Development of candidate *Human papillomavirus* vaccines

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Certification by Supervisors

In terms of paragraph GP9 of the regulations for the degree of Doctor of Philosophy at the University of Cape Town, we certify that we approve of the inclusion in this thesis of material already published, or submitted for publication by candidate Arvind Varsani.

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Abstract

The objective of this thesis was to investigate novel and plant-based vaccines against *Human papillomavirus* type 16 (HPV-16), which is primarily responsible for cervical cancer. As a first study, the L1 gene of a South African variant of HPV-16 (L1 504) and a mutant (504[ΔA266T]), where the alanine at 266 was mutated to a threonine, were expressed in insect cells by recombinant baculovirus, and the resulting virus-like-particles (VLPs) were tested with a panel of well-characterised monoclonal antibodies (Mabs). Both the proteins bound neutralising conformation-specific Mabs H16:V5 and H16:E70, conformation specific Mabs H16:U4 and H16:9A, and Mabs H16:J4, H16:I23 and H16:D9 that recognise a linear epitope. However, the binding of Mab H16:E70 to L1 504 was at a 2 x higher level compared to the mutant 504[ΔA266T]. This set the field for later work with the South African isolate, as it was shown to be antigenically equivalent to other well-characterised HPV L1 genes.

A deletion and mutation study was undertaken using a recombinant baculovirus expression system, to identify elements of the L1 structure that are important for maintaining the correct antigenicity and the ability to form particles. The conformation-specific Mabs bound products resulting from a 21 amino acid C-terminal deletion (ΔC483), a 9 amino acid (residues 2-9) N-terminal deletion (M-NΔ10), and the combination of both N - and C - terminal deletions (M-NΔ10CΔ483) of L1 (L1 504) protein. The protein products assembled into VLPs of ~55nm, however for M-NΔ10 a mixed population of VLPs were observed under the electron microscope. Further C-terminal deletion to the conserved cysteine residue (ΔC465 and ΔC427) did not allow for assembly to VLPs; hence, the conformation-specific Mabs were unable to bind these L1 proteins. Mab H16:D9, whose binding regions had not been characterised, was unable to bind the L1 proteins of ΔC427: this suggests that the amino acid region 428-465 is the putative binding region for this Mab. However, the deletion study showed that apart from L1 504 and ΔC483 the scope of using other deletants and mutant genes for expression in plants was limited and hence these two genes were used in the initial investigation of plant-based HPV vaccines.

The potential for plant-based HPV-16 VLP vaccines was investigated by two different approaches. In the first, transgenic *Nicotiana tabacum* cv. Xanthi plants were prepared by *Agrobacterium tumefaciens*-mediated transformation. The second approach looked at transient

expression using a *Tobacco mosaic virus* (TMV) vector. The plant-derived L1 product from both the expression systems yielded a heterogeneous population of particles that comprised some VLPs amongst more numerous pentamer aggregates. These particles maintained a similar antigenicity when assayed by binding to a panel of Mabs to those expressed in insect cells by recombinant baculovirus. The estimated yield of the transiently expressed L1 was ~10 fold higher that than resulting from transgenic expression, however, the TMV vector was found to be unstable. Rabbits immunised with concentrated extracts of plant-derived L1 gave a poor response. This could be attributed to too low a starting concentration of the antigen in the plants.

Candidate L1 chimaeric vaccines were designed to display a cross-neutralising minor capsid protein (L2) epitope on various surface loops and regions of the L1 structure. These were expressed in insect cells by recombinant baculoviruses. Presentation of the L2 epitope in the internal C-D loop (ChiΔH-L2), the E-F loop (ChiΔA-L2) and h4 helix (ChiΔF-L2) prevented the formation of VLPs, whereas presentation in the D-E loop (ChiΔC-L2) and the coil between the h4 helix and β-J sheet (ChiΔE-L2) did not. All the chimaeras bound the entire panel of Mabs, with the exception of ChiΔH-L2 which did not bind any of the conformation-specific Mabs, and ChiΔC-L2 which did not bind the conformation-specific "neutralising" Mabs H16:V5 and H16:E70. Of the five regions investigated, presentation of the L2 epitope in ChiΔF-L2 and ChiΔE-L2 elicited the greatest anti-L1 immune response after immunisation of mice; however, ChiΔF-L2 elicited a better anti-L2 response.

In summary, this thesis successfully investigated the expression of HPV-16 vaccines in plants by transgenic and transient expression systems. Further, it also investigated the potential of HPV-16 L1 to display foreign epitopes for multivalent vaccine development, and outlines the key elements in the L1 structure that are important for maintaining the structure and correct antigenicity of the L1 particles.

Statement for animal experiments

Animal experiments reported in this thesis were approved by the **Animal Ethics Committee** at the **University of Cape Town.**

The animal work was carried out qualified animal technicians.

Chapter 1

Literature review

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1.1 INTRODUCTION

During the last couple of decades, there has been substantial progress in relation to the aetiology of cervical cancer. Prior to this, sexually transmitted infectious agents, such as syphilis, gonorrhoea, chlamidia, trachomatis and *Herpes simplex virus* type 2, were proposed over the years to be causative of cervical cancer (Bosch & Munoz, 2002). Between 1974 and 1976, Prof. Harald zur Hausen started to postulate and analyse the possible role of *Human papillomavirus* (HPV) in cervical cancer (zur Hausen *et al.*, 1974, 1975, 1977). During the early 1980s development of technology to test for presence of HPV DNA in cellular specimens helped establish the definite aetiological role of HPV in cervical cancer (Bosch *et al.*, 2002; zur Hausen, 1996). The first HPV types isolated from cancer biopsies of the cervix were 16 and 18: these were cloned in 1983 and 1984 respectively, and this initiated the rapid expansion of research into this field (Boshart *et al.*, 1984; Durst *et al.*, 1983).

Papillomavirus infections in humans cause a variety of benign proliferations, these include warts, epithelial cysts, intraepithelial neoplasias, anogenital papillomas, oral laryngeal and pharyngeal papillomas, keratoacanthomas and other types of hyperkeratoses (see Table A.1, Appendix A; zur Hausen, 1996). A compelling body of clinical, molecular and epidemiological evidence has now established that HPV types associated with anogenital neoplasms, including condylomata, cervical dysplasia and cervical carcinoma, are almost always sexually transmitted (IARC, 1999; Lowy *et al.*, 1994). Involvement of HPV in a considerable proportion of cancers of the vulva, vagina, anal canal, perianal skin and penis is currently being addressed in addition to the possible role of HPV in skin cancer, oral cavity cancers and other cancers of the upper aerodigestive tract (Bosch & Munoz, 2002).

1.2 HPV AND CERVICAL CANCER

HPV has been detected in more than 90% of cervical cancer cases and therefore implicated as the main cause of cervical cancer (Munoz, 2000; Wallboomers *et al.*, 1999; zur Hausen, 1996). Clifford *et al.* (2003) recently reported a meta-analysis of HPV types in cervical cancer. They found that in squamous cell carcinoma, HPV-16 was the predominant type (46-63%) followed by HPV-18 (10-14%), 31 (2-7%), 33 (3-5%) and 45 (2-8%) in all regions except Asia where HPV types 58 (6%) and 52 (4%) were more frequently identified whereas in adenosquamous

carcinoma HPV-18 was the predominant type in every region (37-41%), followed by 16 (26-36%) and 45 (5-7%). In addition to these the other HPV types considered carcinogenic are HPV-31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82, in addition, types 26, 53 and 66 are probably carcinogenic (Munoz et al, 2003; Steller, 2002).

An estimated 400 000 women develop cervical cancer each year accounting for almost 12% of all cancers in women (Goldie, 2002; Pisani *et al.*, 1999; zur Hausen 2002). Cervical cancer is a preventable disease, however, it is a major burden on public health resources in Africa, where less than 5% of the women are screened in the very setting where more than 80% of these cases occur due to the lack of screening (Goldie, 2002; Ponten *et al.*, 1995). Countries in sub-Saharan region have some of the world's highest age-standardised death rates from cervical invasive cancer, e.g. 67 per 100 000 people in Harare, Zimbabwe, 40.8 per 100 000 in Kampala, Uganda (Chirenje *et al.*, 2001). In South Africa the age-adjusted incidence rate (ASIR) of cervical cancer in the African population is 35 versus 11.7 per 100 000 in the white population (Sitas *et al.*, 1997). In the year 2001, 23 million women were reported to be living with HIV/AIDS (www.unaids.org). It is estimated by de Sanjose & Palefsky (2002) that 529 000 cases of invasive cervical cancer would develop amongst HIV infected individuals during their life expectancy.

Screening by Papanicolaou (Pap) smears in the United States has resulted in a 70% decline in the mortality from cervical cancer during the past 50 years (Bosch *et al.*, 2002; Plummer & Franceschi, 2002). However, in developing countries women have limited access to these screening programmes and there are many obstacles in setting up effective screening programs in low resource settings. Screening techniques that are more appropriate to low resource settings are currently being investigated as opposed to currently used cytology based methods (Shankaranarayanan *et al.*, 1998).

Cervical cancer develops gradually over time, with precursor lesions or cervical intraepithelial neoplasia (CIN) occurring prior to invasive cancer and the latency period between initial HPV infection and cancer can be more than 10 years (zur Hausen, 2002). The premalignant stages can be identified both clinically by speculum examination and in the laboratory by Pap smear analysis, however, cervical cancer develops in a multi-step process over a fairly long period of time and involves both the presence of oncogenic HPV genotypes and the interaction of various host factors (Table 1.1; zur Hausen, 2002). Even though a high percentage of sexually active women are exposed to and develop productive HPV infections, only a small percentage go on to

develop cervical cancer (Brisson *et al.*, 1996; Elfgren *et al.*, 2000). In most cases the HPV infection is cleared by the immune system mediated cytotoxic T cells by the interaction of the viral epitopes with major histocompatible class (MHC) I molecules (Steller, 2002). Even though HPV infections are common among younger women of the age group 19-35 years, cervical cancer is more common in women over the age of 35 years (Anderson, 2002).

TABLE 1.1: The stepwise progression of high risk HPV – induced lesions (zur Hausen, 1996, 2002).

Stage	Genetic modification	Clinical effect
1	Induction of chromosome instability	No visible modification
2	Disruption of a signalling cascade (CIF 1) interfering with viral oncogene function	Low grade CIN
3	Integration of viral DNA and partial deregulation of E6/E7 oncoprotein expression	High grade CIN
4	Disruption of a signalling cascade under paracrine control (CIF II) suppressing viral oncogene transcription	Carcinoma in situ
5	Failing immunological control of HPV-modified cells	Invasive cancer

Various co-factors have been shown to be associated with cervical cancer. Bosch & Munoz (2002) report these co-factors to be high parity (seven or more pregnancies), long-term use of oral contraceptives (five or more years) and smoking. Factors that are currently being evaluated are infections with Chlamidia trachomatis and *Herpes simplex virus* type 2, co-infection with HIV and to a lesser established level, some nutritional factors (Bosch & Munoz, 2002). HIV-positive women have been shown to be at an increased risk of cervical squamous intraepithelial lesions when compared with their HIV-negative counterparts. The association is stronger in women with low CD4+ T-lymphocyte count (Ahdieh *et al.*, 2000; Palefsky *et al.*, 1999). However, some studies have shown that there is no significant increase in the incidence of invasive cervical cancer as a consequence of the HIV epidemic (Gallagher *et al.*, 2001; Gichangi *et al.*, 2002) and hence this issue is still open to scientific debate.

The most promising means of controlling and reducing the incidence of cervical cancer would be by checking *Human papillomavirus* (HPV) infection which is the major causative agent of cervical cancer (Walboomers *et al.*, 1999). However, in developing countries, the most appropriate intervention to this disease burden would be by immunisation against HPV infection by a prophylactic or therapeutic vaccine.

1.3 CLASSIFICATION OF PAPILLOMAVIRUSES

Recent taxonomic developments have separated papillomaviruses from polyomaviruses. Therefore, papillomaviruses belong to the taxonomic family *Papillomaviridae*, in the a single genus *Papillomavirus*. HPVs are provisionally listed as a single species in the genus (van Regenmortel *et al.*, 2000).

Papillomaviruses are widespread in nature and have been recognised primarily in higher vertebrates including humans, cattle, rabbits, horses, dogs, sheep, elk, deer, non-human primates, mice and some avian species. They are highly species-specific and there is no firm evidence of a papillomavirus from one species causing productive infection in a second species. Most animal papillomaviruses are associated with purely squamous epithelial proliferative lesion (warts), which can be cutaneous or can involve the mucosal squamous epithelium from the oral pharynx, the oesophagus, or the genital tract (Howley, 1995). In humans they infect the skin, mouth, oesophagus, larynx and anogenital tract (Steller, 2002).

Chan *et al.* (1995) divided the PVs into five supergroups, A to E (Figure 1.1). Most of the clinically important viruses were assigned to the subgroup A (mucosal and genital PVs) or B (epidermodysplasia verruciformis PVs). Supergroups C and D comprise of ungulate PVs, C consists of fibropapillomaviruses and D consists of those viruses that cause true papillomas. Supergroup E is the most poorly defined of the five and consists of a mix of animal and human cutaneous PVs (Figure 1.1). PVs are described as "types", and a HPV type is defined as a genome whose L1 gene nucleotide sequence differs from the homologous nucleotide sequence of every other HPV type by at least 10%. However, most pairwise sequence distances between HPV exceed this percentage by a wide margin (Chan *et al.*, 1995, 1997). Similar distance magnitudes are found among animal PVs, and hence this definition applies to them as well (Chan *et al.*, 1997). Independent isolates of the same PV types are referred to as "variants" or "subtypes" and their nucleotide sequences generally differ from one another by less than 5% in most genomic regions (Chan *et al.*, 1992a, 1995).

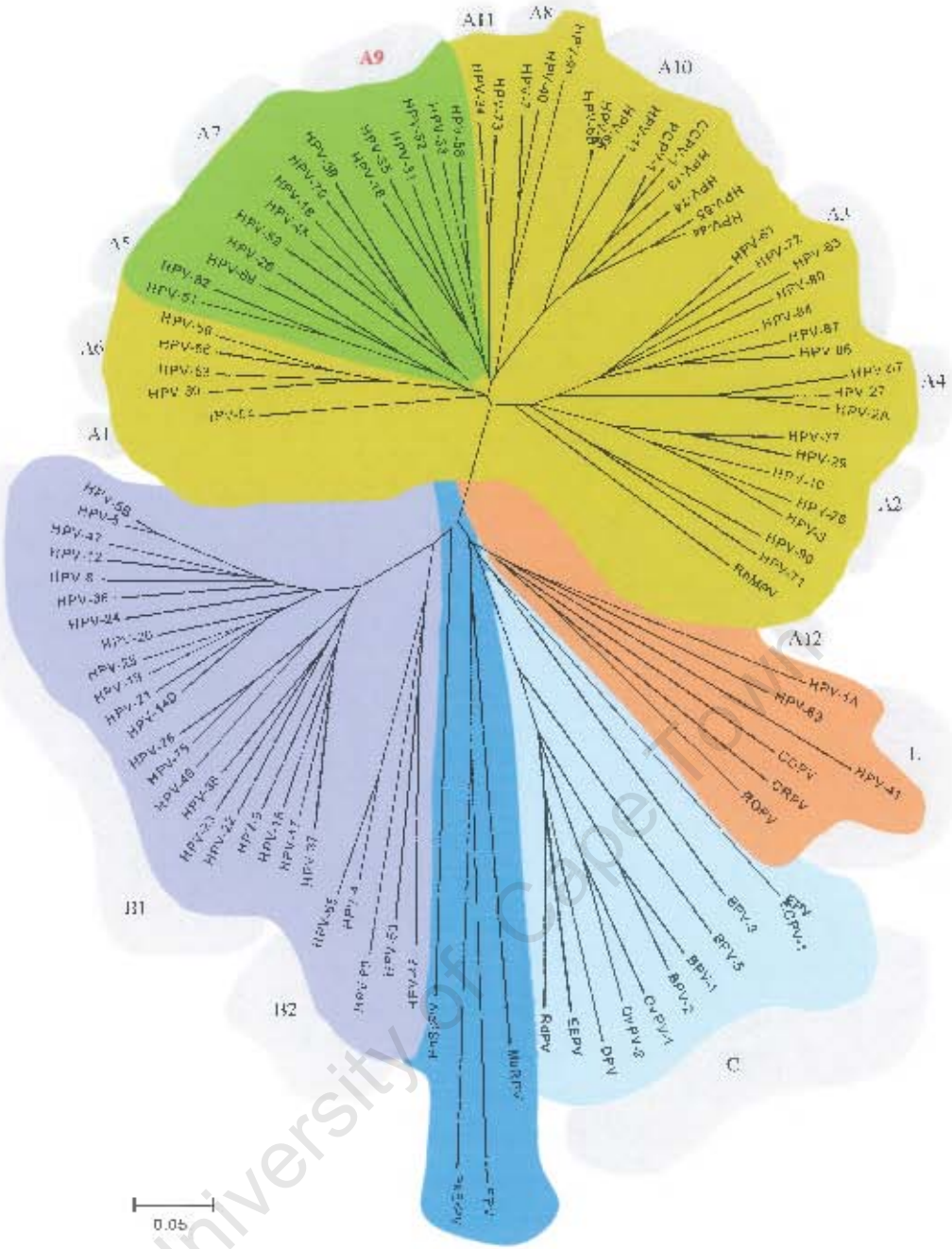


FIGURE 1.1: Neighbour joining phylogenetic tree of 101 PVs based on the nucleotide sequence of the major capsid gene (L1). The PV sequences were downloaded from the GenBank database (see Table A.1 and A.3, Appendix A for accession numbers). These sequences were edited using BioEdit sequence alignment editor (Hall, 1999), the multiple sequence alignments were carried out using Clustal x 1.8.1 software (Thompson *et al*., 1997) and phylogenetic analysis was conducted using *MEGA* version 2.1 (Kumar *et al*., 2001). Groups A5, A7 and A9 consist of the high-risk HPVs types and HPV-16 is part of the A9 group in the supergroup A. Unabbreviated names of the PVs and GenBank accession numbers for the L1 are provided in Table A.1 and A.3 (Appendix A). The distance bar represents 0.05 units.

1.3.1 Evolution and relationships of papillomaviruses

Since PVs are DNA viruses, they are proposed to evolve up to six orders of magnitude more slowly than RNA viruses (Halpern, 2000). This is largely because their replication is linked to the division of the infected host epithelial cells (Shimmin *et al.*, 1993). The molecular epidemiology of variants of several HPV types suggests that HPVs existed at least a few hundred thousand years ago (Bernard *et al.*, 1994). Bernard *et al.* (1994) estimated diversification of most variable parts of the HPV genome occurs maximally at a rate of 0.25% over a period of 10 000 to 20 000 years. In general, HPV isolates have been relatively stable during the period for which sequences are available (Halpern, 2000; Xi *et al.*, 1995). Serial detection of genital HPVs in a single patient at a point in time do not show a quasispecies or mutant spectrum characteristic of rapidly mutating RNA viruses (Ho *et al.*, 1991).

PVs have both animals and humans as hosts, yet viruses that infect animals are found in several separate regions of the phylogenetic tree, intermingling with those infecting humans (Figure 1.1). Interspecies transmission between humans and primates was initially suggested by Meyers & Farmer (1995) especially with respect to group A10, where PCP and CCPV are on the same branch as some HPVs. This has subsequently been challenged by Chan *et al.* (1997) who suggested that interspecies transmission, if at all, would have occured between ancestral species. The mammalian PVs are more closely related to each other than are to other animal PVs and highly divergent PVs such as FPV, PhSpPV, PsErPV and MuRPV are found in more remote hosts (Figure 1.1). Chan *et al.* (1997) suggested that lineages of viruses have been limited to evolving within the lineages of their host by host-linked evolution, and that there have not been any instances of zoonosis and state "the branches in PV evolution have been determined as a response to host speciation. Superimposed on this are mechanisms of drift and selection unrelated to host speciation resulting in the intrahost species diversity we see today". There are isolated observations that might reflect PV evolution in response to change in selection pressure as demonstrated by Roden *et al.* (1997b) where a HPV-16 L1 variant was identified that had lower binding affinity to a neutralising antibody (H16:E70).

1.4 MOLECULAR BIOLOGY OF HUMAN PAPILLOMAVIRUSES

To date over 80 different HPV types have been sequenced and there exist more than a 100 additional HPV types that are less well characterised (IARC, 1999). HPVs are a heterogeneous group of DNA viruses with double stranded closed circular genomes of ~ 8 kilobases (kb) (Seedorf *et al.*, 1985). The HPV genome (Figure 1.2) encodes six early open reading frame (ORF) proteins (E1, E2, E4, E5, E6 and E7) and two late ORF proteins (L1 and L2). The late genes encode the viral capsid proteins whereas the early genes encode proteins involved in a variety of regulatory functions.

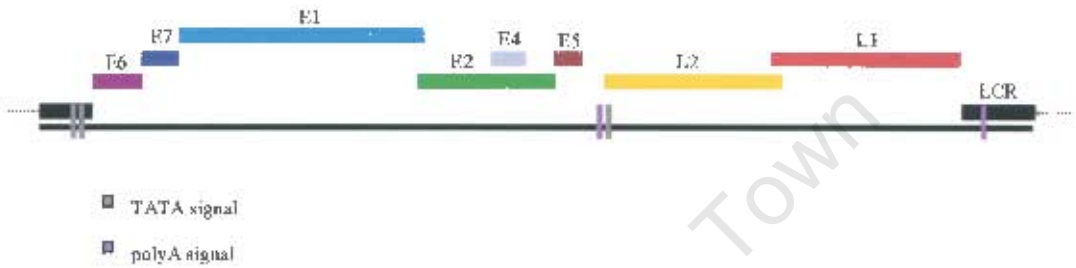


FIGURE 1.2: Organisation of the HPV-16 genome showing the eight ORFs in the three translation frames and the long control region showing the six early genes (E1, E2, E4, E5, E6 & E7), the two capsid genes (L1 & L2) and along control region (LCR).

Mucosotrophic HPVs are grouped into low-risk and high-risk categories on the basis of each genotype's association with benign or malignant disease process. Low risk HPV types 6 and 11 are commonly detected in condyloma accuminata but hardly ever found in cervical carcinomas (Lowy & Schiller, 1998). In contrast HPV types 16 and 18 are detected in over 70% of squamous cell carcinomas of the cervix (Clifford, 2003; de Villiers, 1989; Steller, 2002). The E6 and E7 genes of high-risk HPV types encode oncoproteins that immortalise human keratinocytes whereas those of low-risk are non-transforming (Pecoraro *et al.*, 1989).

1.4.1 HPV pathogenesis

HPV infects and replicates in squamous epithelium and links its life cycle to the differentiation programme of the host epithelial cells (zur Hausen, 1996). HPVs are able to infect basal stem cells and establish their genomes as low copy episomal DNA through minor skin abrasions

(Tindle, 2002). Viral episome replication occurs synchronously with host cell chromosome replication and following cell division, the infected daughter cells follow the normal epithelial cell differentiation pathway, migrating from the basal layer towards the surface epithelium (Tindle, 2002; zur Hausen, 2002). Viral infection and limited replication in the suprabasal layers is accompanied by expression of the HPV early proteins, E1, E2, E5, E6 and E7 (zur Hausen, 1996). E4, although located in the early genomic region, is generally expressed as a late gene (Howley, 1995). The late or coat protein genes (L1 and L2) are expressed giving rise to virions as a result of the distal movement of the infected cells and the virus production is restricted to cells in transition from proliferating undifferentiated (stem) cells to more distal non-dividing differentiated cells (Hines *et al.*, 1995; Tindle, 2002). There is limited host cell death and inflammation associated with HPV infection and the mature virus is shed externally from distal terminally differentiated keratinocytes (Tindle, 2002). Expressions of the early gene products, E2, E6 and E7, determine whether an HPV infection is active or latent, or leads to malignant transformation (Anderson, 2002). The E6 and E7 oncogene protein expression is modulated by transcription regulator encoded by the E2 gene (Anderson, 2002). Integration of HPV into the host chromosome leads to the disruption of the E2 gene and therefore leads to increased expression of E6 and E7 (Anderson, 2002; zur Hausen, 2002). Oncogene E6 and E7 products of HPV-16 and 18 can bind to and inactivate cellular tumour suppressor gene products p53 and pRb thereby cause their degradation (Anderson, 2002; Kaufman *et al.*, 2000). In the development of cervical cancer, this plays an important role by altering host gene expression, releasing cells from cell cycle checkpoints, affecting DNA repair process and /or activating the expression of telomerase (Anderson, 2002).

1.4.2 Host control of HPV infection

Cell-mediated and humoral responses towards HPV infection by the host are important aspects in controlling the infection. Then knowledge of humoral immune response to HPV antigens such as the viral particles composed of the L1 and L2 capsid protein and the E6 and E7 oncoproteins, is limited by the difficulty in measuring specific immunity (Hines *et al.*, 1995; Stern *et al.*, 2000). However, serological assays using virus-like particles (VLPs) indicate that a high proportion of individuals that are exposed to HPV develop systemic antibodies to the L1 major capsid protein (Brown *et al.*, 2001; Carter *et al.*, 1996; 2000; Emeny *et al.*, 2002; Firzlaff *et al.*, 1988; Hagensee *et al.*, 1997; Kirnbauer *et al.*, 1996; Lehtinen *et al.*, 1996; Nonnenmacher *et al.*, 1995; Wideroff

et al., 1995; Wideroff *et al.*, 1999). Numerous studies in animal models have shown antibody-mediated protection against animal papillomavirus infection when VLPs were used as antigens. The antibodies recognised conformational epitopes on the VLPs and were able to neutralise animal viruses (Breitburd *et al.*, 1995; Christensen & Kreider, 1991; Suzich *et al.*, 1995). Further, data from a recent phase II clinical trial showed antibody mediated protection against HPV-16 infection in females immunised with a HPV-16 VLP-based vaccine (Koutsky *et al.*, 2002). This provides proof that neutralising antibodies against HPV are efficient in preventing HPV infection. Neutralising antibodies against HPV are discussed in detail in section 1.6.1.4.

Cellular immunity also plays a key role in the control of HPV infection. Antigenic viral peptides that are recognised by cytotoxic T lymphocytes (CTL) are presented at the cell surface after complexing with MHC I, serve as targets for CTLs (Hines *et al.*, 1995). The CTLs recognise the presented viral peptide within the MHC I molecules and trigger the secretion of cytokines such as tumor necrosis factor (TNF) α , perforin, interferon γ (IFNγ; Hines *et al.*, 1995). Some insight into these early events can be drawn from studies carried out on the regression of genital warts by Coleman *et al.* (1994) where treatment of genital warts with IFNγ and IFNα 2a showed a marked increase, in responders to this treatment, in infiltration of T-helper 1 (Th1) inflammatory cells, macrophages and natural killer cells, with the activation of CD4+ cells indicating a proliferative response to HPV L1 capsid proteins of types 1 and 11. Non-responders had depleted Langerhan cells (LC) with less T-cell infiltration and reduced levels of IL-1, GM-CSF and TNF and high levels of HPV E7 expression (Arany & Tyring, 1996; Hong *et al.*, 1997). These results suggest that the events in lesion destruction are Th1 response dependent and do not necessarily target the E7 protein (Stern *et al.* 2000). Barnard & McMillan (1999) reported that E7 could inhibit the induction of IFNα inducible genes but not IFNγ inducible genes which is similar to a report by Schneider *et al.* (1987) who reported that HPV-16 and 18 lesions showed a reduced response to IFNα treatment and decreased levels of E7 in comparison to HPV-6 and 11 lesions. Hence, high-risk HPV lesions may be relatively refractory to host innate immune responses compared to low risk type infections (Stern *et al.* 2000).

It was demonstrated by Li *et al.* (1999) that HPV-18 E6 binds to Tyk2 and thus preventing it from binding to the IFNα receptor 1, leading to the breakdown of the IFNα Jak-STAT pathway. The binding of E6 to Tyk2 is viral type dependent: for instance, E6 from HPV-18 binds stronger than that of HPV-6, therefore suggesting that lesions as a result of low risk HPV types are more

susceptible to IFNα due to the poor ability of the E6 to interfere with the Jak-STAT pathway; therefore persistence of high risk HPVs may be as a result of viral oncogene mediated protection from interferon induced by innate immunity (Stern *et al.*, 2000).

1.4.3 Function of HPV early proteins and the long control region

1.4.3.1 Long control region (LCR)

The LCR of papillomaviruses contains most of the *cis*-responsive regulatory elements (zur Hausen, 1996). The LCR of HPV-16 is 850bp in length and similar in organisation to other genital HPVs (Figure 1.3). The E6 promoter, the enhancer and the replication origin are collectively flanked by two nuclear matrix attachment regions (MARs), located in the 5' region of the LCR and in the E6 gene itself (Tan *et al.*, 1998). The 3' portion contains the replication origin and the E6 promoter, which has four *cis*-responsive elements (which modulate E6/E7 promoter activity) i.e. a binding site for the transcription factor SP1, two binding sites for the viral factor E2 and a TATA box (Chiang *et al.*, 1992; Desaintes *et al.*, 1997; Dong *et al.*, 1994; Tan *et al.*, 1994).

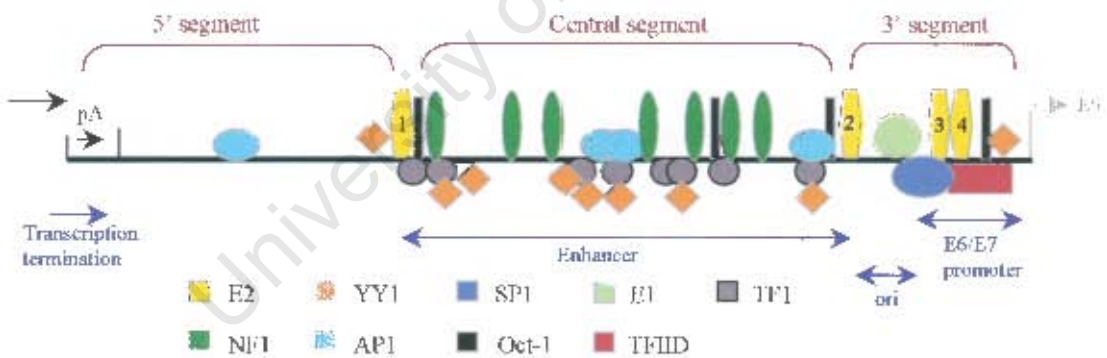


FIGURE 1.3: A schematic representation of the HPV-16 LCR (O'Conner *et al.*, 1995). The four E2 binding sites divide the LCR into functionally distinct segments: 5', the central, and the 3' segment. The 5' segment contains the transcription termination polyA signal (pA); the central segment contains the epithelial specific enhancer that contains the majority of transcription factor binding sites; the 3' segment contains the origin of replication and the p97 E6/E7 promoter. All the transcription factor-binding sites are indicated. Descriptions and functions of the transcription factors are summarised in Appendix B.

Binding of SP1 to its site activates the E6/E7 promoter, but not replication, because SP1 blocks E2 binding and enhances E1-dependent replication initiation and displacement of SP1 by E2 at

specific concentrations activates replication and transcription is partially repressed (Desaintes *et al.*, 1997; Tan *et al.*, 1994). The binding of E2 to the #4 E2 binding site leads to transcription factor IID (TFIID) displacement and further repression, therefore, such a series of events constitute a switch between replication and transcription initiation and would depend upon the local concentrations of E2 (Desaintes *et al.*, 1997).

1.4.3.2 Replication proteins E1 and E2

The E1 protein is highly conserved among HPV types, and recognises and binds to specific DNA elements in the viral origin of replication (*ori*), located in the proximal end of the LCR (Wilson *et al.*, 2002; zur Hausen, 1996). E1 on its own binds with low specificity to the *ori*; the specific and efficient binding recognition is accompanied by cooperative binding of E1 and transcription factor E2 to immediately adjacent sites (Lusky *et al.*, 1993; Mohr *et al.*, 1990; Sedman & Stenlund, 1995). E1 binds as a dimer together with a dimer of E2 to the *ori*, which results in the formation of the E1E2-ori complex, which is highly sequence specific (Sedman *et al.*, 1997). E1 binds to a recognition sequence consisting of the hexanucleotide sequence AACNAT, with relaxed specificity for transitional changes in positions 1 to 3 and where N is any base but G (Chen & Stenlund, 2001). Once the complex is formed, in an ATP-dependent step, E2 can be displaced and additional E1 molecules can be added to the complex (Sanders & Stenlund, 1998).

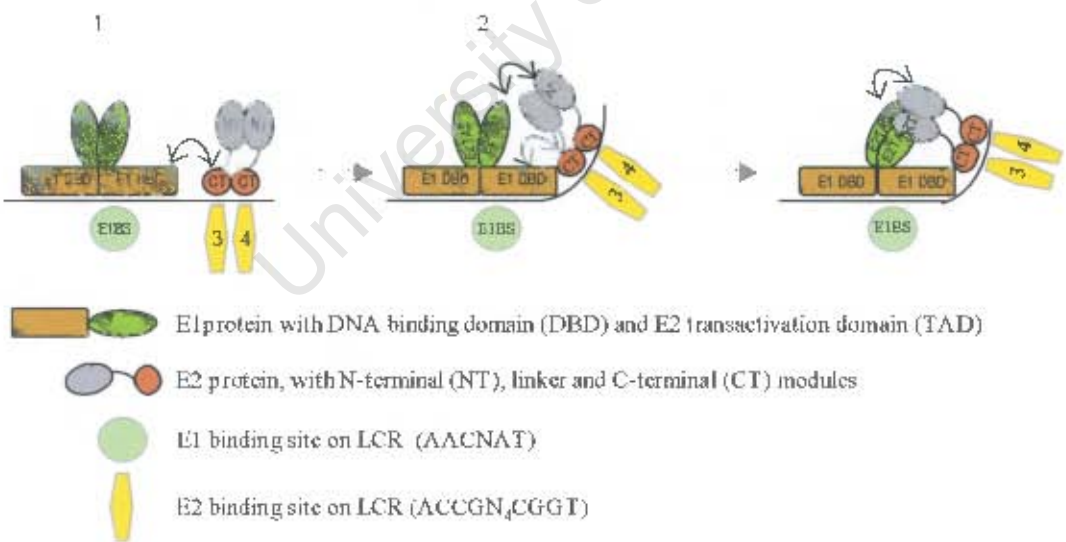


FIGURE 1.4: Model of cooperative binding of E1 and E2 proteins to the ori on the LCR (Chen & Stenlund, 2000). The cooperative binding involves two steps. The first is E1 DBD and E2 C-terminal DBD interaction which causes alteration in the DNA structure, which results in the kinking of the DNA which places the E2 N-terminal activation domain (AD) in a position where it can interact with the E1 helicase domain which forms the second step of the cooperative binding.

The E2 protein has three distinct modules: the sequences of the N-terminal module (E2NT) is responsible for interaction with viral and host-cell transcription factors such as SP1, TFIIB and AMF-1 (Breiding *et al.*, 1997; McBride & Meyers, 1997; Yao *et al.*, 1998). The E2NT is followed by a flexible, proline rich linker module and a C-terminal module (E2CT). E2CT binds tightly as a homodimer to DNA sites with a consensus sequence of $ACCGN_4CGGT$ (McBride & Meyers, 1997). During early stages of viral infection, the E2 protein represses the transcription of the oncogenes E6 and E7. Desaintes *et al.* (Desaintes *et al.*, 1997) reported that E2 proteins of HPV-18 and BPV-1 could induce apoptosis when expressed in HeLa cells, in part by restoring the p53 functions as a result of E2-mediated repression of E6 expression.

1.4.3.3 E4 protein

The E4 protein is located in the early genomic region, yet it is generally expressed as a late gene with a role in productive infection (Howley, 1995). The E4 protein localises and aggregates into cytoplasmic and nuclear inclusion granules within the differentiating layer of the infected epithelium and in the basal layer, implying both an early and late function (Roberts *et al.*, 1993). In warts, levels of E4 expression usually correlate with those of virion coat proteins and hence involvement in virus maturation and/or vegetative viral DNA replication has been suggested (Brown *et al.*, 1995, 1996; Crum *et al.*, 1990; Sterling *et al.*, 1993). Since E4 has been shown to associate with keratin cytoskeleton by promoting cytoskeleton collapse, its role in virus release has been suggested (Doorbar *et al.*, 1997).

1.4.3.4 Oncoproteins E5, E6 and E7

E5 is the major transforming protein in *bovine papillomavirus* (BPV), however, in HPV infections, E5 has only weak transforming activity (Pim *et al.*, 1992, Schiller & Lowy, 1996). Due to the integration of the HPV genome during malignant progression, the E5 gene is not expressed in cervical tumours but large amounts of E5 mRNA and protein have been detected in anogenital low-grade intraepithelial neoplasia (Kell *et al.*, 1994). This may support the possibility that E5 plays a role in early steps of HPV infection but is dispensable for maintenance of malignant transformation (zur Hausen, 1996).

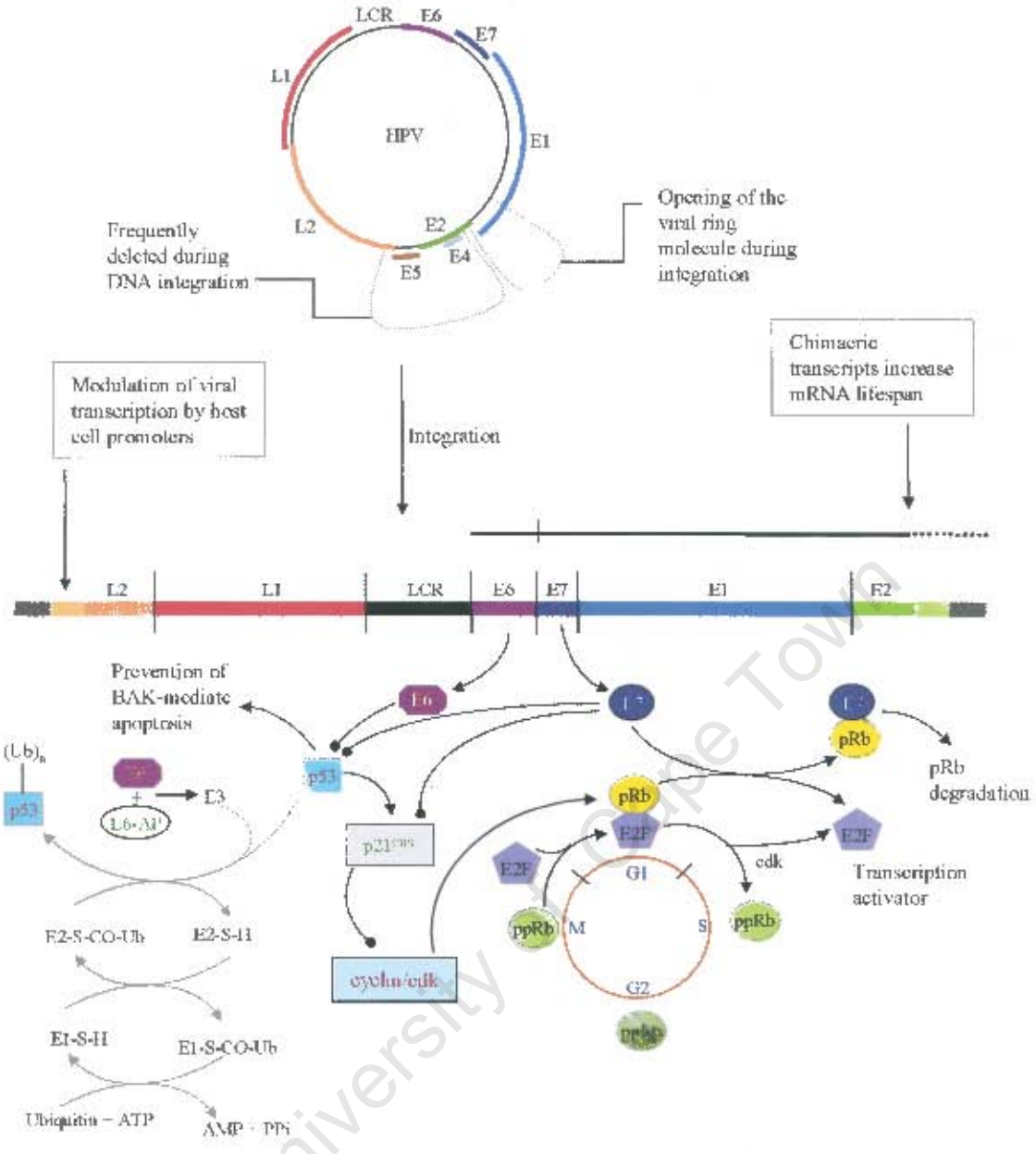


FIGURE 1.5: An illustrated summary of the functions of the E6 and E7 proteins (Munger & Howley, 2002; zur Hausen, 2002). The host cell integrated genes E6 and E7 are expressed and their protein products are responsible for the oncogenesis. E7 interferes with transcriptional activity of p53, inactivates the cdk inhibitor $p21^{CIP1}$ (a major mediator of p53-induced growth arrest) and blocks pRb binding to E2F. The combined inactivation of pRb and p53 tumor suppressor pathways interferes with the integrity of important cell checkpoints and the cellular apoptosis defence programme. The E6 protein can block apoptosis by binding to p53 and targeting this tumour suppressor for proteolysis via the ubiquitin pathway.

