAAGAGCCTTGTCCGTTGTACGGGCATGTGTCACAGTGACAGAT   60
   1 M  D  A  I  R  A  R  A  L  S  V  V  R  A  C  V  T  V  T  D    20

  61 GCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGATTGCAATCAATAGGTAT  120
  21 A  R  V  S  L  D  P  G  V  M  E  T  L  G  I  A  I  N  R  Y    40

 121 AATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACCCAAGCAGAACGAAATGAA  180
  41 N  G  L  T  N  H  S  V  S  M  R  P  Q  T  Q  A  E  R  N  E    60

 181 ATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCGCTGAACGTCCAAATTGGGAATATT  240
  61 M  F  F  M  C  T  D  M  V  L  A  A  L  N  V  Q  I  G  N  I    80

 241 TCACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTCGCAACGACTGAAATTCCA  300
  81 S  P  D  Y  D  Q  A  L  A  T  V  G  A  L  A  T  T  E  I  P   100

 301 TATAATGTTCAGGCCATGAATGACATCGTTAGAATAACGGGTCAGATGCAAACATTCGGA  360
 101 Y  N  V  Q  A  M  N  D  I  V  R  I  T  G  Q  M  Q  T  F  G   120

 361 CCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAGGTGCAACAATCTGGCAGA  420
 121 P  S  K  V  Q  T  G  P  Y  A  G  A  V  E  V  Q  Q  S  G  R   140

 421 TATTACGTACCGCAAGGTCGAACGCGTGGTGGGTACATCAATTCAAATATTGCAGAAGTG  480
 141 Y  Y  V  P  Q  G  R  T  R  G  G  Y  I  N  S  N  I  A  E  V   160

 481 TGTATGGATGCAGGTGCTGCGGGACAGGTCAATGCGCTGCTAGCCCCAAGGAAGCTTGAA  540
 161 C  M  D  A  G  A  A  G  Q  V  N  A  L  L  A  P  R  K  L  E   180

 541 TTAGATAAATGGGCAGAGCTCGACAAGTGGGCAGAGCTAGATAAATGGGCACTCGACAGG  600
 181 L  D  K  W  A  E  L  D  K  W  A  E  L  D  K  W  A  L  D  R   200

 601 GGGGACGCAGTCATGATCTATTTCGTTTGGAGACCGTTGCGTATATTTTGTGATCCTCAA  660
 201 G  D  A  V  M  I  Y  F  V  W  R  P  L  R  I  F  C  D  P  Q   220

 661 GGTGCGTCACTTGAGAGCGCTCCAGGAACTTTTGTCACCGTTGATGGAGTAAATGTTGCA  720
 221 G  A  S  L  E  S  A  P  G  T  F  V  T  V  D  G  V  N  V  A   240

 721 GCTGGAGATGTCGTCGCATGGAATACTATTGCACCAGTGAATGTTGGAAATCCTGGGGCA  780
 241 A  G  D  V  V  A  W  N  T  I  A  P  V  N  V  G  N  P  G  A   260

 781 CGCAGATCAATTTTACAGTTTGAAGTGTTATGGTATACGTCCTTGGATAGATCGCTAGAC  840
 261 R  R  S  I  L  Q  F  E  V  L  W  Y  T  S  L  D  R  S  L  D   280

 841 ACGGTTCCGGAATTGGCTCCAACGCTCACAAGATGTTATGCGTATGTCTCTCCCACATGG  900
 281 T  V  P  E  L  A  P  T  L  T  R  C  Y  A  Y  V  S  P  T  W   300

 901 CACGCATTACGCGCTGTCATTTTTCAGCAGATGAATATGCAGCCTATTAATCCGCCGATT  960
 301 H  A  L  R  A  V  I  F  Q  Q  M  N  M  Q  P  I  N  P  P  I   320

 961 TTTCCTCCGACTGAAAGGAATGAAATTGTTGCGTATCTATTAGTAGCTTCTTTAGCTGAT 1020
 321 F  P  P  T  E  R  N  E  I  V  A  Y  L  L  V  A  S  L  A  D   340

1021 GTGTATGCGGCTTTGAGACCAGATTTCAGAATGAATGGTGTTGTCGCGCCAGTAGGCCAG 1080
 341 V  Y  A  A  L  R  P  D  F  R  M  N  G  V  V  A  P  V  G  Q   360