The role of malignant transformation can be assigned to the E6 and E7 genes and their proteins, which are consistently expressed in malignant tissue. Figure 1.5 summarises the role of E6 and E7 carcinogenesis. In the course of cancer part of the HPV viral DNA integrates into the host-cell

DNA (Figure 1.5). The circular viral DNA is opened within the E2 ORF and the adjacent E4, E5 and part of the L2 ORFs are deleted (zur Hausen, 2002).

Late in the M-phase, ppRb is dephosphorylated to pRb which forms transcription repressor complex with E2F (Munger & Howley, 2002). E7 binds pRb, leading to the release of E2F transcription factors and up-regulation of cyclin-dependent kinase inhibitor $p16^{INK4A}$ (Morris *et al.*, 1993; Munger & Howley, 2002). The increased E2F activity leads to apoptosis in E7 expressing cells and E7 stimulates the S-phase gene cyclin A and cyclin E which seems to block the function of cyclin-dependent kinase inhibitors $p21^{CIP1}$ and $p27^{KIP1}$ (Jones *et al.*, 1997; Zerfass-Thome *et al.*, 1996). HPV E7 interferes with transcriptional activity of p53 and inactivates the cdk inhibitor $p21^{CIP1}$ which is a major mediator of p53-induced growth arrest (Munger & Howley, 2002; Wang *et al.*, 1996). Therefore, the combined inactivation of pRb and p53 tumour suppressor pathways interferes with important cell checkpoints and cellular apoptosis defence programme (Munger & Howley, 2002).

E6 binds E6-AP (recognises p53) and catalyses the formation of an isopeptide bond between the carboxy-terminal glycine of ubiquitin and a lysine side chain of p53 (Scheffner *et al.*, 1993). In catalysing the ubiquitination of p53, HPV-16 E6 also induces self-ubiquitination and proteolysis of E6-AP (Munger & Howley, 2002). In addition, E6 also degrades the pro-apoptotic protein BAK, which results in resistance to apoptosis and an increase in chromosome instability (Jackson *et al.*, 2000). Further, E6 prevents E7-induced apoptosis by degrading the apoptosis-inducing proteins p53 and BAK (Thomas & Banks, 1999).

1.4.4 PV capsid proteins

Papillomaviruses encode two viral structural proteins, the major capsid protein (L1) and the minor capsid protein (L2). The HPV-16 L1 capsid protein comprises 504 residues and has a molecular weight (MW) of 55 KDa. The L2 has 473 residues and has a MW of 50 KDa. PV capsid proteins have nuclear localisation signals (NLS); HPV-16 L1 has two NLS sequences (as do most PV L1s) consisting of clusters of basic amino acids (Zhou *et al.*, 1991a). The first is situated at the carboxy-terminal of L1 and consists of the sequence KRKKRK (amino acids 499 to 504) and another bipartite NLS (separated by 12 amino acids) overlaps this site, situated at amino acids 484 to 486 (KRK) and 499 to 500 (KR; Zhou *et al.*, 1991a). The L2 NLS is located

between residues 456 to 461 (RKRRKR; Zhou *et al.*, 1991a). The NLS is recognised by adapters of the karyopherin α (Kapα) family and by import receptors of the karyopherin β (Kapβ) family that shuttle between the nucleus and cytoplasm (Nelson *et al.*, 2002). Nelson *et al.* (2002) showed that HPV-16 L1 capsomers are translocated into the nucleus by forming complexes with Kap α2β1 heterodimers. The HPV16 L1 major capsid protein enters the nucleus via Kap α2β1 mediated pathway to assemble the virions during the productive stage of infection and also inhibits the Kap β2-mediated nuclear import of host hnRNP A1 protein thereby favoring virion formation (Nelson *et al.*, 2002).

HPV-16 L1 pseudovirions are capable of binding cell receptors but L1-L2 pseudovirions achieve higher infectivity, suggesting that L2 may affect infectivity at a postadsorption level (Kawana *et al.*, 2001). The residues 108-120 of the HPV-16 L2 have been shown to dramatically reduce pseudovirion infectivity in competition assays carried out in monkey COS-1 cells, which suggests the presence of cell surface receptors for the HPV-16 L2 region 108-120 (Kawana *et al.*, 2001). Furthermore, saturation of the surface L2 receptors with the L2 peptide (108-120) lowered the measured level of infectivity of the L1-L2 pseudovirion to that of L1 pseudovirions (Kawana *et al.*, 2001). Recent studies have shown that the L2 localises to nuclear structures called nuclear domain 10 (ND10) or promyelocytic leukaemia protein (PML) oncogenic domains (PODs), and recruits L1 into these domains (Day *et al.*, 1998). Okun *et al.* (2001) have identified interaction domains in L2 of BPV-1 (residues 129-246 and 384-460) necessary for encapsidation of the viral genome but it is not known at what stage in the viral assembly, L1 and L2 interact. Day *et al.* (1998) from their studies on the interaction of L1 and L2 suggest that virion assembly is triggered by interaction of L2 with ND10 and the colocalisation of the L1. It is likely that the association of the L1 with ND10 is as a result of direct interaction of L1 with L2, since stable L1-L2 complexes form in both fully assembled VLPs *in vivo* and also in partially assembled capsomers *in vitro* (Okun *et al.*, 2001). Although L1 can self-assemble into virus-like particles (VLPs) in the absence of L2 (Kirnbauer *et al.*, 1992; 1993), addition of L2 increases VLP formation by 4-fold in insect cells and 100-fold in mammalian cells (Hagensee *et al.*, 1993; Kirnbauer *et al.*, 1993; Zhou *et al.*, 1993). This greater efficiency could be the result of an increased rate of capsid assembly as a consequence of the L2-mediated concentration of the L1 in ND10 domains. Florin *et al.* (2002) suggest that synthesis of L2 is initiated prior to L1 in the terminally differentiated keratinocytes. L2 is translocated into the nucleus independently of the L1, inducing the reorganisation of ND10 whereas L1 is assembled into capsomers in the cytoplasm and the

capsomers are then translocated into the nucleus and recruited to ND10 after L2-induced release of Sp100, a major ND10-associated protein, which is subsequently degraded (Florin et al., 2002). L2 and the L1 capsomers in the nucleus then further their assembly into capids (Florin et al., 2002).

1.4.4.1 DNA packaging of viral genomes

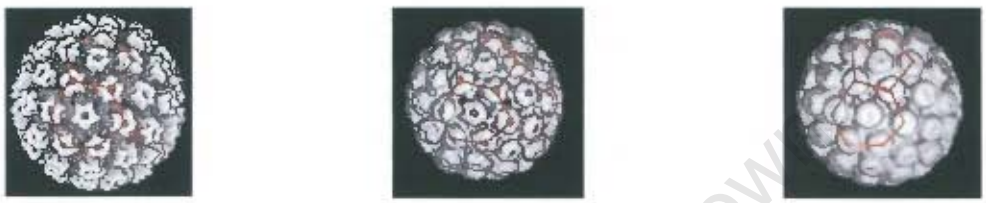
Zhou *et al.* (1994a) reported that basic amino acids (Arg and Lys) at the N-terminal (12 amino acids) were required for the interaction and exhibited no DNA sequence specificity. The positively charged side-chains of these amino acids appear to interact with the phosphate backbone of the DNA. Day *et al.* (1998) speculate that the viral genome encapsulation is due to the L2-dependent translocation of the E2-genome complex to the N10 domains since E2 binds multiple sites on the viral genome as demonstrated by Androphy *et al.* (1987) for BPV. However, a recent study by Zhao *et al.* (1999) demonstrated that deletion of the E5, E4, E3, E2 and E8 regions of BPV did not affect DNA packaging efficiency by BPV VLPs but the deletion of the E1 gene resulted in no DNA packaging. Similar observations were made for HPV-6b, clearly indicating that the E1 region is important for DNA packaging (Zhao *et al.*, 1999).

Packaging of plasmid DNA by PV VLPs (5.4kb - 8kb) have been extensively studied either by disassembling the particles and then assembling them in the presence of plasmid DNA, or by direct expression of the L1 and L2 genes together with the plasmid DNA (Fligge *et al.*, 2001b) (Kawana *et al.*, 1998b; Stauffer *et al.*, 1998; Touze & Coursaget, 1998; Unckell *et al.*, 1997; Zhao *et al.*, 1998). Fligge *et al.* (2001b) reported that the DNA encapsidated by the prolonged expression of HPV-33 L1 and L2 in insect induced a higher degree of disulphide cross-linking making the VLPs less sensitive to trypsin and remained infectious.

1.5 ARCHITECTURE OF PV VIRIONS

The overall structural organisation of the PVs resembles that of murine polyomavirus and SV40 (Figure 1.6; Baker *et al.*, 1991; Belnap *et al.*, 1996). PV virions are ~55nm in diameter, nonenveloped and comprise 360 molecules of the major capsid protein L1, arranged as 72 pentamers or capsomers in a T=7*dextro* (right handed) icosahedral lattice where sixty of the pentamers are hexavalent and 12 are pentavalent (Baker *et al.*, 1991; Belnap *et al.*, 1996;

Hagensee *et al.*, 1994). Three dimensional image reconstruction of cryo-electron micrographs of quench-frozen BPV virions revealed the capsid architecture to 9-Å resolution and a protein density within the pentamer cavity of the pentavalent capsomers was detected (Baker *et al.*, 1991). Kirnbauer *et al.* (1993) suggested that the minor capsid protein L2 was associated with these 12 vertex capsomers in a ratio of 12 molecules of L2 to 360 (72 pentamers) molecules of L1. Further, L2 must be surface exposed since neutralising antibodies can be generated by L2 immunisation (Chandrachud *et al.*, 1995; Christensen & Kreider, 1991; Heino *et al.*, 1995b; Roden *et al.*, 1994). In addition, the distinct manner in which the L1 monoclonal antibodies bind to the capsids, strongly suggests that conformational differences exist between hexavalent and pentavalent capsomeres (Booy *et al.*, 1998).



BPV-1 Polyomavirus SV40

FIGURE 1.6: Reconstructed images of cryo-electron micrographs taken of BPV-1, polyomavirus and SV40 virions by Belnap *et al.* (1996; http://bilbo.bio.purdue.edu/~baker/projects/papova/papilloma/papilloma.html). The hexavalent and pentavalent capsomers in the virions are indicated with red line frames.

Structural analysis of papillomavirus particles has been limited by the inability to obtain large amounts of virus and grow large amounts of the virus in culture in addition to the difficulty in crystallising the virions purified from warts (Chen *et al.*, 2000b). However, two recent reports by Angeletti *et al.* (2002) and Zhoa & Frazer (2002) have demonstrated the ability of PV genomes to replicate in *Saccharomyces cerevisiae* (*S. cerevisiae*) and this may possibly provide for a means of generating large amounts for virions for further structural work. In non-mammalian cells the expression of L1 on its own results in the efficient self-assembly into virus like particles (VLPs) that resemble authentic viral capsids, both structurally and antigenically (Hagensee *et al.*, 1993; Kirnbauer *et al.*, 1992; Le Cann *et al.*, 1994; Rose *et al.*, 1993; Touze *et al.*, 1996, 1998a). The co-expression of L1 and L2 leads to formation of VLPs that contains both proteins, with the ratio of L1 to L2 similar to that of authentic virions (Buonamassa *et al.*, 2002; Kirnbauer *et al.*, 1993; Roden *et al.*, 1996; Volpers *et al.*, 1994). Chen *et al.* (2000b) were able to express a truncated HPV-16 L1 in *Escherichia coli* (*E. coli*) and determined the crystal structure to 3.5-Å where a 10 residue N-terminal truncation (ΔN10L1) favoured I2 pentamer T=1 particle

formation and it is thought that this N-terminal polypeptide acts as a switch between 72 pentamer VLPs and 12 pentamer particles. The crystal structure from the truncated L1 was used to generate an atomic model of the full length HPV-16 L1 by Modis *et al.* (2002).

1.5.1 Structure of HPV-16 L1 virions

The secondary structure of PV L1 (Figure 1.7) was determined from the crystal structure of N-terminal truncated HPV-16 L1 (ΔN10L1; Chen *et al.*, 2000b). The N-terminal region of the L1 (residues 1-27) of various PVs and the β-sheets and helices h3, h4 and h5 are highly conserved whereas the C-terminal region that harbours the NLS is very variable (Figure 1.7).

FIGURE 1.7: Major capsid protein sequence alignments of nine HPV types from groups A7, A9 and A10 (see Table A.1 and A.3, Appendix A for GenBank accession numbers). Alignments were conducted using the DNA software analysis program DNAMAN (Lynnon BioSoft, Quebec, Canada) The secondary structural elements identified from the crystal structure of N-terminal truncated HPV-16 L1 (ΔN10L1) are shown above the sequence; β-sheets are represented in blue and α-helices in red. Basic residues that constitute the NLS are indicated in pink and the conserved cysteine residues 175 and 428 are indicated in yellow.

The PV L1 monomer folds in a classical 'jelly roll' β sandwich that is similar to that of polyomavirus and SV40, made up of the residues 20- 382 with α helices on the C-terminus (Figures 1.8 and 1.9; Chen *et al.*, 2000b). The C-terminal arm is proposed to interact with pentamers either in a pentavalent environment (where a pentamer is surrounded by 5 pentamers) or in a hexavalent environment and the h5 helix anchor the C-terminal arms back to the jelly roll (Modis *et al.*, 2002). This interaction is termed 'invading arm' since each pentamer receives five invading arms, which is similar to what is observed in SV40 and murine polyomavirus (Stehle *et al.*, 1994; Liddington *et al.*, 1991).

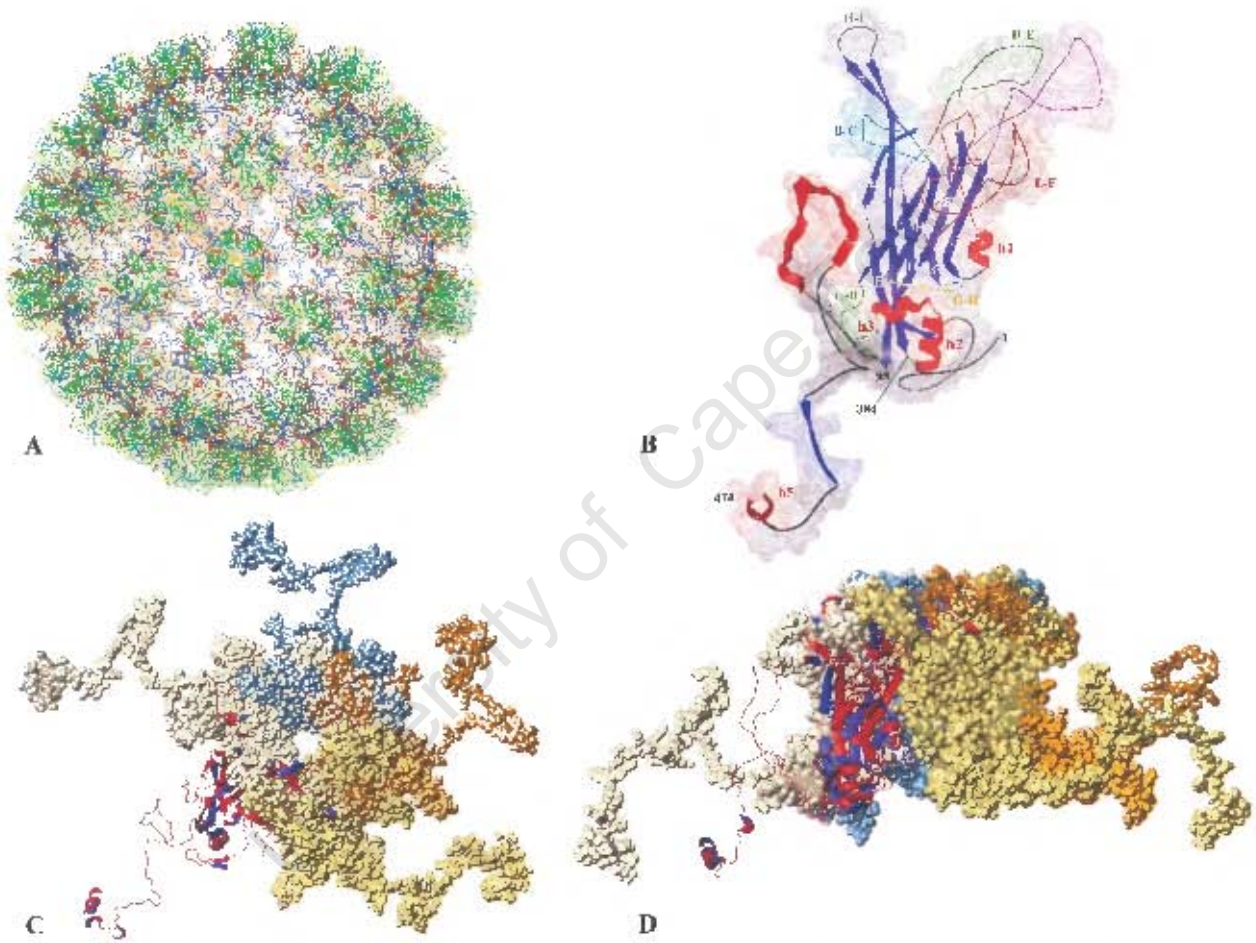


FIGURE 1.8: Structural models of HPV-16 L1 protein, capsomers (pentamers) and capsid, based on the atomic structure of HPV-16 L1 determined by Modis *et al.* (2002). Images were drawn using Rasmol (**A**; Sayle & Milner-White, 1995), PyMol (**B**; DeLano Scientific) and MolMol (**C** & **D**; Koradi *et al.*, 1996) structural analysis software. **A:** C-α backbone of a HPV-16 T=7 particle showing the organisation of the L1 pentamers (72 pentamers) and their basic interaction with neighbouring pentamers. **B:** Atomic structure of HPV-16 L1 monomer showing the β-sheets (jelly roll β sandwich) and the α-helices. **C:** L1 pentamer viewed along the five-fold axis showing the outer surface. **D:** Side view of the L1 pentamer.

The L1 monomer orientation along the pentamer axis, creates an inward facing conical hollow that opens to the exterior of the particle through a narrow neck, about 14Å in diameter (Figure

1.8; Chen *et al.*, 2000b). The polypeptide chains of the adjacent chains interact directly, the β-G sheet at the inner margin of the β-B, β-D, β-G sheets, augments the β-C, β-H, β-E, β-F sheets of the neighbouring monomer in a clockwise fashion before returning to its own subunit (Chen *et al.*, 2000b). The loops are also elaborately intertwined: the II-I loop of one monomer extends outwards and inserts between the F-G and E-F loops of the anticlockwise neighbour (Chen *et al.*, 2000b). The five points of the start shaped cap of the pentamer are created by part of the E-F loop projecting outwards to the edge of the pentamer (Figure1.8).

The N-terminal arm structure of PV was unknown; however, based on the β-hairpin in polyomaviruses, Modis *et al.* (2002) were able to reconstruct the first 19 residues of HPV-16 L1. They gave it three different orientations with respect to its subunit of origin: one for the β-hairpins nearest to the icosahedral 2-fold axis; the second for β-hairpin nearest to the icosahedral 3-fold, and a third to all the others (Figure 1.9). Near the 2-fold axis, the modelled β-hairpins lie roughly parallel to the viral surface on the 2-fold axis whereas around the 3-fold axis, the modelled β-hairpins extend out towards the symmetry axis, and in the remaining subunits it wraps snugly around the h2 helix (Modis *et al.*, 2002).

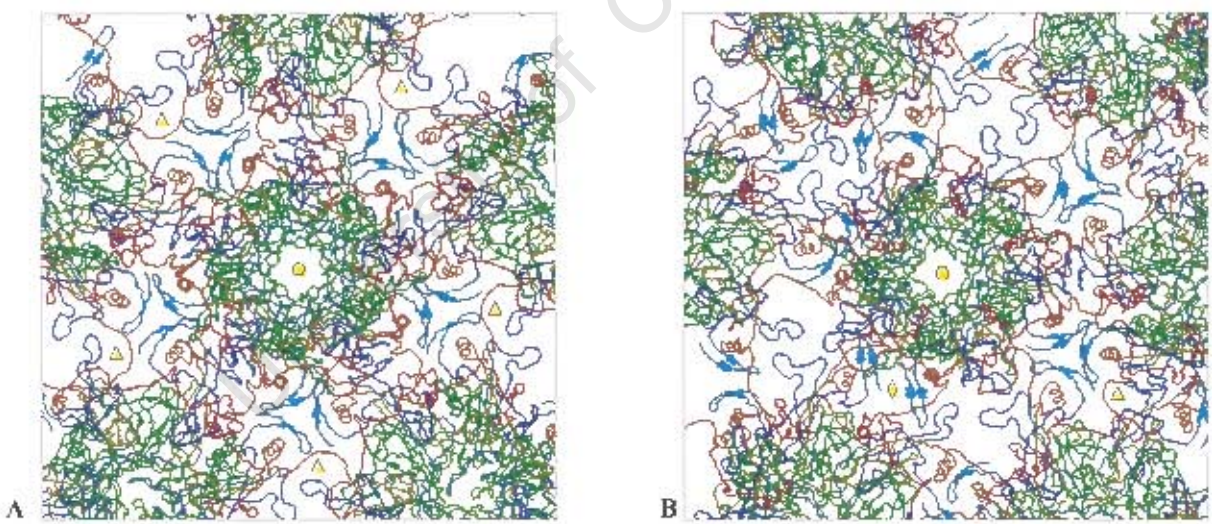


FIGURE 1.9: Close view showing interaction of the N-terminal arms between pentavalent pentamers and hexavalent pentamers. Images drawn using RasMol (Sayle & Milner-White, 1995) structural analysis software. **A:** The β-hairpin N-terminus in pentavalent pentamers extends out towards the symmetry 3-fold axis in a triangular fashion. **B:** In the hexavalent pentamers, two β-hairpins either lie in a parallel fashion or perpendicular to each other near the 2-fold axis.

The C-terminal arms form the major interpentamer contacts, by extending away from their subunits of origin and invading a subunit in the adjacent clockwise pentamer (Modis *et al.*, 2002). Modis *et al.* (2002) elegantly describe the C-terminal interaction between pentamers in the following manner:

"The particular relative orientation of the hexavalent and pentavalent pentamers leads to just three kinds of interpentameric interactions: a 3-fold cluster and two different 2-fold clusters. Each type of cluster is defined by the pattern of interchange of C-terminal arms. The 3-fold cluster includes one pentavalent pentamer and two hexavalent pentamers. The C-terminal arm invades the cloackwise-neighbouring pentamer. Thus, the pentamers in a 3-fold cluster exchange C-terminal arms in a cyclical fashion. Both kinds of 2-fold clusters contain two hexavalent pentamers. In one kind, pentamers related by the icosahedral 3-fold axis exchange C-terminal arms; in the other, pentamers related by an icosahedral 2-fold axis exchange arms." (Figure 1.9; Modis *et al.*, 2002, page 4760)

Modis *et al.* (2002) propose that residues 403-413 act as a flexible hinge or adapter, bridging the gaps between the donor and acceptor pentamer at the base of the protein shell. The flexibility of this hinge, which is proline- and glycine – rich, allows a given pentamer to donate C-terminal arms either in a pentavalent environment or in a hexavalent environment, and the conformation of the C-terminal arm is different for each subunit (Modis *et al.*, 2002). The C-terminus of the h4 helix inserts into the space between the B-C and E-F loops (Figure 1.10), and hence the two highly conserved cysteine residues (residues 175 and 428) lie opposite each other to form an intermolecular disulphide bond, thereby strengthening the C-terminal attachment (Figure 1.9). The residues 430-446 extend around the circumference of the neighbouring pentamer, linking the h4 with the C-terminal segment (Modis *et al.*, 2002). The region between the β-J sheet and h5 helix (residues 447-474) inserts into the receiving pentamer at the interface between two L1 subunits, firmly linking the invading and receiving pentamers and is capped by residues 402-414 of yet another C-terminal arm, thereby blocking access of the h5 helix and locking the C-terminus to the outer viral surface (Figure 1.10; Modis *et al.*, 2002). The variable region of the C-terminus (residues 473-504 of HPV-16) is disordered and projects towards the interior of the particle (Chen *et al.*, 2000b; Modis *et al.*, 2002). This region, which is rich in basic residues including threonine and serine, can be easily cleaved away by proteolysis before assembly (Chen *et al.*, 2000b).

The highly conserved C-D loops (Figure 1.7) form important contacts between subunits in pentavalent pentamers and adjacent subunits in hexavalent pentamers (Figure 1.10). The four C-D loops in hexalavent pentamers that do not interact with pentavalent pentamers possibly move out to plug the remaining gaps in the protein shell and hence these loops form additional interpentameric contacts, in particular near the 3-fold icosahedral axis (Modis *et al.*, 2002)

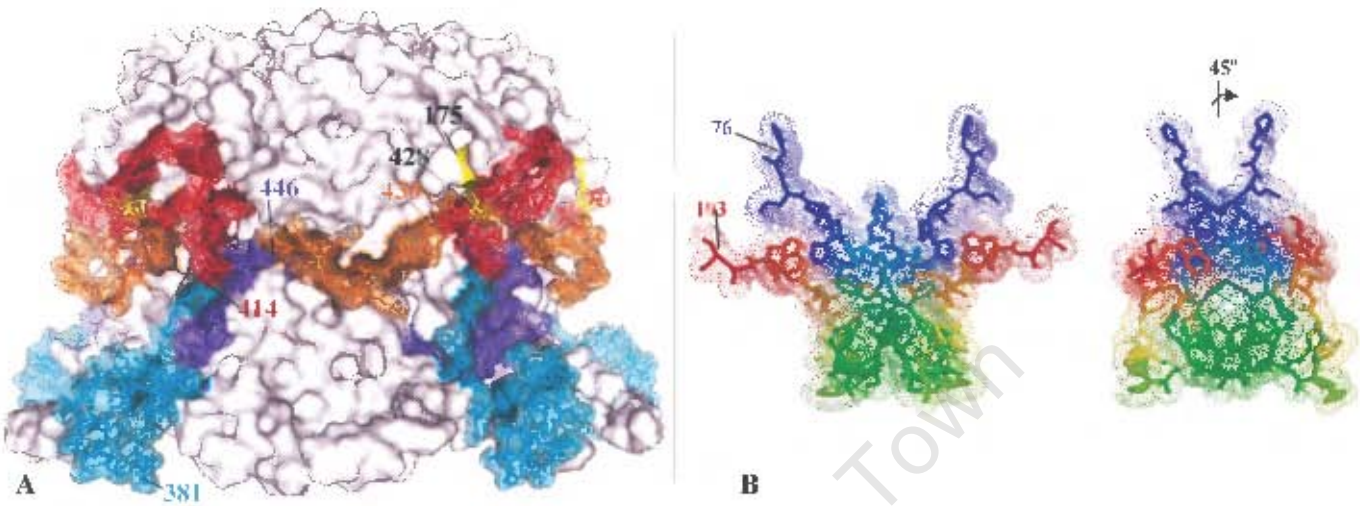


FIGURE 1.10 : Illustration of interpentameric interactions. Images were drawn using PyMol (**A**; DeLano scientific) and RasMol (**B**; Sayle & Milner-White, 1995) structure analysis software. **A:** HPV-16 pentamer showing the 'invading' C- terminal arms from the donor subunits, the residues 447-474 can be seen inserting into the receiving pentamer and the C-terminal donor arm is capped by residues 402-414 by another C-terminal arm. **B:** Interactions of the C-D loops in subunits between pentavalent and hexavalent pentamers. The C-D amino acids structures are coloured in a rainbow fashion (N-to C-terminus) to illustrate the perfect opposing interaction between two C-D loops.

The N-terminus residues (1-20) are highly conserved in all PVs (Figure 1.7). Chen *et al.* (2000b; 2001) reported that deletion of the first 10 residues (ΔN10L1) results in the formation of T=1 particles for L1 expressed in *E. coli*, and the addition of a glycine or a threonine to the N-terminus of the ΔN10L1 results in the reversion to T=7 particles. The T=1 particles are made up of 12 pentamers and have a diameter of 30nm; they were used by Chen *et al.* (2000b) to determine the crystal structure of HPV-16 L1. The monomers of ΔN10L1 assemble in a similar manner to those of full length, except for the C-terminal portion (Figure 1.11). The C-terminal portion in ΔN10L1 monomers (β-J and h5) anchors the projection back to the jellyroll. The C-terminus (383-474) of ΔN10L1 forms a projecting structure that presents acceptor and donor interaction surfaces while remaining anchored to the jellyroll domain (Chen *et al.* 2000b).

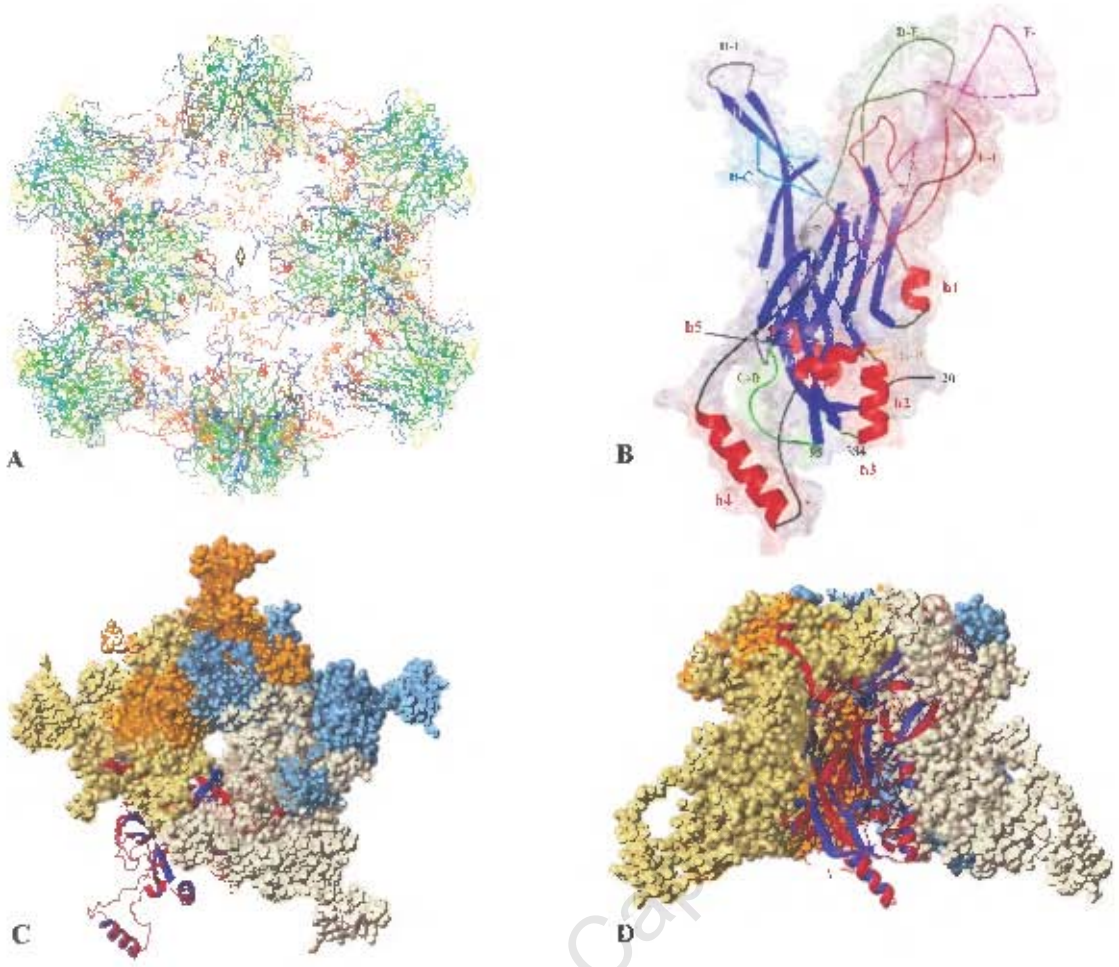


FIGURE 1.11: Structural models of HPV-16 ΔN10L1 protein, capsomers (pentamers) and capsid based on the crystal structure of truncated HPV-16 L1 determined by Chen *et al.* (2000b). Images were drawn using Rasmol (**A**; Sayle & Milner-White, 1995), PyMol (**B**; Delano Scientific) and MolMol (**C** & **D**; Koradi *et al.*, 1996) structural analysis software. **A:** C-α backbone drawing of HPV-16 T=1 particle showing the organisation of the L1 pentamers (12 pentamers) and their basic interaction with neighbouring pentamers. **B:** Atomic structure of HPV-16 L1 monomer showing the β-sheets (jelly roll β sandwich) and the α-helices. **C:** L1 pentamer viewed along the five-fold axis showing the outer surface. **D:** Side view of the L1 pentamer.

The h2, h3 and h4 helix form the surface contacts between monomers of neighbouring pentamers, unlike in the full-length L1 particles (Figure 1.12). Chen *et al.* (2000b) describe the interpentameric contacts as being modest, leaving significant gaps in the vicinity of the 2-fold axis. The absence of the 10 N-terminal residues possibly allows for the C-terminal arm to fold back into the jellyroll.

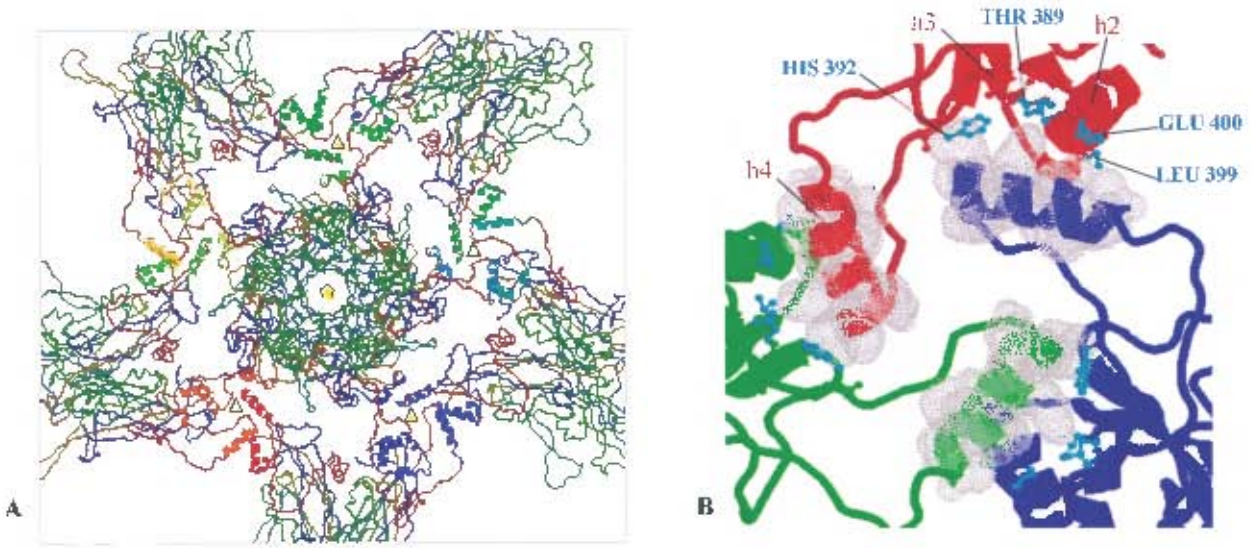


FIGURE 1.12: Five fold and three fold interactions of HPV-16 ΔN10 L1 subunits in T=1 particles. Images were drawn using RasMol (Sayle & Milner-White, 1995) and Protein Explorer (Martz, 2002) structural analysis software. **A:** Image showing the predominantly helical (h2, h3 and h4) interaction in T=1 particles. B: Detailed view of the interaction of h2 and h4 (residues 399-400), h3 and h4 (residue 389, 392) around the 3-fold axis.

C-terminal deletions that include any residues in the in the h5 helix make the L1 extremely protease sensitive, indicating that an ordered h5 is important for stability (Chen *et al.*, 2000b, 2001). There are two highly conserved cysteines in the L1 of all PVs, and mutations of these cysteines results in capsomer formation rather than formation of VLPs (Fligge *et al.*, 2001a; Li *et al.*, 1998). McCarthy *et al.* (1998) reported that the disulphide bonds are essential in maintaining the long-term structure of L1 VLP particles at physiological ionic strengths, and that a high NaCl concentration (0.5M) inhibited disassembly. In ΔN10L1 the two cysteines are not close enough to make disulphide bonds and do not contribute to interpentameric bonds. Free pentamers are the only product of *in vitro* assembly for deletion of the residues 408-431 of HPV-16 L1, indicating the importance of this region for interpentameric bonds (Chen *et al.*, 2001). Chen *et al.* (2001) have demonstrated that acidic pH (4 - 6.2) in addition to high ionic strength (0.5M NaCl) favour particle assembly.

1.6 VACCINE-BASED STRATEGIES FOR HPV PREVENTION

There are two basic approaches to the development of anti-HPV vaccines: these are prophylactic and therapeutic vaccines. The prophylactic vaccines would elicit a humoral immune response by elicidation of antibodies that are capable of neutralising the viral antigen before it enters the cell and also induce immune response to infected cells; the target of choice for these would be the PV

capsid proteins. The major capsid protein (L1) on its own can assemble into VLPs when expressed in various expression systems, hence would be ideally suited for prophylaxis. However, various studies have shown that VLPs from the co-expression of L1 and L2 are generally more stable (Hagensee *et al.*, 1993; Kirnbauer *et al.*, 1993; Zhou *et al.*, 1993).

Therapeutic vaccines would induce cellular components of the immune system to recognise and attack HPV infected cells including malignant tissue. Thus viral oncoproteins E6 and E7 are considered the appropriate therapeutic agents since they are expressed through all stages of cervical neoplasia. Viral protein E2 may also be a suitable immune target for vaccines aimed at premalignant lesion prevention and therapy (Stern *et al.*, 2000).

1.6.1 Prophylactic capsid protein vaccines

Traditionally most prophylactic vaccines have constituted of live attenuated virus or formalin inactivated virus. However, due to the risk involved in generating large quantities of these traditional vaccines, the development of a capsid protein-based subunit vaccine is of great interest. In the case of HPV prophylactic vaccine development the focus has been on recombinant subunit preparations consisting of L1 and L2 capsid proteins. The advantage of papillomavirus virions is that they induce high titre (>10 000) neutralising antibodies (Kirnbauer *et al.*, 1992; Dvoretzky *et al.*, 1980) and the response has been shown to be type-specific but long term (Carter *et al.*, 1996, 2000; Wang *et al.*, 1997). Small quantities of VLPs administered to rabbits and mice have been effective immunogens, even in the absence of adjuvants (discussed in detail in section 1.6.1.2). The ordered repetitive display of neutralising epitopes on the VLP surface, together with their stability, possibly contributes to the highly immunogenic nature of PV VLPs (Schiller, 1999). The interaction of VLPs with B cells could potentially induce their activation through the oligomerisation of VLP-specific, cell surface immunoglobulins (Igs) as proposed for hepatitis B core particles (Milich, 1987; 1991). PV capsomers have also been shown to elicit neutralising immune responses (Fligge *et al.*, 2001a; Yuan *et al.*, 2001), suggesting that intact VLP preparations are not that critical.

The development of neutralising antibody assays for the analysis of PV VLPs is important. Early neutralisations were limited to those virus types that could be propagated in the athymic mouse xenograft system, and required stocks of infectious HPV, specialised animal facilities and a

source of human genital tissue (Bonnez *et al.*, 1998; Brown *et al.*, 1998; Christensen *et al.*, 1990, 1997; Kreider *et al.*, 1995). Recent innovations include a transient *in vitro* infection assay using xenograft-derived virus (Smith *et al.*, 1993, 1995; White *et al.*, 1998), organotypic raft culture-derived virions (Frattini *et al.*, 1997; Meyers *et al.*, 1992, 2002), infectious pseudovirions (Roden *et al.*, 1996; Touze & Coursaget, 1998; Unckell *et al.*, 1997; Yeager *et al.*, 2000). Recently, HPV-16 DNA and BPV-1 DNA has been shown to replicate in *Saccharomyces cerevisiae* (Angeletti *et al.*, 2002; Zhao & Frazer, 2002) with formation of VLPs in the case of the latter (Rossi *et al.*, 2000). Assays are now in place to assess virus neutralisation for most HPV types.

1.6.1.1 Route of VLP administration and adjuvants

The generation of appropriate local immunity, ease of vaccination and longevity of memory are critical for effective vaccination - and the route of immunisation may be the main factor in influencing these factors. Subcutaneous delivery of antigens results in the generation of systemic humoral immune responses and high titres of serum IgG that could be sufficient for protection against genital tract infection; however, it would be ideal to induce secretory IgA in the genital mucosal secretions. Bouvet *et al.* (1994) demonstrated that intramuscular systemic immunisation of women with tetanus toxoid vaccine can induce a systemic-derived antibody release in the vaginal fluid. Systemic immunisation of African green monkeys with HPV-11 VLPs induced significant levels of HPV neutralising antibodies in the cervico-vaginal secretions (Lowe *et al.*, 1997). Therefore, systemic vaccinations would be efficient at the genital level and thus could reinforce or even replace a local vaccine. However, a few studies in mice and monkeys have shown that systemic IgG but no secretory IgA was induced after parenteral administration of the VLPs (Balmelli *et al.*, 1998; Lowe *et al.*, 1997). The evidence that that nasal immunisation with VLPs in mice can induce neutralising antibodies throughout the oestrous cycle (Nardelli-Haefliger *et al.*, 1997), and also activate specific CD4+ and CD8+ T cells in the vagina (Dupuy *et al.*, 1999), suggests that vaccination protocols to elicit local mucosal immunity could be advantageous for prophylaxis.

When recombinant attenuated *Salmonella* expressing HPV-16 VLPs was administered to BALB/c mice by oral and nasal routes, anti-VLP IgG and IgA was elicited (Nardelli-Haefliger *et al.*, 1997). However, in the comparison of parental and nasal vaccination with regard to mucosal immune response, Nardelli-Haefliger *et al.* (1999) suggest that mucosal immunization might be

more efficient than parenteral immunization at inducing continuous protection of the female genital tract. This is supported by the data by Liu *et al.* (1998) where intranasal immunization of mice with BPV-1 VLPs elicited a high local IgA response in the lungs. Oral vaccination, by gavage with HPV-11 VLPs has been shown to induce production of serum anti-VLP IgG and IgA, and faecal and vaginal IgA, as well as a cytotoxic T-cell response (Rose *et al.*, 1999). Gerber *et al.* (2001) showed that HPV-16 and HPV-18 VLPs are immunogenic when administered orally, and that oral co-administration of these antigens with *Escherichia coli* (*E. coli*) heat-labile enterotoxin mutant (LT) R192G or CpG DNA to mice can significantly improved anti-VLP humoral response in peripheral blood and in genital mucosal secretions.

Incomplete Freund's adjuvant (IFA) and alum adjuvant have been commonly used to induce local inflammatory responses during VLP immunisation (Breitburd *et al.*, 1995; Bryan *et al.*, 1997; Fligge *et al.*, 2001a; Liu *et al.*, 2000b; Lowe *et al.*, 1997). A microfluidised oil-in-water emulsion (MF59) has been successfully tested for use with VLPs by Greer *et al.* (2000) and Buonamassa *et al.* (2002). HPV-16 VLPs have been shown to induce both systemic IgG and genital mucosal IgA after low dose intranasal immunisation in mice but this response was greatly enhanced with the use of a cholera toxin (CT) as a mucosal adjuvant (Balmelli *et al.*, 1998). LTR72, mutated LT with only has residual toxicity, when co-administered with HPV-6b VLPs intranasally induced specific vaginal IgA and IgG responses in addition to high IgG and IgA serum titres (Greer *et al.*, 2000).

1.6.1.2 Prophylactic vaccine studies in animal models

Papillomavirus infections are species-restricted, and therefore there are no animal models for HPV infection with the exception of human xenografts in immunocompromised mice. Hence, animal studies of prophylactic VLP-based vaccines have involved animal papillomavirus infections of their corresponding host. The models investigated include the Shope cottontail rabbit papillomavirus (CRPV) which causes cutaneous papillomas (Breitburd *et al.*, 1995), bovine papillomavirus 4 (BPV-4; Kirnbauer *et al.*, 1996) and canine oral papillomavirus (COPV) which induces oral and alimentary tract neoplasia (Suzich *et al.*, 1995). The preclinical animal studies have provided sufficient evidence that VLP vaccination can induce sterilising immunity against experimental papillomavirus challenge.

All the trials in animal models have been carried out in a similar manner: the animals were parenterally primed with nanogram to microgram quantities of purified VLPs and boosted with a similar dose of VLPs. The animal were subsequently challenged by the application of a high dose of purified virus to epithelium that was abraded to expose the proliferating basal cells to virus infection. Complete protection was reported for COPV under conditions where 100% of control animals developed papillomas (Suzich *et al.*, 1995). Immunisation of rabbits with CRPV VLPs induced an anti-VLP immune response that dropped significantly over time, but a strong protection was seen when the animals were challenged with high dose virus one year after vaccination, indicating a long term protective immune response that required only low titres of anti-VLP antibodies (Christensen *et al.*, 1996b). Breitburd *et al.* (1995) showed that rabbits immunised with CRPV VLPs and subsequently challenged with CRPV were not preferentially resistant to lesions that lacked malignant potential, since no malignancies developed in the vaccinated animals whereas half the control rabbits underwent malignant progression. Systemic immunisation with BPV-4 VLPs protected calves against BPV-4 infection of the oral mucosa (Kirnbauer *et al.*, 1996; Suzich *et al.*, 1995). Protection has been passively transferred in immune sera or purified IgG from immunised rabbits or dogs, conferring protection against subsequent challenge with homologous virus (Breitburd *et al.*, 1995; Suzich *et al.*, 1995) suggesting that cell-mediated effectors responses are not required for protection (Schiller, 1999). Embers *et al.* (2002) immunised rabbits with synthetic peptides consisting of two overlapping sequences each in the residue region of 94 to 122 of the ROPV and CRPV L2 protein. Following viral challenge, cutaneous papilloma growth was completely absent in rabbits immunized with CRPV peptide, and ROPV peptide-immunized rabbits were protected from oral papillomatosis. The mechanism of protection by VLPs is as a result of neutralising antibodies, as challenge of CRPV peptide-immune rabbits with the naked viral genome resulted in efficient papilloma growth (Embers *et al.*, 2002).

1.6.1.3 Variants in HPV-16 major capsid protein

Anti-HPV neutralising antibodies in general recognise conformational epitopes and are type specific (Christensen *et al.*, 1996a). Thus, it is important to understand something about the variability of the specific types for purpose of adequate vaccine coverage. Numerous nucleotide sequence variants of HPV-16 have been identified and most of these have been isolated from different geographical locations (Chan *et al.*, 1992b, 1992a; Ho *et al.*, 1991, 1993; Touze *et al.*,

1998b). Of the 50 HPV-16 variant L1 sequences analysed only seventeen variants were found (Table 1.2). It is interesting to note that of these 17 variants, the residues highlighted in Table 1.2 are highly conserved among different HPV types (Figure 1.7), suggesting that these supposed variants may reflect sequencing errors or errors during amplification of the native L1 gene sequence.

Roden *et al.* (1997a) described pseudovirions of a HPV-16 variant, Z-1194, whose neutralisation by Mab H16:E70 was two orders of magnitude weaker than that of variant 114K. Despite the existence of various HPV-16 variants, different HPV-16 serotypes do not exist (Cheng *et al.*, 1995). Touze *et al.* (1998b) tested sera HPV-16 positive sera from 11 different countries against HPV-16 L1 VLPs derived from 3 different variants from Senegal, Algeria and the Philippines. They observed a strong correlation between the reactivity of the 3 different VLP variants independent of the geographical origin of the sera, and the 3 strains were serologically cross-reactive despite 14 differences in their amino acid sequence (Touze *et al.* (1998b). Sasagawa *et al.* (1995) reported that two HPV-16 L1 variants (16 B27L1 and 16 T3L1), isolated from benign cervical samples, respectivly produced up to 68- and 14-fold more VLPs than the prototype L1 (16 PL1) when expressed in fission yeast *Schizosaccharomyces pombe (S. pombe)*.

In a study where the L1 protein sequences of the 10 variants differed by up to 15 residues, the reported relative VLP assembly yield ranged from 1 to 79 (Touze *et al.*, 1998b). In the 10 variants studies, Touze *et al.* (1998b) reported that residues 83 and 97 were critical for higher levels of L1 protein expression in insect cells using recombinant baculoviruses. These residues are part of the C-D loop which is highly conserved (Figure 1.7) and forms important intrapentameric contacts: therefore, mutations in this region could hinder VLP assembly. The variant that resulted in the highest VLP yield, from the above study by Touze *et al.* (1998b) was Phi1, whose amino acid sequence differs from that of 114K by a residue (Thr) a position 266 (Table 1.2) and also to the prototype sequence with residue 202 corrected from His to Asp, as published by Seedorf *et al.* (1985). This sequence would be ideally suited for prophylactic HPV-16 L1-VLP-based vaccines – moreover, data from the variation of PVs discussed earlier (1.2.1) suggest that the use of only one HPV-16 strain would possibly be sufficient for the development of an L1-based prophylactic vaccine against the virus. However, data from characterisation of antigenicity of variants using neutralising antibodies (described in detail in section 1.6.1.4) suggests that a L1 sequence that achieves higher yield of VLPs in baculovirus expression system does not necessarily have the correct antigenicity profile.

TABLE 1.2: Summary of sequence differences found from analysing 50 HPV-16 variant L1 sequences. Sequences were downloaded from GenBank and amino acid alignments were carried out using Clustal x 1.8 software (Thompson *et al*., 1997). The alignments were batch translated and analysed using BioEdit sequence alignment editor (Hall, 1999). Highlighted residues are highly conserved among the various HPV types therefore suggesting either mutations during amplification of the native genes or sequencing errors.

HPV-16 variant	Major capsid (L1) protein residue position																			
	41	50	76	78	83	97	159	176	181	191	206	258	266	282	316	353	380	389	442	443	474
AY177679	Arg	Phe	His	Pro	Phe	Arg	Ile	Thr	Asn	Ile	Gly	Arg	Ala	Ser	Ala	Thr	Lys	Thr	Lys	Lys	Leu
NC001526													Thr								
AF134177		Leu	Tyr														Asn				
AF134178	Gly		Tyr					Asn	Tyr					Pro		Pro					Phe
U34190			Tyr					Asn	Thr							Pro		Ser			Phe
AF134175			Tyr					Asn	Thr		Ser					Pro		Ser			Phe
U37217			Tyr					Asn	Thr					Pto		Pro					Phe
U34176			Tyr					Asn						Pro		Pro					Phe
U34174			Tyr					Asn													Phe
U34183			Tyr					Asn											Asn		Phe
AF043287				Arg				Asn										Ser			
U34169							Leu						Thr								
U34187									Thr												
U34167										Leu											
AF140365												Gly									
AF084952															Thr						
U34193																				Gln	
Alg1*						Trp							Thr								Phe
Fra23*					Ser																Phe

* Variant sequences reported by Touze *et al*. (Touze *et al*., 1998b); sequences not found in GenBank. Protein sequences AY177679 is identical to 114K; NC001526 to Phi1; AF134177 to GU-2, AF134178 to GU-1, AF134175 to Rochester-1K and NM.T529; U34174 to Sen32 and OR.7587; U37217 to Z-1194; AY177679 is to prototype and 114B; U34167 to NM.T197; U34187 to OR7574

1.6.1.4 Prophylactic vaccines that induce neutralising antibodies

Antibody neutralisation of PVs has been proposed to occur by two distinct mechanisms: these are blocking of cell binding receptor sites on the virus (presumably by steric interference) and inhibition of uncoating (Booy *et al.*, 1998; Christensen *et al.*, 1995a; Roden *et al.*, 1994). Neutralising epitopes are present in the L1 major capsid protein (Christensen *et al.*, 1990, 1995a, 1995b, 1996a; Christensen & Kreider, 1990, 1991, 1993; Combita *et al.*, 2002; Fligge *et al.*, 2001a; McClements *et al.*, 2001; Rose *et al.*, 1998; Smith *et al.*, 1995) and the L2 minor capsid protein (Gaukroger *et al.*, 1996; Kawana *et al.*, 1999; Roden *et al.*, 1994, 2000). Neutralising antibodies are highly type specific, despite the significant sequence conservation in the L1 genes of different genotypes (Figure 1.7). Protection by HPV VLPs was initially thought to be mediated by neutralising antibodies that recognise conformational epitopes (Breitburd *et al.*, 1995; Christensen & Kreider, 1990; Kirnbauer *et al.*, 1992) however, recent reports suggest that linear epitopes can also elicit neutralising antibodies (Combita *et al.*, 2002; Kawana *et al.*, 1999; Roden *et al.*, 2000). All the characterised cross reactive epitopes are linear (Christensen *et al.*, 1996a, 2001; Kawana *et al.*, 1999).

Monoclonal antibodies (Mabs) raised to HPV-16 VLPs have aided in the determination of the both conformational and linear epitopes. Some important neutralising antibodies raised against HPV-16 VLPs are summarised in Table 1.3.

TABLE 1.3: Summary of neutralising monoclonal antibodies (Mabs) raised to HPV-16 capsid proteins and their characterised epitopes

Mab name	Capsid protein/ Epitopes	Epitope type	Reference
H16:V5	L1: F50, A266, S282	Conformational / neutralising	Christensen *et al.*, 1996a; Roden *et al.*, 1997; White *et al.*, 1999; Combita *et al.*, 2002
H16:E70	L1: F50, A266, S282	Conformational / neutralising	Christensen *et al.*, 1996a; Roden *et al.*, 1997; White *et al.*, 1999; Combita *et al.*, 2002
H16:U4	L1: unknown	Conformational / neutralising	Christensen *et al.*, 1996a; Roden *et al.*, 1997; White *et al.*, 1999; Combita *et al.*, 2002
H16:J4	L1: 268-281	Linear / neutralising	Christensen *et al.*, 1996a; Combita *et al.*, 2002
H16:I23	L1: 111-130 (L152)	Linear / neutralising	Christensen *et al.*, 1996a; Combita *et al.*, 2002
H16:9A	L1: 1-173	Conformational / neutralising	Christensen *et al.*, 2000
MAb5 and MAb13	L2: 108-120	Linear / neutralising	Kawana *et al.*, 1999

Mab H16:V5 was found to block serological reactivity of human sera with HPV-16 capsids; out of the 352 human serum samples tested for the presence of IgG against HPV-16, more than 75% of the reactive sera were completely blocked by this Mab (Wang *et al.*, 1997). Mab H16:9A was raised to against hybrid HPV-16/11 VLPs where the backbone was genetically made up of HPV-11 and the residues 1-173 were from HPV-16 (Christensen *et al.*, 2001). Mabs MAb5 and MAb13 were raised against the HPV-16 L2 peptide 108-120 and both cross-neutralised HPV-16 and HPV-6 L1/L2 pseudovirions (Kawana *et al.*, 1999). Since pseudovirions of HPV-31, 33 and 58 (phenylalanine at residue 152) were not neutralised by Mab H16:I23, it is suggested that residue 152 (leucine) is important for the cross-neutralising by Mab H16:I23 of HPV-16, 18, 45 and 59 pseudovirions, whereas Mab H16:J4 was found to cross-neutralise pseudovirions of HPV-16, 31, 33, 58 and 45 (Combita *et al.*, 2002). Therefore it is evident that the L1 protein contains common linear cross-neutralising epitopes, suggesting that some degree of cross protection could occur. However, there is no virological or epidemiological evidence of natural cross-protection between HPV types.

1.6.1.5 Clinical trials of prophylactic HPV vaccines

HPV-16 VLPs were found to be generally well tolerated during vaccination in human subjects and to induced high levels of anti-HPV-16 antibodies (Pastrana *et al.*, 2001). More recently, a double blind, placebo-controlled, dose-escalation trial to evaluate the safety and immunogenicity of baculovirus-produced HPV-16 L1 VLP vaccine in healthy adults was reported by Harro *et al.*, (2001). Volunteers were given intramuscular injections with placebo or with 10- or 50-μg doses of HPV-16 L1 VLP vaccine administered without adjuvant, or with alum or MF59 as adjuvants, at 0, 1, and 4 months, and the immune responses were measured by an HPV-16 L1 VLP-based enzyme-linked immunosorbent assay (ELISA) and by an HPV16 pseudovirion neutralization assay (Harro *et al.*, 2001). With the higher dose, the geometric means of serum ELISA antibody titers (95% confidence intervals) to purified VLP one month after the third injection were 10,240 (1499 to 69 938) without adjuvant, 10,240 (1114 to 94 145) with MF59, and 2190 (838 to 5723) with alum (Harro *et al.*, 2001). Harro *et al.* (2001) reported that the HPV-16 L1 VLP vaccine was well tolerated and was highly immunogenic even without adjuvant; the majority of the recipients achieved serum antibody titers that were approximately 40-fold higher than what is observed in natural infection.

In the most recent double-blind clinical study, 2392 young women (defined as females 16 to 23 years of age) received three doses of placebo or *S. cerevisiae*-produced HPV-16 VLP vaccine (40 μg per dose), administered at day 0, month 2, and month 6 (Koutsky *et al.*, 2002). Koutsky *et al.* (2002) monitored genital samples for HPV-16 DNA by polymerase chain reaction (PCR) at enrollment, one month after the third vaccination and every 6 months thereafter. Of the women who received the vaccine, Koutsky *et al.* (2002) observed 99.7% seroconversion. The women were monitored for a median of 17.4 months after completing the vaccination regimen and the incidence of persistent HPV-16 infection was 3.8% in the placebo group whereas 0% in the vaccinated group, thereby suggesting 100 % efficacy of the vaccine (Koutsky *et al.*, 2002). Nine cases of HPV-16 related CIN were reported in the course of the trial by Koutsky *et al.* (2002), however, these occurred only in the placebo group further supports the efficacy of the vaccine. Therefore, this study demonstrated that administration of HPV-16 VLP-based vaccine reduced the incidence of both HPV-16 infection and HPV-16-related CIN, thus allowing for the conclusion that immunising HPV-16-negative women could potentially reduce the incidence of them developing cervical cancer (Koutsky *et al.*, 2002).

1.6.2 Therapeutic vaccines

Therapeutic vaccines are administered to reduce or eradicate existing disease or infections by targeting cells expressing tumour-associated or tumour-specific antigens on their surface (Steller, 2002). Protein-based strategies for disease prevention require APC engulfment of exogenous proteins and the presentation of processed peptide fragments to T cells in a MHC-restricted manner (Steller, 2002). Endocytosis of denatured protein by APCs (consisting of a heterogeneous population of leukocytes including Langerhan cells, macrophages, B cells and dendritic cells) effectively induces CD8+ CTLs (Martinez-Kinader *et al.*, 1995; Schirmbeck *et al.*, 1995). The use of full-length E6 and E7 proteins for therapeutic vaccines has the advantage of including all the putative immunogenic epitopes for every MHC haplotype, whereas peptides are attractive vaccine candidates because they can be synthesised in large quantities, albeit expensively, and are largely non-toxic. A variety of approaches have been used to design and test potential therapeutic vaccines: these include oncogenic protein and peptide-based vaccines, virus vector-based vaccines, DNA vaccines and cell-based vaccines.

Vaccination with the HPV-16 E7 protein administered together with an adjuvant, PROVAX, protected mice against E7-transfected K1735 metastatic melanoma cell line C3 and elicited an E7-specific cytotoxic T lymphocyte response in the vaccinated mice. The effect of vaccination was abolished by depletion of CD8+ and CD4+ cells (Hariharan *et al.*, 1998). A fusion protein vaccine comprising *Mycobacterium bovis* hsp65/HPV-16 E7 induced regression of E7 positive TC-1 tumours, which were obtained after co-transfection of murine lung cells with E6/E7 HPV-16 and activated Ha-ras DNA. This fusion protein conferred protection against tumour re-challenge and allowed long-term survival of the vaccinated mice (Chu *et al.*, 2000). Vaccination with HPV-16 L2, E6 and E7 as a single fusion protein has been shown to elicit HPV-16-specific cytotoxic T lymphocytes, T-helper cells, and antibodies in a mouse model (van der Burg *et al.*, 2001). Fernando *et al.* (1999) demonstrated that vaccination with HPV-16 E7 fused to glutathione-S-transferase protects mice against subsequent lethal challenge with an HPV16 E7 DNA-transfected C3 cell line.

Studies by Selvakumar *et al.* (1995) have demonstrated that rabbits immunised with CRPV E1 and E2 proteins, expressed in bacteria, promoted the regression of CRPV-induced skin papillomas. Similarly, Jasen *et al.* (1997) showed that vaccination of rabbits with recombinant *Listeria monocytogenes* expressed CRPV E1 also resulted in papilloma regression in majority of the vaccinated animals. In another study by Han *et al.* (1999) reported that intramuscular immunisation of rabbits with naked DNA encoding CRPV E1, E2, E6 and E7 induced CD4+ T cell-mediated immune response, however no humoral immune response was detected and the immunity induced did not protect against subsequent challenge. Intracutaneous DNA vaccinations of rabbits with CRPV E6 by Sundaram *et al.* (1998) provided partial protection from subsequent viral challenge. However, the efficacy of the E6 vaccine was improved by priming the sites of vaccination with granulocyte-macrophage colony-stimulating factor (GM-CSF; Leachman *et al.*, 2000). A follow up study by Leachman *et al.* 2002, showed highly significant protection against papilloma formation by immunisation with E1 + E2, E6 and E7 DNA vaccines administered individually and in combinations. Ubiquitin fused E6 (Ub-E6) vaccine induced significant protection whereas UbE1 + UbE2 + UbE7 combination vaccination induced complete regression (Leachman *et al.* 2002).

The identification of peptides that bind to MHC molecules and activate T-cells have suggested the possibility of using E6/E7 synthetic peptides for successful vaccination against HPV16-associated tumours (Bubenik, 2002). Human cytotoxic T lymphocytes induced by using the

HPV-16 E7 peptides 11-20 or 86-93 are capable of recognising and lysing CaSki cervical cancer cells that contain integrated HPV-16 DNA (Alexander *et al.*, 1996; Ressing *et al.*, 1995, 1999; 2000; Steller, 2002). Two HPV-16 E7 peptides (aa 21-28, aa 48-55) were found *in vivo* to induce cytotoxic T lymphocytes against EL-E7 target cells derived from murine EL-4 thymoma by co-transfection with HPV16 and activated ras DNA (Bauer *et al.*, 1995). HPV16 E7 peptide (aa 49-57) was shown to induce cytotoxic T lymphocyte responses which was able to eradicated established E7 positive C3 tumors in mice (Bubenik, 2002; Feltkamp *et al.*, 1993, 1995; Kast *et al.*, 1996).

1.6.2.1 Clinical trials of therapeutic HPV vaccines

Several therapeutic vaccines have been tested in phase I/II clinical trials and these include HPV-16 E6/E7 (with ISCOMATRIX adjuvant) and HPV-16 L2/E6/E7 fusion proteins (de Jong *et al.*, 2002; Rudolf *et al.*, 1999, 2001; van der Burg *et al.*, 2001), peptide based vaccines (HPV-16 $E7_{11-20}$, lipidated $E7_{86-93}$, synthetic peptides representing two HPV-16 E7-encoded, HLA-A*0201-restricted cytotoxic T lymphocyte epitopes and a pan-HLA-DR-binding T-helper epitope, PADRE, in adjuvant (Muderspach *et al.*, 2000; Ressing *et al.*, 2000; Steller *et al.*, 1998) recombinant vaccinia viruses (HPV-16 and -18 expressing E6 and E7 oncoproteins; Adams *et al.*, 2001; Borysiewicz *et al.*, 1996) and DCs pulsed with autologous or allogenic cervical tumor lysate (Adams *et al.*, 2001; de Jong *et al.*, 2002). In general, these vaccine have been proven safe, and vaccination of cervical cancer patients resulted in the detection of a vaccine-induced T-cell response against HPV (Adams *et al.*, 2001; Ressing *et al.*, 2000; Steller *et al.*, 1998).

1.6.3 Chimaeric vaccines

Despite the development of HPV VLPs as a candidate prophylactic vaccine, chimaeric VLPs are increasingly being researched and synthesised to accommodate more viral antigens targets for CTL responses (Greenstone *et al.*, 1998; Steller, 2002). An extensive study investigating chimaeras of HPV-16 and L1 and E7 was conducted by Muller *et al.* (1997), where either the 34 residue-C-terminus of L1 was replaced with various segments of E7, or the E7 segments were inserted into the L1 residue region 295-302. Most of the chimaeric VLPs were able to induce a neutralising immune response (Muller *et al.*, 1997). HPV-16 E7 chimaeras of both L1 and L2 have been shown to elicit a CD8+ CTL response against E7 in mice, and to protect against E7-

expressing tumors (Greenstone *et al.*, 1998). Similar results have been obtained with chimaeric HPV-16 L1 fused at the C-terminus with the truncated E7 protein (Schafer *et al.*, 1999), E7 peptides (Peng *et al.*, 1998) and a string of HIV CTL epitopes (Liu *et al.*, 2000b).

The development of PV VLPs as DNA delivery vectors for genetic immunisations is at a relatively early stage. BPV-1 and HPV-33 VLPs, expressed by recombinant vaccinia virus, have been shown to encapsidate a plasmid containing the *β*-galactosidase (*β*-Gal) reporter gene (Unckell *et al.*, 1997; Zhao *et al.*, 1998). BPV genomes autonomously replicating in a mammalian cell line have been encapsidated by BPV-1 and HPV-16 L1 protein expressed by recombinant *Semliki forest virus* (Roden *et al.*, 1996). DNA encapsidated in pseudovirions can also be generated from assembly of previously disassembled particles in the presence of different nonrelated plasmids containing a reporter gene (Kawana *et al.*, 1998b; Touze & Coursaget, 1998). Assembly of pseudovirions and encapsidation of plasmid DNA in *S. cerevisiae*-expressed HPV-16 L1/L2 has recently been shown, and *in vitro* infection of mammalian cells with these pseudovirions resulted in the delivery of the reporter gene (Rossi *et al.*, 2000). These studies support the possibility of generating PV pseudovirions that package DNA *in vitro* and that such pseudovirions are able to deliver the packaged DNA to various cell lines.

VLPs have been extensively investigated for the display of foreign epitopes on the surface of capsid proteins (Jenkins *et al.*, 1990; Nieland *et al.*, 1999; Pumpens *et al.*, 2002). Slupetzky *et al.* (2001) investigated the prospect of displaying the HIV-1 gp41 envelope protein on the surface loops and the C-terminus of HPV-16 and BPV-1 L1 capsid protein: the resulting chimaeric capsomers were found to bind some of the conformational neutralising antibodies, and mice immunised with these chimaeras elicited a HIV-1 gp41 epitope specific immune response. Liu *et al.* (2002) prepared BPV-1 chimaeras with sequences from the HIV-1 gp120 (V3 loop) and a shorter peptide incorporating a CTL epitope (HIVP18I10). Immunisation with these chimaeras induced antibodies specific for BPV-1 VLPs and P18 peptide and CTL precursors specific for the P18 CTL epitope were recovered from spleen (Liu *et al.*, 2002).

An alternative strategy that has recently been tested for PV vaccines involves production of hybrid recombinant viruses and VLPs that contain capsid antigen determinants from multiple HPV types. Christensen *et al.* (2001) showed that a chimaeric VLP containing the hyper variable F-G and H-I loops (H11:16[266-97+339-65]) of HPV-16 L1 in an HPV-11 L1 backbone induced

neutralising activity against both HPV-11 and HPV-16. In addition, conformation-dependent and type-specific Mabs to both HPV-16 and 11 VLPs were obtained from mice immunised with the chimaeric VLPs (Christensen *et al.*, 2001).

1.7 POTENTIAL EXPRESSION SYSTEMS FOR HPV VACCINES

Vaccination is thought to be the most cost effective way of preventing infectious diseases and as a result of the worldwide vaccination programmes, the incidence of many fatal diseases has drastically decreased (Liljeqvist & Stahl, 1999). Majority of the routinely used vaccines today as part of childhood immunisation programmes are whole organism vaccines, consisting of live attenuated vaccines or killed whole bacteria or viruses (Liljeqvist & Stahl, 1999; Plotkin, 1993). However, the risk of reversion to virulent wild-type strains that can lead to disease when using attenuated bacteria or virus exists, more so in immunocompromised hosts (Liljeqvist & Stahl, 1999). The emphasis on recombinant vaccine, therefore, has increased with the sophistication of techniques available to produce them. The first recombinant subunit vaccine investigated by Valenzuela *et al.* (1982) was the hepatitis B surface antigen vaccine produced in *S. cerevisiae* and was licensed in 1986 (Liljeqvist & Stahl, 1999). The advantages of subunit vaccines are numerous, for example the pathogen is entirely excluded from the production of the vaccine, which eliminates risks associated with production, as well as risks of contamination with toxic compounds, and risks of reversion to virulent genotypes or of incomplete inactivation of whole-cell vaccines (Liljeqvist & Stahl, 1999). Further, the delivery systems could be optimised in addition to the vectors for their delivery to specific sites, for instance, many recombinant subunit vaccines are being investigated for mucosal delivery, using the appropriate vectors.

1.7.1 Recombinant subunit vaccine production hosts

Commonly used systems for recombinant protein production and their characteristics summarised by Awram *et al.*, 2002 are shown in Table 1.4. Each expression system offers its own advantage, however there are also limitations which have to be considered when choosing the expression system. One potential drawback of using prokaryotes as production hosts is that they are unable to carry out posttranslational modification; however, their cost effective production makes bacteria the dominating hosts for production of candidate subunit vaccines

(Liljeqvist & Stahl, 1999). However, purification of the protein does often pose a substantial problem.

TABLE 1.4: Comparison of various systems for expression of foreign proteins (Awram *et al.*, 2002).

	Growth media/ expense	Equipment costs	Level of protein production	Purification difficulty	Sensitivity to growth conditions *	Post translational Processing #
Bacteria	Moderate	Moderate	High	Easy to moderate	Moderate	Poor
Insect cells	Moderate to high	Moderate	Moderate to high	Easy to moderate	Moderate	Moderate
Animal cells	High	High	Low	Moderate to difficult	High	Excellent
Yeast cells	Moderate	Moderate	High	Easy to Moderate	Moderate	Moderate to excellent
Plants	Low	Low	Low	Moderate to difficult	Low	Moderate

*Shear forces, pH, temperature, oxygen; #expression of mammalian proteins

1.7.2 Options for recombinant vaccines for developing countries

A vaccine strategy employing purified VLP preparations, prepared using conventional methods, is probably too costly for widespread vaccination to be implemented in developing countries, where the highest incidence of cervical cancer occurs (Schiller, 1999). Sanders and Taira (2003) estimate that the cost of vaccinating an individual, in a 3 injection protocol with a booster administered every 10 years, would cost US$100 per injection. Therefore, strategies applicable to these underdeveloped settings are thus under investigation.

1.7.2.1 Live viral vectors

Genes encoding relevant antigens can be spliced into recombinant expression vectors, allowing for increased cellular production of the antigen and induction of humoral and cellular immuneresponses (Mackett *et al.*, 1982). The advantages of vaccinia virus systems are the wide host range of mammalian cells that can be infected, the high expression levels, as well as the ease of virus stock production, however, requires biosafety level 2 for production (Carroll & Moss, 1997). The vaccinia virus system has been successfully used for large scale (1000L) production

of different proteins such as HIV-1 rgp160 and human pro-thrombin by (Wurm & Bernard, 1999). Live recombinant vaccinia viruses have been used to express PV L1 and L2 capsid proteins which form VLPs in mammalian cells (Browne *et al.*, 1988; Fang *et al.*, 1999; Hagensee *et al.*, 1993; Volpers *et al.*, 1994; Zhou *et al.*, 1991b; Zhou *et al.*, 1991a, 1993) and also for delivery of therapeutic antigens (Chen *et al.*, 2000a; Davidson *et al.*, 2001; Hoos *et al.*, 1996; Lamikanra *et al.*, 2001; Nindl *et al.*, 1996). Recombinant vaccinia virus expressing modified E6 and E7 genes was shown to protect against subsequent tumour challenge in a variety of experimental systems (Boursnell *et al.*, 1996; Gao *et al.*, 1994; Meneguzzi *et al.*, 1991). Mice inoculated with a vaccinia viral vector encoding an E7/lysosomal-associated membrane protein-1 (LAMP-1) fusion proteins were protected against subsequent challenge with an HPV-induced tumour model (Lin *et al.*, 1996) and mice with small-established tumours that could not be cured by wild-type E7 vaccinia were cured by the same treatment (Ji *et al.*, 1998).

Sindbis virus or Semliki Forest virus (SFV) are ssRNA (+) genome alphaviruses commonly used for heterologous expression (Lundstrom, 1997). The broad host range of SFV has made it particularly attractive for production of recombinant proteins (Berglund *et al.*, 1993). Additionally, the extremely efficient SFV 26S RNA subgenomic promoter used in these SFV expression vectors and the high number of copies of RNA of foreign protein (200 000) per infected cell leads to high expression (Berglund *et al.*, 1993; Liljestrom, 1994; Zhou *et al.*, 1994b). PV capsid proteins have been successfully expressed using SFV and have been shown to assemble into VLPs (Heino *et al.*, 1995a; Roden *et al.*, 1996). Recombinant SFV expressing HPV16 E6/E7 proteins induced efficient *in vivo* priming of HPV16-specific CTL activity and protection from TC-1 tumor challenge in the vaccinated mice (Daemen *et al.*, 2000). Augmentation of helper T cell activity by potentiation of CD4+ T cell responses has also been explored using the lysosomal-associated membrane protein-1 (LAMP-1) system (Wu *et al.*, 1995). A Venezuelan equine encephalitis (VEE) alphavirus vector delivering the HPV16 E7 RNA was shown to induced class I-restricted CD8+ T-cell responses in an E7+ tumour model (Wurm & Bernard, 1999). Scale-up of production for alphaviruses has been established for SFV; however, it requires high biosafety level production facilities (Lundstrom, 1997).

Adenoviral vectors are not pathogenic in humans, can be made replication competent or deficient, and can be administered orally (Imler, 1995). Humoral, cell-mediated and mucosal immunity can be elicited to the heterologous antigens delivered by adenoviruses (Liljeqvist & Stahl, 1999). Several viral antigens such as HBV surface antigen, the measles virus nucleocapsid,

and glycoproteins from herpes simplex virus and rabies virus have been expressed and delivered by adenoviral vectors. Liu *et al.* (2000a) investigated the potential of the HPV-16 E5 protein as a vaccine candidate by means of a single intramuscular immunization of mice with a recombinant adenovirus. They showed that the E5 vaccine-induced tumour protection occurred by means of CD8+ T cells and not CD4+, indicating E5 as a tumor rejection antigen.

Recombinant polioviruses have used to express a variety of viral antigens, including HBV surface antigen and SIV proteins (Crotty *et al.*, 2001; Tang *et al.*, 1997; Yim *et al.*, 1996). A recent study showed that trasfection of cells with replication-competent recombinant Mahoney 1 poliovirus DNA expressing HPV-16 L1 gene resulted in expression and assembly of the HPV-16 major capsid protein into VLPs in Vero cells (van Kuppeveld *et al.*, 2002). Unfortunately immunization of mice with the recombinant virus induced a very weak anti-HPV-16 L1 immune response in mice.

Despite the numerous studies, including clinical, conducted using live recombinant viral vectors, no such vaccine candidate has progressed past phase II evaluation, since profiles of the immune responses elicited were not considered ideal (Liljeqvist & Stahl, 1999). Liljeqvist & Stahl (1999) speculate that until the safety and immunological questions are solved, only certain specific vaccines such as those for HIV and cancer, in addition to veterinary vaccines, would be allowed applications for virus-based vectors.

1.7.2.2 Live bacterial vectors

Live bacterial vaccines are relatively inexpensive to manufacture and are well suited to large-scale administration in both developed and developing countries. They have the added advantage that they can be administered orally, which is practical and reliable in large-scale vaccination programmes (Shata *et al.*, 2000). All field and clinical trials with *Salmonella* as a delivery system have been limited to attenuated *Salmonella typhi*; however, a promising alternative as a vaccine vector for heterologous antigen delivery is *S. typhimurium*. Nardelli-Haefliger *et al.* (1997) and Benyacoub *et al.* (1999) have demonstrated the assembly of HPV-16 VLPs, as have Hopkins *et al.* (1995) for hepatitis B virus core antigen, in attenuated *S. typhimurium*. Intranasal immunisation of mice with recombinant *Salmonella* induced systemic neutralising and mucosal anti-HPV-16 VLP antibody responses. Recombinant *Salmonella* has also been developed to

express HPV-16 E6 and E7 oncoproteins, and antibody responses to the recombinant have been demonstrated (Krul *et al.*, 1996; Londono *et al.*, 1996).

The use of tuberculosis vaccine Bacille Calmette-Guerin (BCG) as a vaccine vector has the advantages of its established safety in humans and its capacity to accommodate large fragments of foreign DNA (Jacobs, Jr. *et al.*, 1990; Lugosi *et al.*, 1989). Jabbar *et al.* (2000) investigated recombinant BCG (rBCG) vaccines which expressed the HPV-6b L1 protein and the HPV-16 E7: the magnitude of the responses observed were less than those elicited by native L1 and E7 protein vaccines. Similar results were observed by Matsumoto *et al.* (1998) with their rBCG vector expressing the C-termal fragment of the *Plasmodium yoelii* merozoite surface protein 1 ($MSP1_{15}$). In a Phase I clinical trial, 24 volunteers immunised intradermally with rBCG expressing *Borrelia burgdorferi* outer-surface-protein A (rBCG-OspA) did not elicit a detectable serum antibody response to OspA (Edelman *et al.*, 1999). The rBCG vaccines expressing HPV-6b L1 or HPV-16 E7 persisted at low levels in the immunised mice suggesting that rBCG vaccines may be beneficial to prime or retain memory responses to antigens, but are unlikely to be useful as a single component vaccine strategy (Jabbar *et al.*, 2000).

In addition to using attenuated pathogens, non-pathogenic commensals of the genital tract such as lactobacilli and streptococci may be manipulated to express HPV proteins. A recombinant *Streptococcus gordonii* has been constructed, expressing an HPV-16 E7 fusion protein on its surface (Pozzi *et al.*, 1992). Immune responsiveness was not investigated, but another recombinant *Streptococcus* has been shown to induce a specific local and systemic response (Medaglini *et al.*, 1995).

1.7.2.3 Recombinant baculoviruses as expression vectors for insect cells

Recombinant baculovirus (rBV) expression vectors are extensively used for the expression of a variety of recombinant proteins in insect cells. These include cytosolic, nuclear, mitochondrial, membrane-bound and secreted proteins (Kost & Condreay, 1999). Co-expression of viral proteins has been used to achieve higher yields and stability of the particles (Kirnbauer *et al.*, 1993; Xi & Banks, 1991). Recombinant BVs have been extensively used to express a variety of viral proteins such as VP1, VP2 and VP3 of polyomavirus (An *et al.*, 1999; Chang *et al.*, 1997), Norwalk virus capsid protein (Bertolotti-Ciarlet *et al.*, 2002), HIV-1 Gag protein (Nermut *et al.*,

1994; Paolazzi *et al.*, 1999; Zhoa *et al.*, 1994) and HPV capsid proteins (Benincasa *et al.*, 1996; Christensen *et al.*, 1994a, 1994b; Kirnbauer *et al.*, 1993; Le Cann *et al.*, 1994, 1995; McCarthy *et al.*, 1998; Rose *et al.*, 1993; Schafer *et al.*, 1999; Touze & Coursaget, 1998; Xi & Banks, 1991). Despite the high level of expression of recombinant proteins, the baculovirus system is relatively expensive. Baculovirus expression systems provide a great tool nonetheless for the investigation of functional and antigenic properties of rBV expressed proteins (Possee, 1997). Baculovirus-produced HPV L1 VLP vaccines are also currently leading the field, along with yeast produced VLPs, in clinical trials (Harro *et al.*, 2001, Koutsky *et* al, 2002)

1.7.2.4 Expression in *E. coli*

The advantage of using *E. coli* for heterologous protein production is its ability to grown rapidly at high density on inexpensive substrates, its well characterised genetics and the availability of a large number of cloning vectors (Baneyx, 1999). However, the main concern with using *E. coli* is that overproduction of heterologous protein is their misfolding and segregation into insoluble aggregates known as inclusion bodies (Baneyx, 1999). The arginine codons AGA and AGG are rarely found in *E. coli* genes, the presence of such codons in cloned genes affects protein accumulation levels and mRNA and plasmid stability; in extreme cases it inhibits cell growth and hence protein synthesis (Zahn, 1996).

In general, expression of HPV L1 in *E. coli* has generally produced modest quantities of assembly-competent pentamers (Banks *et al.*, 1987; Kelsall & Kulski, 1995; Li *et al.*, 1997; Zhang *et al.*, 1998). Yields have recently been enhanced by using GST-L1 fusions, which facilitate purification and the separation of L1 from GroEL-bound complex by the addition of ATP-$MgCl_2$ to cell lysate followed by treatment with urea (Chen *et al.*, 2001). The purified HPV L1 was subsequently assembled using buffer containing 0.2M sodium acetate and 1M NaCl at low pH (Chen *et al.*, 2000b, 2001).