1081 ATTAACAGAGCTCTTGTGCTAGCAGCCTACCATTAG                         1116
 361 I  N  R  A  L  V  L  A  A  Y  H  *                          372
```

Fig. 3.3 (c) Nucleotide and amino acid sequence of S7-177Tri-E.

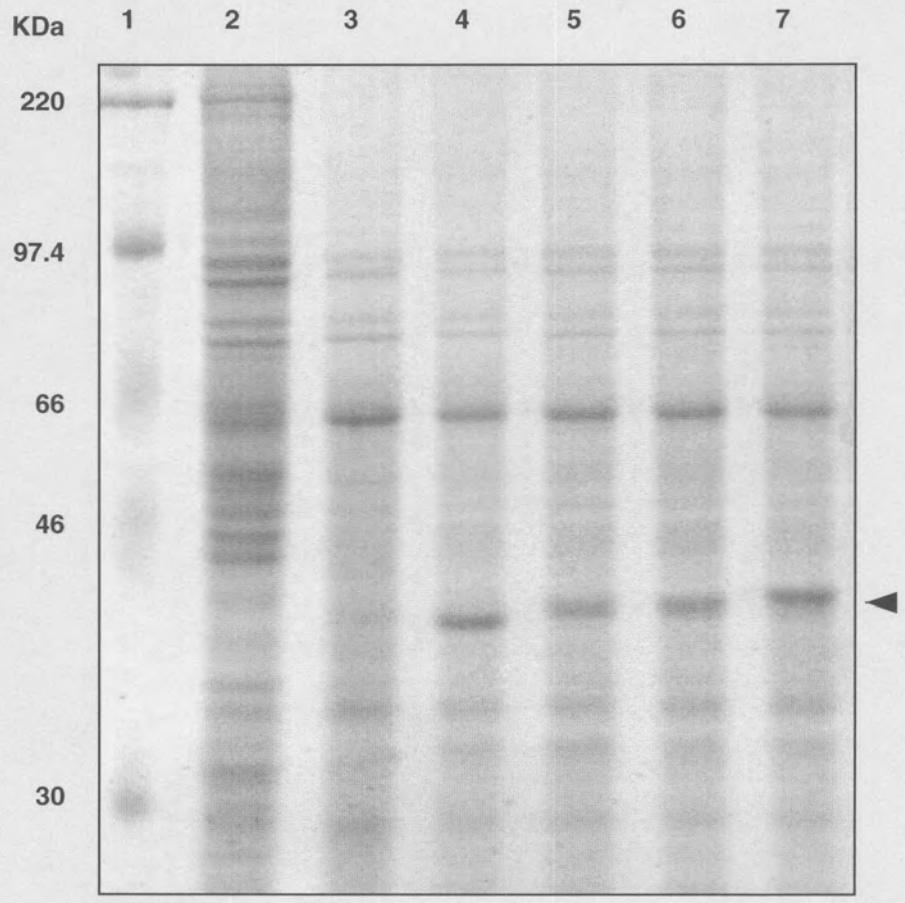


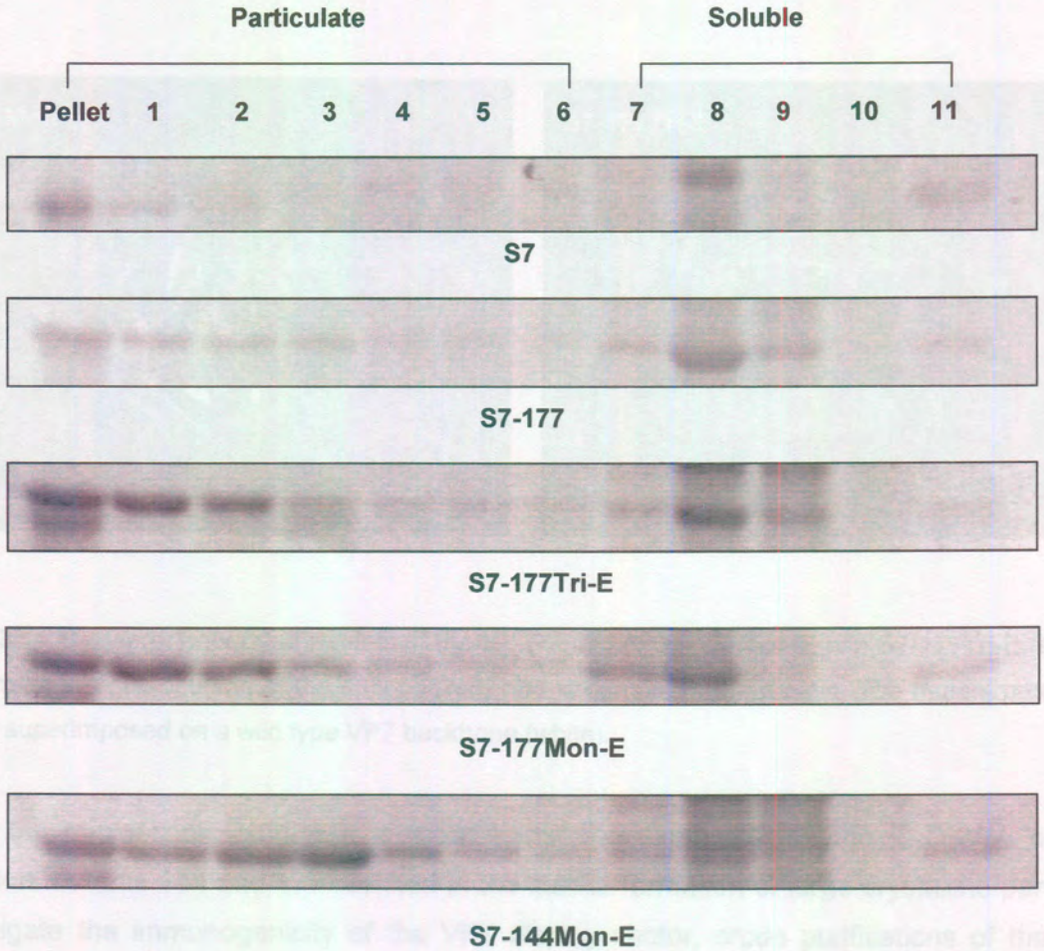
Fig. 3.4 A whole cell fraction of each insertion mutant was loaded onto a 10% polyacrylamide gel and subjected to overnight electrophoresis at 50 volts. Lane1) Molecular weight size marker. 2) Uninfected Sf9 cells. 3) Sf9 cells infected with wild type baculovirus. 4) Wild type VP7 (38KDa). 5.) S7-144Mon-E (40KDa). 6) S7-177Mon-E (40KDa). 7) S7-177Tri-E (41KDa). The position of VP7 in indicated by the black arrow.

3.3.4 Solubility studies

To investigate the effect of the epitope insertion on the solubility of the insertion mutants, a solubility assay was preformed as described in chapter 2 section 2.2.18.1. The results of the sucrose density gradient is presented in Fig. 3.5. The insertion of both the single and triple repeats of the epitope resulted in a considerable decrease in the solubility of insertion mutant S7-177. The single repeat insertion into mutant 144 also resulted in a recombinant protein that is quite insoluble. It is evident that the majority of S7-144Mon-E, S7-177Mon-E and S7-177Tri-E is present in the pellet and bottom fractions of the gradient, compared to S7-177. A rough estimation of soluble VP7 protein confirms the observed decrease, S7-177 (45%), S7-144Mon-E (20%), S7-177Mon-E (25%) and S7-177Tri-E (25%) (Fig. 3.6).

3.3.5 Microscopic analysis

Sf9 cells were infected with recombinant baculoviruses expressing S7-177, S7-144Mon-E, S7-177Mon-E and S7-177Tri-E and viewed under 40 to 100X magnification with a Nikon light microscope (Fig. 3.7). The effect of the decrease in solubility is visible as both S7-177Mon-E and S7-177Tri-E shows the formation crystalline particles. The increased solubility of S7-177 and the resluting lack of visible crystal formation, was discussed in chapter 2. It is evident that the observed decrease in solubility caused by the insertion of the epitopes into mutant 177, resulted in the re-emergence of the characteristic large VP7 crystals. Phenotypically these crystals resemble wild type VP7 crystals. Sucrose gradient purified crystals were prepared and viewed under an electron microscope (Fig. 3.8). S7-144Mon-E, S7-177Mon-E and S7-177Tri-E all showed the formation of hexagonal crystalline structures, these particles were subsequently used as immunogens to test their ability to elicit an epitope specific antibody response to the ELDKWA epitope.



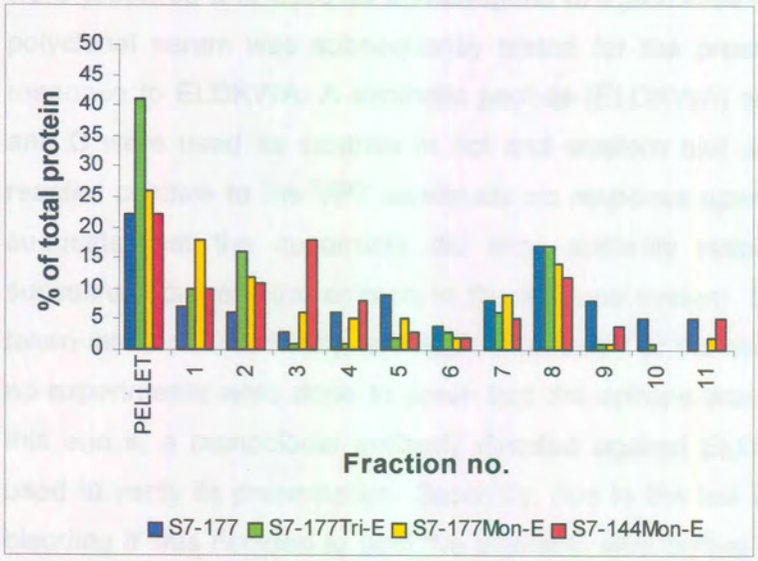


Fig. 3.5 The results of the 40-70%(w/v) sucrose density gradient for S7, S7-177, S7-177Mon-E, S7-177Tri-E and S7-144Mon-E are shown. The gradients were fractionated and a sample of each fraction was resolved by 10% PAGE . (Left) a graph depicting the separation of the VP7 constructs crystalline particles in the gradient.

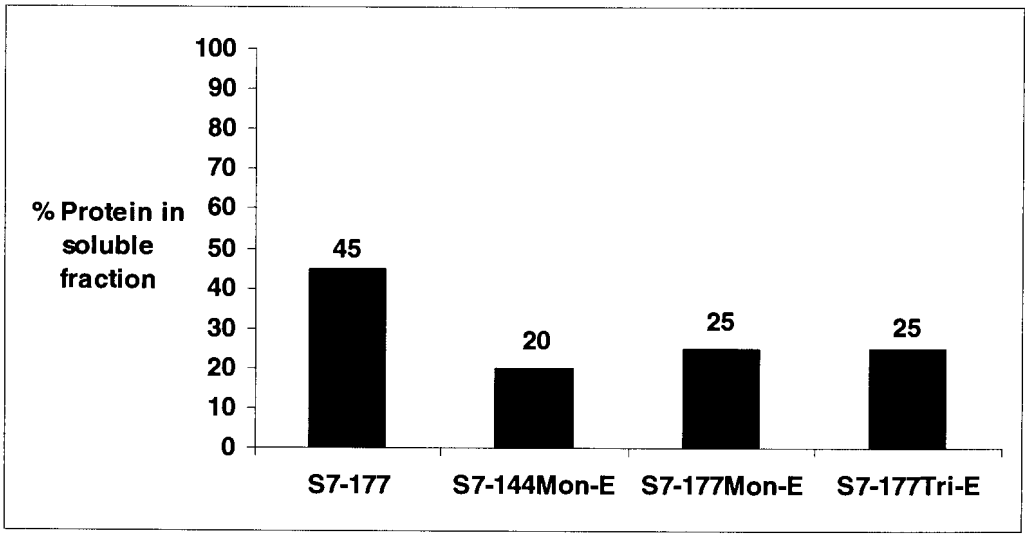


Fig. 3.6 Whole cell fractions of S7-177, S7-177Mon-E, S7-177Tri-E and S7-144Mon-E were prepared and loaded onto a 40-70% discontinuous sucrose gradient. After fractionation, a sample of each fraction was resolved on a 10% polycrylamide gel. The relative amount of VP7 in each band was determined using the Sigma Gel™ software package, and an estimation of the amount of protein remaining in the soluble fraction was obtained.

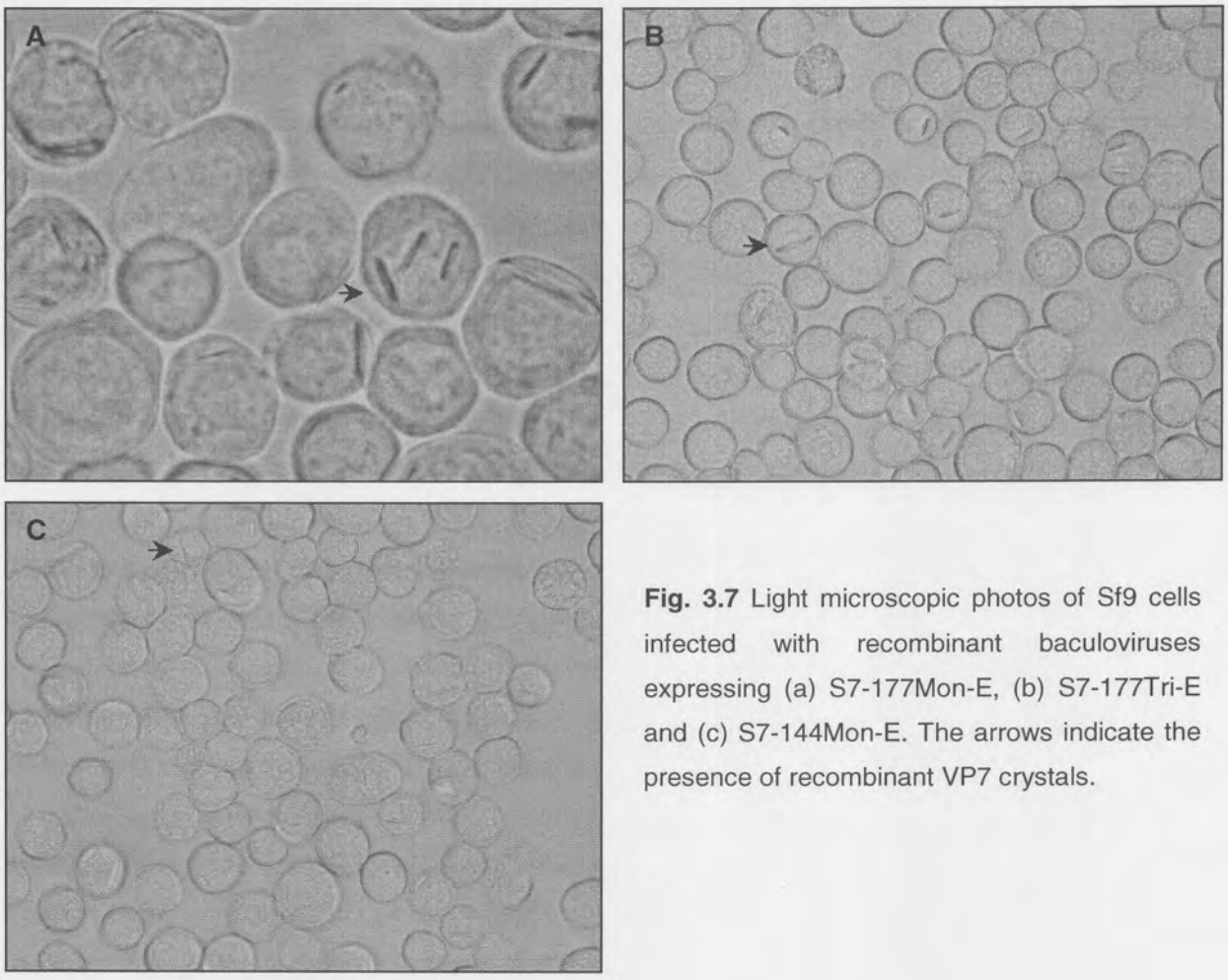


Fig. 3.7 Light microscopic photos of Sf9 cells infected with recombinant baculoviruses expressing (a) S7-177Mon-E, (b) S7-177Tri-E and (c) S7-144Mon-E. The arrows indicate the presence of recombinant VP7 crystals.

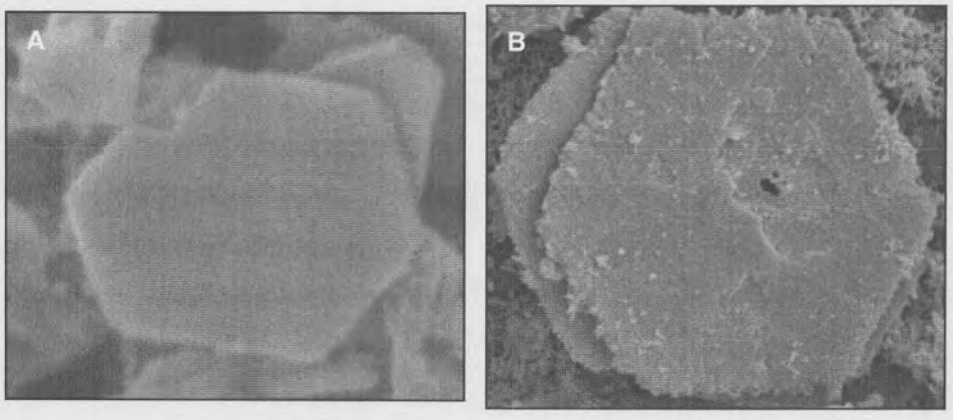


Fig 3.8 Electron microscope photos of a) S7-144Mon-E and b) S7-177Tri-E crystalline particles. S7-177Mon-E also assemble into similar crystals (result not shown)

3.3.6 Induction of immune response against recombinant VP7 constructs

The aim of this experiment was to determine the ability of the VP7 crystalline particles to present the epitope to the immune system. To this end, the VP7 constructs were purified and injected into a group of test animals. The resulting antiserums were subsequently screened for the presence of an epitope specific antibody response to the ELDKWA epitope. The VP7 constructs were purified by 40-70%(w/v) sucrose density centrifugation as described in section 2.2.18.1, the particles recovered from the pellet was used for the immunization of the test animals, consequently only the largest structures were investigated for its immunogenicity. For each of the three constructs a group of five Balb/c mice were immunized over a six week period. For the first immunization all the samples were administered in Freunds complete adjuvant. Two booster immunizations were given every two weeks in Freunds incomplete adjuvant and the antiserum recovered on day 42. The antiserum obtained for each construct was pooled before it was tested for the presence of an epitope specific antibody response.

3.3.7 Chemilumanesence blot

To test the antiserum for the presence of a response against the ELDKWA epitope, a dot blot was performed. The antibody responsible for recognizing the ELDKWA sequence is commonly referred to as monoclonal antibody 2F5 (Muster *et al*, 1993). To this end a seven amino acid synthetic peptide, ELDKWA (American peptide company) as well as subtype B and C HIV-1 viral lysates were blotted onto a PVDF membrane (lysates were kindly provided by the National Institute of Virology). The subtype B lysate has the conserved ELDKWA sequence in its transmembrane protein gp41 and together with the synthetic peptide should yield a positive signal if 2F5 is present in the pooled antiserum, while the subtype C was included as a negative control since its equivalent of the epitope, ALDSWK will not be recognized by the 2F5 antibody. Pooled antiserum was diluted to 1/1000 and used to detect the presence of the antibody. As a negative control a membrane was also developed in the absence of primary antibodies. Fig 3.9 shows the results of the dot blot. None of the antiserums tested showed a reaction to the synthetic peptide, while signals visibly higher than the background signal were obtained against both HIV viral lysates. The absence of a signal against the synthetic peptide suggests that no specific response was induced against the ELDKWA epitope. Possible explanations for the positive signals against both subtype B and C viral lysates are non specific binding of the polyclonal serum against the viral proteins, or merely an artefact as a result of the low stringency during incubation of the primary antibodies as the incubation was done at room temperature for an hour. To verify the nature of the cross-reaction signals and to confirm the

absence of a specific response to ELDKWA the antiserums were tested by western blot analysis.

3.3.8 Western blot analysis