1.7.2.5 Expression in *S. cerevisiae*

The advantages of using yeast as an expression systems are that they are easy to culture on inexpensive media (Sudbery, 1996). They also generally provide an appropriate environment for eukaryotic post-translational processing and secretion, resulting in a product that is often

identical or very similar to the native protein (Sudbery, 1996). The classic brewer's yeast *S. cerevisiae* has been widely used for the production of VLPs of many different viruses, including PVs (Angeletti *et al.*, 2002; Cook *et al.*, 1999; Hofmann *et al.*, 1996; Jansen *et al.*, 1995; Joyce *et al.*, 1999; Lowe *et al.*, 1997; Neeper *et al.*, 1996; Rossi *et al.*, 2000; Zhao & Frazer, 2002). Other antigens of interest expressed in *S. cerevisiae* include HIV-1 Gag protein (Jacobs *et al.*, 1989; Sakuragi *et al.*, 2002), antimalarial antigens (Brady *et al.*, 2001), poliovirus subviral proteins (Rombaut & Jore, 1997) and hepatitis B virus surface antigen (Valenzuela *et al.*, 1982). The recent prophylactic HPV-16 vaccine clinical trials involving 2392 women (section 1.5.4.1) used VLPs produced in *S. cerevisiae* (Koutsky *et al.*, 2002). Due to its application in the bread and alcoholic beverage industries, industrial scale fermentation facilities are already in place for large-scale production and sterility may not be of critical importance (Sudbery, 1996). Expression of PV VLPs in *S. cerevisiae* definitely has enormous potential for the production of relatively cheap vaccines.DNA vaccines

DNA vaccines have emerged as an attractive vaccine strategy because they are stable and relatively easy to produce. The DNA can be administered by a variety of routes, all of which result in DNA uptake by APCs and other cells as well as expression of the DNA-encoded antigen (Steller, 2002). Expression of foreign proteins within host cells from naked DNA constructs allows for the induction of humoral and cell-mediated responses, whilst protein subunit vaccines may only induce antibodies (Ulmer *et al.*, 1996b, 1996a). DNA can be propagated as plasmids in bacteria and purified with ease and at a low cost (Lijeqvist & Stahl, 1999). This approach is a promising one, as demonstrated with CRPV (Donnelly *et al.*, 1996). Rabbits injected with CRPV L1 DNA intramuscularly were completely resistant to papilloma development by CRPV challenge, while all control unimmunised or L2 DNA-immunised animals developed papillomas (Donnelly *et al.*, 1996). Neutralising antibodies were induced, and titres persisted at a stable level of more than 32 weeks, demonstrating potential long-lasting protection implying that L1 was presented to the immune system in the correct conformation for a protective response (Donnelly *et al.*, 1996).

Since the E6 and E7 proteins are capable of transforming cells, in the E6/E7 DNA vaccines the oncogenes are altered to non-transforming type by deletions, insertions or mutations (Steller, 2002). E7 plasmid DNA vaccines have been shown to induce E7-specific CTL responses, and to completely protect the vaccinated mice against a challenge with E7-transfected murine sarcoma C3 (Shi *et al.*, 1999; Tuting *et al.*, 1999). Vaccination with chimaeric DNA constructed by

linking E7 DNA to LAMP-1 (Sig/E7/LAMP-1 DNA) generated stronger antitumour immunity than did corresponding wild-type E7 DNA in tumour prevention and tumour regression assays (Ji *et al.*, 1998, 1999). The Sig/E7/LAMP-1 DNA was capable of generating potent antitumour immunity in the TC-1 model of tumour which metastasizes to liver and lung after i.v. or intrahepatic injection (Chen *et al.*, 1999). Chen *et al.* (2000a) found that priming with Sig/E7/LAMP-1 DNA and boosting with vaccinia virus expressed Sig/E7/LAMP-1 generated a stronger immune response than either of the two on their own.

1.7.2.6 Cell-based vaccines

Preclinical models have shown that administration of peptide with an appropriate adjuvant is important for enhancing the immunogenic stimulus and dendritic cells (DCs) represent a usefull adjuvant (Steller, 2002; Vitiello *et al.*, 1995). DCs are significantly more efficient in inducing antitumour protection than immunisation with peptides alone (Celluzzi *et al.*, 1996; Paglia *et al.*, 1996). DC pulsed with the whole E7 protein elicited MHC class I-restricted CTL responses and protected against growth of the C3 tumour in syngeneic mice (Tuting *et al.*, 1997). Intramuscular administration of HPV16 E7 gene-transfected murine DC generated strong immunity also against another HPV16 E7 positive tumour, TC-1 (Wang *et al.*, 2000). Vaccines based on dendritic cells pulsed with HPV16-associated tumour oncoproteins, as well as genetically modified DC-based vaccines, have significance in the HPV16-associated tumour systems (Bubenik *et al.*, 2001).

1.7.2.7 Plant-based vaccines

Interest in vaccine production in plants has expanded rapidly due to the considerable advantages of expressing antigenic proteins in plants. Plants can be growth locally and cheaply using standard methods, thus reducing the issues with distribution, storage and transport (Koprowski & Yusibov 2001). Plants as bioreactors are of interest since they allow for the production of large quantities of recombinant proteins at relatively low cost (Kusnandi *et al.*, 1997). The whole production is flexible and easily changed to market needs (Herbers & Sonnewald, 1999). The cultivation, harvesting, storage, and processing of a transgenic crop would also use an existing infrastructure and require relatively little capital investment (Ganz & *et al*, 1996; Pen, 1996; Whitelam, 1995). Kusnandi *et al.* (1997) have estimated that the cost of producing recombinant proteins in plants could be 10- to 50- fold lower than in *E. coli*. Plant derived products, whether

purified or not, are less likely to be contaminated human pathogenic microorganisms than those derived from mammalian cell cultures since plants do not harbour human infectious pathogens therefore sterility is not a big concern.

There are two main systems for expressing foreign proteins in plants: these are transgenic and transient strategies. In the first, stably transformed transgenic plants are produced using *Agrobacterium*-mediated biolistic or other standard transformation techniques. *Nicotiana tabacum* is widely used as a model expression system, but various other plants including *Nicotiana benthamiana*, *Arabidopsis thaliana*, tomato, banana, oilseed rape, Ethiopian mustard, lettuce, rice, wheat and maize have been used. The second strategy is to infect non-transgenic plants with recombinant plant viruses that express transgenes during their replication in the host. The two-host virus systems frequently used are cowpeas with *Cowpea mosaic virus* (CPMV), tobacco with *Potato virus X* (PVX), *Alfalfa mosaic virus* (AIMV) and *Tobacco mosaic virus* (TMV) (Table 1.5). The product yields are generally much higher than those from transgenic plants. Another mode of transient expression is making chimaeric plant viruses that display epitopes / peptides on the surface of the virus capsid. A summary of foreign antigens produced in plants is given in table 1.5.

TABLE 1.5: Summary of foreign antigens expressed in plants

Potential application	Plant	Protein	Expression system	Reference
Hepatitis B virus	Tobacco	Recombinant Hepatitis B surface antigen	AMT	Mason *et al.* 1992, Thanavala *et al.* 1995; Tsuda *et al.*, 1998; Richter *et al.*, 2000;
	Lettuce			Kapusta *et al.*, 1999
	Potato			Kong *et al.*, 2001
Hepatitis C virus	Tobacco	HVR1 mimotopes/CTB	TMV	Nemchinov *et al.*, 2000
Murine hepatitis virus	Tobacco	Murine hepatitis epitope	TMV	Koo *et al.* 1999
Dental caries	Tobacco	Streptococcus mutant surface protein SpaA	AMT	Tacket *et al.*, 1999;
Autoimmune diabetes	Potato	*Vibrio cholera* toxin B subunit-human insulin fusion	AMT	Arakawa *et al.*, 1998 Lam *et al.*, 2000
		Glutamic acid decarboxylase	AMT	Ma *et al.*, 1997
Transmissible gastroenteritis coronavirus	Maize	Spike protein of TGEV	AMT	Streatfield *et al.*, 2001
	Potato	Antigenic N-terminus of glycoprotein S (N-gS)		Gomez *et al.* 2000
Cholera and *E. coli* diarrhoea	Tobacco/potato	*E.Coli* heat-labile enterotoxin LT-B	AMT	Lauterslager *et al.* 2001; Richter *et al.*, 2000; Tacket *et al.*, 1998
	Maize			Streatfield *et al.*, 2001
Oral vaccine against cholera	Potato	*V. cholera* toxin CtoxA and CtoxB subunits	AMT	Fischer *et al.*, 2000

Table 1.5 cont.

Potential application	Plant	Protein	Expression system	Reference
Mucosal vaccines not requiring adjutants	Cowpea	D2 pepride of fibronectin-binding protein B of Staphylococcus aureus	CPMV	Brennan *et al.*, 1999
Norwalk virus	Tobacco/potato	Coat protein	AMT	Dixon *et al.*, 1997; Tacket *et al.*, 2000
Rabies	Tobacco/spinach	Rabies virus glycoprotein	AMT	McGarvey *et al.*, 1995
	Tobacco	Rabbies virus B-cell epitope	AIMV/TMV	Yusibov *et al.* 1997
HIV	Tobacco/ blackeyed bean	HIV epitope (gp120)	CPMV/AMT	Doran *et al.*, 2000; Mushegian *et al.*, 1995
	Cowpea	HIV epitope (gp 41)	CPMV	Brennan *et al.*, 1999
	Tobacco		*PVX	Marusic *et al.*, 2001
	Tobacco		AIMV/TMV	Yusibov *et al.* 1997
	Tobacco	p24	ATM	Zhang *et al.* 2002
	Tobacco		TBSV	Zhang *et al.*, 2000
Rhinovirus	Blackeyed bean	Human rhinovirus epitope (HR Doran *et al.*, 2000;)	CPMV	Tacket *et al.*, 1999
Foot and mouth disease virus	Blackeyed bean	Foot and mouth virus epitope (VP1)	CPMV	Tacket *et al.*, 1999; Beachy *et al.*, 1999
	Alfalfa		ATM	Dus Santos *et al.*, 2002 Wigdorovitz *et al.*, 1999b
	Arabidopsis		ATM	Carrillo *et al.*, 1998
	Tobacco		TMV	Wigdorovitz *et al.*, 1999b
Canine parvovirus	Blackeye beans	VP2 peptide	*CPMV	Langeveld *et al.*, 2001 Nicholas *et al.*, 2002
	Arabidopsis	VP2 peptide (2121)	AMT	Gill *et al.*, 2001
Mink enteritis virus	Blackeyed bean	VP2 epitope	*CPMV	Dalsgaard *et al.*, 1997
Malaria	Tobacco	Malaria B-cell epitope	*TMV	Tacket *et al.*, 1999; Turpen *et al.*, 1995
Influenza	Tobacco	Haemagglutinin	TMV	Beachy *et al.*, 1999
Cancer	Tobacco	c-Myc	TMV	Beachy *et al.*, 1999
Bovine rotavirus A	Potato	VP6	AMT	Matsumura *et al.*, 2002
Human rotavirus	Tobacco		PVX	O'Brien *et al.*, 2000
Rabbit haemorrhagic disease virus	Tobacco	VP60	PPV	Fernandez-Fernandez *et al.*, 2001
***Pseudomonas aeruginosa* infections**	Cowpea	Outer membrane F protein peptide	*CPMV	Brennan *et al.*, 1999a; Brennan *et al.*, 1999b
	Tobacco		*TMV	Gilleland *et al.*, 2000
			*TMV	Staczek *et al.*, 2000
Staphylococcus aureus	Cowpea	D2 peptide of fibronectin-binding protein B (FnBP)	*CPMV	Brennan *et al.*, 1999

*Chimaeric plant virus
PPV: *Plum pox virus*; **TMV**: *Tobacco mosaic virus*; **CPMV**: *Cowpea mosaic virus*; **PVX**: *Potato virus X*; **AMT**: Agrobacterium mediated transformation; **TBSV**: Tomato bushy stunt virus; **AIMV**: *Alfalfa mosaic virus*; **CTB**: cholera toxin subunit B

Initial attempts to determine the immunogenicity of plant-derived antigens were disappointing, which could be attributed to the impurities and low concentration of the plant-produced antigen. Mason *et al.*, (1992) showed that Hepatitis B surface antigen (HBsAg) expressed in transgenic tobacco formed VLPs, and a crude extract containing 3% of the HBsAg was used to immunise

mice. However, the immune response was observed to be lower than that induced by the yeast-derived protein (Mason *et al.*, 1992). The expression was slightly higher (5 ng/g fresh weight) in lettuce and induced low levels of serum antibodies in humans (Kapusta *et al.*, 1999). Expression of HBsAg in transgenic potatoes achieved a higher yield and oral administration of raw potatoes induced an antibody response that was greater than those required for protection (Kong *et al.*, 2001).

About 25-50% assembly was achieved for Norwalk virus capsid protein expressed in transgenic potato and immunisation of volunteers with 150 g doses (215-751μg of VLPs depending on the batch) resulted in modest serum IgG increase after 3 weekly oral immunisations (Tacket *et al.*, 2000). Good protection against *E. coli* LT-B and transmissible gastroenteritis virus (TGEV) has been achieved using transgenic corn produced LT-B (5 or 50 μg) in mice and spike protein of TGEV (2μg) in piglets (Streatfield *et al.*, 2001). Immunisation with a crude extract of transgenic *Arabidopsis* plants expressing FMDV VP1 protein conferred complete protection against FMDV (Carrillo *et al.*, 1998) whereas that expressed in alfafa protected 12 of the 17 mice immunised (Wigdorovitz *et al.*, 1999). A human trial on plant-produced LT-B antigen of *E. coli* using 50-100μg of raw transgenic potatoes per dose showed that antibodies to LT-B were detected in 10 of 11 volunteers fed with the plant-produced antigen, and the antibody levels were similar to those obtained when volunteers were subjected to 10^6 infectious *E. coli* (Tacket *et al.*, 1998). Chimaeric plant-produced vaccines displaying a parvovirus epitope (17 residue peptide from the S protein) on the coat protein of *Cowpea mosaic virus* (CPMV) expressed in black-eyed beans (yield of 1-1.2mg of virus particles per g fresh plant material) were able to protect 11 of 12 animal immunised and subsequently challenged with MEV (Dalsgaard *et al.*, 1997). Transiently expressed FMDV VP1 produced in tobacco plants using TMV protected 30 that were mice immunised and subsequently challenged (Wigdorovitz *et al.*, 1999).

These reports show the considerable potential of plant-expressed vaccines as a cheap alternative to vaccine production systems. Evidence has not shown than any particular plant-based expression of foreign antigen method is best, and success may depend more on the choice of antigen than on the method of expression and delivery (Awram *et al.*, 2002).

1.8 PROJECT AIMS

The major objective of this thesis was to investigate plant-based expression systems for producing cheap prophylactic HPV vaccines for developing countries, where HPV is the leading cause of cancer-related mortality. The rapid development of plant biotechnology during the past 15 years has made possible to use plants as a viable alternative to cell-culture systems for the production of subunit vaccines. In theory, scale up would not require large investments in hardware and culture media and could used existing agricultural resources to produce vaccines where sterility is not a great concern since plants are generally free of human / animal pathogens.

HPV-16 is the most prevalent type associated with cervical cancer, found in over 50% of cervical cancer cases (Clifford *et al.*, 2003) and hence this was the genotype chosen for this study. HPV-16 variants have been shown to escape recognition by certain Mabs (Roden *et al.*, 1997b; Sasagawa *et al.*, 1995), therefore, it was essential to check with a panel of well characterised anti HPV-16 L1 Mabs that indeed the South African isolated HPV-16 L1 retained its antigenicity when expressed in well characterised expression systems such as *Sf*21 insect cells by recombinant baculovirus, before being tested for expression in plant-based expression systems. In addition to this, a study was undertaken, using the baculovirus system, to study the effects on the antigenicity and particle formation of various C-terminal deletions as far the conserved cysteine at reside 428 in combination with N-terminal deletions (residues 2-10) and mutation of the cysteine residue 428 to a glycine. The aim of this study was to identify suitable deletion mutants for expression in plants. The magnificent work on the crystal structure of the mutant HPV-16 L1 (10 residue N-terminal deletion of the L1 gene; ΔN10 L1) by Chen *et al.* (2000b) provided a great insight into the effects of the various deletant and mutant constructs on assembly.

Once the antigenicity of the L1 and its deletion mutants had been established, the next major objective was to investigate plant-based expression of HPV-16 L1. Two different plant expression systems were tested, transgenic expression in *Nicotiana tabacum* cv. Xanthi using *Agrobacterium tumefaciens* mediated transformation and transient expression in *Nicotiana benthamiana* using a tobamovirus vector. The transgenic systems clearly have the advantage of ease of scale up from transgenic seed; however, the expression levels reported for foreign

proteins are low. The transient expression of foreign proteins is reported to attain higher yields than transgenic expression, however stability of the virus vectors remains an issue.

Further, analysis of the structure of HPV-16 L1 and multiple sequence alignments revealed a variety or regions and surface loops that could potentially be used for displaying foreign epitopes in a bid to create novel chimaeric / multivalent HPV-based vaccines. Kawana *et al.* (1998a, 1999) reported on a HPV-16 minor capsid L2 epitope (resides 10-120) that has common-neutralising properties for HPV-6 and 16. Since the immune response to HPV is generally type specific, a chimaeric HPV vaccine that offered a broad serotype protection would further reduce the cost of candidate vaccine in cervical cancer prevention. Therefore, the L2 epitope was thought to be a suitable epitope for the initial investigation of potential display sites in the HPV-16 L1 structure in *Sf*21 cells using recombinant baculovirus.

In summary the main objectives of this thesis were:

- To analyse various deletant and mutant HPV-16 L1 gene products for further use in plant-based expression systems, predominantly from an antigenic perspective.

- To investigate the prospect of expressing the selected deletant and mutant genes of HPV-16 L1 in plants by transgenic and transient expression, the latter by using TMV as a vector. Once the expression was confirmed, the next major objective was to analyse antigenicity and potentially the immunogenicity of the isolated L1 product.

- To investigate the potential of using HPV to deliver foreign epitopes and possibly investigate the prospects of an HPV L1/L2 vaccine.

Chapter 2

Deletion and point mutation study of the HPV-16 L1 major capsid protein gene

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ABSTRACT

Recombinant HPV virus-like particles (VLPs) are promising vaccine candidates for use as a prophylactic vaccine against HPV infection. Variants within a genotype have been shown to have different binding affinity to neutralising monoclonal antibodies (Mabs) raised against HPV-16 L1 VLPs and the expressed L1 genotypes have varied protein yields in recombinant expression systems. This chapter analyses, by means of a panel of well characterised Mabs, the effects of mutating an alanine at residue 266 to a threonine (504[ΔA266T]), various C- and N-terminal deletions of HPV-16 L1 (504) and the mutation of the conserved cysteine at position 428 to a glycine in the deletion constructs on the antigenicity of protein product. The antigenicity data indicate that the binding of Mab H16:E70 to 504[ΔA266T] was reduced by almost half in comparison to 504. The C-terminal region 428-483 was found to be critical for maintaining the correct conformation for the binding of conformation specific Mabs (H16:V5, H16:E70, H16:U4 and H16:9A) whereas the deletion of the nuclear localisation signal (NLS) or the mutation of the cysteine at residue 428 to a glycine or the N-terminal (residues 2-9) deletion had no effect on the antigenicity. A N-terminal deletion (residues 2-9) resulted in a mixed population of VLPs that were 30nm and 55nm in diameter whereas pentamer aggregates were observed as products from the deletion of the 428-465 region. The mutation of the cysteine 428 resulted in capsomer formation but not VLPs.

2.1 INTRODUCTION

The virions of papillomaviruses are highly immunogenic, inducing high titres of neutralising antibodies when systemically inoculated (Dvoretzky *et al.*, 1980; Kirnbauer *et al.*, 1992). Papillomavirus like-particles (VLPs), made from the major capsid protein L1 alone, or by the co-expression of L1 and the minor L2 capsid protein, have been proven to induce protective immunity in animal models (Breitburd *et al.*, 1995; Jansen *et al.*, 1995; Suzich *et al.*, 1995) and *S. cerevisiae*-produced HPV-16 VLPs used in a recent clinical trial resulted in a 99.7% seroconversion in those patients that received the vaccine (Koutsky *et al.*, 2002).

Viruses that are controlled efficiently by neutralising antibodies are able to mutate to give rise to serotypes or strains that do not necessarily react with neutralising antibodies elicited by other strains (Bachmann & Zinkernagel, 1996). The serotype concept has proven useful in

characterising human viral pathogens and in designing protective vaccines against them. Numerous HPV variants have been isolated from different geographical locations and evidence suggests the evolution of the variants coincides with the migration patterns of early humans (Ho *et al.*, 1991). The variants have been grouped according to homology into five major phylogenetic branches: these are European, African 1, African 2, North American and Asian American (Chan *et al.*, 1992; Yamada *et al.*, 1995). Nucleotide differences that result in amino acid changes for the L1 in the variants closely map to the principal neutralisation epitopes of Mab H16:V5 and H16:E70 (Chen *et al.*, 2000), which suggests that variants had evolved to escape neutralisation (Pastrana *et al.*, 2001). In support of this hypothesis, the H16:E70 Mab was able to neutralise a European (114K) but not an Africa 2 (Z-1194) pseudotype virions (Roden *et al.*, 1997) and a single amino acid change resulted in the loss of H16:V5 binding (White *et al.*, 1999) (see Table 1.2, Chapter 1). However there is also evidence of cross-reactivity of sera between variants e.g. sera from women naturally infected with HPV-16 recognised VLPs of HPV-16 variants 114K and Z-1194 (Cheng *et al.*, 1995). It is therefore important to determine the effects of mutations on the neutralisation, since the immunodominant neutralising antibodies mainly recognise conformational epitopes (Christensen *et al.*, 1994; Christensen & Kreider, 1990; Roden *et al.*, 1994).

A study on expression levels of six of HPV-16 L1 variants in insect cells using recombinant baculovirus demonstrated that the yield varied in a ratio range from 1 to 79 depending on the HPV-16 L1 gene sequence (Touze *et al.*, 1998b). Similarly a study by Sasagawa *et al.* (1995) reported that of the three variants expressed in *S. pombe*, the two variants 16 B27L1 and 16 T3L1 produced 64- and 14 fold more VLPs than the prototype sequence.

Chen *et al.* (2000b) showed that a 10 residue N-terminal deletion for L1 expressed in *E. coli* results in formation of T=1 particles and addition of a glycine to the N-terminus deletion results in reversion of the T=1 particles to T=7 particles. The ΔN10 T=1 and full length L1 T=7 particles were found to be stable at high ionic strength over a pH range of 4-7.5; the L1 T=7 particles were slightly unstable at pH7.5 (Chen *et al.*, 2001). The PVs have a bipartite nuclear localisation signal (NLS; $KRK_1atpttssttstaKRKKRKL_2$) and that of HPV-16 L1 is located between residues 484-504 (Zhou *et al.*, 1991). Particles resulting from a 30 residue C-terminal deletion of the HPV-16 L1 gene have been shown to assemble into T=7 particles (Chen *et al.*, 2001). The cysteine at residue 428 is highly conserved among all papillomaviruses and forms disulphide bonds with residue 175 in T=7 particles (Figure 1.10, Chapter 1 section 1.1.1). This is not the

case in T=1 particles since the two cysteines are too far apart (Figure 1.12, Chapter 1): the interpentameric interaction here is attributed to helices h3, h4 and h5. Mutations of these cysteines results in the formation of capsomers rather than VLPs (Fligge *et al.*, 2001; Li *et al.*, 1998; McCarthy *et al.*, 1998; Sapp *et al.*, 1998). Sapp *et al.* (1998) have demonstrated that two cysteines at position 175 and 428 form disulphide bonds that yield a L1 trimer. In the T=7 particles the C-terminal arms form the principal interpentameric contacts, by extending away from the subunit of origin towards another subunit from a neighbouring pentamer (see chapter 1, section 1.1.1).

A deletion study on *Canine oral papillomavirus* (COPV) L1 indicated that a C-terminal deletion of 67 residues and an N-terminal deletion of 25 residues allowed for VLP formation, but that the conformation-specific antibodies only bound particles resulting from C-terminal deletion of 26 residues or less (Chen *et al.*, 1998). A study by Chen *et al.* (2000b) that solved the crystal structure of HPV-16 L1 looked at N- and C-terminal deletions. The report indicated that N-terminal deletions of 15 and 20 residues and C-terminal deletions of 46 and 86 residues rendered the gene product sensitive to trypsin, therefore more prone to proteolytic degradation. Li *et al.* (1997) demonstrated that digestion of the recombinant HPV-11 L1 and protein with trypsin yielded two major cleavage products: a ~ 42kDa product resulting from the cleavage at R416 (R420 for HPV-16) and an intermediate species of ~ 48kDa likely to result from the cleavage at R462 (R466 for HPV-16). Thiol reduction renders the HPV VLPs trypsin sensitive where only a 42kDa cleavage product is observed.

This chapter first addresses an investigation of the HPV-16 L1 variants in terms of antigenicity for use in subsequent studies for this thesis. The HPV-16 L1 sequence that was available for this work (GenBank accession no. AY177679) is identical in amino acid sequence to 114K (see Table 1.2, Chapter 1 for sequence variations) and differs from the Phi sequence published by Touze *et al.* (1998b) at residue 266 (Ala in 114K; Thr in Phi1). Since Phi1 has been demonstrated to yield more VLP product than 114K, it was necessary to investigate this variant from an antigenicity perspective. Second, it reports investigation of the effects of various deletions, both C- and N- terminal, together with mutation of the conserved cysteine at residue 428, on the antigenicity of the gene products. Both these variant and deletion/mutation studies were carried out to prospect for suitable HPV-16 L1 gene sequences for use in the plant-based candidate vaccine development (Chapters 3 and 4).

2.2 MATERIALS AND METHODS

2.2.1 Synthesis of deletion and mutant constructs

The HPV-16 L1 gene (South African isolate; GenBank accession no. AY177679) was PCR amplified (primers listed in Table 2.1) from a biopsy sample by Wendy Burgers (Division of Medical Virology) and cloned into pSK (Stratagene Cloning Systems) and sequenced. The HPV-16 L1 (referred to as 504 throughout this chapter) gene in pSK plasmid (pSK-L1-SA) was mutated so as to change amino acid residue 266, from an alanine to a threonine (504[ΔA266T]), by polymerase chain reaction (PCR) mutagenesis using *Pfu* DNA polymerase using overlapping primers described in Table 2.1. The PCR mix from the mutagenesis was digested with *Dpn*I restriction endonuclease in order to digest methylated template DNA. DH5α *E. coli* cells were transformed with the amplified mutated DNA and the mutant DNA product was sequenced.

TABLE 2.1: List of primers used to synthesise the deletion and mutation constructs pf HPV-16 L1.

Construct	Primers
***504**	Forward 5'-*CCCGGG*ATGTCTTTGGCTGCCTAG-3'
	Reverse 5'-*GCCTCGAC*TTACAGCTTACGTTTTTGC-3'
§504[ΔA266T]	Forward 5'-TTATAGGGCTGGTACTGTTGGTGAAAATGTACCAGACG-3'
	Reverse 5'-GGTACATTTTCACCAACAGTACCAGCCCTATTAAATAAATG-3'
ΔC483	Forward 5'-*TTAATTAA*ATGTCTCTTTGGCTGCCTAGTGAGG-3'
	Reverse 5'-*CTCGAG*TTATCCTAATGTAAATTTTGGTTTGGCC-3'
ΔC465	Forward 5'-*TTAATTAA*ATGTCTCTTTGGCTGCCTAGTGAGG-3'
	Reverse 5'-*CTCGAG*TTATCCTAAAGGAAACTGATCTAGGTCTGC-3'
ΔC427	Forward 5'-*TTAATTAA*ATGTCTCTTTGGCTGCCTAGTGAGG-3'
	Reverse 5'-*CTCGAG*TTAAGCAATTGCCTGGGATGTTACAAACC-3'
M-ΔN10	Forward 5'-*TTAATTAA*ATGGTCTACTTGCCTCCTGTCCCAG-3'
	Reverse 5'-*CTCGAG*TTACAGCTTACGTTTTTTGCGTTTAGCAGTTGTAG-3'
M-ΔN10ΔC483	Forward 5'-*TTAATTAA*ATGGTCTACTTGCCTCCTGTCCCAG-3'
	Reverse 5'-*CTCGAG*TTATCCTAATGTAAATTTTGGTTTGGCC-3'
M-ΔN10ΔC465	Forward 5'-*TTAATTAA*ATGGTCTACTTGCCTCCTGTCCCAG-3'
	Reverse 5'-*CTCGAG*TTATCCTAAAGGAAACTGATCTAGGTCTGC-3'
M-NΔ10CΔ427	Forward 5'-*TTAATTAA*ATGGTCTACTTGCCTCCTGTCCCAG-3'
	Reverse 5'-*CTCGAG*TTAAGCAATTGCCTGGGATGTTACAAACC-3'
#pen	Forward 5'-GTAACATCCCAGGCAATTGCT**GGA**CAAAAACATACACCTC-3'
	Reverse 5'-GCTGGAGGTGTATGTTTTTG**TCC**AGCAATTGCCTGGGATG-3'

* Primers used to amplify the HPV-16 L1 from a patient biopsy for cloning into pSK vector
§ Mutation of alanine residue 266 to threonine
# Mutation of cysteine residue 428 to glycine
CTCGAG: *Xho*I; *TTAATTAA*: *Pac*I; *CCCGGG*: *Xma*I / *Sma*I; *CTCGAC*: *Sal*I
5'-**GGA**-3' / 5'-**TCC**-3': glycine

The deletion constructs were prepared by PCR amplification (primers described in Table 2.1) of the appropriate region of the HPV-16 L1 gene in the pSK-L1-SA using Expand Long Template PCR system (Roche molecular Biochemicals). The amplified deletion PCR products were cloned into a pGEM® T Easy vector system (Promega) and sequenced.

The mutation of residue 428 from cysteine to glycine of the of the various deletion mutants in pGEM® T Easy vector and of L1 504 in pSK vector were carried out using overlapping primers (primers described in Table 2.1) and *Pfu* DNA polymerase as described above for the mutagenesis of residue 266.

The mutant and deletion constructs prepared for this study are summarised in Figure 2.1.

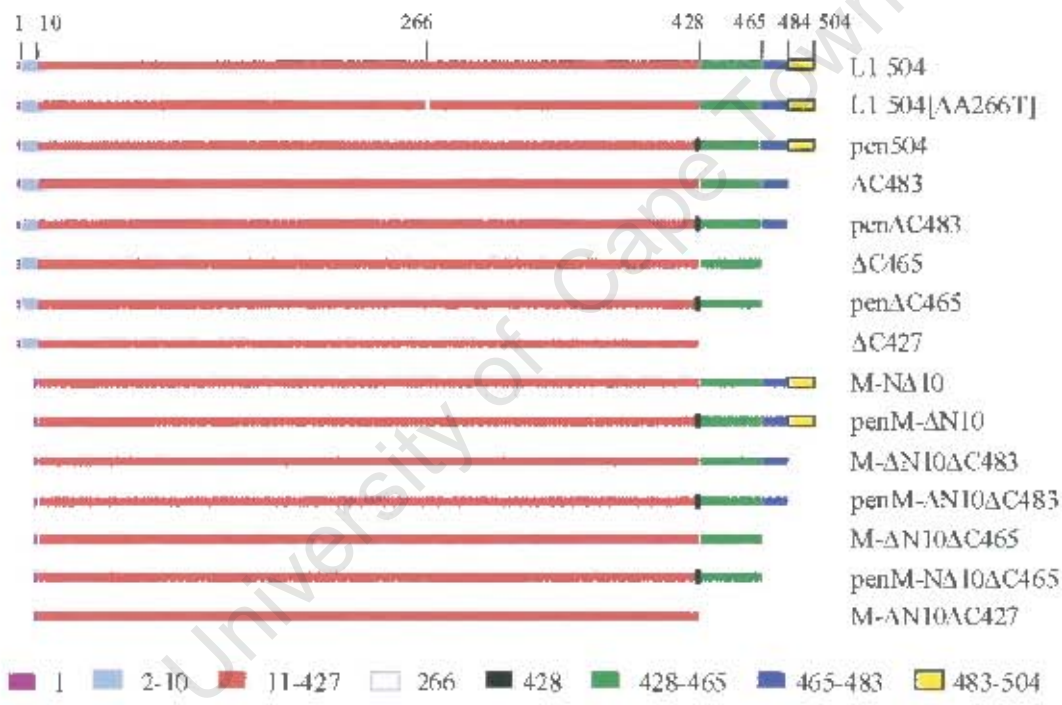


FIGURE 2.1: Illustration of the HPV-16 L1 deletant and mutant constructs.
pen: mutation of cysteine at 428 to glycine
M-ΔN: N-terminal deletion of residues 2-10
ΔC: C-terminal deletions
L1 504: South African isolated L1
L1 504[ΔA266T]: mutation of alanine at residue 266 in L1 504 to threonine

2.2.2 Expression of the deletant and mutant HPV-16 L1s in *Sf*21 insect cells using recombinant baculovirus

The deletion and mutation products ΔC483, ΔC465, ΔC427, M-ΔN10, M-ΔN10ΔC483, M-ΔN10ΔC465, M-ΔN10ΔC427, penΔC483, penΔC465, penM-ΔN10, penM-ΔN10ΔC483 and penM-ΔN10ΔC465 cloned in the pGEM® T Easy vector were directionally (orientation checked with *Bam*HI restriction digests) sub-cloned into pFastBac1® vector (Life Technologies) into the *Not*I restriction site. The full length HPV-16 L1 (504), pen504 and 504[ΔA266T] were directionally cloned into pFastBac1 using *Sal*I / *Xho*I restriction sites. The DNA from the resulting pFastBac1® constructs was used to transfect DH10bac *E. coli* cells for the preparation of bacmid clones. The resulting Bacmid DNA was subsequently transfected into *Spodoptera frugiperda* (*Sf*21) cells (Life Techologies) using Cellfectin (Life Techologies).

The following slightly modified Bac-to-Bac® protocol was followed to amplify the recombinant virus. Petri dishes (35mm x 10mm) were seeded with approximately 1 x 10^6 *Sf*21 cells in 2 ml of complete insect cell growth media (TC100 / 10% foetal calf serum / antibiotics) and incubated overnight at 27°C. 5µl of recombinant bacmid DNA was added to 100µl of TC100 and 6µl of Cellfectin to 100µl of TC100 in two separate sterile Eppendorf tubes. The contents of the tubes were mixed gently and incubated at room temperature for 15-45 minutes and the volume was made up to 1ml with TC100. The monolayers of *Sf*21 cells were washed twice with TC100 and to this the 1ml of lipid-DNA complex was added. The Petri dishes were incubated for 5 hours at 27°C followed by the addition of 1ml of complete media. The Petri dishes were incubated in a moist environment (in a moist sandwich box) at 27°C for 48-72 hours. The recombinant virus (1^{st} sup) was harvested from the supernatant of the infected cells in the Petri dishes was stored away. 2ml of complete media was added to the cells and incubated for a further 48-72 hours to obtain the working recombinant virus stock (2^{nd} sup). 200µl of 1^{st} sup and 300µl of complete media was added to 25cm^2 flasks seeded with 1.5 x 10^6 *Sf*21 cells. This was allowed to incubate for an hour at room temperature and the virus was removed and the cells were incubated with 4ml of fresh complete media at 27°C for 48-72 hours into order to generate the amplified recombinant virus stock for use in all further infections.

For analysis of the deletion and mutant HPV-16 L1 gene products, five insect cell tissue culture 75 cm^2 flasks on average per recombinant baculovirus construct, were seeded with 6 x 10^6 *Sf*21

cells and infected with 40µl of the respective recombinant baculovirus amplified stock in a total of 10ml of complete media. The flasks were incubated at 27°C for 48-72 hours. The infected cells were harvested by washing them off them flask surface, and spinning them down at ~ 4 000 x g.

2.2.3 Extraction of deletion and mutant HPV-16 L1s expressed in *Sf*21 cells

The harvested infected *Sf*21 cells were spun down at ~ 4 000 x g, resuspended in high salt phosphate buffered saline (PBS with 0.5M NaCl) and sonicated 4 times at 5 second intervals. The sonicated material was overlaid onto a 40% sucrose cushion and pelleted at 100 000xg for 3 hours. The pellet was resuspended in CsCl buffer (PBS with 0.4g/ml CsCl) with sonication (4 times with 5 second intervals). The suspension was centrifuged at 100 000 x g at 10°C for 24 hours in a Beckman SW50.1 rotor. Two distinct bands were observed in most cases and the top bands were extracted and dialysed overnight against PBS at 4°C. In cases where no clear band but a diffused zone was observed, 500µl fractions were collected and analysed by ELISA using Mab H16:J4 (binds linear epitope 261-280), the positive fractions were pooled and dialysed as above.

2.2.4 Synthesis, expression and purification of *Cottontail rabbit papillomavirus* (CRPV) L1 by recombinant baculovirus in *Sf*21 cells

The CRPV L1 gene (kindly provided by Dr. N Christensen, Penn State University) was PCR amplified using the following set of primers:

forward 5'TTAATTAAATGGCAGTGTGGCTGTCTACG-3' (TTAATTAA - *Pac*I)

reverse 5'-CTCGAGTTAAGTACGTCTCTTGCGTTTAGATGATTTC-3' (CTCGAG - *Xho*I)

The amplified gene was cloned into pGEM® T Easy vector and sequenced. The CRPV L1 gene was directionally cloned into pFastBac1® vector into the *Eco*RI restriction sites and the procedure outlined above (section 2.2.1 and 2.2.2) was followed to amplify the recombinant baculovirus and purify the CRPV L1 VLPs.

2.2.5 Indirect immunofluorescence microscopy of the deletant and mutant HPV-16 L1s

Glass sides were soaked overnight in 70% ethanol / 1% HCl, washed thoroughly with distilled water (twenty times) and baked for 20 minutes at 100°C. The slides were transferred to sterile petri dishes and seeded with 0.5 x 106 *Sf*21 cells in 500µl complete media. The cells were allowed to settle for an hour and the complete media was replaced with 200µl of the various recombinant amplified virus stocks and incubated for a further hour at room temperature. The inoculum was removed and replaced with 500ml of complete media and incubated overnight at 27°C in a moist environment.

Two controls were set up, one uninfected and the second infected with recombinant baculovirus expressing GUS (prepared by Eric van der Walt, University of Cape Town). The infected *Sf*21cells on the slides were fixed in methanol at - 20°C for 5 minutes. The slides were washed with PBS and incubated for 1 hour at room temperature in a moist environment with Mabs H16:V5 and H16:J4 (500µl per slide) diluted 1:250 in 1% blocking solution (non-fat milk powder in PBS). Detailed description of the Mabs is provided in Table 1.3, Chapter 1). The slides were washed for 10 minutes in PBS and incubated with FITC labelled secondary goat anti-mouse antibody (150µl per slide) diluted 1:40 in blocking solution with Evans Blue dye (1:20 dilution in blocking solution) for an hour in a dark moist environment. The slides were then washed three times with PBS over 20 minutes and mounted with a drop of Vector Shield (Vector Laboratories). The slides were viewed with a Diaphot (TMD) inverted microscope using the Nikon DM510 B2 filter and Fluor 20 Ph3DL lens. Images were captured using an Zeiss AxioCam digital camera.

2.2.6 Antibody characterisation of the deletant and mutant HPV-16 L1s by ELISA and western blot analysis

A dilution series of the deletant and mutant proteins resulting from expression ofHPV-16 L1 (504), pen504, ΔC483, penΔC483, M-ΔN10, penM-ΔN10, M-ΔN10ΔC483, ΔC465, penΔC465, M-ΔN10ΔC465 and ΔC427 genes or constructs, were tested against Mab H16:J4, and dilutions that gave similar absorbances by ELISA were selected in order to normalise the concentrations. The normalised dilutions were used for further antibody characterisation by ELISA.. The protein products were coated onto ELISA plates overnight at 4°C. The plates were washed four times

with PBS and blocked with 1% non-fat milk. The monoclonal antibodies were diluted at 1:500 in PBS/milk and allowed to bind to the protein products for 2 hrs at room temperature followed by four stringent washes. The bound primary antibody was probed with the alkaline phosphatase labelled goat anti-mouse secondary antibody. The binding was detected using p-nitrophenyl phosphate (Sigma) and the ELISA plate absorbance was read at 405nm using a Titrex ELISA plate reader.