Samples of wild type VP7, S7-144Mon-E and S7-177Tri-E protein were separated with the HIV viral lysates on a 12% polyacrylamide gel. Both HIV lysates were included to detect the non-specific response obtained by the dot blot. HIV lysate subtype B was also included as a control to verify the absence of a specific response to the ELDKWA epitope. The result of the western blot is presented in Fig. 3.10. The polyacrylamide gels show the presence and position of the VP7 constructs as well as the presence of the viral lysates. The blot results shows no response to any of the HIV proteins, it is therefore concluded that the non-specific response obtained during the dot blot is as a result of the low stringency of the primary antibody incubation in combination with the background signal of the secondary antibodies. During the western blot the primary antibody was incubated overnight at 4°C on a shaker, resulting in the occurrence of less non-specific binding. The absence of a response against ELDKWA was again confirmed. The blot results also indicate the detection of both wild type VP7 and the ELDKWA expressing VP7 constructs, S7-144Mon-E and S7-177Tri-E. This response is due to the non-specific recognition of VP7 by the polyclonal antiserum, as the signal intensity is comparable to that of the other background signals.

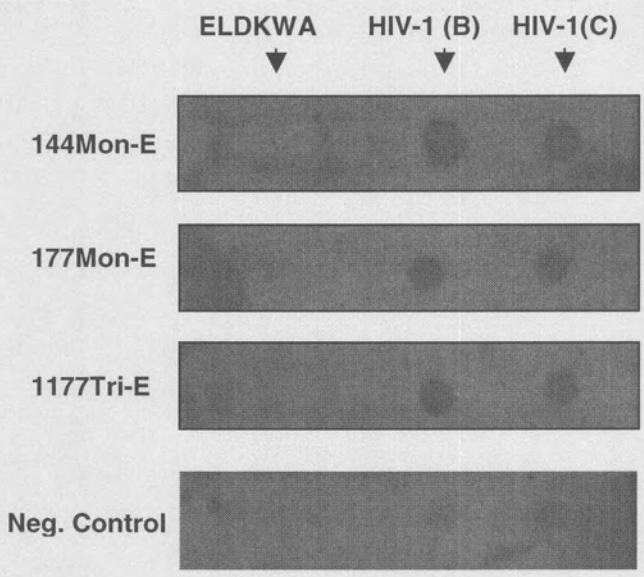


Fig. 3.9 The antiserum obtained against the recombinant VP7 crystals were tested for the presence of an epitope specific antibody response to the ELDKWA epitope via a dot blot. As controls synthetic peptide (ELDKWA) and HIV-1 subtype B and C lysates were used to test the antiserum. A negative control with no primary antibody was also included.

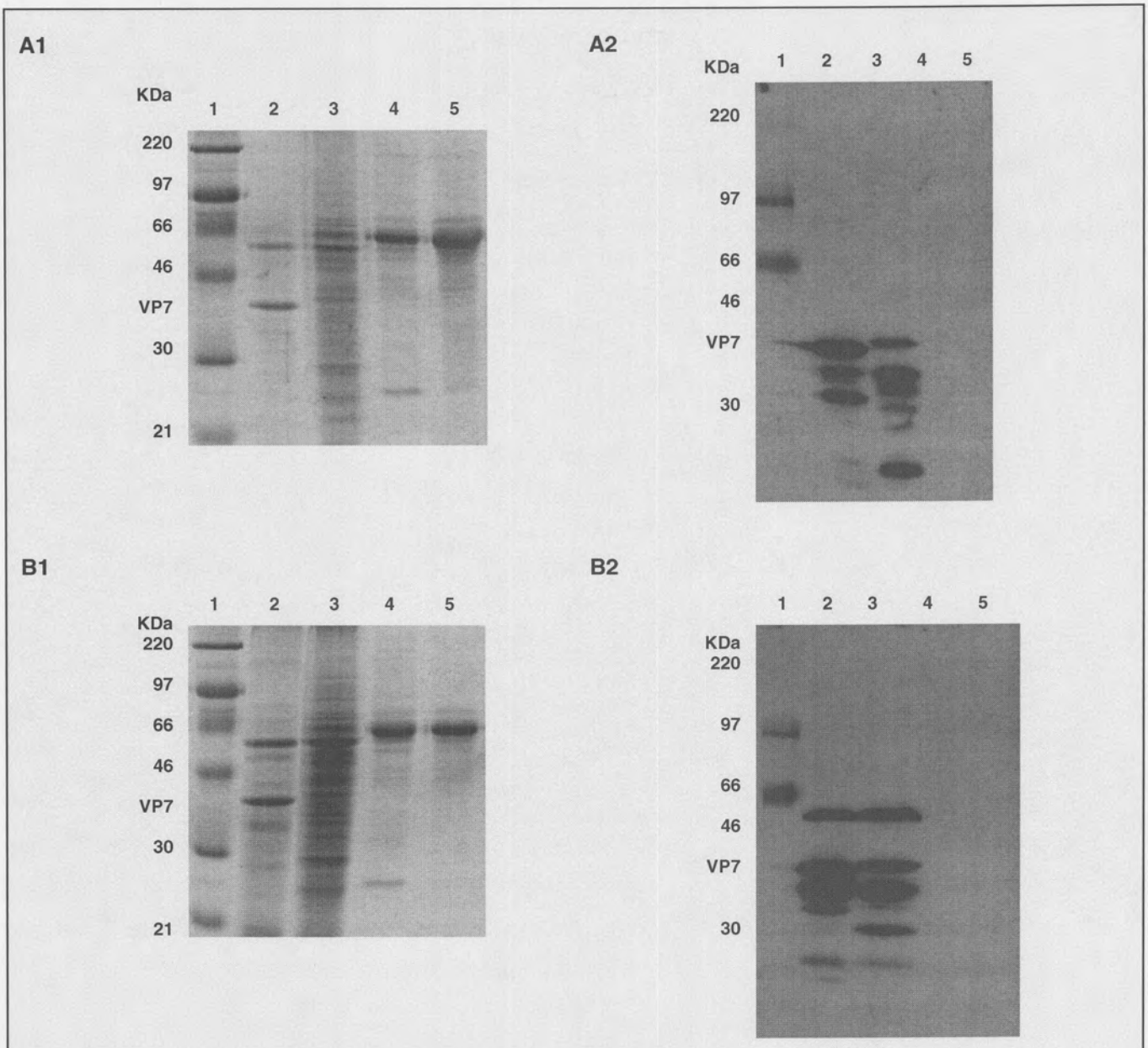


Fig 3.10 12% polyacrylamide gels (A1 and B1) and the western blot results (A2 and B2) are shown. A1 and A2) Lane 1) Molecular weight marker. Lane 2) Wild type VP7. Lane 3) S7-177Tri-E. Lane 4) HIV-1 subtype B lysate. Lane 5) HIV-1 subtype C lysate. B1 and B2) Lane 1) Molecular weight marker. Lane 2) Wild type VP7. Lane 3) S7-144Mon-E. Lane 4) HIV-1 subtype B lysate. Lane 5) HIV-1 subtype C lysate.

3.4 Discussion

The relative ease with which large quantities of VP7 crystals can be produced and purified led our laboratory to look at the development of these structures as vectors for the presentation of epitopes to the immune system. Based on crystallographic data (Basak *et al*, 1996) and physico-chemical profiles of VP7, four hydrophilic regions suitable for the presentation of foreign peptides were identified (Maree, 2000). These sites are located on exposed loops on the surface of the VP7 trimeric molecule at amino acid positions 144,177, 200 and 237 (Maree, 2000). Three insertion mutants were subsequently constructed for the presentation of epitopes, at positions 144, 177 and 200 (Maree, 2000; Riley personal communication).

In this study mutants 144 and 177 were investigated for its ability to display a linear HIV-1 neutralizing antibody epitope (ELDKWA) to the immune system. This epitope is located in the HIV-1 subtype B transmembrane protein gp41, at amino acid position 671 to 676 (Xu *et al*, 1991). As we are interested in testing the ability of these large VP7 structures to effectively display epitopes, this epitope was chosen due to its highly immunogenic nature (Muster *et al*, 1993; Conley *et al*, 1994; Katinger *et al*, 1998; Broliden *et al*, 1992). The results obtained therefore reflect the efficacy of VP7 particles as a display vector for the epitope.

Three recombinant VP7 proteins, each expressing the gp41 epitope were constructed. Two of the mutant proteins present a single copy repeat of the epitope while the third presents a triple repeat. There have been numerous reports of an amplified immune response to synthetic peptide vaccines consisting of several repeats of the epitope (Lu *et al*, 2000; Liao *et al*, 2001; Chen *et al*, 1999). We were consequently interested in exploring this approach in combination with the VP7 display system.

An important part in the development of the VP7 display system is investigating to what extend the insertion of foreign peptides effect particle formation. As we are interested in using the crystalline particles, complete abolishment of structure assembly is undesirable. To this end, each of the recombinant proteins was investigated for its solubility and ability to form stable particulate structures. It had been reported previously that insertion mutant 177 is more soluble than wild type VP7. Approximately 50% of mutant 177 trimers remain in solution (Maree, 2000). Although mutant177 aggregates into particulate structures there is no sign of the characteristic large crystals in the insect cells during expression. The absence of these large structures that were visible under the light microscope probably reflects the increase in solubility. The insertion of both the single and triple repeats of the epitope at position 177 resulted in a marked

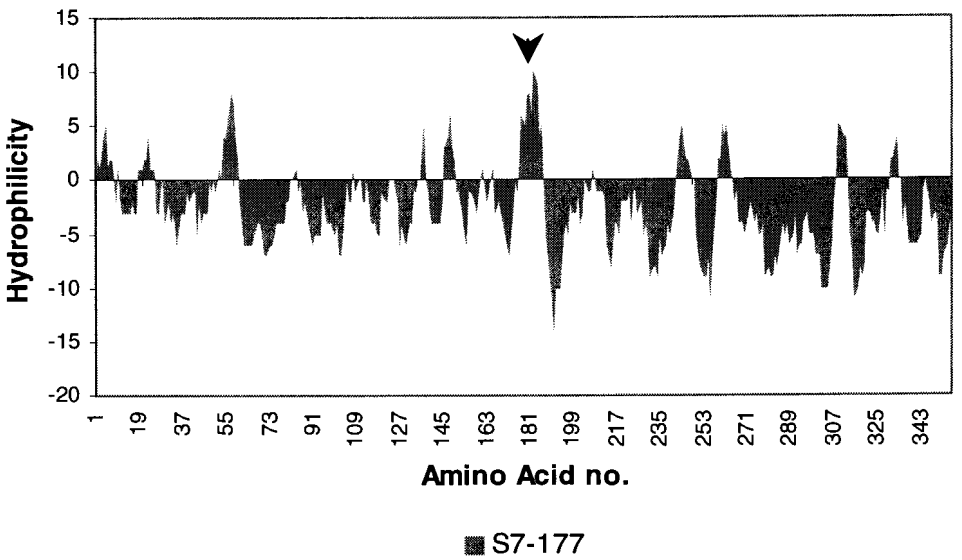
decrease in solubility of the protein. Only 25% of the protein was present in soluble form. This decrease in solubility is significant as it resulted in the re-appearance of the characteristic large hexagonal crystals. These crystals are phenotypically similar to the wild type VP7 crystals previously described (Chuma *et al*, 1992; Burroughs *et al*, 1994). The formation of VP7 crystals is desirable since we are interested in utilizing these structures for immunization experiments.

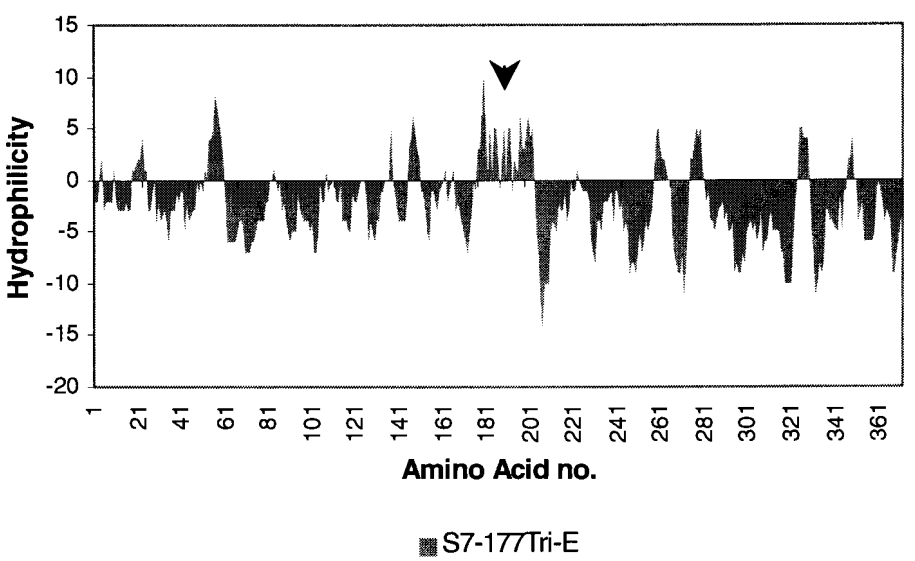
The importance of the top domain in the solubility of VP7 has been studied extensively (Basak *et al*, 1996; Monastyrskaya *et al*, 1997). To investigate the mechanism involved in the reformation of the mutant 177 crystals, hydrophilicity profiles were generated by the algorithm of Hopp and Woods (1981). From the profiles a decrease in the local hydrophilicity at the area of insertion can be observed (Fig. 3.11). Hydrophobic interaction amongst VP7 trimeric molecules results in its aggregation into crystals. In the case of mutant t177, an increase in the local hydrophilicity at position 177 probably resulted in a decrease in the hydrophobic effect that stabilizes trimer interaction. Subsequently the mutant protein was observed to be more soluble and less likely to aggregate into large crystals. As a result of mutant t177 trimers favouring the solution, it assembles into smaller crystalline particles not visible under the light microscope. For the mutant 177 trimers presenting ELDKWA the opposite is true. As a result of the predicted decrease in hydrophilicity, VP7 assembly into large particulate structures are probably favoured as crystallization appears to be stabilized by stronger hydrophobic interaction amongst the trimers.

Analogous to mutant 177 insertion mutant 144 also showed an increase in solubility, although not to the same extend as mutant 177. The introduction of the six amino acid insertion site did not effect crystal formation, however the structures also seem to be somewhat smaller (J Riley, personal communication). The insertion of a single copy repeat of the epitope resulted in reduced hydrophilicity in the area of insertion; again a decrease in protein solubility and the formation of large crystals was observed (Fig. 3.12).

It had been suggested that the insertion of extra amino acids at position 177 results in the distortion of the crystalline shape due to steric effects (Maree, 2000). No distortion was however observed in this study (Fig. 3.8). There appears to be enough space within the crystal layers to accommodate the insertion of the extra amino acids. It remains to be determined exactly how many amino acids can be successfully presented without abrogating particle formation completely. Maree (2000) reported the construction of insertion mutants presenting 105 amino acids. These constructs were reported to form structures visible under the electron microscope, although not in the characteristic shape of VP7 particles.