The cell lysates from the expression of the HPV-16 L1 gene constructs L1 504, ΔC483, ΔC465 and ΔC427 in addition to a negative control (ROPV L2) were denatured for 10 minutes at 100°C in SDS disruption solution. The denatured lysates (10µl) were resolved on a 10% SDS PAGE gel. The resolved gel was transferred onto a nitrocellulose membrane by semi-dry electrophoresis (BioRad) for 25mins at 25V. The membrane was blocked using 1% non-fat milk for 2 hours and incubated with Mab H16:D9 at a dilution of 1:250 overnight at 4°C. The membrane was washed with PBS/0.05% Tween-20 and probed with alkaline phosphatase-labelled secondary goat anti-mouse antibody diluted 1:2000, for 1 hour at room temperature. Reaction was detected colorimetrically using 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) and 4-nitro blue tetrazolium chloride (NBT) substrates.

2.2.7 Transmission electron microscopy of particles

The deletant and mutant particles were viewed either after direct adsorption onto carbon coated copper grids, or after immunotrapping with preadsorbed H16:J4 Mab (binds linear epitope residues 261-280) at a dilution of 1:50 onto carbon coated copper grids. The Mab was incubated with the carbon coated copper grids for 15 minutes, washed and the incubated with the purified deletant and mutant gene products for 10 minutes. The grids were washed with water and stained with 2% uranyl acetate and viewed using a JEOL 200CX transmission electron microscope.

2.3 RESULTS

2.3.1 Expression and analysis of VLPs resulting from the expression of HPV-16 L1 504 and 504 [ΔA266T] genes by recombinant baculoviruses

The Phi1 sequence reported by Touze *et al.* (1998b), which differs from L1 504 by A→T change at amino acid position 266, generated the highest yield of VLPs, of the HPV-16 L1 variants tested, when expressed in insect cells by recombinant baculovirus. However, White *et al.* (1999) have mapped the epitopes of the neutralising Mabs H16:V5 and H16:E70 to the phenylalanine at residue 50, alanine at 266 and serine at 282. Therefore, it was necessary to investigate the effect of the A→T 266 change from an antigenicity perspective with regard to neutralising conformation-specific Mabs H16:V5 and H16:E70, if this gene sequence were to be used for candidate vaccine development. Therefore, the A at position 266 in HPV-16 L1 504, which is identical to that of 114K (amino acid differences in HPV-16 variants are outlined in Table 1.2, Chapter 1) was mutated to a T (504[ΔA266T]) using overlapping primers.

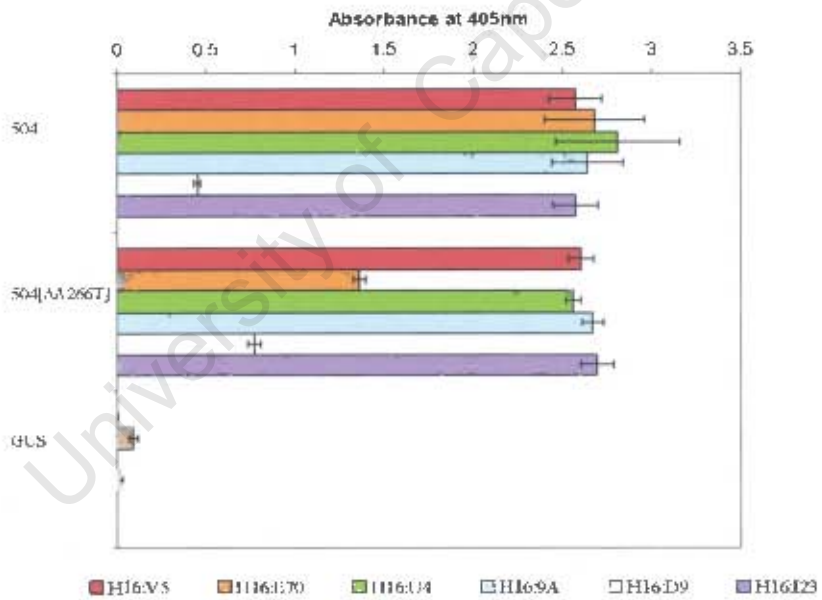


FIGURE 2.2: Antibody characterisation by ELISA of VLPs resulting from the expression on of HPV-16 L1 504 and 504[ΔA266T] in insect cells by recombinant baculovirus using a panel of well characterised Mabs. A two fold higher binding to H16:E70 is observed for 504 compared to 504[ΔA266T].

The VLPs resulting from the expression of the L1 proteins 504 and 504[ΔA266T] in insect cells by means of recombinant baculovirus were analysed for antigenicity using a panel of well characterised Mabs (Table 1.3, Chapter 1) that bind neutralising, conformational and linear epitopes by direct ELISA assays (Figure 2.2).

The binding affinity to Mabs H16:V5, H16:U4, H16:9A and H16:I23 to HPV-16 L1 (504) and 504[ΔA266T] VLPs were almost identical. The most notable difference was observed for the binding of Mab H16:E70: L1 504 had almost twice the binding affinity to H16:E70 than L1 504[ΔA266T] VLPs. The higher binding affinity of Mab H16:D9 to 504[ΔA266T] VLPs indicates that there was probably a larger amount of denatured L1 in this extract since H16:D9 has been shown to only bind denatured particles (Christensen *et al.*, 1996a).

No significant difference in the particle morphology was observed in the two different kinds of VLPs when observed under the electron microscope. Due to the greater binding affinity for H16:E70, it was decided to use the HPV-16 L1 504 gene sequence for the deletion and point mutation study.

2.3.2 Synthesis and expression of deletion and point mutation constructs of HPV-16 L1

Deletant and mutant constructs were prepared and expressed to determine their antigenicity so that suitable selections could be tested in plant-based expression systems (Chapters 2 and 3). Four potential deletion regions were identified, one at the N-terminus and three at the C-terminus. A 10 residue N-terminal deletion of the HPV-16 L1 gene expressed in *E. coli* resulted in particles that were 30nm in diameter with a T=1 icosahedral lattice (Chen *et al.*, 2000). The PV L1s have a bipartite nuclear localisation signal (NLS; NLS; KRK_1atpttssts$ttaKRKKRKL_2$) and that of HPV-16 L1 is located between residues 484-504 (Zhou *et al.*, 1991). The h5 helix (residues 462-473) docks into the interior hollow of the HPV-16 L1 pentamer. Residue 428 is a highly conserved cysteine (see Figure 1.7, Chapter 1) that has been shown to be important for intrapentameric bonds (Li *et al.*, 1998; Sapp *et al.*, 1998). Based on some of these key features of the HPV-16 L1, seven deletion constructs were synthesised, covering various permutations and combinations of deletion, by PCR amplification using various sets of primers. These are shown in Table 2.1. The conserved cysteine, residue 428, was mutated using a set of overlapping

primers for five of the deletion constructs and the full-length construct (Table 2.1). The various deletion and mutated constructs synthesised by PCR amplification are summarised in Figure 2.1.

The expression in insect cells was confirmed by indirect immunofluorescence using Mabs H16:J4 and H16:V5 (Figure 2.3-2.6). Results of the indirect immunofluorescence of the deletion and mutant constructs are summarised in Table 2.2. Constructs 504, ΔC483, M-ΔN10, M-ΔN10ΔC483, pen504, penΔC483, penM-ΔN10 and penM-ΔN10ΔC483 bound both the conformationally dependent neutralising Mab H16:V5 and Mab H16:J4 (recognises a linear epitope residues 261-280). The rest of the constructs bound only H16:J4. This data indicated that the C-terminal region residues 428-483 are important for maintaining the correct conformation of the particles formed, if any, as a result of the C-terminal deletions. Since the binding of H16:V5 is retained by the mutation of the cysteine at residue 428 in the constructs 504, ΔC483, M-ΔN10, M-ΔN10ΔC483, pen504, penΔC483, penM-ΔN10 and penM-ΔN10ΔC483, this indicates that the quaternary structure, probably at pentameric level, is not altered by this mutation.

TABLE 2.2: Summary of the indirect immunofluorescence microscopy of the deletion and mutant HPV-16 L1s

	Mabs		Localisation	
Construct	**H16:J4**	**H16:V5**	**Nuclear**	**Cytoplasmic**
504	+	+	+	-
pen504	+	+	+	-
M-ΔN10	+	+	+	-
penM-ΔN10	+	+	+	-
Δ483	+	+	-	+
pen Δ483	+	+	-	+
M-ΔN10 Δ483	+	+	-	+
penM-ΔN10 Δ483	+	+	-	+
Δ465	+	-	-	+
penΔ465	+	-	-	+
M-ΔN10 Δ465	+	-	-	+
penM-ΔN10 Δ465	+	-	-	+
Δ427	+	-	-	+
M-ΔN10 Δ427	+	-	-	+
GUS	-	-	n/a	n/a
Negative	-	-	n/a	n/a

n/a not applicable

All the deletion and mutation constructs were successfully expressed in insect cells using recombinant baculoviruses and the gene products were purified using CsCl gradient centrifugation and used in the antibody characterisation and electron microscopy analysis.

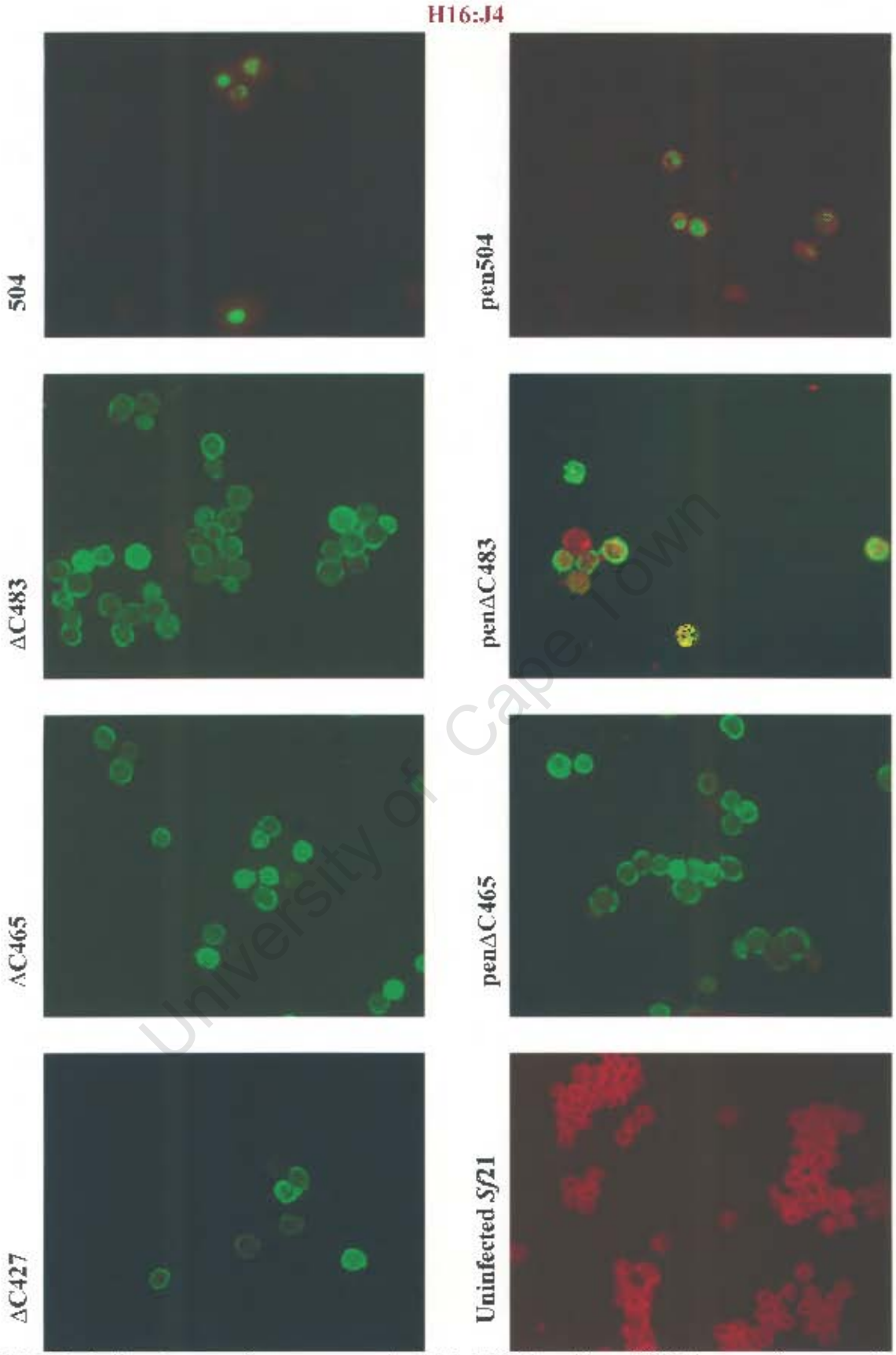


FIGURE 2.3: Indirect immunofluorescence using Mab H16:J4 probing of *Sf*21 insect cells expressing the various deletant and mutant HPV-16 L1 gene products. *Sf*21 cells were counterstained with Evan's blue. The Mab H16:J4 binding to the 504, pen504, ΔC483, penΔC483, ΔC465, penΔC465 and ΔC427 gene products was detected using FITC labelled goat anti-mouse secondary antibody. No H16:J4 binding was detected in uninfected negative control cells. Digital images captured at x20 magnification.

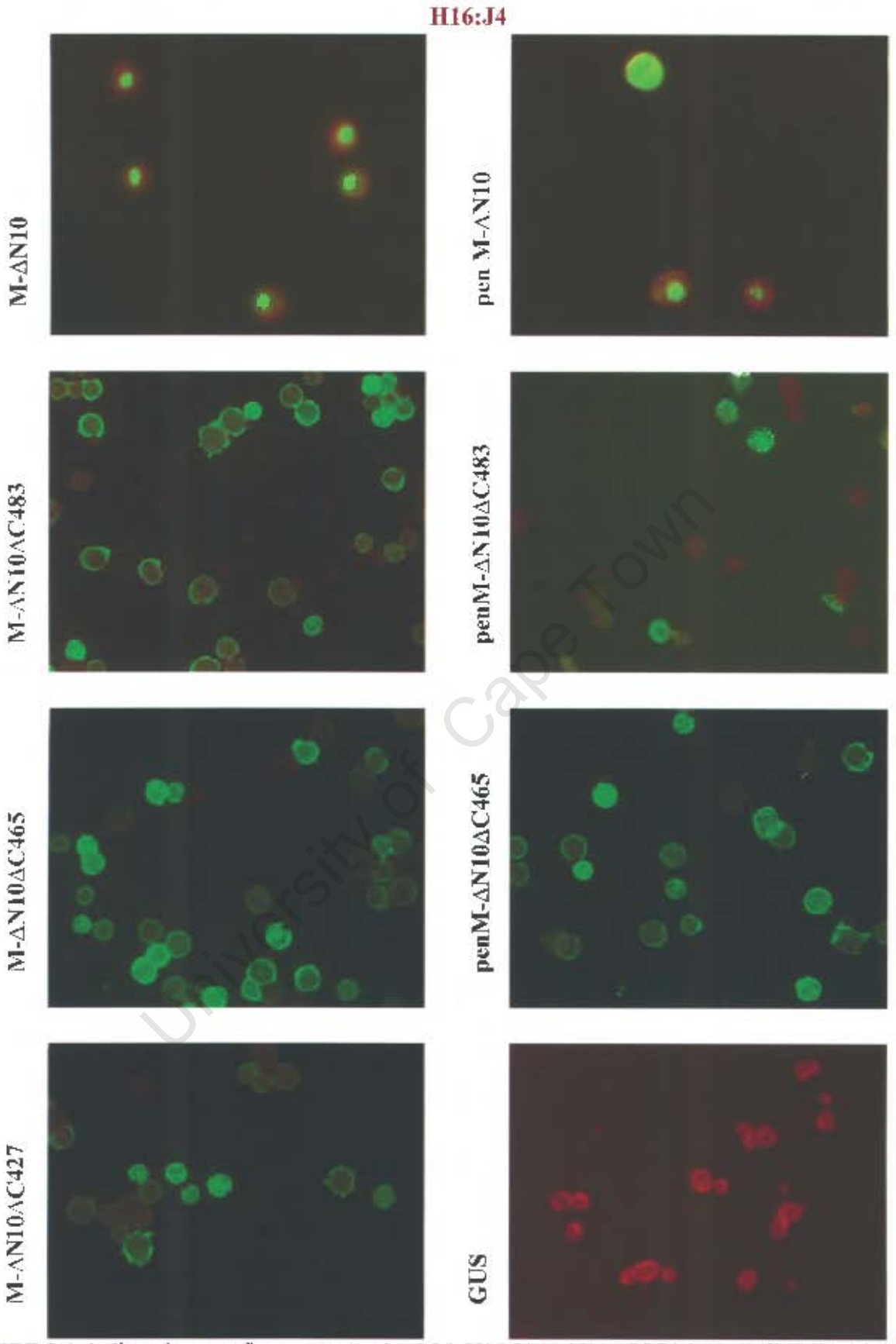


FIGURE 2.4: Indirect immunofluorescence using Mab H16:J4 probing of *Sf*21 insect cells expressing the various deletant and mutant HPV-16 L1 gene products. *Sf*21 cells were counterstained with Evan's blue. The Mab H16:J4 binding to the M-ΔN10, penM-ΔN10, M-ΔN10ΔC483, pen M-ΔN10ΔC483, M-ΔN10ΔC465, pen M-ΔN10ΔC465 and M-ΔN10ΔC427 gene products was detected using FITC labelled goat anti-mouse secondary antibody. No H16:J4 binding was detected in cells infected with the GUS expressing recombinant baculovirus negative control. Digital images captured at x20 magnification.

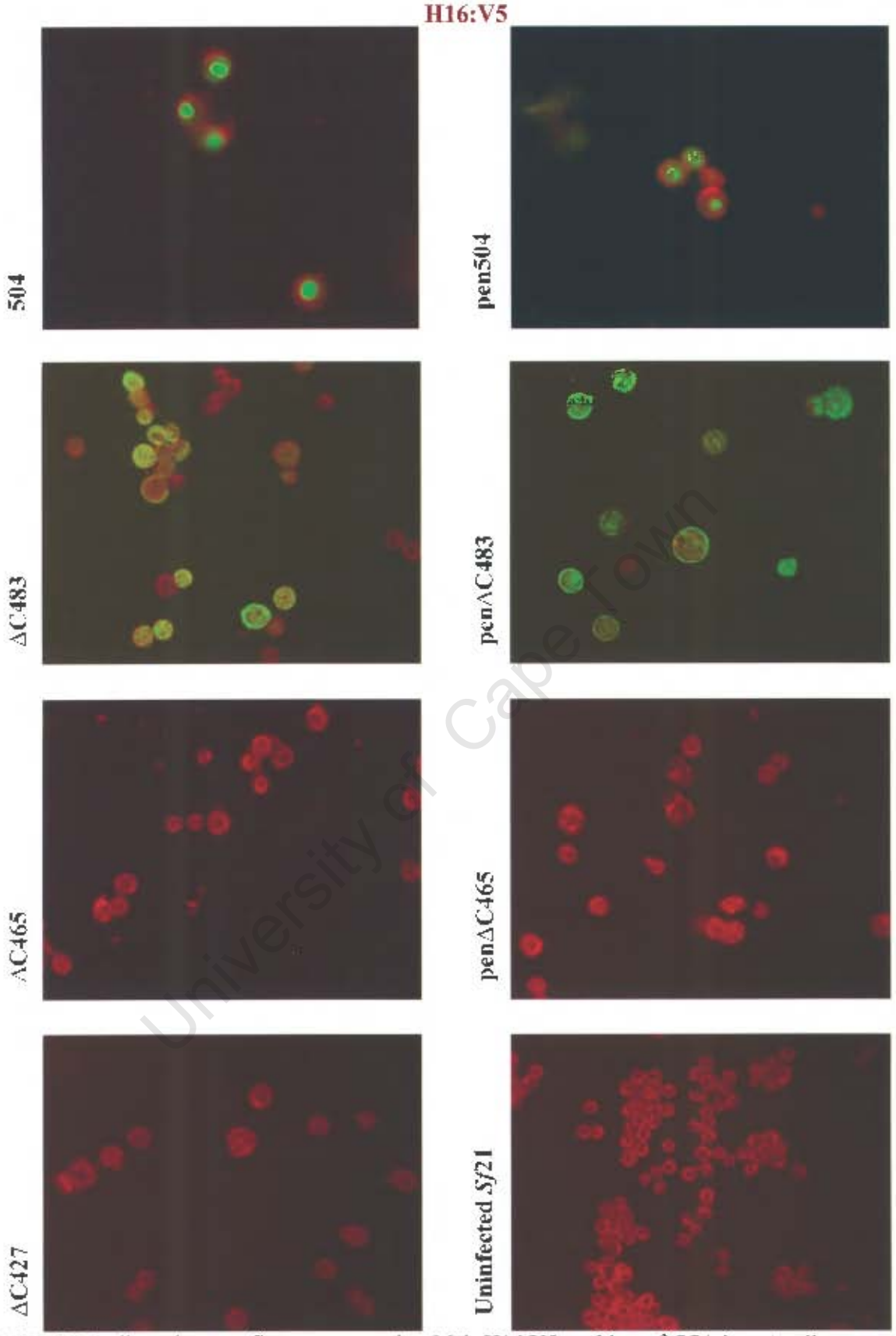


FIGURE 2.5: Indirect immunofluorescence using Mab H16:V5 probing of *Sf*21 insect cells expressing the various deletant and mutant HPV-16 L1 gene products. *Sf*21 cells were counterstained with Evan's blue. The Mab H16:V5 binding to the 504, pen504, ΔC483 and penΔC483 gene products was detected using FITC labelled goat anti-mouse secondary antibody. No binding of H16:V5 was detected for gene products of ΔC465, penΔC465 and ΔC427 in addition to the uninfected the negative control cells. Digital images captured at x20 magnification.

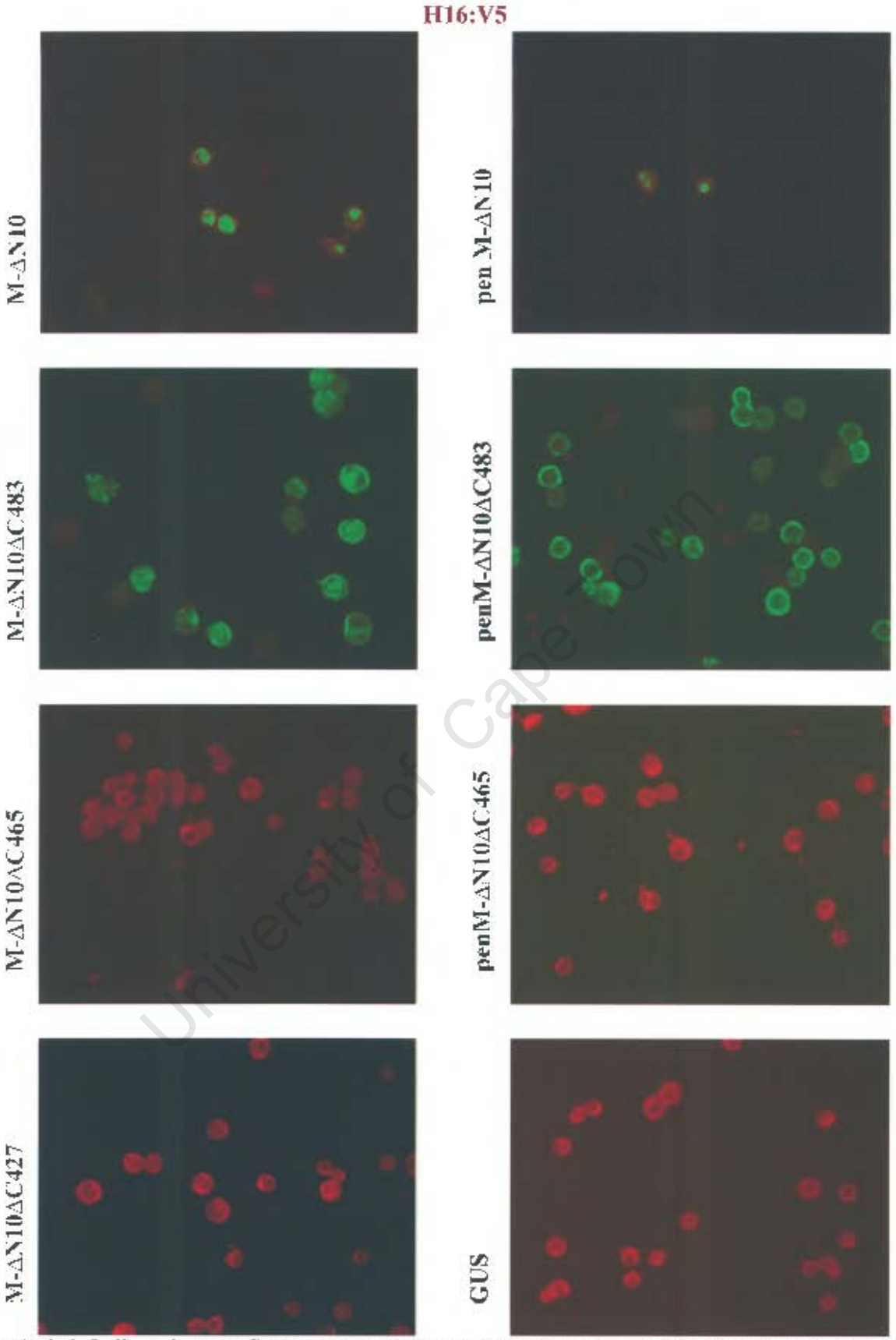


FIGURE 2.6: Indirect immunofluorescence using Mab H16:V5 probing of *Sf*21 insect cells expressing the various deletant and mutant HPV-16 L1 gene products. *Sf*21 cells were counterstained with Evan's blue. The Mab H16:V5 binding to the M-ΔN10, penM-ΔN10, M-ΔN10ΔC483 and pen M-ΔN10ΔC483 gene products was detected using FITC labelled goat anti-mouse secondary antibody. No binding of H16:V5 was detected for gene products of M-ΔN10ΔC465, pen M-ΔN10ΔC465 and M-ΔN10ΔC427. No H16:J4 binding was detected in cells infected with GUS expressing recombinant baculovirus negative control. Digital images captured at x20 magnification.

The indirect immunofluorescence results show that the L1 product in the case of 504, M-ΔN10, pen504 and penM-ΔN10 was localised in the centre of the *Sf*21cells (Figures 2.3-2.5), presumably in the nucleus. For the constructs that did not have the NLS, the L1 product seemed to be diffused throughout the cell. It was interesting that the infected *Sf*21 insect cells were almost twice the size (Figures 2.4 and 2.6) of uninfected cells (Figures 2.3, 2.5) clearly indicating a morphological change due to infection.

2.3.3 Antibody characterisation of the deletant and mutant HPV-16 L1s using a panel of Mabs

The indirect immunofluorescence gave base line data of the antigenicity of the various deletant and mutant HPV-16 L1 gene products as well as of localisation in the *Sf*21 insect cells. It was nonetheless important to determine the full antigenicity profile of some of these deletant and mutatant products (504, pen504, ΔC483, pen ΔC483, M- ΔN10, penM- ΔN10, M- ΔN10 ΔC483, ΔC465, pen ΔC465, M- ΔN10 ΔC465 and ΔC428) The ELISA binding results for these purified deletion and mutant HPV-16 L1 gene products as well for as a CRPV L1 as a control with a panel of Mabs, are summarised in Figure 2.7. Gene products from constructs 504, ΔC483, M-ΔN10, M-ΔN10ΔC483, pen504, penΔC483, penM-ΔN10 and M-ΔN10ΔC483 bound all the Mabs used in this study (H16:V5, H16:E70, H16:U4, H16:9A, H16:D9, H16:J4 and H16:123) whereas ΔC465, penΔC465 and M-ΔN10ΔC465 bound only the Mabs that recognise linear epitopes (H16:D9, H16:J4 and H16:I23). ΔC427 only bound two, H16:J4 and H16:I23, of the three Mabs that recognise a linear epitopes. Furthermore the significant binding of H16:D9 to all the deletion and full length constructs except ΔC427 indicates the presence of unassembled L1 protein in the preparations.

The ΔC427 protein bound Mab H16:D9 to a level that was barely detectable; thus it is possible to speculate that the binding region of Mab H16:D9, which has not been characterised, is between residues 428-465. Since H16:D9 has been reported to only bind denatured HPV-16 L1 protein, western blot analysis of the cell lysate from the expression of 504 (56.2kDa), ΔC483 (53.7kDa), ΔC465 (51.7kDa) and ΔC427 (47.3kDa) genes in *Sf*21 cells using recombinant baculovirus was performed. The western blot results show the loss in binding for H16:D9 ΔC427 protein (Figure 2.8).

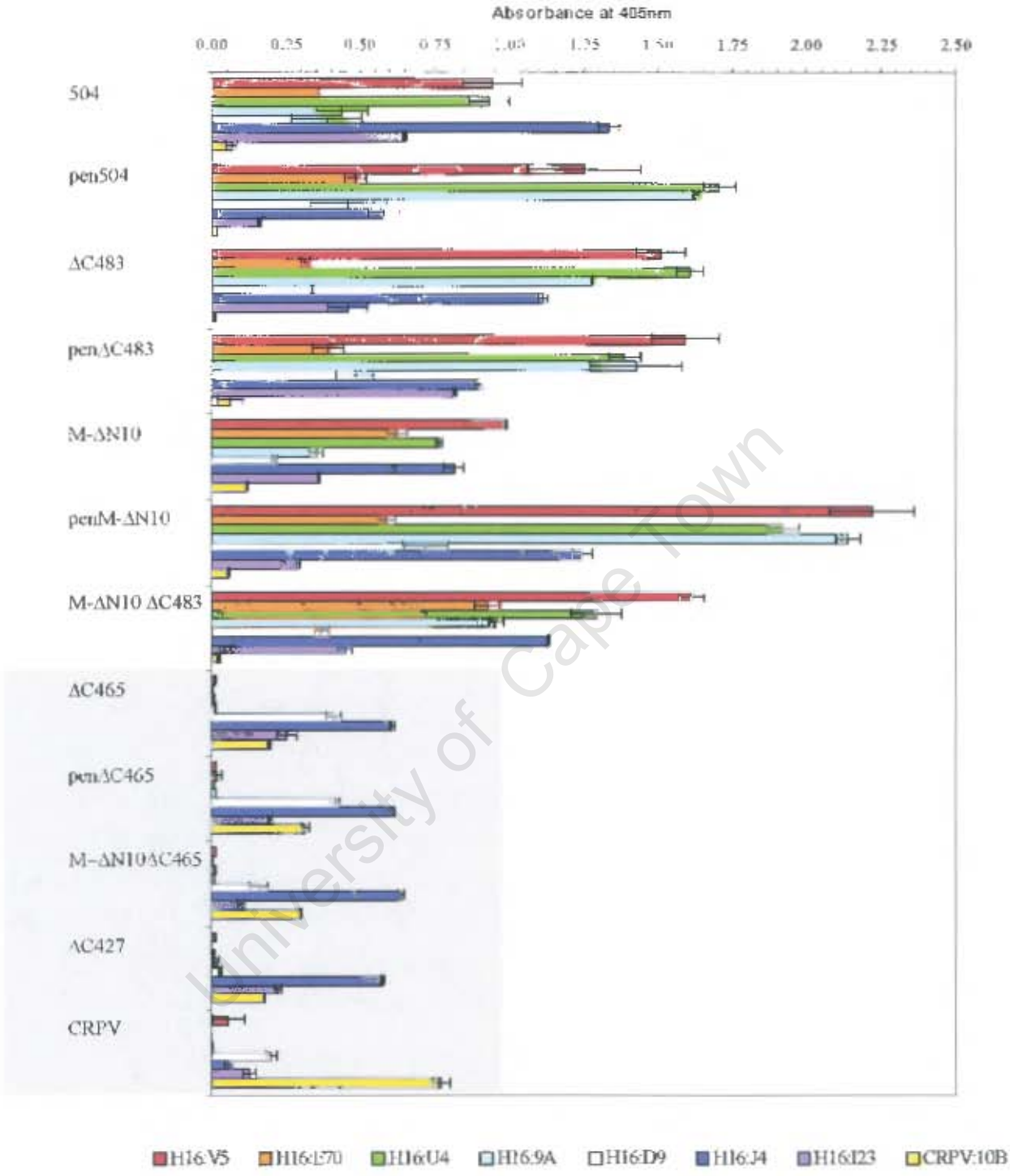


FIGURE 2.7: Antibody-binding characterisation by ELISA of some of the purified deletant and mutant HPV-16 L1 proteins, using a panel of Mabs. CRPV L1 was used as a negative control. ELISA data highlighted by the grey box show the deletant and mutant constructs that did not bind any of the conformational-specific Mabs (H16:V5, H16:E70, H16:U4 & H16:9A)

The Mab CRPV:10B binds to both denatured and intact VLPs since it recognises a surface linear epitope (Christensen *et al.*, 1996a). Some level of cross reactivity was observed by Mabs H16:D9, H16:J4 and H16:I23 between CRPV L1 VLPs and the deletion gene products. This can be attributed to the fairly conserved binding regions (see Figure 1.7, Chapter 1) for Mabs H16:J4 (residues 261-280) and H16:I23 (111-130).

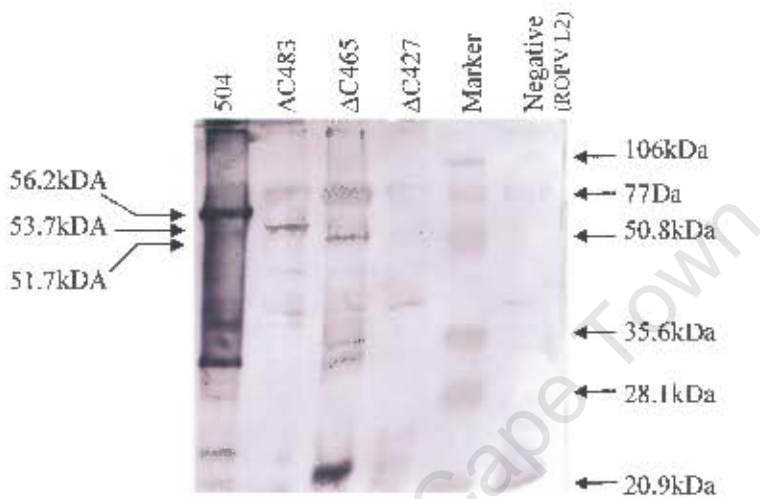
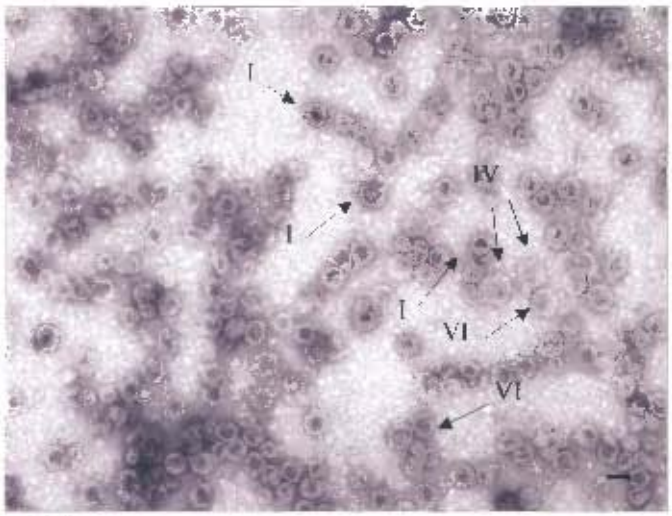


FIGURE 2.8: Western blot analysis probing with Mab H16:D9 of the cell lysates from the expression of the 504 (56.2kDa), ΔC483 (53.7kDa), ΔC465 (51.7kDa) and ΔC427 (47.3kDa) proteins in Sf21 cells using recombinant baculovirus. The Mab H16:D9 does not bind to the ΔC427 gene products.

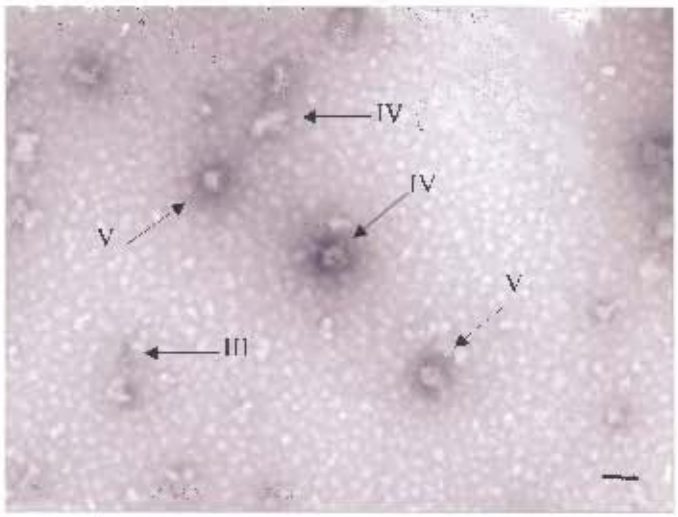
2.3.4 Electron microscopy of the deletant and mutant HPV-16 L1s

The purified deletant and mutatant proteins were viewed under the electron microscope by immunotrapping with H16:V5 and H16:J4 Mab. In general H16:J4 was found to be better for immunotrapping the products.

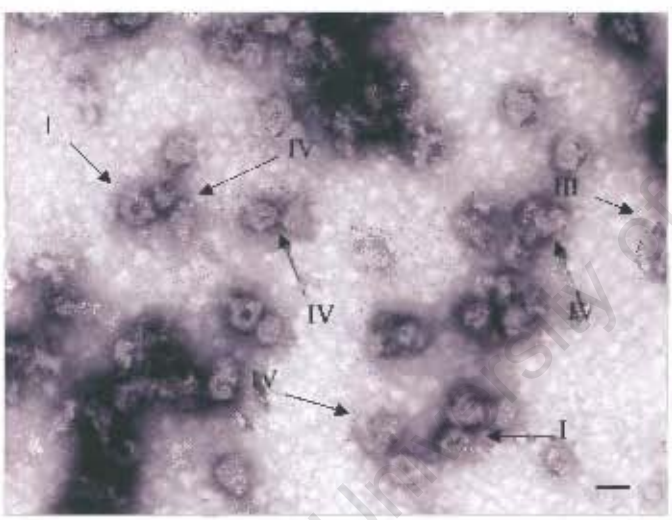
VLPs of 55nm diameter were observed for the L1-derived proteins 504, M-ΔN10, ΔC483 and M-ΔN10ΔC483 (Figures 2.9). In the case of M-ΔN10 and M-ΔN10ΔC483 (Figure 2.10) a mixed population of VLPs comprising of 55nm and 30nm diameter particles were observed. Most of the particles observed for ΔC465 and M-ΔN10ΔC465 (Figure 2.11) were aggregates of pentamers and / or partially disassembled particles. Predominantly aggregates of pentamers were observed for ΔC427 and M-ΔN10ΔC427 (Figure 2.12). The mutation of the cysteine at residue 428 generally favoured the formation of capsomers that were approximately 30nm in diameter for pen504, penM-ΔN10, penΔC483 and penM-ΔN10ΔC483 whereas for pen465 and penM-ΔN10ΔC465 aggregates of pentamers were observed (Figures 2.9-2.11).