A comparison of the Hydrophilicity profiles of S7-177, S7-177Mon-E and S7-177Tri-E





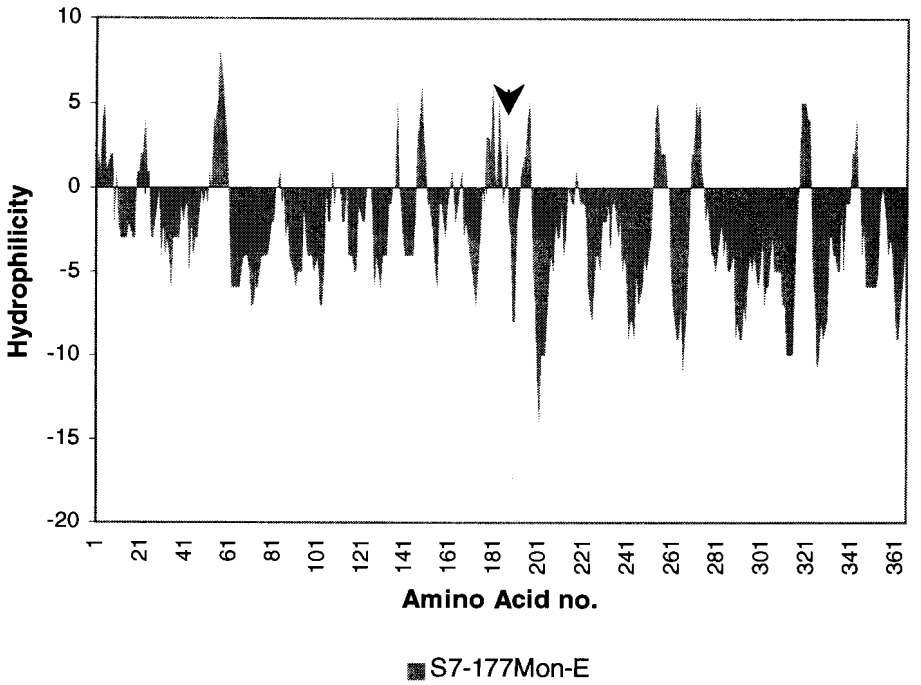
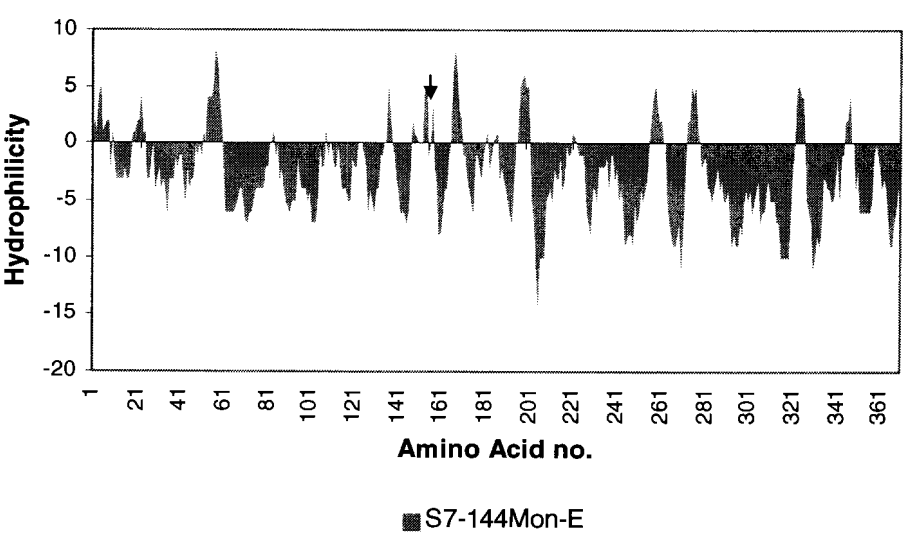


Fig. 3.11 Comparison of the hydrophilicity plots of insertion mutant 177 before and after the single and triple repeat epitope insertions according to the algorithm of Hopp and Woods (1981).

A comparison of the Hydrophilicity profiles of S7-144 and S7-144Mon-E



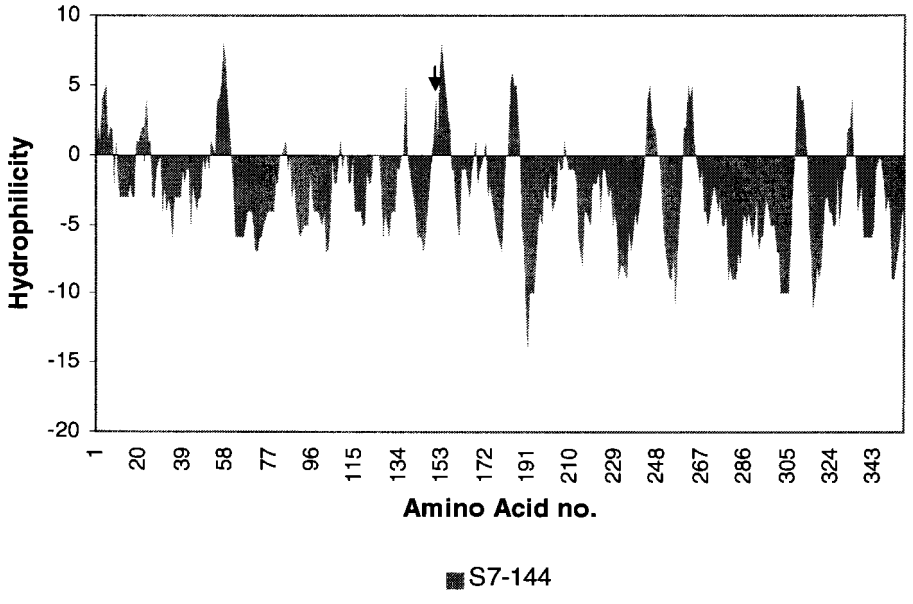


Fig. 3.12 Comparison of the hydrophilicity plots of insertion mutant 144 before and after the single repeat epitope insertion according to the algorithm of Hopp and Woods (1981).

Solvent accessibility profiles generated for the insertion mutants reflect the inherent physico-chemical properties of the epitope sequence. The hydrophobic nature of the inserted peptide results in a low solvent accessibility prediction for these regions (Fig 3.13). The plots therefore suggest that the epitope presenting regions of VP7 is likely to be poorly presented to the environment. For the epitope to elicit a response from the immune system it must be presented in such a way that it is exposed to the surrounding environment. It is evident that the size and the chemical nature of the epitope plays a central role in its own presentation in the VP7 display vector.

Swissprot models generated for the recombinant VP7 proteins, predict the exposure of the epitope to the environment (Fig. 3.14). It should be noted that this model only represents the top domain of a monomer, and once it is assembled into a biologically active trimer the epitope is less likely to be as well exposed.

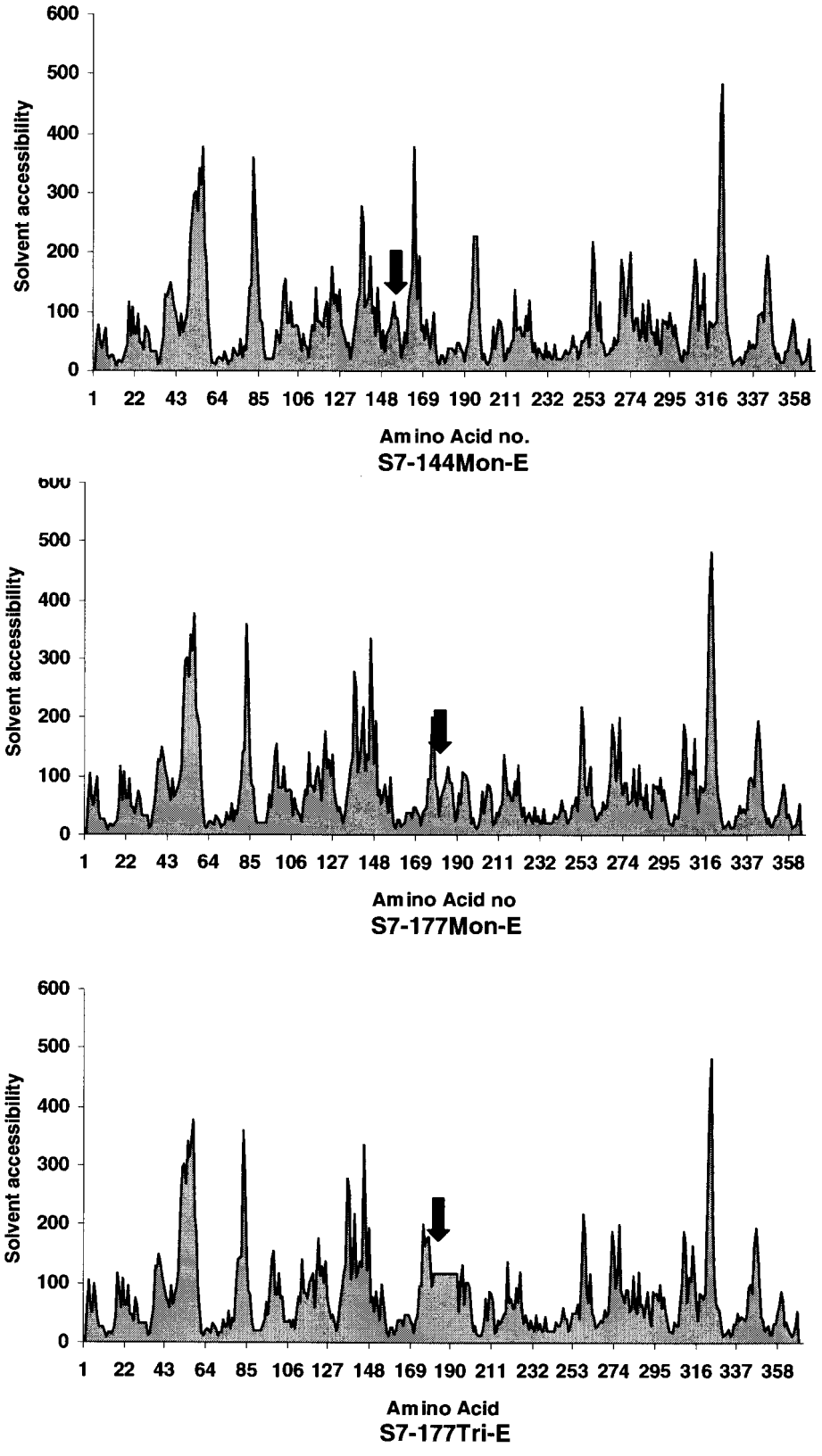


Fig. 3.13 Solvent accessibility profiles generated for insertion mutants S7-144Mon-E, S7-177Mon-E and S7-177Tri-E. The black arrow indicates the region presenting the epitope.

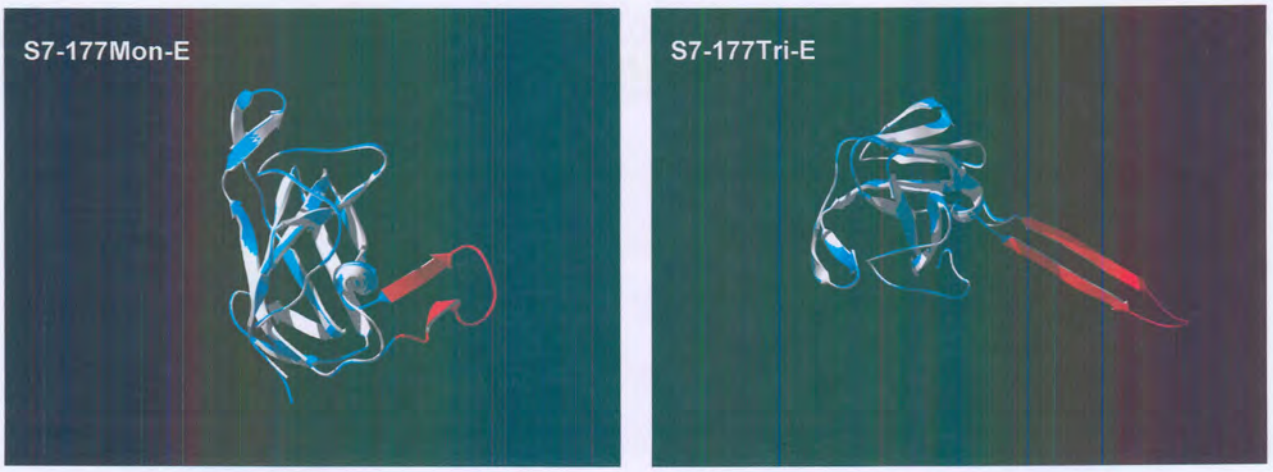


Fig. 3.14 Swissprot generated models of the top domain of S7-177Mon-E and S7-177Tri-E (blue) illustrates the presentation of the epitope (red) on the surface of the protein. The mutant proteins were superimposed on a wild type VP7 backbone (white).

From the microscopic studies it is evident that the expression of the ELDKWA epitope in insertion mutants 144 and 177 resulted in the stabile formation of large crystalline particles. To investigate the immunogenicity of the VP7 display vector, crude purifications of the crystals were prepared and used as immunogens to inject mice over a six week period. The obtained polyclonal serum was subsequently tested for the presence of an epitope specific antibody response to ELDKWA. A synthetic peptide (ELDKWA) as well as HIV-1 lysates of subtype B and C were used as controls in dot and western blot analysis. Although the pooled serums reacted positive to the VP7 constructs no response specific for ELDKWA was detected. This suggests that the constructs did elicit antibody responses but was possibly unable to succesfully present the epitope to the immune system. Several factors however needs to be taken into account. Firstly, although expression of the epitope bearing constructs were shown no experiments were done to prove that the epitope was in fact presented on the surface. To this end it, a monoclonal antibody directed against ELDKWA or HIV+ human sera could be used to verify its presentation. Secondly, due to the low amount of mouse sera obtained after bleeding it was decided to pool the available sera before testing. It is possible that some mice may have provided a better response to ELDKWA than others by pooling the sera the activity was diluted out.

The use of chemically synthized ELDKWA peptides conjugated to a carrier protein, has been shown repeatedly to induce a very strong epitope specific humoral immune response under similar immunization conditions as reported here (Lu *et al*, 2000; Liao *et al*, 2000). In most cases the response resulted in antiserum capable of neutralizing HIV-1 isolates. Other attemps to present the ELDKWA epitope were less succesful. The display of ELDKWA on the surface of the *E. coli* MalE protein was unsuccessful in elicting a response (Coeffier *et al*, 2001). This reflects the importence of the molecular context in which this particular epitope is displayed, as the MalE system has been shown previously to be a powerful display vehicle for foreign epitopes (Martineau *et al*, 1992).

The failure of VP7 crystals to successfully present ELDKWA to the immune system although dissapointing was not entirely unexpected. The hydrophillic and solvent accesibility profiles predicted that these regions were likely to be less well exposed to the environment. Further more due to the hydrophobic nature of the VP7 immunogens it seems reasonable to conclude that the epitopes could be further sheltered from the environment if the crystals aggregates with other crystal particles or other hydrophobic molecules once it is injected.

It should also be noted that at the time of the immunization trial no synthetic ELDKWA peptide was available to inject as a proper ELDKWA control. An immune response (or lack their of) to this control would have provided a better indication as to the reason for the lack of response to the VP7 crystal vector, primarily the efficacy of the immunization protocol.

CHAPTER 4

CONCLUDING REMARKS

4.1 Summary of findings

CHAPTER 2

- The introduction of site-specific mutation L345R in the bottom domains of both wild type VP7 and insertion mutant 200 resulted in an increase in protein solubility.

- The introduction of mutation L345R in mutant 177 did not result in a further increase in protein solubility.

- Introduction of the L345R mutation did not effect the ability of the VP7 L345R mutant proteins to form stable trimers.

- VP7 L345R mutant trimers still assemble into particulate structures.

- A correlation seems to exist between increased VP7 solubility and the absence of the formation of the characteristic large VP7 crystals.

The increase in protein solubility and the resulting absence of large VP7 crystals can be best explained by the inceased hydrophilicity of the botom domains of the VP7 L345R mutants. Although an increase was observed it is unlikely to significantly influence core-like particle yield. The insolubility of VP7 therefore remains an obstacle for the use of core-like particles as an epitope delivery system. It is unlikely that site-specific mutations in VP7 will result in a significant increase in solubility, to the extend that a drastic increase in core-like particle yield will be obtained. The use of core-like particles are therefore considered to be non-viable approach at this time.

CHAPTER 3

- The expression of the HIV-1 neutralizing epitope ELDKWA in insertion mutants 144 and 177 resulted in a marked decrease in protein solubility.

- The decrease in solubility was accompanied by the formation of large VP7 crystalline particles.

- None of the ELDKWA presenting particles was able to induce an epitope specific antibody response in mice.

It is apparent that the physico-chemical nature of the epitope efffects both the solubility of VP7 as well as its display to the environment. Less soluble VP7 protein molecules favours assembly into large crystalline structues. The hydrophobic nature and size of the epitope probably results in poor presentation of the insertion region. Therefore, the inability of VP7 particles to succesfully dislay ELDKWA reflects the impact of the epitope size and chemistry on the system. Further more, in this study neither of the insertion sites proved to be more effective for epitope display.