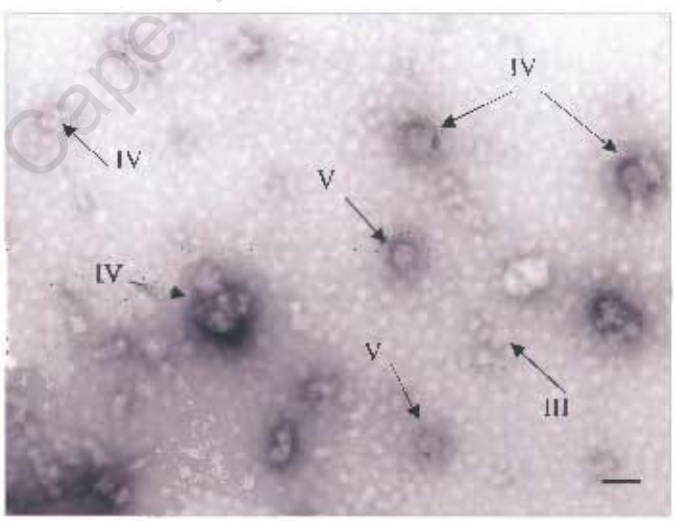
504



pen504

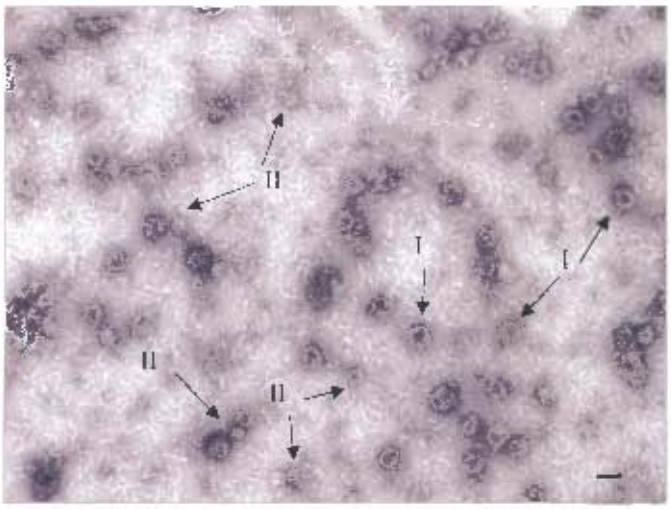


ΔC483

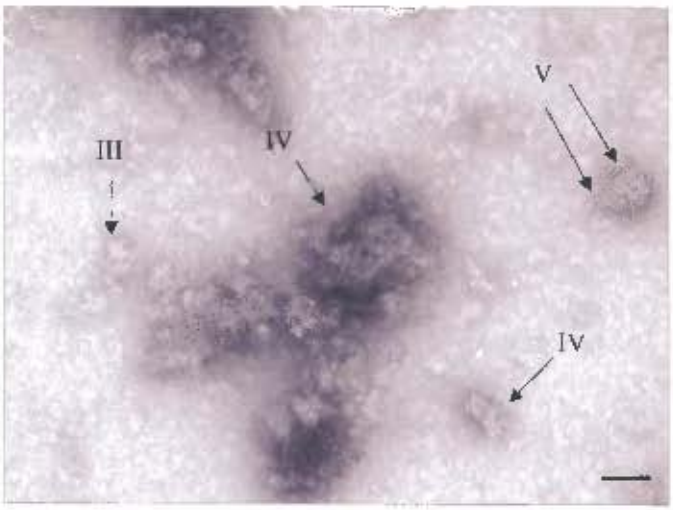


penΔC483

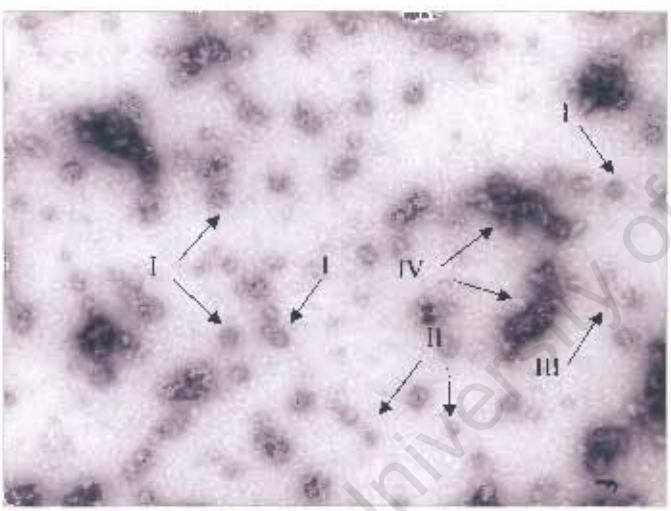
FIGURE 2.9: Electron micrographs of purified proteins of L1s 504, pen504, ΔC483 and penΔC483 immunotrapped with Mab H16:J4.
Bar = 50nm.
I: VLPs (55nm diameter)
II: Small VLPs (30nm diameter) characterised by Chen *et al.* (2000)
III: free pentamers (approximately 10nm diameter); **IV**: pentamer aggregates / disassembled particles
V: capsomers of approximately 30nm diameter
VI: VLPs in a state of disassembly / assembly.



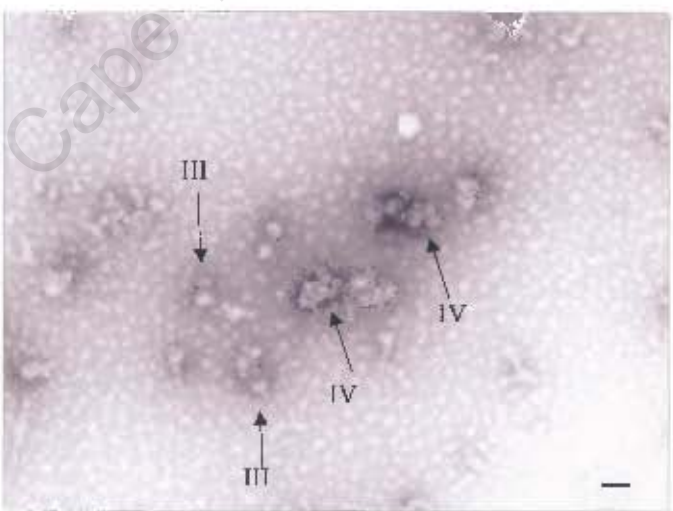
M–ΔN10



penM-ΔN10

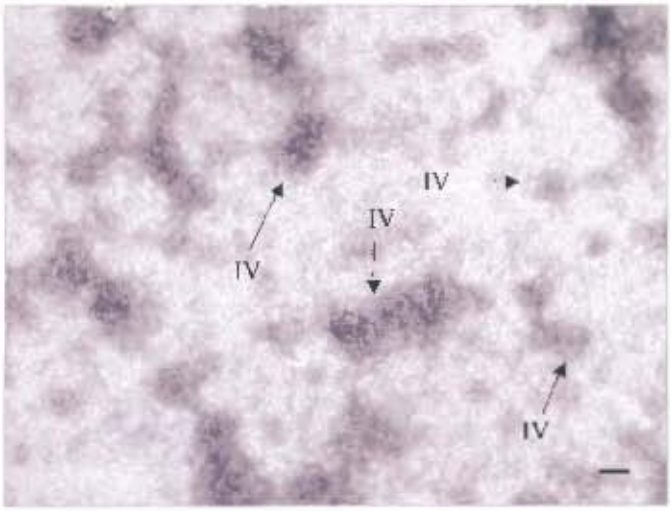


M–ΔN10ΔC483

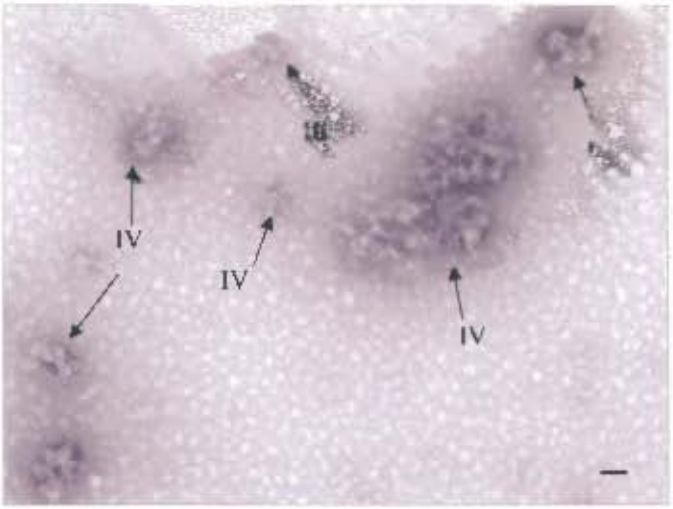


penM-ΔN10ΔC483

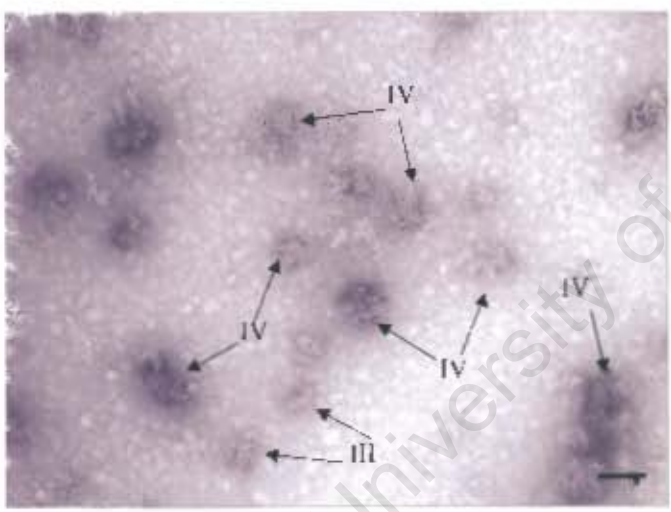
FIGURE 2.10: Electron micrographs of purified proteins of L1s M-ΔN10, penM-ΔN10, M-ΔN10ΔC483 and penM-ΔN10ΔC483 immunotrapped with Mab H16:J4.
Bar = 50nm.
I: VLPs (55nm diameter)
II: Small VLPs (30nm diameter) characterised by Chen *et al.* (2000)
III: free pentamers (approximately 10nm diameter); IV: pentamer aggregates / disassembled particles
V: capsomers of approximately 30nm diameter
VI: VLPs in a state of disassembly / assembly.



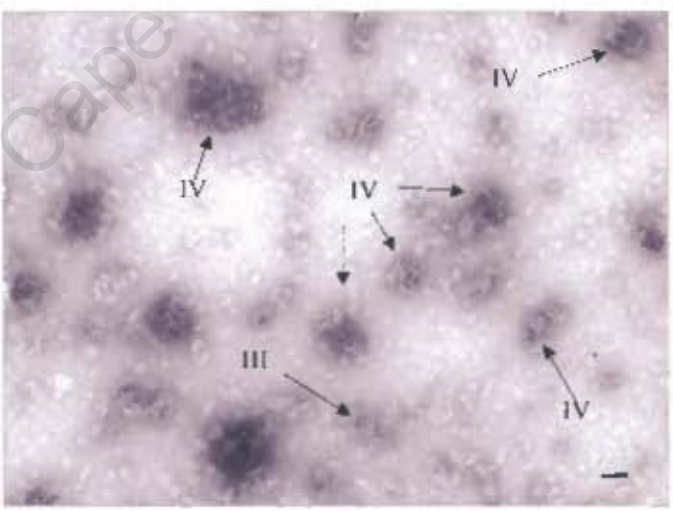
ΔC465



penΔC465

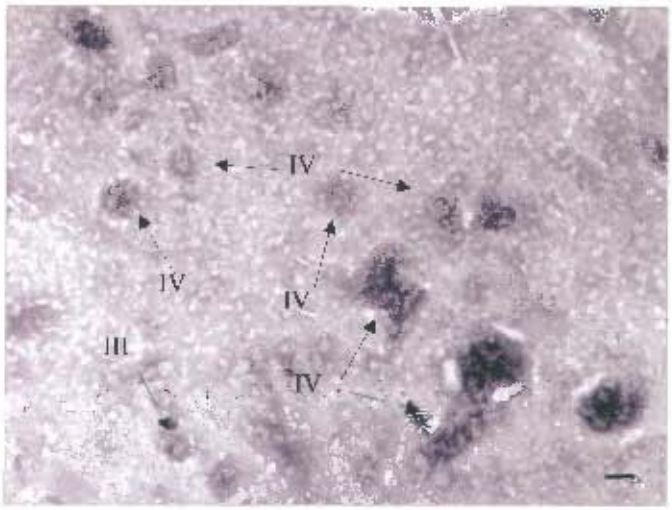


M–ΔN10ΔC465

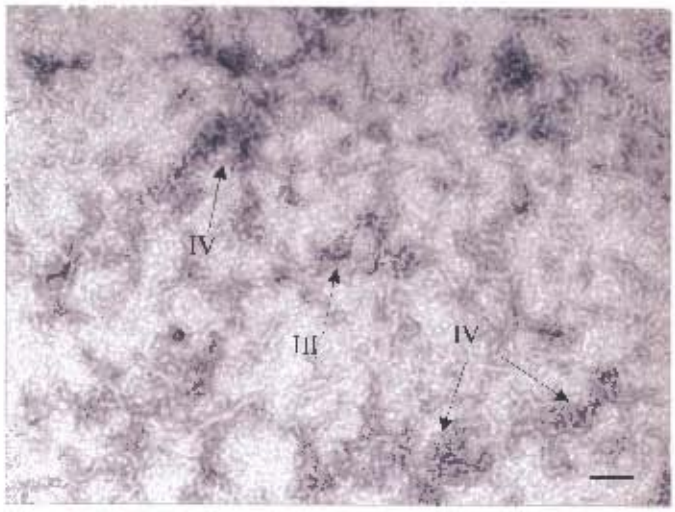


penM-ΔN10ΔC465

FIGURE 2.11: Electron micrographs of purified proteins of L1s ΔC465 and penΔC465, M-ΔN10ΔC465 and penM-ΔN10ΔC465 immunotrapped with Mab H16:J4.
Bar = 50nm.
I: VLPs (55nm diameter)
II: Small VLPs (30nm diameter) characterised by Chen *et al.* (2000)
III: free pentamers (approximately 10nm diameter); **IV**: pentamer aggregates / disassembled particles
V: capsomers of approximately 30nm diameter
VI: VLPs in a state of disassembly / assembly.



ΔC427



M-ΔN10ΔC427

FIGURE 2.12: Electron micrographs of purified proteins of L1s M-ΔC427 and M-ΔN10ΔC427 immunotrapped with Mab H16:J4.
Bar = 50nm.
I: VLPs (55nm diameter)
II: Small VLPs (30nm diameter) characterised by Chen *et al.* (2000)
III: free pentamers (approximately 10nm diameter); IV: pentamer aggregates / disassembled particles
V: capsomers of approximately 30nm diameter
VI: VLPs in a state of disassembly / assembly.

The electron microscopic observations of immunotrapped particles are summarised in Table 2.2.

TABLE 2.2. Summary of the observations made under the electron microscope of the immunotrapped particles resulting from the expression of the various deletion and mutant gene products.

	T=7 55nm	T=1 30nm	Pentamers 10nm	Comments
504		-		Predominantly T=7 particles
pen504	-	-	−	Capsomers and pentamer aggregates
ΔC484		+	+	Predominantly T=7 particles
penΔC484	-	-	+	Capsomers and pentamer aggregates
M–ΔN10	+	+	+	Mixed population of T=7 and T=1 particles
PenM-ΔN10	-	-	+	Capsomers and pentamer aggregates
M ΔN10ΔC484	−	+	+	Mixed population of T=7 and T=1 particles
penM-ΔN10ΔC484	-	-	+	Capsomers and pentamer aggregates
ΔC465	-	-	+	Pentamer aggregates
penΔC465	-	-	+	Pentamer aggregates
M–ΔN10ΔC465	-	-	+	Pentamer aggregates
penM-ΔN10ΔC465	-	-	+	Pentamer aggregates
ΔC428	-	-	+	Pentamer aggregates
M–ΔN10ΔC428	-	-	+	Pentamer aggregates

2.4 DISCUSSION

The primary objective of a prophylactic HPV-16 VLP-based vaccine is to elicit a protective immune response as a result of the induction of virus neutralising antibodies. A variety of neutralising epitopes of the HPV-16 major capsid protein L1 have been characterised. Christensen *et al.* (1996a) generated a whole panel of monoclonal antibodies against HPV-16 to analyse surface and buried capsid epitopes. The epitopes of many of these Mabs were identified using synthetic peptides. Of the various Mabs, H16:V5, H16:E70 and H16:U4 were found to be type-specific and conformation-dependent Mabs and H16:V5 and H16:E70 neutralised pseudotype HPV-16 virions (Roden *et al.*, 1997). Mab H16:V5 was found to completely block more than 75% of the serological reactivity of human sera (HPV-16 infected) to HPV-16 L1/L2 VLPs whereas H16:E70 achieved considerably less blocking of human sera (Wang *et al.*, 1997). This suggested that the binding site of the H16:V5 is similar to the immunodominant site recognised by the human sera following infection. Residues F50, A266 and S282 were found to be critical for the binding of Mabs H16:E70 and H16:V5 (Roden *et al.*, 1997; White *et al.*, 1999).

Recently, Combita *et al.* (2002) demonstrated that linear neutralising epitopes can be found on the HPV-16 L1 capsid protein: these are the epitopes that bind the Mabs H16:J4 (residues 261-280) and H16:I23 (residues 111-130). The sera raised to the two linear epitopes were also found to be cross neutralising: antibodies to 111-130 reacted with HPV-31, 33, 58 and 45 whereas antibodies to 261-280 reacted with HPV-45 and 59. The various epitopes of the neutralising Mabs and other Mabs produced by Christensen *et al.*, 1996a, 2001) are summarised in Table 1.3 (see Chapter 1) and illustrated on the models generated from the crystal structure of mutant HPV-16 ΔN10L1 (Chen *et al.*, 2000b) and the HPV-16 L1 atomic structural model (Modis *et al.*, 2002) in Figure 2.13. The epitopes of Mabs H16:V5, H16:E70, H16:U4, H16:J4 and H16:I23 are all surface exposed on the head and crown of the pentamer.

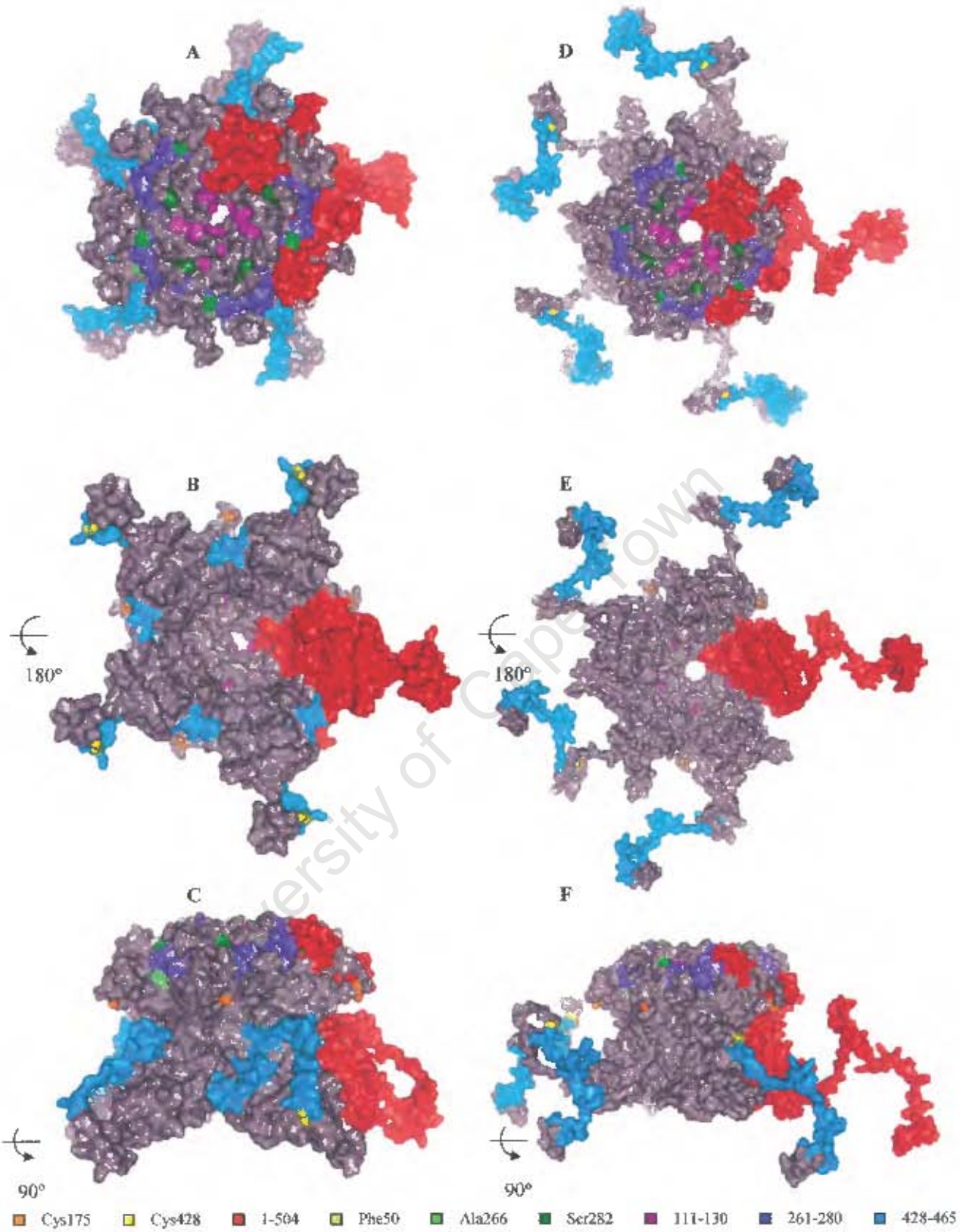


FIGURE 2.13: Structural models of pentamers of HPV-16 ΔN10 L1 (A, B, C) and HPV-16 L1 (D, E, F) highlighting the binding regions of the Mabs H16:V5 and H16:E79 (50, 266, 282), H16:I23 (111-130), H16:J4 (261-280) and H16:D9 (possibly 428-465). The two conserved cysteines (175, 428) are also highlighted. The models are based on the crystal structure of HPV-16 ΔN10 L1 by Chen *et al.* (2000b) and the atomic model of HPV-16 L1 by Modis *et al.* (2002) and drawn using the PyMOL structural software (DeLano Scientific). A and D show the top surface of the pentamer; B and E show the pentamers rotated at 180° along the X axis; C and F show the rotation of 90° along the X axis.

In a magnificent study to determine the yield of VLPs from the expression of six HPV-16 variants by recombinant baculovirus in insect cells, Touze *et al.* (1998b) showed that Phi1 sequence generated the greatest yield of VLPs, 79 times more relative to Fra25 (see Table 1.2, Chapter 1, amino acid sequences of HPV-16 L1 variants). Therefore, this indicates that there are certain elements in the HPV-16 variant gene sequences that play a key role in the assembly of major capsid particles. The HPV-16 L1-SA gene (from a South African isolate; GenBank accession no. AY177679) is identical to 114K in predicted amino acid sequences, and varied from the Phi1 sequence by one residue (A in South African isolate, T in Phi1)at position 266.

Roden *et al.* (1997) demonstrated that a HPV-16 variant (Z-1194) with seven residues differing from AY177679 (Y76, D176, T181, A266, P282, P353 and F475) lost Mab H16:E70 binding; further analysis of various mutants showed that the serine at residue 282 was necessary to maintain the binding of H16:E70. It was therefore thought necessary to analyse the antigenicity of the VLPs resulting from the L1 504 and a mutant 504[ΔA266T] which encodes identical amino acid sequence to Phi1. All the amino acid residue differences of HPV-16 L1 variants described in Table 1.2 (Chapter1, section 1.5.3.1) are illustrated in Figure 2.14. Residue 50 is not fully surface exposed, whereas the other key residues involved in the binding of neutralising Mabs H16:V5 and H16:E70 are surface exposed, and majority of the other amino acid variants are on the surface (Figures 2.13-2.14). This supports the notion proposed by Roden *et al.* (1997) that papillomaviruses may be subject to evolutionary pressure to escape neutralisation. The evolution of greater than 80 HPV genotypes is consistent with this hypothesis.

The comparison of the antigenicity (Figure 2.2) of the two variants 504 and 504[ΔA266T] clearly indicates that alanine at position 266 enhances the binding of H16:E70 by almost twofold compared to threonine at the same position. White *et al.* (1999) carried out a study to map the H16:V5 and H16:E70 epitopes and showed that the variant GU-1 (differs from AY177379 at G41, Y76, N176, T181, P282, P353 and F474) was unable to bind Mab H16:E70, whereas GU-2 (differs from AY1773979 at L50, T266 and N380) was unable to bind both H16:E70 and H16:V5 Mabs. The mutation of L50 to F in GU-2 completely restored Mab H16:V5 and partially restored Mab H16:E70 binding; full binding of the latter was achieved by mutating the T 266 to A (White *et al.*, 1999).

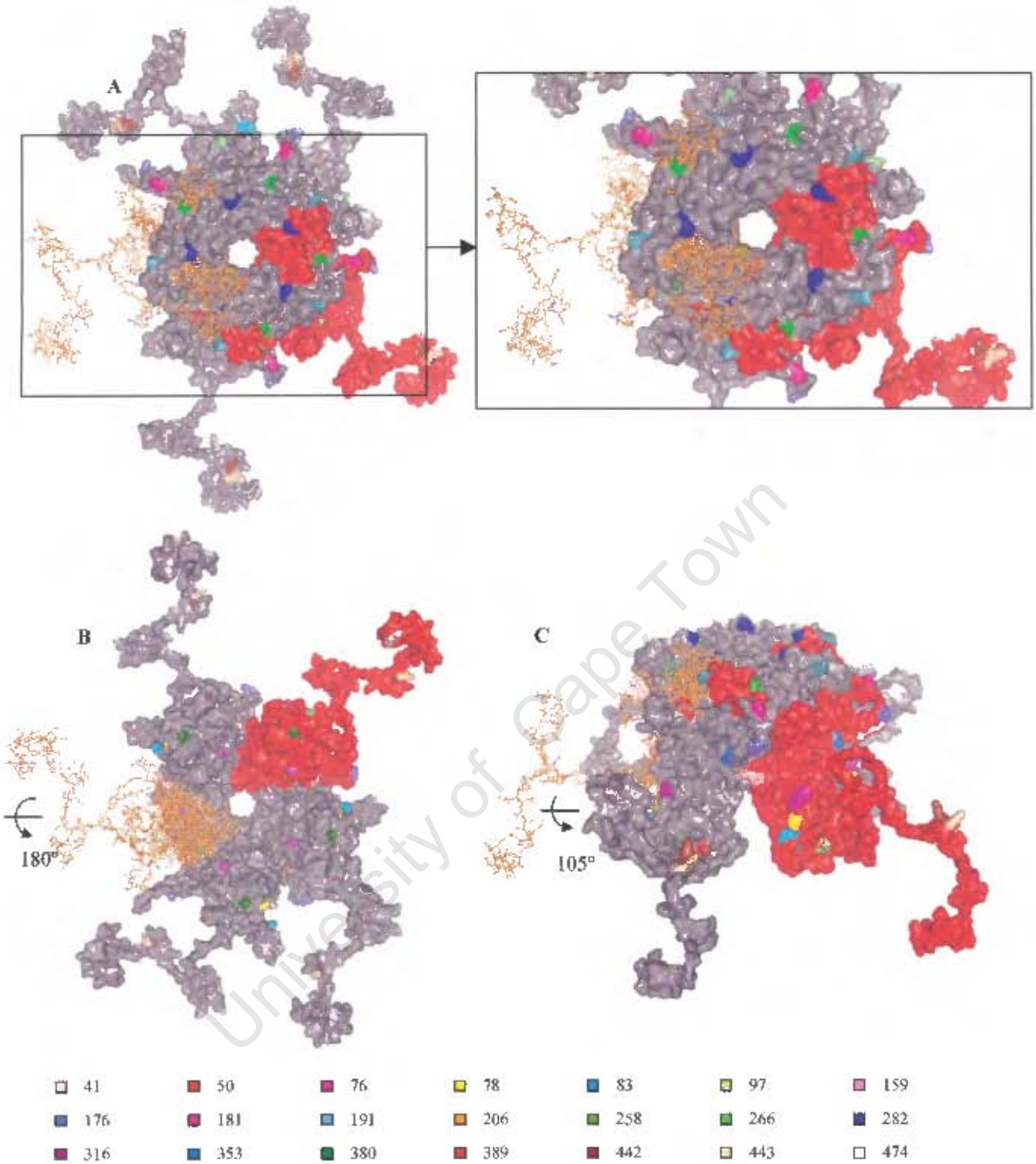


FIGURE 2.14: Structural models of pentamers of HPV-16 L1 highlighting the amino acid residues that differ from AY177679 gene sequence in the various HPV-16 L1 variants (Table 1.2, Chapter 1, section 1.5.1.3). The models are based on the atomic model of HPV-16 L1 by Modis *et al.* (2002) and drawn using the PyMOL structural software (DeLano Scientific). **A** show the top surface of the pentamer; **B** shows the pentamer rotated at 180° along the X axis; **C** shows the rotation of 90° along the X axis.

Based on the antigenicity of the HPV-16 L1 (504), it was therefore decided to use this gene sequence for the deletion study and based on the various features of the HPV-16 L1 in terms of structure, various deletant and mutant gene constructs were prepared and expressed in *Sf*21 insect cells using recombinant baculovirus. A variety of deletions at the N- and C-termini of HPV16 L1 504 were studied to determine their effect on the antigenicity of the particles formed and their structure in a baculovirus expression system. The deletion regions of the 504 gene are shown in Figure 2.1; these are also shown on structural pentameric models of T=1 and T=7 particles in Figure 2.15.

The indirect immunofluorescence data for *Sf*21 cells infected with recombinant baculoviruses provides a good basis in terms of antigenicity of the deletion and mutant gene products. One obvious result of the deletions and mutations L1 genes is that the proteins ΔC465, M-ΔN10ΔC465, ΔC428 and M-ΔN10ΔC428 have disrupted H16:V5 binding sites. Another is that the N-terminal deletion had no affect on the binding of Mab H16:V5, and that the deletion of the NLS clearly prevents the localisation of the protein L1 protein product into the nucleus (Figures 2.3-2.7) confirming work by Zhou *et al.* (1991). Further, mutation of the C 428 to G does not affect the Mab H16:V5 binding, indicating that capsomeric pentamers maintain the same epitope conformation as intact VLPs. It is possible that these protein products could elicit a neutralising immune response similar to that reported for HPV-33 L1 capsomers (Fligge *et al.*, 2001). The indirect immunofluorescence is unique tool for rapid qualitative analysis of gene expression since the gene products are not lost during purification.

The antibody characterisation of the various purified deletants and mutants revealed that the conformation of proteins ΔC465, penΔC465, penM-ΔN10Δ465 and ΔC428 is altered since none of the conformation specific Mabs (H16:V5, H16:E70, H16:U4 and H16:9A) bind significantly to them. These results, in addition to those from the indirect immunofluorescence of the infected *Sf*21 cells, suggests that the region 428-475 is critical for maintaining the correct conformation. In both the T=1 and T=7 models of HPV-16 L1, the residues 445-474 interact with the core of the pentamer, the interaction being intrapentameric for T=1 and interpentameric for T=7, thus providing stability to the pentamers in the former case. The C-terminal deletion ΔC465 results in the partial destruction of the h5 helix that docks into the intersubunit gap, and the loss of these important residues definitely seems to distort the overall stability of the pentamers.

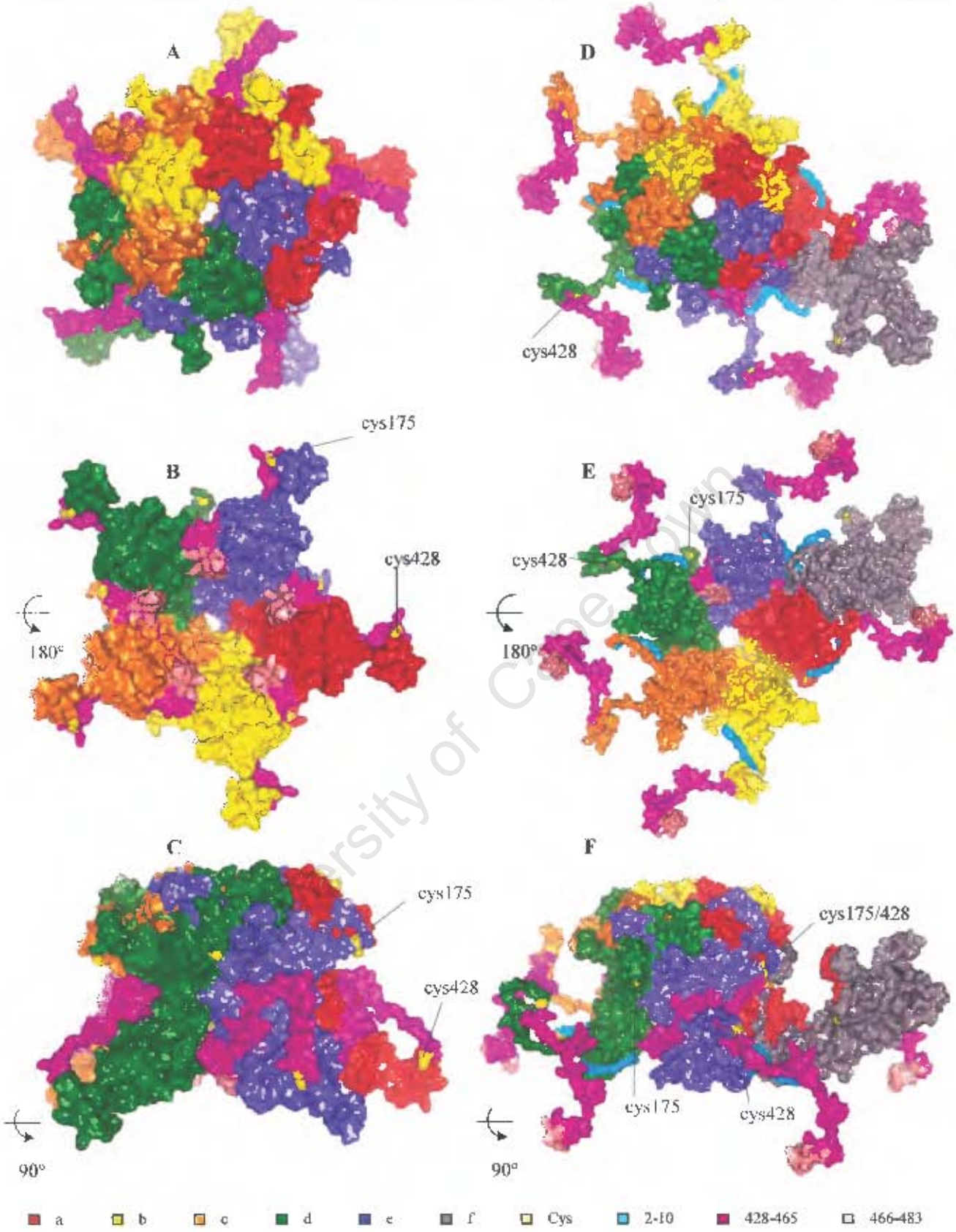


FIGURE 2.15: Structural models of pentamers of HPV-16 ΔN10 L1 (**A, B, C**) and HPV-16 L1 (**D, E, F**) highlighting the arrangement of the subunits / monomers chains (**a,b,c,d,e & f**), the conserved cysteines (175, 428) and residues 1-10, 428-465 and 466-475. The models are based on the crystal structure of HPV-16 ΔN10 L1 by Chen *et al.* (2000) and the atomic model of HPV-16 L1 by Modis *et al.* (2002) and drawn using the PyMOL structural software (DeLano Scientific). **A** and **D** show the top surface of the pentamer; **B** and **E** show the pentamers rotated at 180° along the X axis; **C** and **F** show the rotation of 90° along the X axis.

Further C-terminal deletions up to residue 428 destabilise the pentamers to a greater extent since the disulphide bond between cysteine residues 175 and 428 is destroyed; the invading C-terminal arm in the T=7 particles therefore has minimal contact with the neighbouring pentamer. As a result the pentamers are probably distorted, thus the loss in binding to all the conformation specific Mabs.

A degree of antigenic cross reactivity between the CRPV L1 VLPs, HPV-16 L1 VLPs and the deletion / mutant proteins was observed for the anti-HPV-16 L1 Mabs that bound linear epitopes and the anti-CRPV L1 Mab (Figure 2.7). Multiple sequence alignments of various PV genotypes at the binding regions of Mabs H16:J4 and H16:I23 show conserved regions (Figure 2.15), thus possibly explaining the cross-reactivity. The epitopes for Mabs H16:D9 and CRPV:10B have not been characterised so far but the antibody binding data (Figure 2.7) with support from the western blot analysis (Figure 2.8) suggests that the inability of ΔC427 to bind Mab H16:D9 and indicates that the epitope is located in the region 428-465.

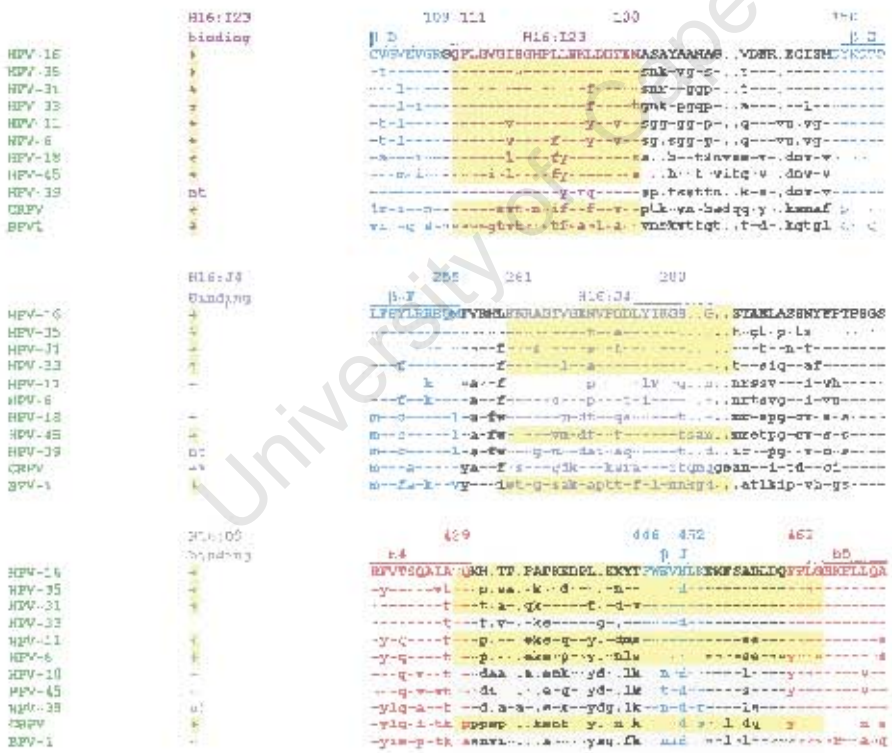


FIGURE 2.16: Multiple sequence alignments of PV genotypes at the Mab H16:I23, H16:J4 and the possibly H16:D9 binding regions. The + / - binding data is from Christensen *et al.* (1996a). * indicates binding observed in this chapter.

It is difficult to suggest a target for Mab H16:D9, based on its binding to CRPV L1, the various constructs in this chapter and to various PV types (HPV-6, 11, 16, 31, 35 and CRPV L1; Christensen *et al.*, 1996a): multiple sequence alignments (Figure 2.16) do not provide as clear an answer within the 428-465 sequences as so other comparisons.

The electron microscopy data clearly indicates that for VLP assembly the C-terminal region 428-465 and the complete h5 helix are required. The majority of ΔC465, penΔC465, ΔC427, M-ΔN10ΔC465, penM-ΔN10ΔC465 and M-ΔN10ΔC427 (Figures 2.9-2.12) form pentamers, and these pentamers, based on the antigenicity data, have an altered conformation. Li *et al.* (1997) showed that HPV-11 L1 particles resulting from the cleavage of the R415 had a pentameric morphology but were unable to assemble further. This cleavage in the case of HPV-16 would be at residue R420 therefore supporting the observation of pentamers for deletants ΔC427 and M-ΔN10ΔC427.

Chen *et al.*, 2000b, 2001) demonstrated that a 10 residue N-terminal deletion in L1 protein expressed in E. coli and assembled at low pH and high ionic strength results in T=1 particles of 30nm diameter, and that the addition of either a glycine or a threonine residues to the NΔ10 results in T=7 particles of 55nm. The particles of M-ΔN10 and M-ΔN10ΔC483 - surprisingly - assemble in two different conformations, T=1 and T=7 (Figure2.10). This observation therefore apparently contradicts what was demonstrated by Chen *et al.*, 2000b; 2001) since the M-ΔN10, based on their data, should assemble into T=1 particles. The main question that arises from this contradictory observation is what is the switch between the T=1 and T=7 particle assembly preferences? Analysis of the atomic structure indicates that the 10 N-terminal residues only interact between pentavalent pentamers (Figure 1.9, Chapter 1, section1.4.1). The first 20 N-terminal residues form a β-hairpin and deletion of 10 N-terminal residues would result in a loss of the β-hairpin's structure. Of the 20 residues in the β-hairpin only residues 10-12 of one subunit interact with 1-4 of the other (Figure 2.16). Therefore, deletion of N-terminal residues 1-4 would prevent the β-hairpin interaction; so, why does deletion of 9 residues still favour T=7 particles and a further deletion of one residue results to T=1 particles (Chen *et al.*, 2000b)? The only other explanation for this observation is the conditions under which the assembly takes place: low pH (4-5.6) and high salt conditions were used to assemble the T=1 particles in the other study (Chen *et al.*, 2000b), and this probably alters the conformation of the N-terminal residues to accommodate for T=1 assembly.

By contrast, in this study assembly will have occurred under essentially physiological conditions. A few smaller size particles were observed for native L1 504 (Figure 2.9) therefore, another question arises: do HPV-16 VLPs expressed *in vivo* have multiple assembly conformations, with T=7 being the predominant one, and if so, why?

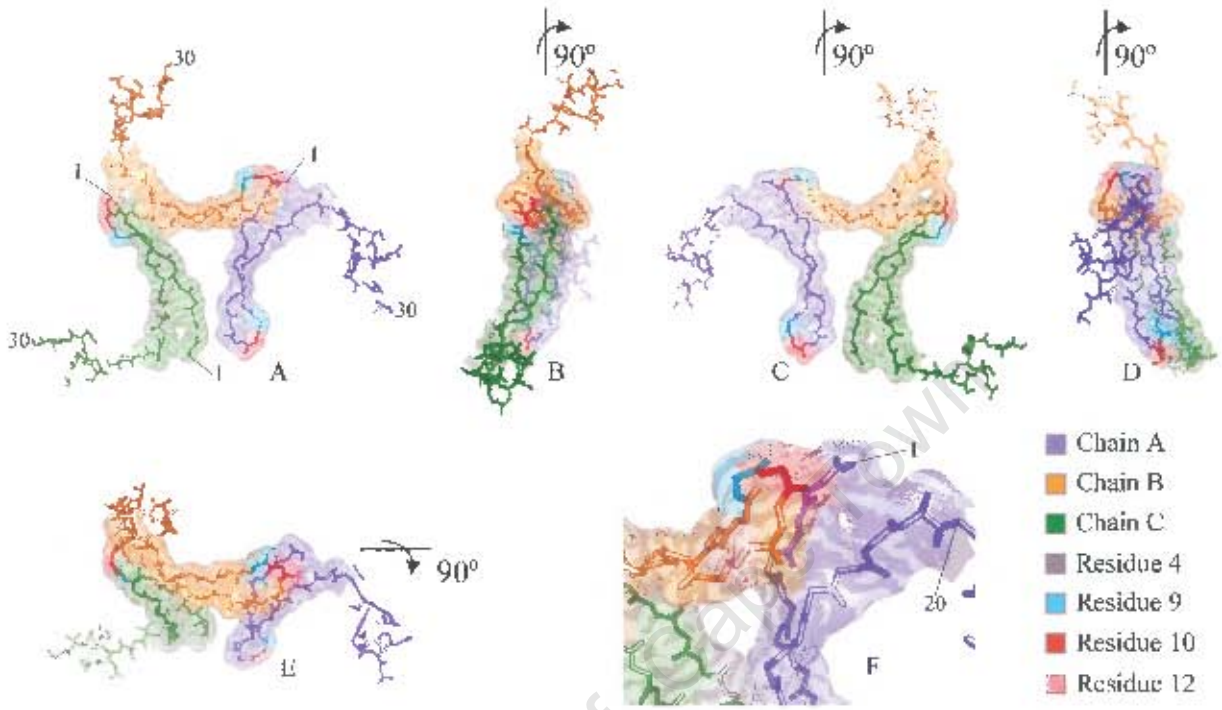


FIGURE 2.17: Structural models showing the interaction of the pentavalent N-terminal regions in the atomic model of HPV-16 L1. Models are drawn using PyMOL structural software (DeLano Scientific). Models represent stick diagrams with the electron density. **B, C** and **D** show the interaction region of model **A** rotated 90° along the y axis in succession whereas **E** is a 90° rotation along the x axis. **F** shows a magnified view of the interaction of residues 1-4 of chain A with 10-12 of chain B.

The mutation of the cysteine to a glycine at residue 428 doesn't affect the antigenicity of the particles, thereby implying that the capsomers and pentamers are potentially appropriately antigenic for elicitation of neutralising antibodies. This mutation probably destabilises the T=7 particles, although doesn't prevent assembly since capsomers of approximately 30nm in diameter were observed (Figures 2.9-2.11). The invading arm hypothesis that was used to generate the atomic model of full length HPV-16 L1 theoretically provides for quite a stable assembly without the disulphide bond at cysteine 428. The model for T=1 particles clearly indicates that the key interpentameric contacts are between the h2, h3 and h4 helices (Figure 1.12, Chapter 1). The capsomers observed as a result of the C428 to G428 mutation are approximately 30 nm in diameter similar to those shown by Li *et al.* (1998). The final question that arises from electron microscopy data of pen504, penCΔ483, penΔN10 and penΔN10ΔC483 gene products, is whether the lack of disulphide bond favours T=1 particle formation? If so, then it is possible that free HPV pentamers, that have a full length L1 gene, are conformationally identical to the pentamers

of T=1 particles (Figures A, B, C of 2.13 and 2.15) and it is only during assembly the C-terminal arms "unlocks" from the pentamer and invades the neighbouring pentamer.

To conclude, the deletion and mutation study provided a very good understanding of the antigenicity of the various gene products, as well as a source of reagents for the rest of the thesis. A trade-off exists between higher yield versus appropriate antigenicity in relation to HPV-16 L1 variants. The 10 residue N-terminal region has no affect on the antigenicity of the particles as assessed by the panel of Mabs, but would result in a mixed population of VLPs. Large scale purification of these mixed population of 55 and 30nm particles could be possibly laborious. However, deletion of the NLS has no impact on the assembly or the antigenicity and hence could potentially be used for further candidate HPV vaccine development.

What is the switch that controls T=1 and T=7 particle assembly? Do HPV-16 VLPs expressed *in vivo* have multiple assembly conformations, with T=7 being the predominant one, and if so, why? Does the destruction of the disulphide bind between cysteine residues 175 and 428 (in HPV-16) favour T=1 particle formation? It is not possible to answer these three questions that arise in this chapter since these are invariably structurally related and potentially constitute a project on their own and are outside the scope of this thesis. Ideally, these questions could be answered by two techniques: firstly by single particle analysis and 3D reconstruction of the purified particles (expressed in *Sf*21 cells using recombinant baculovirus) imaged by electron microscopy (Ruprecht & Nield, 2001), or by 3D reconstruction of the particles from cryo-electron micrographs (Baker *et al.*, 1999).

Chapter 3

Expression of the HPV-16 L1 major capsid protein in transgenic *Nicotiana tabacum* cv. Xanthi

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ABSTRACT

The production of vaccine antigens in plants is a safe and potentially very cost-effective alternative to traditional expression systems. An investigation was undertaken into the possibility of transgenic expression of the HPV-16 L1 major capsid protein, with and without nuclear localisation signal (L1 and L1ΔC483), in *Nicotiana tabacum* cv. Xanthi plants. The genes were stably integrated into the *N. tabacum* cv. Xanthi genome, and both the expressed proteins were capable of assembling into capsomers and virus-like particles. The proteins in concentrated leaf extracts were tested for antigenicity using a panel of characterised monoclonal antibodies (Mabs). Neutralising and conformation-specific Mabs (H16:V5 and H16:E70) were shown to bind to both types of the plant-produced particles. The product yield from plants was estimated to be 2-4μg per kg of fresh leaf material. Rabbits immunised with small doses of plant-produced particles produced a weak anti HPV-16 L1 immune response.

3.1 INTRODUCTION

Traditionally most prophylactic vaccines for human viruses have consisted of live attenuated or inactivated virus. Due to the difficulties and risks involved in generating large quantities of these traditional vaccines there has recently been increasing emphasis on developing viral protein subunit vaccines. With the advent of recombinant technologies, subunit vaccines based on protein expression in bacteria, yeast and insect cells have therefore become popular. However, subunit vaccines produced in this way tend to be expensive and with the recent advances in molecular biology there has been considerable interest in developing new methods for producing cheap subunit vaccines.

Over the last decade plants have become a popular choice for the production of recombinant proteins. Production of foreign proteins in plants has many economic and qualitative benefits (Ganz & *et al*, 1996). The cultivation, harvesting, storage, and processing of transgenic crops could use existing infrastructure and would require relatively little capital investment (Ganz & *et al*, 1996; Pen, 1996; Whitelam, 1995). Kusnandi *et al*. (1997) have estimated that the cost of producing recombinant proteins in plants could be 10 to 50 fold lower than in *E. coli*. Two main strategies have been employed in achieving recombinant protein expression in plants: these are

transient expression using plant viruses, and transgenic expression (Table1.5). SpaA protein from *Streptococcus mutans*, one of the main bacterial agents causing tooth decay, was first expressed in transgenic plants for the purpose of vaccine production in 1990 with a low yield of 0.02% of total soluble protein (Curtiss & Cardineau, 1990). Subsequently, there have been several reports demonstrating that antigens derived from various pathogens can be synthesised in their authentic forms in plants (Arakawa *et al.*, 1997; Gomez *et al.*, 1998; Mason *et al.*, 1992, 1996; Richter *et al.*, 2000). When administered orally, by feeding, antigens such as hepatitis B surface antigen (HBsAg), *Norwalk virus* capsid protein and *E. coli* heat-labile enterotoxin (LT-B) were able to induce an immune response (Haq *et al.*, 1995; Kong *et al.*, 2001; Mason *et al.*, 1996), and in some cases, confer protection against subsequent challenge by LT-B and cholera toxin B (Arakawa *et al.*, 1998; Mason *et al.*, 1998). As a result, certain antigens such as LT-B and *Norwalk virus* capsid protein expressed in transgenic plants have been tested in clinical trials (Tacket *et al.*, 1998, 2000).

The ability to make stable transgenic plants by integration of DNA into the chromosome is the result of the discovery that *Agrobacterium tumefaciens* can insert foreign genetic material into the plant genome and the resulting transformed cells can be regenerated into whole plants (Awram *et al.*, 2002). Although foreign DNA can be introduced into plants by biolistic bombardment, *Agrobacterium* remains the preferred choice for its consistent integration of foreign DNA (Awram *et al.*, 2002). *Agrobacterium* is a plant pathogen that can transform a large range of hosts including plants, fungi and animal cells. Tobacco and *Arabadopsis* have proven to be easy to work with in the laboratory, whereas others, such as rice and maize, are not nearly as amenable to genetic manipulation although they would be better suited for vaccine production. *Agrobacterium*-mediated transformation allows the integration of a gene cassette (T-DNA) that usually has been engineered to contain a strong promoter, and a terminator in addition to selection markers, into the plant genome (Awram *et al.*, 2002).

In this chapter, the transgenic expression of HPV-16 L1 (full length) and L1ΔC483 (without C-terminal nuclear localisation signal) genes in *Agrobacterium*-transformed *N. tabacum* cv. Xanthi plants was investigated. These two genes were chosen for this study since their gene products maintain the conformational epitopes that bind the neutralising Mabs H16:V5 and H16:E70 (Chapter 2). The data from this study indicates that a transgenic plant-based expression system for the production of the HPV-16 L1 VLPs has potential for low-cost vaccine production.

3.2 MATERIALS AND METHODS

3.2.1 Cloning of HPV-16 L1 and L1ΔC483 genes into the binary vector pART27 and *Agrobacterium* transformation

The PCR amplification and cloning of the L1 (504) and L1ΔC483 genes into pSK (pSK-L1-SA) and pGEM®-T Easy (pGEM- L1ΔC483) vectors are described in Chapter 2 and the primers are listed in Table 2.1 (Chapter 2).

The L1 504 and L1ΔC483 genes were directionally subcloned into the multiple cloning site of the plasmid pART7 (Gleave, 1992), *Cla*I / *Xba*I and *Eco*RI restriction sites respectively (Figure3.1; see Appendix C for map of pART7 vector). This placed the L1 genes downstream of the CaMV 35S promoter and upstream of the octopine synthase gene terminator (*ocs* 3'). The CaMV 35S promoter, HPV-16 L1 504 / L1ΔC483 ORF and *ocs*3' cassette was excised and cloned into the binary vector pART27 (*Not*I). The binary vector pART27 (Gleave, 1992) carries the neomycin phosphotranferase (npt) gene for kanamycin resistance on the T-DNA as a selection marker in both *E. coli* and *Agrobacterium*, and a lacZ' region immediately 3' of the right T-DNA border and 'overdrive' enhancer element. The pART27 (see Appendix C for map of pART27 vector) backbone has the ColE1 origin of replication for high copy maintenance in *E. coli*, the Tn7 spectinomycin/streptomycin resistance gene for bacterial selection, the RK2 minimal replicon for replication and maintenance in *E. coli*, and the *Agrobacterium* and RK2 derived origin of transfer (*ori*T) for conjugal transfer.

Agrobacterium tumefaciens C58C1 competent cells were transformed with L1 504- and ΔC483-pART27 plasmid DNA using the freeze-thaw method of (Hooykaas, 1988). The transformed cells were selected on Luria agar plates containing kanamycin (40μg/ml) and rifampicin (100μg/ml) at 30°C and screened by PCR.

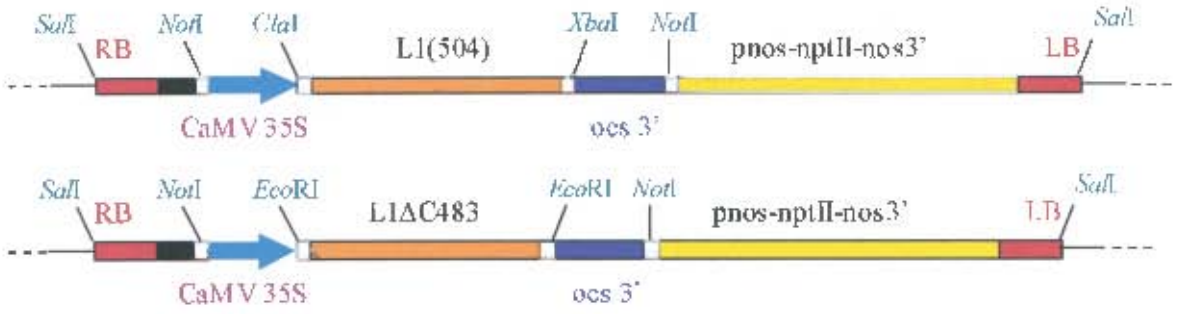

FIGURE 3.1: T-DNA cassettes containing the expression cartridge for the HPV-16 L1/ L1ΔC483 genes under the CaMV 35S promoter, the nptII selection gene under the nopaline octapine synthase promoter (pnos) and the left and right borders.

3.2.2 Leaf disk transformations of *N. tabacum* cv. Xanthi and selection on tissue culture media

N. tabacum cv. Xanthi leaf disks were transformed with *Agrobacterium* constructs as described by Horsch *et al.* (1985). In brief, *N. tabacum* cv. Xanthi leaves were sterilised (using 10% bleach) and washed, cut into small disks (approximately 5-10mm^2) and immersed, lower epidermis down, for a few minutes in an overnight culture of *Agrobacterium tumefaciens* constructs (HPV-16 L1 / L1ΔC483). The immersed leaf disks were transferred to co-cultivation plant tissue culture media with no selection, and kept in the dark in a plant tissue culture room for 3 days at 25°C. The leaf disks were the transferred to regeneration media for callus formation under kanamycin (for *N. tabacum* transformants) and cefotaxim selection (inhibiting *Agrobacterium* growth). The kanamycin resistant calli were allowed to differentiate into shoots and then roots on shooting and rooting plant tissue culture media. The plant tissue culture room was maintained at 22°C with 16 hours of light per day. Following root induction, the plantlets were transferred to soil and grown to maturity in plant growth rooms maintained at 25°C, at 80% relative humidity, with 16 hours of light per day. Flowers of the R_0 generation were self-pollinated by placing a small paper bag on the flower and seeds were screened on plant tissue culture containing 300µg/ml kanamycin. The resistant germinated seeds were transplanted to soil, once the 3rd leaves had grown and grown to maturity.

3.2.3 Screening of transgenic plants for HPV-16 L1 and L1ΔC483 genes

Plant genomic DNA was extracted from the *N. tabacum* leaves using the method outlined by Dellaporta *et al.* (1983).

The transformed *N. tabacum* (cv. Xanthi) plants were screened by PCR for the HPV-16 L1 and L1ΔC483 genes using the following primer pairs:
forward 5'-ATGTCTCTTTGCTGCCTAGTGAG-3'
reverse 5'-TTACAGCTTACGTTTTTTGCGTTTAGCAGTTG-3' (for L1)
reverse 5'-TTATCCTAATGTAAATTTTGGTTTGGCCTTCAATC-3' (for L1ΔC483).

3.2.4 Analysis of total mRNA extracted from transgenic plants

Total RNA was extracted from fresh or stored (-70°C) leaves from L1 and L1ΔC483 transgenic *N. tabacum* plants and non-transgenic *N. tabacum* plants using the TRIzol™ reagent (Life Technologies). The L1 and L1ΔC483 mRNA was detected by RT-PCR amplification using the Access RT-PCR system (Promega) using primers that amplified an internal L1 fragment of 500 bp (forward 5'-GGTCCATTAGGTGTGGG-3', reverse 5'-AGCTGTCGCCATATGGTTCTG-3').

3.2.5 Concentration of transgenic plant material

Transgenic *N. tabacum* plants were homogenised in 1:2 (w:v) cold high salt (0.5M NaCl) phosphate buffered saline (PBS). The homogenate was strained through cheesecloth and centrifuged at low speed (~4 300 x g; 10 minutes). The supernatant was ultracentrifuged at ~77 000 x g for 6 hrs. The resulting pellet was resuspend in 1/5 initial volume of PBS and the suspension was clarified by centrifuging at ~27 000 x g for 20 minutes. The supernatant was subjected to a second round of ultracentrifugation at ~178 000 x g for 3 hours. The pellet was resuspended in PBS and for the antibody characterisation of the L1 and L1ΔC483 capsomers / VLPs, the protein was further concentrated using the Centricon® YM-30 (30 000 kDa molecular weight cut off) centrifugal filters (Amicon Separations). A non-transgenic negative control was prepared using the method outlined above.

3.2.6 Antibody characterisation of the transgenic plant-produced HPV-16 $L1_{Tr}$ and $L1\Delta C483_{Tr}$ protein

Transgenic plant-produced HPV-16 L1 ($L1_{Tr}$) and L1ΔC483 ($L1\Delta C483_{Tr}$) protein and non-transgenic negative control concentrate were tested with the panel of monoclonal antibodies (Mabs) H16:V5, H16:E70, H16:U4, H16:9A, H16:D9, H16:I23 and H16:J4 (see Table 1.3, Chapter 1) by capture ELISA. Mabs H16:V5 (binds aa 50, 266 and 282), H16:E70 (binds aa 50, 266 and 282), H16:U4 (epitope unknown), H16:9A (unknown but in region aa 1-172), all recognise conformational epitopes, while H16:D9 (binds aa 428-465), H16:I23 (binds aa 111-130), H16:J4 (binds aa 261-280), recognise linear epitopes (Christensen *et al.*, 1996a, 2001). Guinea pig anti-HPV-16 serum was diluted 1:500 and coated overnight onto ELISA plates. The plates were blocked with 1% non-fat milk in PBS. The extract was diluted 1:10 and allowed to bind for 2 hrs. The captured antigen was probed with the Mabs (1:200) for 2 hrs. Anti-mouse-alkaline phosphatase conjugated secondary antibody (1:2000) was allowed to bind to the Mab for 1 hour at 37°C. The secondary antibody was detected using p-nitrophenyl phosphate and the absorbance was measured using a Titrex ELISA plate reader at 405nm.

3.2.7 Electron microscopy of transgenic plant extracts

The plant extract was viewed by immunotrapping the particles with either of Mabs H16:J4 and H16:V5 diluted 1:50 (PBS, 0.1%BSA) on carbon coated copper grids. The Mab was incubated with the carbon coated copper grids for 15 minutes. Grids were washed and then incubated with the extract for 30 minutes. The grids were then washed with distilled water and stained with 2% uranyl acetate.

For immunogold labelling, the coated grids were incubated with guinea pig anti-HPV-16 L1 serum raised against HPV-16 L1 VLPs produced in insect cells by recombinant baculovirus, diluted 1:100 in PBS, 0.1% BSA for 15 minutes. The transgenic and non-transgenic control plant extracts were immunotrapped onto grids by incubation (room temperature) for 60 minutes, washed thoroughly (4 X 1 minute) with PBS and then blocked with 1% BSA in PBS for 30 minutes. The grids were then washed thoroughly (4 X 1 minute) with PBS, probed with Mab H16:V5 diluted 1:50 dilution in PBS with 1% BSA for 60 minutes and thoroughly washed before incubating with secondary gold-labelled (10nm gold particles) anti-mouse polyclonal antibodies

diluted 1:100 in PBS for 60 minutes. The grids were thoroughly washed (5 X 2 minutes) with distilled water and then stained with 2% uranyl acetate.

All copper grids were viewed using a JEOL 200CX transmission electron microscope at 120kV.

3.2.8 Immunisation of rabbits with transgenic HPV-16 $L1_{Tr}$ plant extracts and serum analysis

Concentrated (65 fold) transgenic HPV-16 $L1_{Tr}$ *N. tabacum* sap extract was injected into three white New Zealand rabbits. For each rabbit the plant extract (with approximately 0.2 µg of L1 antigen) was diluted to 500µl in PBS and injected 1:1 with Freund's incomplete adjuvant. The HPV-16 $L1_{Tr}$ antigen was administered to two subcutaneous sites and an intramuscular site at days 1, 21, 96 and 118. The rabbit serum at a dilution of 1:50 was analysed for VLP specific antibodies by ELISA against baculovirus-produced HPV-16 $L1_{Bac}$ VLPs (100µl per well coated at 1µg/ml concentration, method identical to Chapter 2) and by western blotting.

The HPV-16 $L1_{Bac}$ protein was denatured for 10 minutes at 100°C in SDS loading dye (without reducing agents). The denatured $L1_{Bac}$ protein was resolved on a 10% SDS PAGE gel. The resolved gel was transferred onto nitrocellulose membrane by semi-dry electrophoresis (BioRad) for 25mins at 25V. The membrane was blocked using 1% non-fat milk for 2 hours and incubated with rabbit serum at a dilution of 1:50 overnight at 4°C. The membrane was washed with PBS/0.05% Tween-20 and probed with alkaline phosphatase-labelled secondary goat anti-rabbit antibody diluted 1:2000, for 1 hour at room temperature. Reaction was detected colorimetrically using 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) and 4-nitro blue tetrazolium chloride (NBT) substrates.

3.3 RESULTS

3.3.1 Production and genetic analysis of transformed transgenic plants

Of all the various transformants selected on kanamycin tissue culture media and subsequently grown on soil, twenty plant lines (ten per construct) resulting from the transformation of leaf discs with *A. tumefaciens* containing HPV-16 L1 and L1ΔC483 genes were screened by PCR for the HPV-16 L1 and ΔC483 genes. The highly specific PCR primers used for the screening amplify the entire HPV-16 $L1_{Tr}$ and $L1\Delta C483_{Tr}$ genes, therefore the amplification products are 1.5kb and 1.45kb respectively. Eight plants were found to be positive for each of the HPV-16 L1 and L1ΔC483 genes (Figure 3.2).

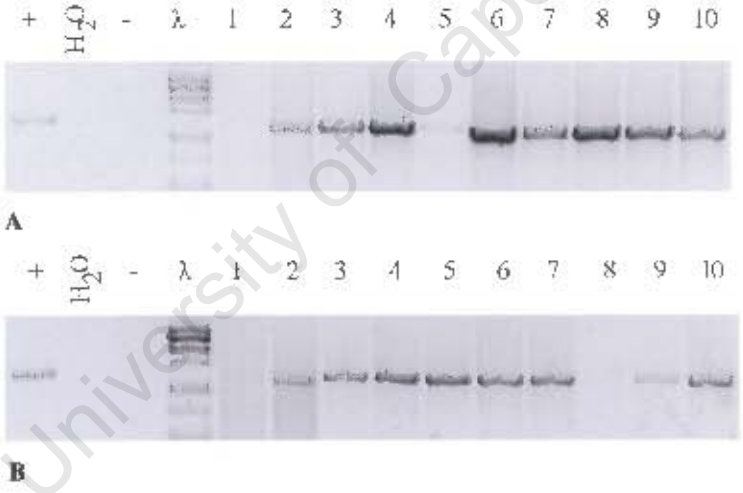

FIGURE 3.2: DNA analysis of the transgenic *N. tabacum* cv. Xanthi (R_0) by PCR for HPV-16 L1 **(A)** and L1ΔC483 **(B)** genes. Lanes 1-10 represents the different lines of transgenic plants. Transgenic lines 2-4 & 6-10 are positive for HPV-16 L1 and 2-7 & 9-10 are positive for L1ΔC483 as shown by the 1.5kb and 1.45kb amplification product respectively.

The total RNA extracts from PCR positive transgenic plants were analysed by RT-PCR for expression of the integrated HPV-16 L1 and L1ΔC483 genes. The RNA from the PCR positive transgenic plants was found to contain the L1 and L1ΔC483 mRNA as indicated by the 500bp DNA fragment observed as a result of the RT-PCR amplification (Figure 3.3), suggesting transcription of the integrated L1 genes.

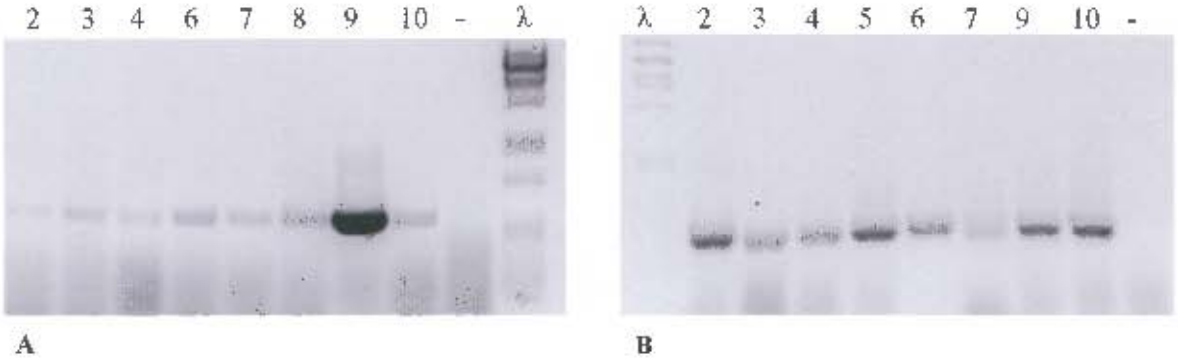

FIGURE 3.3: RNA analysis of the transgenic *N. tabacum* cv. Xanthi. **A**: RT-PCR analysis of the total mRNA extracted from the PCR-positive transgenic plants for HPV-16 L1 and L1ΔC483 **(B)**. All respective the PCR-positive transgenic plants expressed the L1 and L1ΔC483 genes as shown by the 500bp amplification product.

Once the stable integration and expression of the HPV-16L1 and L1ΔC483 foreign genes in transgenic *N. tabacum* had been established it was important to assess the stability of these genes in the subsequent self-pollinated generations. Four of the R_0 (parent) transgenic plants (lines 2, 4, 6 and 10 for L1 and lines 3, 4, 6 and 10 for the L1ΔC483) were self-pollinated. The seeds generated were collected and screened on kanamycin tissue culture media and transplanted to soil. Random PCR screening of up to four T_1 (first generation) plants per line of the four the L1 and L1ΔC483 *N. tabacum* cv. Xanthi transgenic revealed that all these plants were positive, suggesting stable integration into the *N. tabacum* cv. Xanthi genome and transfer of the transgene to the next generation. Since the T_1 seedlings were initially selected in a kanamycin tissue culture media, the ideal 3:1 Mendelian inheritance of the transgene was not observed in the plants screened by PCR.

3.3.2 Analysis of the antigenicity of HPV-16 L1 protein produced in transgenic plants

The HPV-16 $L1_{Tr}$ and $L1\Delta C483_{Tr}$ protein products could not be detected in the initial analysis of the crude leaf sap of the transgenic plants (R_0) by western blot or ELISA. The expression of the genes had been confirmed by mRNA analysis (Figure 3.3) in the PCR-positive transgenic plants; therefore, it was assumed that the expression and accumulation of both the $L1_{Tr}$ and $L1\Delta C483_{Tr}$ proteins was below detectable limits. In a bid to detect the $L1_{Tr}$ and $L1\Delta C483_{Tr}$ proteins, and possibly also the correctly folded capsomers or particles, approximately 500 g of fresh transgenic *N. tabacum* leaf material from the T_1 transgenic plants (all lines pooled together for each

construct) was homogenised in PBS / 0.5M NaCl and concentrated 100 fold by a combination of ultra centrifugation and the use of a YM-30 Centricon filter devices that retain >30kDa protein. The $L1_{Tr}$ and $L1\Delta C483_{Tr}$ protein production in the transgenic plants was confirmed by ELISA and the antigenicity of the protein product was tested with a panel of characterised monoclonal antibodies (see Table 1.3, Chapter 1). The binding of the conformation-specific antibodies H16:V5, H16:U4 and H16:E70 in the assays indicated that $L1_{Tr}$ and $L1\Delta C483_{Tr}$ apparently fold correctly since the four conformation-specific Mabs (H16:V5, H16:E70, H16:U4 and H16:9A) bind the antigens (Figure 3.4).

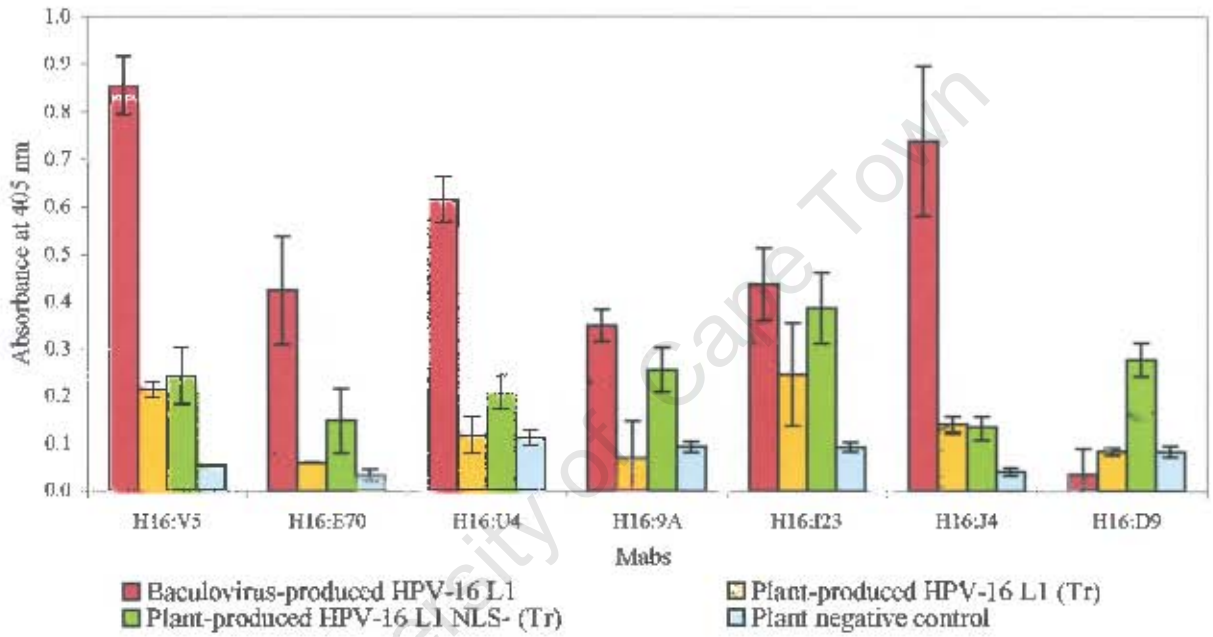

FIGURE 3.4: Monoclonal antibody characterisation by capture ELISA of the HPV-16 $L1_{Tr}$ and $L1\Delta C483_{Tr}$ expressed in *N. tabacum* and HPV-16 $L1_{Bac}$ expressed in insect cells by recombinant baculovirus.

The binding of neutralising Mabs H16:V5 and H16:E70 further suggests the potential of these antigens to elicit a neutralising immune response. Despite similar amounts of capsomers / VLPs of $L1_{Tr}$ and $L1\Delta C483_{Tr}$, based on H16:V5 and H16:J4 binding, there is a difference in the relative binding of the Mabs H16:E70, H16:U4, H16:9A, H16:I23 and H16:D9 to the two plant-produced antigens coupled to baculovirus-produced VLPs. The greater binding of H16:D9 to $L1\Delta C483_{Tr}$ and the minimal binding to $L1_{Bac}$ (despite the high binding of $L1_{Bac}$ to Mab H16:V5), suggest that there is a large population of unassembled / denatured protein since this Mab has been shown to only bind denatured VLPs *in vitro* (Christensen *et al.*, 1996a).

3.3.3 Estimation of the HPV-16 $L1_{Tr}$ and $L1\Delta C483_{Tr}$ protein yield

The baculovirus-produced VLPs ($L1_{Bac}$) at a concentration of 0.63μg/μl were diluted to 0.13 ng/μl for characterisation of the antigenicity of HPV-16 $L1_{Tr}$ and $L1\Delta C483_{Tr}$. Therefore, based on the absorbance and dilution of the $L1_{Tr}$ and $L1\Delta C483_{Tr}$, an approximate concentration of the capsomers could be worked out. Table 3.1 shows the summary estimated concentrations based on absorbance resulting from the binding to capsomers of neutralising conformation specific Mabs H16:V5, H16:E70 and H16:U4 and linear epitope recognising Mab H16:J4 to the capsomers.

TABLE 3.1: Summary of calculated concentrations of $L1_{Tr}$ and $L1\Delta C483_{Tr}$ capsomers based on the binding of the various Mabs to $L1_{Bac}$.

	Concentration of capsomers per kilogram of fresh transgenic leaf material			
Antigen	**H16:V5**	**H16:E70**	**H16:U4**	**H16:J4**
HPV-16 $L1_{Tr}$	3.2 μg	1.8 μg	2.4 μg	2.4 μg
HPV-16 $L1\Delta C483_{Tr}$	3.6 μg	4.4 μg	4.3 μg	2.3 μg

The yield based on H16:V5 and H16:J4 is similar for both $L1_{Tr}$ and $L1\Delta C483_{Tr}$. It is important to note that there is not a great variation in the yields determined using different Mabs.

3.3.4 Electron microscopy of the plant extracts

The plant extracts containing $L1_{Tr}$ and $L1\Delta C483_{Tr}$ were viewed under the electron microscope by immunotrapping the particles with Mabs H16:J4 and H16:V5 onto carbon coated copper grids and negative staining. The majority of the particles observed by immunotrapping with Mab H16:J4 were pentameric capsomers (10nm) with a few examples of higher order structures resembling VLPs (Figure 3.5). None of these kinds of particles were observed in the non-transgenic control. The full size particles/VLPs ($L1_{Tr}$) observed by immunotrapping with H16:V5 (Figure 3.6) were very similar in size and structure to those produced in insect cells by recombinant baculovirus (Figure 3.7). Similar full size particles were not seen in the immunotrapped $L1\Delta C483_{Tr}$ transgenic plant extract. The particles shown in Figure 3.6 (except particle IV) seems to have encapsidated some nucleic acid since the centres of the particles are not heavily stained unlike those seen for $L1_{Bac}$ VLPs in Figure 3.7 (A). These particles are very similar in morphology to those that have apparently encapsidated nucleic acid from expression in insect cells (Figure 3.7, particles B and D).

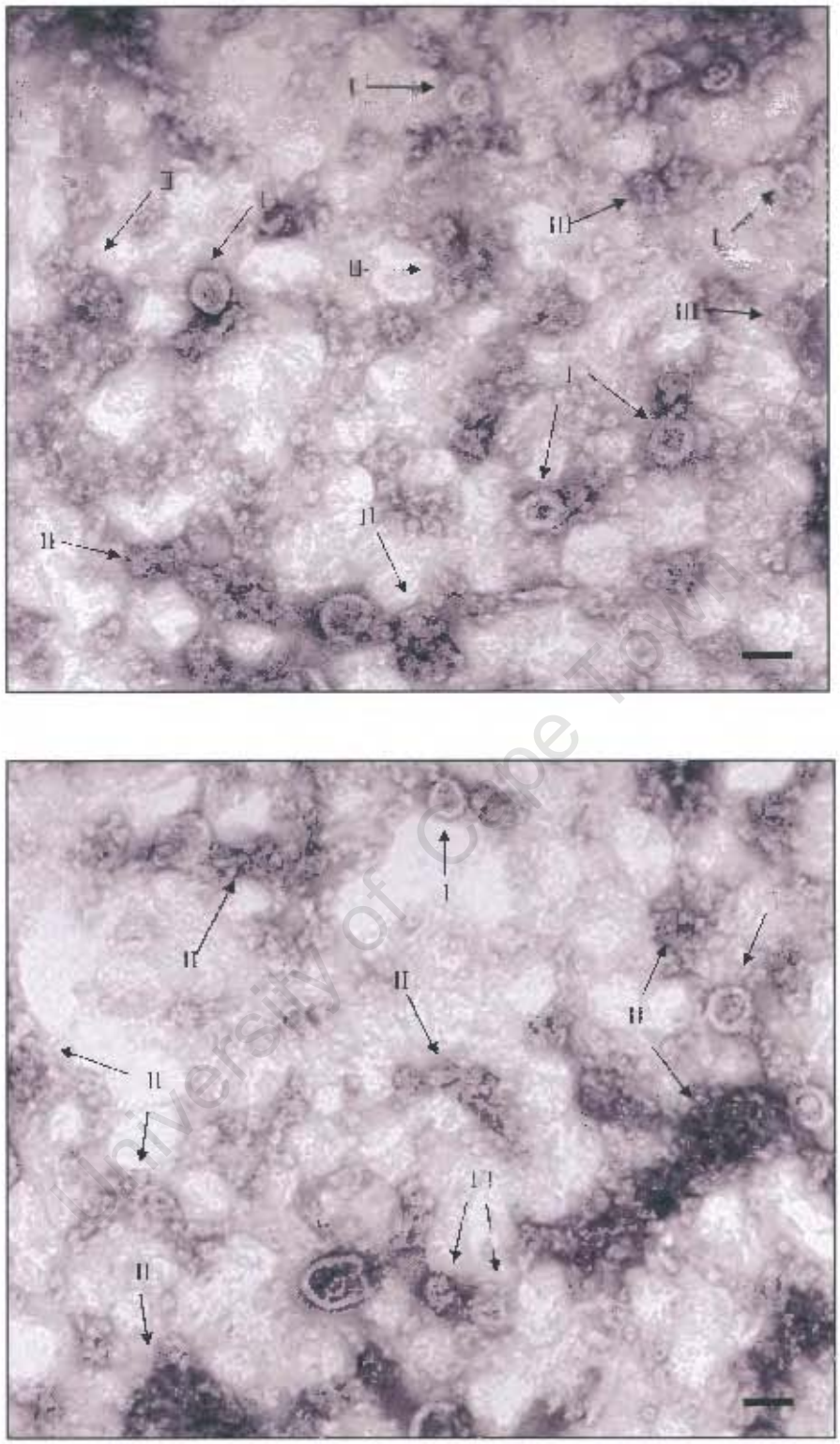

FIGURE 3.5: Electron micrographs of $L1_{Tr}$ (**A**) and $L1\Delta C483_{Tr}$ (**B**) immunotrapped particles (Mab H16:J4) in concentrated transgenic plant extract (**Bar = 50nm**). Mixed population of full size VLPs (**I**), capsomers (**II**) and disassembled/ broken down particles (**III**) are observed in both extracts.

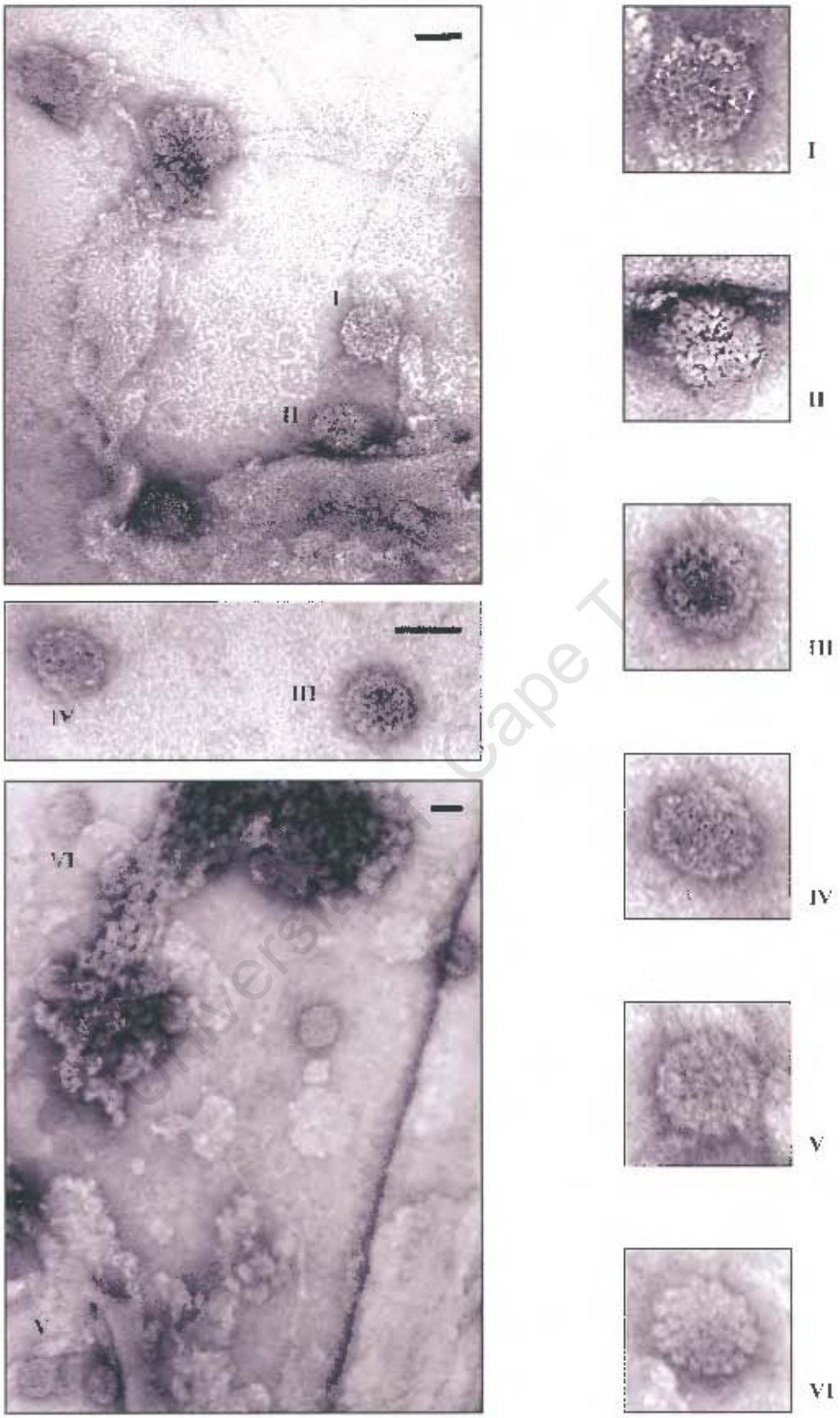

FIGURE 3.6: Electron micrographs of HPV-16 $L1_{Tr}$ VLPs immunotrapped particles with Mab H16:J4 from concentrated plant extracts (**Bar = 50nm**). Particles **I, II, IV, V** and **VI** probably contain nucleic acid whereas particle III is probably an empty capsid VLP.