4.2 Future objectives

There are several other studies currently conducted in our laboratory investigating different aspects of the VP7 particulate display system. One of the questions looked at invloves the insertion of large protein fragments in an effort to determine the maximum insertion range. Construction of VP7 mutants displaying 50, 100 and 150 amino acid fragments of the HIV-1 subtype C envelope protein is underway. In two other studies VP7 double and triple insertion mutants were constructed allowing the simultaneous presentation of several epitopes on one VP7 trimer. There is particular interest in combining these constructs with the multiepitope approach.

In order to gain a better understanding of the VP7 display vector the following questions needs to be answerd.

- The maximum fragment size that can successfully be displayed in each of the insertion sites without completely abbrogating particle formation.

- The ability of larger insert fragments to induce epitope specific immune responses.

- The immunogenicity of smaller crystalline particles needs to be investigated.

- The use of different immunization strategies and test animals.

In conclusion, probably the most important factor determining the successfull presentation of a short linear epitope in the VP7 particulate display system is the inherent chemical nature of the epitope. Through protein modelling relatively good guidelines can be obtained that can help with the future choice and design of epitopes. As there is no indication of the impact the epitope size will have on presentation it is quite possible that fragments in a range large enough to guarantee proper exposure and assembly into particulate structures may be the determining factor for good immune responses. In instances were there is a need for the presentation of short epitopes such as the multiepitope approach, hydrophillic adapters to increase the hydrophillic profile of the insertion region and to increase the size may be a viable option. It is evident that many questions remained unanswered and should receive attention if VP7 is to be developed into a usefull display system.

PAPERS AND CONGRESS CONTRIBUTIONS

Publications in peer-reviewed journals

Maree, F.F., Meiring, T., Riley, J., Meyer, Q.C. and H. Huismans. EFFECTS of SITE DIRECTED INSERTION MUTAGENESIS on the CRYSTAL FORMATION, SOLUBILITY and CLP FORMATION of AFRICAN HORSESICKNESS VIRUS VP7. (In preparation)

International congress participation

Posters

Meyer, Q.C., van Rensburg, R., Riley, J. and H. Huismans. 2001 The effect of a variety of small amino acid insertions in the top domain of African horse sickness virus core protein VP7 on crystal formation, and solubility. XVII BIENNIAL CONFERENCE ON PHAGE/VIRUS ASSEMBLY. Hansaari Congress Center, 02100 Espoo, Finland, June 30 – July 5, 2001.

Maree, F.F., Riley, J., Meyer, Q.C. and H. Huismans. 2000 EFFECTS of SITE DIRECTED INSERTION MUTAGENESIS on the CRYSTAL FORMATION, SOLUBILITY and CLP FORMATION of AFRICAN HORSESICKNESS VIRUS VP7. 7th Conference on double stranded RNA viruses. Aruba, December 2-7, 2000.

Local congress participation

Posters

Meyer, Q.C., Maree, F.F. and H. Huismans. 2000 Effect of site directed mutations in the major core protein, VP7 of African horse sickness virus on solubility and core-like particle assembly. Seventeenth Congress of the South African Genetics Society. Pretoria. June 2000.

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