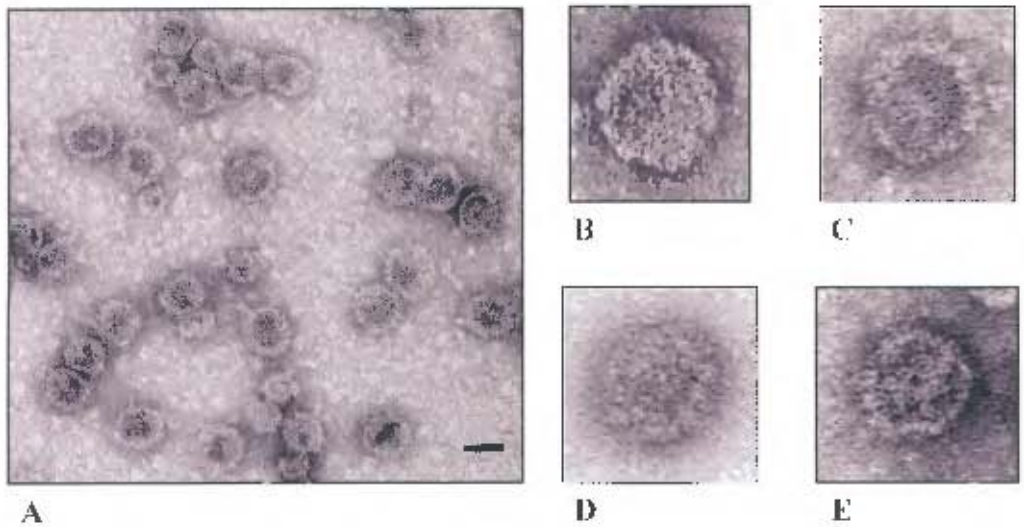

FIGURE 3.7: Electron micrographs of baculovirus produced HPV-16 $L1_{Bac}$ VLPs (**Bar = 50nm**). Empty VLPs shown in micrograph **A** and magnified particles in **C** and **E**. Capsids **B** and **D** probably contain nucleic acid since there is no evident heavy staining from the uranyl acetate as would be in the case of empty VLPs (**C** and **E**).

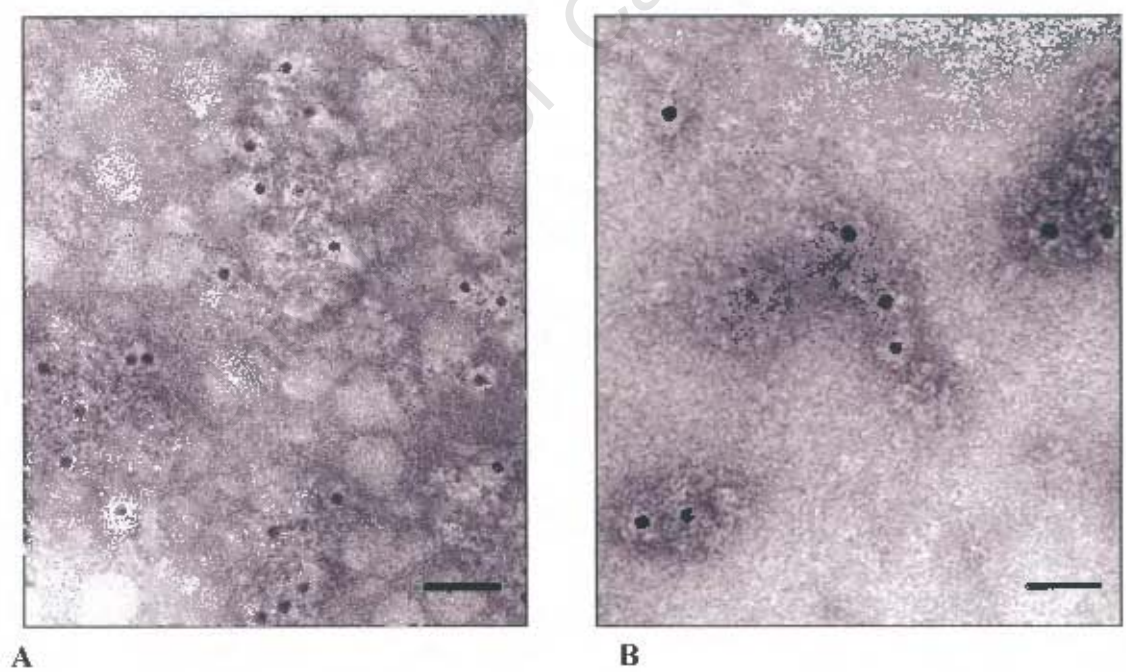

FIGURE 3.8: Electron micrographs of immunogold labelled capsomers of HPV-16 L_{Tr} (**A**) and $L1\Delta C483_{Tr}$ (**B**) that were trapped onto the copper grids with anti HPV-16 L1 guinea pig serum and probed with Mab H16:V5 (**Bar = 50nm**). The gold particles are approximately 10nm in diameter.

There was no marked difference observed in the particles resulting from the transgenic plant expression of L1ΔC483 to that of L1 (Figure 3.5, A and B). To confirm the observation of true HPV-16 $L1_{Tr}$ and $L1\Delta C483_{Tr}$ capsomers, the particles were immunotrapped using guinea pig anti-HPV-16 L1 serum onto carbon coated copper grids and probed with H16:V5 gold-labelled secondary anti-mouse antibodies (10nm gold particles). Gold labelling was observed at the centres of putative capsomers (Figure 3.8). No binding was observed in the negative control.

3.3.5 Analysis of the immune response to HPV-16 $L1_{Tr}$

No adverse effects were observed in the immunised rabbits as a result of immunisations with concentrated transgenic HPV-16 $L1_{Tr}$ plant extract. The rabbit sera were analysed for VLP specific antibodies by indirect ELISA using baculovirus-produced HPV-16 $L1_{Bac}$ VLPs. ELISA plates were coated overnight at 4°C with 100µl of HPV-16 $L1_{Bac}$ VLPs at a concentration of 1µg/ml, and probed with rabbit sera diluted 1:100 in PBS with 1% non-fat milk powder added. An unexpected complication was that the pre-immunised (day 0) sera were found to be highly reactive to HPV-16 $L1_{Bac}$, almost certainly as a result of baculovirus and /or insect cell contamination of the HPV-16 $L1_{Bac}$ preparation as indicated by the western blots (Figure 3.8). This rendered interpretation of the ELISA results difficult; however, a weak but generally increasing immune response was observed for the two of the three rabbit sera analysed (Figure 3.7). The weak immune response could be attributed to the low concentration of antigen administered in each inoculation (approximately 50ng).

Day 15 sera from all three rabbits were checked by western blotting against denatured baculovirus-produced HPV-16 $L1_{Bac}$ VLPs (Figure 3.8). The prebleed serum and the putative L1-immune sera and the H16:J4 Mab positive control bound a number of protein bands in common in the denatured HPV-16 $L1_{Bac}$ preparations, including in the expected region for L1 monomers (50–60 kDa); however, the three L1 sera also bound to at least one additional higher MW band in common with the H16:J4 Mab positive control, while the rabbit ID12 serum binds very similarly to the H16:J4 antibodies. This suggests that the preimmune and immune rabbit sera bind common insect cell or baculovirus proteins, while the immune sera and the control Mab also bind L1 protein bands.

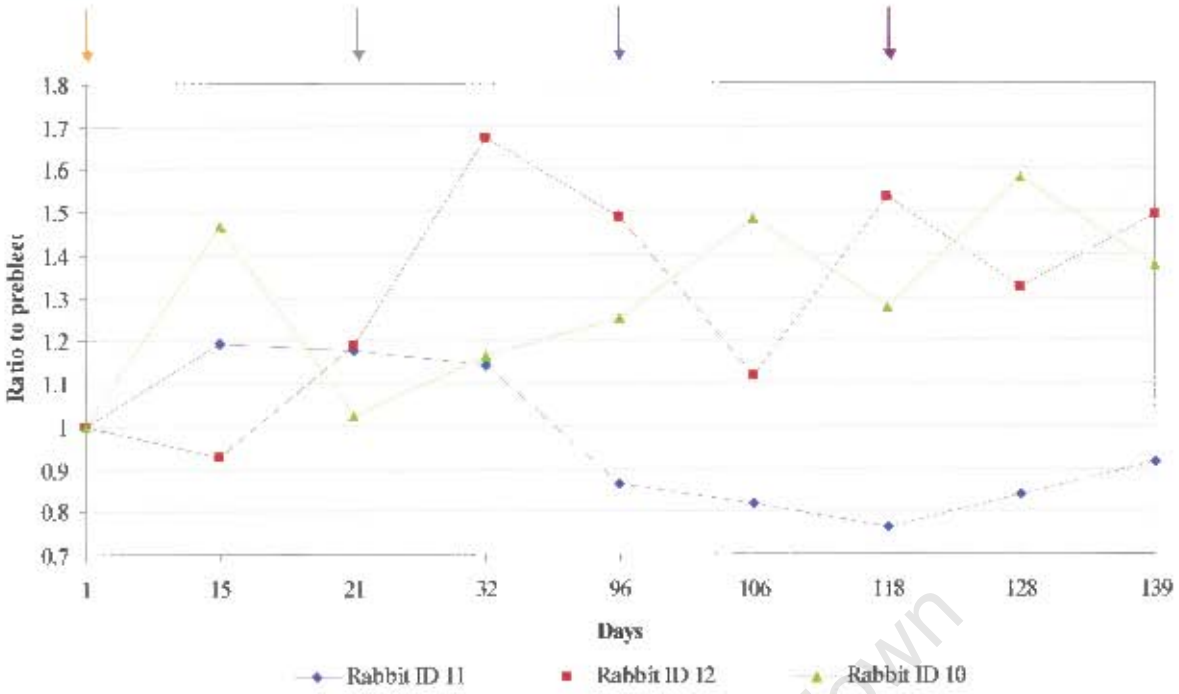

FIGURE 3.7: Analysis of sera from New Zealand white rabbits immunised with plant produced HPV-16 L1 antigen. Sera tested against recombinant baculovirus-produced HPV-16 $L1_{Bac}$ VLPs, 100µl of 1µg/ml coated per well onto ELISA plates. The rabbits were immunised on days **1, 21, 96** and **118.**

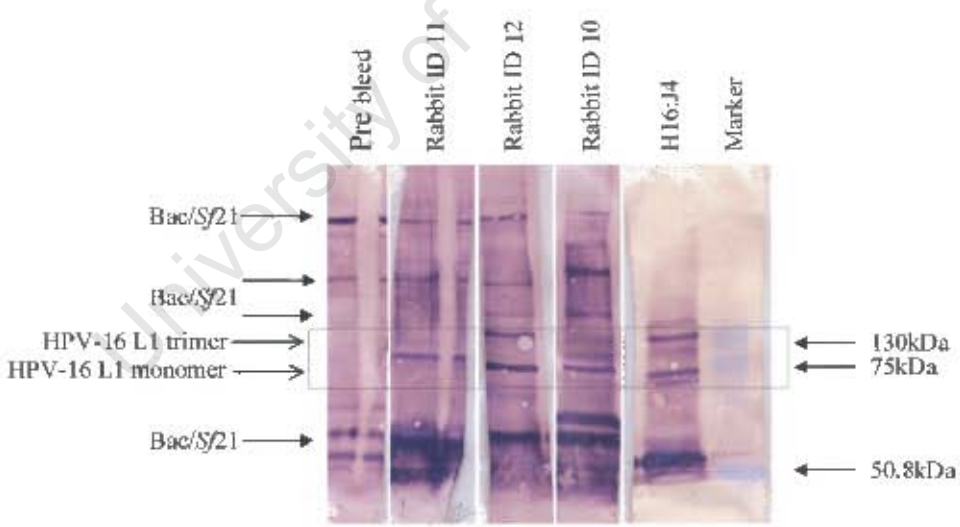

FIGURE 3.8: Western blot analysis of the rabbit sera against denatured HPV-16 $L1_{Bac}$. Binding of rabbit antibodies from the pre-bleed to certain baculovirus / *Sf*21 protein is evident but one predominant band (L1 monomer) at the same position as those in the H16:J4 control is shown in the dotted box for sera from rabbits all the three rabbits and another band, possibly a trimer is observed for sera from rabbit ID 12.

The western blots were performed on proteins that had been denatured with detergent, but not reduced: this allows retention of intermolecular S–S bridges, which results in dimer and trimer bands for L1 (Chen *et al.*, 2001). Thus, the H16:J4 antibodies and probably also at least the ID12 serum bound to putative L1 dimers and trimers in addition to monomers, with binding to the latter being masked by interfering anti-baculovirus and/or *Sf*21 cell contaminating proteins which also bound antibodies from preimmune sera. Thus, an HPV-16 L1-specific response was elicited in rabbits immunised with concentrated extracts from L1 transgenic plants.

3.4 DISCUSSION

A variety of expression systems have been investigated for the expression of HPV capsid protein as potential systems for producing vaccines: these include prokaryotic (Li *et al.*, 1997; Nardelli-Haefliger *et al.*, 1997), baculovirus (Kirnbauer *et al.*, 1992, 1996; Rose *et al.*, 1993; Suzich *et al.*, 1995; Touze *et al.*, 1998; Volpers *et al.*, 1994), yeast (Buonamassa *et al.*, 2002; Greer *et al.*, 2000; Jansen *et al.*, 1995; Sasagawa *et al.*, 1995) and mammalian expression systems (Hagensee *et al.*, 1993, 1994; Zhou *et al.*, 1993). All these recombinant cell expression systems require stringent purification and sterile protocols to obtain sufficient PV L1 antigen. Even with technological improvements, most of these technologies are expensive for developing countries that urgently require these vaccines. Reports have suggested that transgenic plants that express antigens could be used for inexpensive vaccine production systems (Arntzen *et al.*, 1994; Mason & Arntzen, 1995). There are several advantages of expressing foreign proteins in transgenic plants: the storage of genes and gene products in plants are stable, it is easy to scale up plant production in which large quantities of recombinant protein could be produced and cultivation of plants is much easier and requires limited skilled labour.

A variety of foreign antigens have been expressed in transgenic plants (see Chapter 1, Table 1.5). Mason *et al.* (1992, 1996) have demonstrated that *Hepatitis B virus* surface antigen and *Norwalk virus* capsids can assemble into VLPs by expression in transgenic plants. About 25-50% assembly was reported for Norwalk virus capsid protein expressed in transgenic potato (Tacket *et al.*, 2000).

This chapter reports the initial investigation into the use of a transgenic plant system to express the HPV-16 L1 and L1ΔC483 capsid protein. The results from this study suggest that the HPV

major capsid protein L1 can be expressed in transgenic *N. tabacum* cv. Xanthi. This protein folds into capsomers as shown by electron microscopy, and forms VLPs that are identical to those produced by recombinant baculovirus in insect cells. A high proportion of capsomers are observed by electron microscopy (Figure 3.5): this suggests that the concentration of L1 protein is low in plants, at a higher concentration of capsomers would presumably drive the equilibrium towards VLP formation. It is interesting to note that the binding affinity of Mab H16:D9 to L1ΔC483 is almost twice that of full length L1 (Figure 3.4), yet the binding of both of these proteins to Mabs H16:V5 and H16:J4 is similar. It can be deduced from the latter that there is a similar quantity of capsomers or higher order L1 structures that result from the transgenic expression, but that there is more denatured protein or unassembled protein in the case of L1ΔC483, since H16:D9 has a high affinity for denatured VLPs and almost doesn't bind intact VLPs (Christensen *et al.*, 1996a). Capsomers have been shown to be immunogenic and can elicit neutralising antibodies (Fligge *et al.*, 2001; Giroglou *et al.*, 2001; Unckell *et al.*, 1997). The electron microscopy data also indicated that there is no marked morphological difference in the particles formed from the expression of the HPV-16 L1 gene with or without the nuclear localisation signal (L1ΔC483). PV virion assembly takes place in the nucleus of the infected cells (Pfister & Fuchs, 1987) and the nuclear localisation signal (NLS) of HPV-16 L1 was identified by Zhou *et al.* (1991) to be bipaprtite and located on the C-terminus (aa 484-504; KRK_1atpttsststtaKRKKRKL_2). There is a strong body of evidence to suggest that C- terminus deletion up to the h5 helix allows the L1 protein to fold into VLPs (Chen *et al.*, 2000b, 2001).

Based on the ELISA data the HPV-16 L1 yield is estimated to be 2-4 μg per kilogram of fresh leaf material. Similar yields were achieved by Kapusta *et al.* (1999) in their expression of hepatitis B surface antigen in lettuce. In general, the expression levels of foreign proteins in transgenic plants have been variable, ranging from 0.001% of total soluble protein for rabies virus glycoprotein (McGarvey *et al.*, 1995), to 0.37% for *Norwalk virus* capsid protein (Mason *et al.*, 1996). This variation occurs since the integration of transgene is random and copy number, positional effects and gene silencing can affect subsequent protein expression. As a result, expression of the foreign DNA will vary depending on where it is integrated into the plant genome. Therefore if the system is to be scaled up for large scale transgenic protein expression, it is necessary to screen a number of different regenerated plants to determine the expression levels.

Sera from rabbit ID 12 reacted to two distinct bands similar to those of the positive control (Figure 3.8). HPV-16 L1 under nonreducing conditions results in 50% monomers and 50% trimers when denatured and run on a SDS PAGE gel (Sapp *et al.*, 1998). Therefore the higher band in the western blots is clearly that of the L1 trimer since the HPV-16 $L1_{Bac}$ protein was denatured in SDS PAGE loading dye without a reducing agent such as β-mercaptoethanol. It is interesting to note that this trimer band is only observed for the sera from rabbit ID 12 which of the three had best anti HPV-16 $L1_{Tr}$ response (Figure 3.7). Gerber *et al.* (2001) have shown that intramuscular immunisation of mice with 0.3μg of HPV-16 VLPs produced in insect cells elicited a strong immune response to L1. However, the anti – HPV-16 $L1_{Tr}$ immune response observed in the rabbit experiments was low. This could almost certainly be attributed to a low concentration of L1 capsomers / VLPs in the plant extracts and that the response can be improved by using a plant extract that has a higher concentration of L1 antigen: this can be done by developing a bulk extraction protocol from a larger source of plant material in addition to increasing expression levels in the transgenic plants. Various approaches have been suggested to increase the expression levels in transgenic plants. These include codon optimisation, modification of plant promoters for transcription of the genes, and insertion of 5'- or 3'-untranslated regions to increase mRNA stability (Mor *et al.*, 1998; Richter *et al.*, 2000), introduction of a 5'-untranslated *Tobacco mosaic virus* sequence or other translational enhancer (Mitsuhara *et al.*, 1996), and the use of a CaMV 35S-dual enhancer promoter (Mason *et al.*, 1992). Leder *et al.* (2001) demonstrated that codon optimising the HPV-16 L1 gene for expression in mammalian cells resulted in an increased efficacy of the L1 DNA vaccines. Therefore, codon optimising the L1 gene towards plant codons may enhance the expression of the L1 / L1ΔC483 transgenes in *N. tabacum* cv. Xanthi.

Exceptionally high levels of protein expression have been achieved by integrating genes into the chloroplast genome where recombinant protein constitutes as much as 46% of the total soluble protein (De Cosa *et al.*, 2000). Each plant cell contains thousands of chloroplasts, resulting a high copy expression of the transgene (De Cosa *et al.*, 2000). This method of transformation avoids positional and silencing effects because the foreign DNA is integrated by homologous recombination within a spacer region in the chloroplast genome. Polycistrons can be expressed in the chloroplast, allowing the expression of multiple proteins. Chloroplasts have relatively fewer proteolytic pathways than the remainder of the cell and as a result, the foreign protein may be subjected to limited degradation (Adam, 2000; Bock, 2001; Ruf *et al.*, 2001). The cholera toxin B

subunit has been expressed in tobacco chloroplasts with a yield of 4.1% of total plant-soluble protein (Daniell *et al.*, 2001). In addition to high level expression, chloroplast integration should decrease transgene spread via pollen since chloroplast are maternally inherited in most plants (Awram *et al.*, 2002; Daniell, 1999) and is therefore worth considering for the expression of HPV-16 L1 capsid protein.

In summary, the HPV-16 L1 and L1ΔC483 genes were introduced into the *N. tabacum* cv. Xanthi plant genomes by *Agrobacterium*-mediated gene transfer. The data in this chapter confirms the successful and stable *in planta* production of HPV-16 L1 particles that are capable of binding conformation-specific neutralising monoclonal antibodies (Mabs). However, preliminary attempts at demonstrating immunogenicity of partially purified extracts from plants were only partially successful. Electron microscopy showed that the particles that result from the transgenic expression in *N. tabacum* cv. Xanthi plants were similar to those produced in insect cells by using recombinant baculovirus. It therefore appears quite feasible that transgenic plants could be used for the production of HPV vaccines if sufficient protein can be harvested.

Chapter 4

Transient expression of the HPV-16 L1 major capsid protein in *Nicotiana benthamiana* using a tobamovirus vector

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ABSTRACT

A *Tobacco mosaic virus* (TMV)-based vector was used to express a *Human papillomavirus* type 16 (HPV-16) L1 gene in *Nicotiana benthamiana*. *In vitro* RNA transcripts of the recombinant TMV vector (pBSG-L1) were inoculated into *N. benthamiana* plants. Detectable amounts of HPV-16 L1 protein were identified by enzyme linked immunosorbent assays (ELISA) after concentrating the plant extract. The concentrated extract was tested for antigenicity with well-characterised monoclonal antibodies (Mabs). Neutralising and conformation-specific Mabs, H16:V5 and H16:E70, were shown to bind to the plant-produced HPV-16 L1 particles. The particles observed by transmission electron microscopy were mainly capsomers but a small population of VLPs, similar to those produced in insect cells by recombinant baculovirus, were present. Immunisation of rabbits with the plant extract that contained a small amount of HPV-16 L1 induced a weak immune response. The yield estimated by ELISA was approximately 20-30μg per kg of fresh leaf material.

4.1 INTRODUCTION

Advances of our understanding of the molecular biology of plant viruses has enhanced the development of expression systems that use viral vectors to produce large amounts of protein in a short period of time. By early 1990s it was apparent that certain plant viruses would tolerate the insertion of foreign gene sequences and support heterologous gene expression *in vivo* (Takamatsu *et al.*, 1990). The general approach has been to insert the foreign gene into the viral RNA genome under the control of a subgenomic promoter, and to introduce the resulting recombinant virus into an appropriate host plant by mechanical inoculation of RNA or even plasmid DNA. Virus vectors are powerful tools for the expression of foreign genes in plants since they offer an alternative to stable transformation for the production of pharmaceutical antigens in plants. Much research has focused on their use for production of complete viral proteins or of peptides fused to plant viral proteins for vaccine production (see Chapter 1, Table 1.6). A major advantage lies in the relatively rapid transient expression possible in plants, ranging from 1-4 weeks after initial cloning.

Unlike conventional recombinant vaccines, plant viruses are generally recognized to be non-pathogenic in humans and other animals. Plant viruses that express transgenes during their replication in the host generally achieve much higher product yields than those achieved in transgenic plants.

TMV is a positive strand RNA virus that has been developed into a flexible and useful expression system for plants (Dawson, 1999; Yusibov *et al.*, 1999). The genome of TMV is a monopartite, positive sense, 6.4 kb ssRNA that contains 4 ORFs (Figure 4.1). The 5'-terminius is capped and the 3' end bears a t-RNA-like configuration that in fact accepts histidine. The 3' end also contains 5 pseudoknots, 2 of which are in the tRNA-like structure. The first ORF encodes a 126 kD protein, but read-through of the UAG termination codon generates a 183 kD protein. These two proteins are involved in replication of the viral RNA with the 126 kD protein containing consensus methyltranferase and helicase motifs and the read through part of the 183 kD protein contain amino acid sequences typical of viral RNA-encoded polymerases (Lewandowski & Dawson, 2000). The last five codons of the 183 kD reading frame overlap the 30 kD reading frame, and the latter terminates two nucleotides prior to the initiation codon of the ORF that encodes the 17.5 kD coat protein. The 30 kD and coat proteins are translated from two 3'-coterminal subgenomic RNAs.

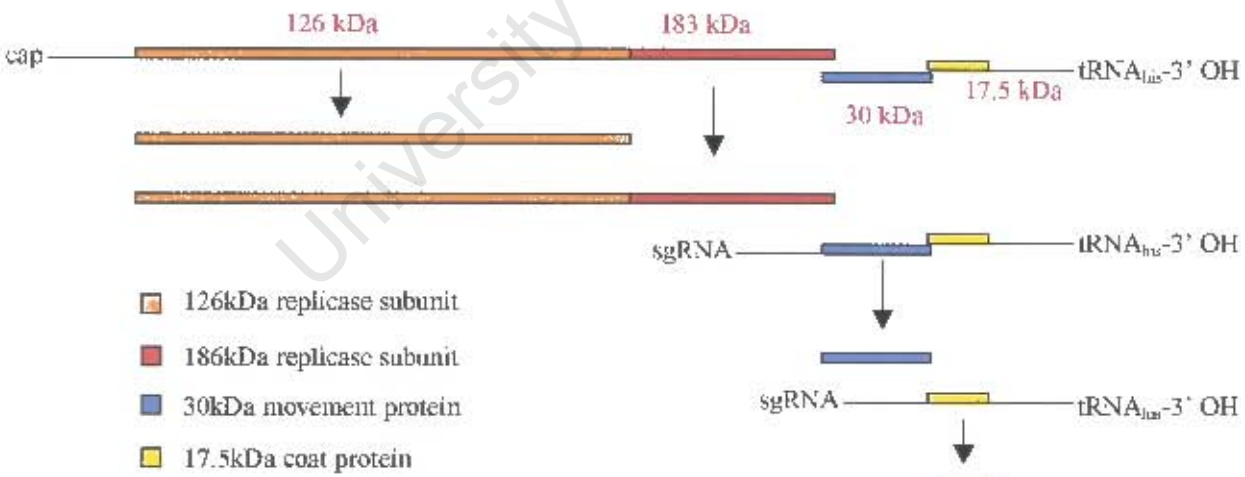

Figure 4.1: Organisation and expression of the TMV genome (Heinlein, 2002). The 126kDA and 183kDa subunits of replicase are translated from the genomic RNA of the virus, the 30kDa movement protein and 17.5kDa coat protein are translated from the subgenomic RNA produced during replication. The genomic RNA and coat protein subgenomic RNA, but not the movement protein subgenomic RNA, are capped and the viral RNA terminates with t-RNA-like structure that accepts histidine.

TMV is transmitted mechanically by physical contact between plants and virus contaminated surfaces, and has no known vectors (Heinlein *et al.*, 1998; Heinlein, 2002). The coat protein of TMV can accumulate to as much as 10% of the dry weight of an infected leaf. First generation TMV vectors did not express proteins throughout the plants since the sequence encoding the coat protein - which is required for movement - was removed and replaced with the open reading frame of the foreign genes. Another problem with genes inserted into TMV genomes was intramolecular recombination due to repeated subgenomic promoters resulting in removal of the sequences (Donson *et al.*, 1991). Subsequently, constructs containing heterologous coat proteins and subgenomic promoters from closely related viruses have reduced these problems and have enabled foreign proteins to be expressed to levels as high as 2% of the total soluble plant protein (Shivprasad *et al.*, 1999). The structural protein VP1 from foot and mouth disease virus has been expressed using TMV (Wigdorovitz *et al.*, 1999). The relatively stable TMV vectors are now used frequently for foreign protein expression in plants (Artnzen, 1997; Hamamoto *et al.*, 1993; Koo *et al.*, 1999; Nemchinov *et al.*, 2000; Takamatsu *et al.*, 1990; Turpen *et al.*, 1995; Verch *et al.*, 1998).

This chapter reports the investigation of transient expression of the HPV-16 L1 gene in *Nicotiana benthamiana* using a TMV-based vector. The potential for production of VLPs, the immunogenicity in rabbits, and the binding of a panel of Mabs known to bind virions and baculovirus-produced VLPs, were examined. Observation of necrosis in *N. benthamiana* as a result of infection with the TMV vector pBSG1057 is a clear sign of recombination where the insert is deleted (personal communication with Dr Kenneth Palmer of Biosource Technologies Inc, Vacaville, USA). Initial work with expressing L1ΔC483 (L1 without the NLS) in *N. benthamiana* using the TMV vector resulted in severe necrosis of the infected plants which was indicative of recombination of the TMV vector where possibly the L1ΔC483 gene was deleted. Further, data from chapter 3 showed that there was no major difference as a result of the deletion of the NLS (L1ΔC483) in terms of yield and antigenicity; therefore, only the full length HPV-16 L1 gene was used for the rest of this study.

4.2 MATERIALS AND METHODS

4.2.1 Construction of the TMV vector encoding the HPV-16 L1 gene

The TMV-based transient expression Geneware™ Vector pBSG1057 (Biosource Technologies Inc, Vacaville, USA) contains sequences from the *Tobacco mild green mosaic virus* U5 isolate, including the coat protein subgenomic mRNA promoter and the coat protein ORF. The replication and movement protein ORFs are from TMV isolate U1. This heterologous vector was found by be the most stable by Shivprasad *et al.* (1999). The 30B-GFPC3 mutant gene (GeneBank accession no. U62637) was replaced with the HPV-16 L1 to obtain the clone, L1-pBSG (Figure 2.2).

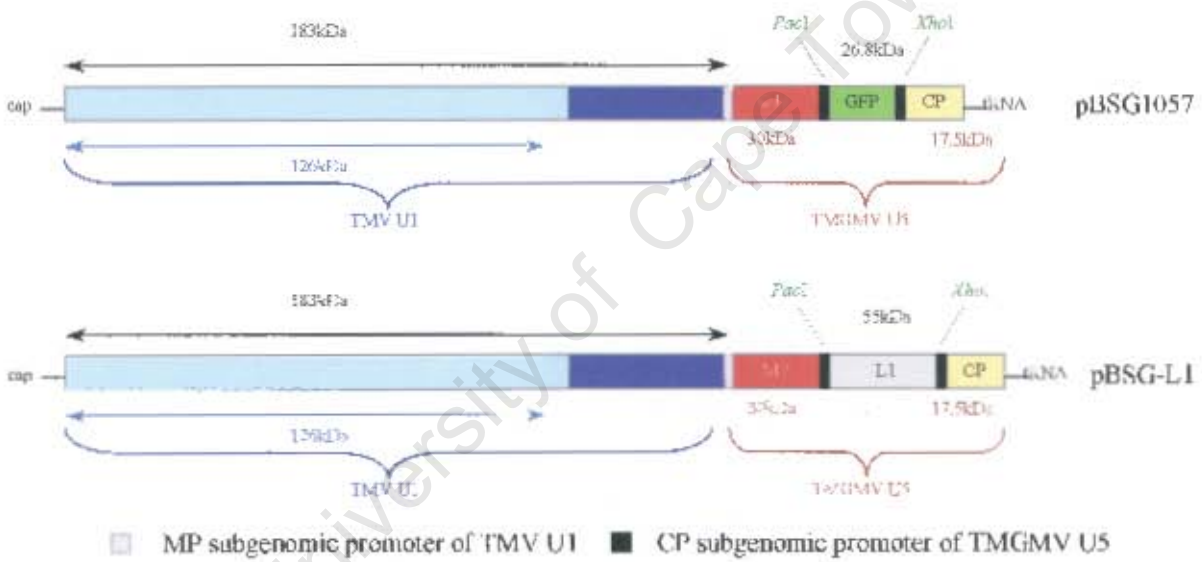

FIGURE 2.2: Schematic diagram of the genomic organisation of pBSG-L1 vector cassette and Geneware™ Vector pBSG1057. The replicase and movement protein genes are derived from TMV U1 whereas the coat protein and the subgenomic coat protein promoter are derived from TMGMV U5. The HPV-16 L1 gene and the 30B GFPC3 mutant gene are placed under the control of the coat protein subgenomic promoter of TMGMV U5.

The HPV-16 L1 gene (South African isolate; Genbank accession no. AY177679; pSK-L1-SA) was PCR amplified (Expand Long template PCR system, Roche) using the following set of primers:

forward 5'-*TTAATTAA*ATGTCTCTTTGGCTGCCTAGTGAGG-3'

reverse 5'-*CTCGAG*TTACAGCTTACGTTTTTTGCGTTTAGCAGTTGTAG-3'

These introduced restriction sites *Pac*I (3'-TTAATTAA-5') and *Xho*I (3'-CTCGAG-5') sites at the 5' and 3' end of the HPV-16 L1 gene respectively. The amplified gene was cloned into pGEM®-T Easy vector (Promega) and sequenced.

The sequenced HPV-16 L1 gene was sub-cloned into the *Pac*I / *Xho*I restriction sites of the Geneware™ Vector pBSG1057 (see Appendix C for map of pBSG1057 vector) by replacing the 30B-GFPC3 mutant (Crameri *et al.*, 1996). This put the HPV-16 L1 gene upstream from the coat protein under the coat protein subgenomic promoter of TMGMV U5. The vector has a pUC backbone for replication in *E. coli* and a ribozyme sequence 3' downstream of the TMV sequence, to allow autocatalytic truncation of *in vitro* synthesised transcripts.

4.2.2 pBSG-L1 and pBSG1057 *in vitro* transcriptions and inoculation of *Nicotiana benthamiana* plants

In vitro transcripts were made using the T7 RNA polymerase (RiboMAX™ Large Scale RNA production System-T7, Promega) and capped using RNA cap-structure analogue m7G(5)ppp(5)G (New England Biolabs). RNA transcripts of pBSG-L1 and pBSG1057 (expressing GFP) vector were prepared using 5 µg of L1-pBSG and pBSG1057 DNA in a 100µl reactions and diluted to 1ml with DEPC treated water for inoculations. The diluted transcripts were mechanically inoculated with cotton buds and Celite onto the bottom leaves of 3-week-old *N. benthamiana* plants. Plants were grown at 22°C with 16-hours light and 8-hours darkeness and monitored for signs to systemic infection.

4.2.3 Analysis of the total mRNA extracted from the transient expression of HPV-16 $L1_{TMV}$ in *N. benthamiana* plants and western blot analysis of the protein products

Total RNA was extracted from fresh or stored (-70°C) leaves using the TRIzol™ reagent (Life Technologies). The L1 and TMV coat protein (CP) mRNAs were detected by RT-PCR amplification using the Access RT-PCR system (Promega) using the following set of primers:

Forward 5'-GGTCCATTAGGTGTGGG-3' / reverse 5'-AGCTGTCGCCATATGGTTCTG-3' for the HPV-16 L1 gene, amplifying an internal 500bp fragment.

Forward 5'-GTTCCGCTTATGCAGATCCTG-3' / reverse 5'-AAGTAGCCGGAGTTGTGGTCC-3') for the TMV CP gene, amplifying an internal 500bp fragment.

For the analysis of the expressed protein products the plant crude extracts made by grinding leaf material in PBS were denatured for 10 minutes at 100 C in SDS PAGE gel loading dye (without reducing agents). The denatured extract was resolved on 12% (for TMV) or 7% (for HPV-16 $L1_{TMV}$) SDS-PAGE gels. The separated resolved protein was transferred onto a nitrocellulose membrane by semi-dry electrophoresis (BioRad) for 25 mins at 25V. The membrane was blocked using 1% non-fat milk for 2 hours and incubated with the primary antibody overnight. For the TMV CP (17.5 kDa), I used anti-TMV CP rabbit polyclonal sera (provided by Prof.E Rybicki) at 1:1000 dilution, and for L1 (55kDa) Mab H16:J4 at 1:200 dilution. The membrane was washed with PBS / 0.05% Tween-20 and probed with alkaline phosphatase-labelled goat anti-rabbit and goat anti-mouse secondary antibodies, respectively, at dilutions of 1:2000. The antibody binding was detected by a colorimetric reaction using 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) and 4-nitro blue tetrazolium chloride (NBT).

4.2.4 Polyethylene glycol (PEG) precipitation of TMV particles

Twenty-five 3-week old *N. benthamiana* plants were inoculated with dried leaf material from *N. benthamiana* plants, initially infected with RNA, and checked for systemic infection (leaf sap of top leaved analysed by electron microscopy) before being harvested 9 days post inoculation. The plant material was weighed (~100g) and homogenised with 300ml of cold PBS (0.5M NaCl). The homogenate was strained through cheesecloth and centrifuged at 6000 x g for 10 minutes. To the supernatant was added 4% (w/v) PEG and stirred overnight at 4°C. The mixture was plant extract was centrifuged at 6000 x g for 20 minutes to pellet the precipitate. To the supernatant was added 10% (w/v) PEG, the precipitate was resuspended in 60ml PBS buffer and this process of precipitation was repeated. Samples of treated extract were collected at most of the steps. Figure 4.3 illustrates the treatment of the homogenised plant material with PEG and the collection various samples. The PEG precipitated pellets were resuspended in $1/10^{th}$ of the supernatant volume. All the samples collected were analysed by western blot for both the HPV-16 $L1_{TMV}$ protein and TMV coat protein as described in section 4.2.3.

4.2.5 Concentration of infected plant material for antigenicity and immunogenicity studies

The RNA transcript was prepared as described in section 4.2.2. Twenty-five plants were inoculated with the freshly prepared RNA transcripts of pBSG-L1, ~40µl was used per plant and three bottom leaves were inoculated. The *N. benthamiana* plant material was harvested 14 dpi (~80g) and homogenised in 1:2 (w/v) ice cold PBS/0.5M NaCl. The homogenate was strained through cheesecloth and centrifuged at 6000 x g for 10 minutes. The TMV was precipitated out of the supernatant using 4% PEG. Following the PEG treatment, the supernatant was concentrated by ultracentrifugation at ~77 000 x g for 6 hours and resuspended in cold PBS. For antibody characterisation of the L1 capsomers the protein was further concentrated using the Centricon YM-30 (30 000kDa cut off) centrifugal filters (Amicon Separation).

4.2.6 Analysis of the antigenicity of the HPV-16 $L1_{TMV}$ protein

Transiently expressed HPV-16 $L1_{TMV}$ protein and uninfected negative control concentrate was tested with a panel of monoclonal antibodies (H16:V5, H16:E70, H16:U4, H16:9A, H16:D9, H16:I23 and H16:J4; see Table 1.3, Chapter 1) by capture ELISA, capturing the particles with guinea pig anti-HPV-16 L1 serum. A similar method to that used in Chapter 3 (section 3.2.3) was followed. Briefly, the diluted (1:500) guinea pig serum was coated overnight onto ELISA plates. The ELISA plate was blocked with 1% non-fat milk in PBS. The plant extract (~100 fold concentrated) was diluted 1:10 and allowed to bind to the serum for 2 hrs. The captured antigen was probed with the Mab (1:200) for 2 hrs. Goat anti-mouse-alkaline phosphatase secondary antibody (1:2000) was allowed to bind to the Mab for an hour at 37°C. The secondary antibody was detected p-nitrophenyl phosphate (Sigma) and the absorbance was measured using a Titrex ELISA plate reader at 405nm.

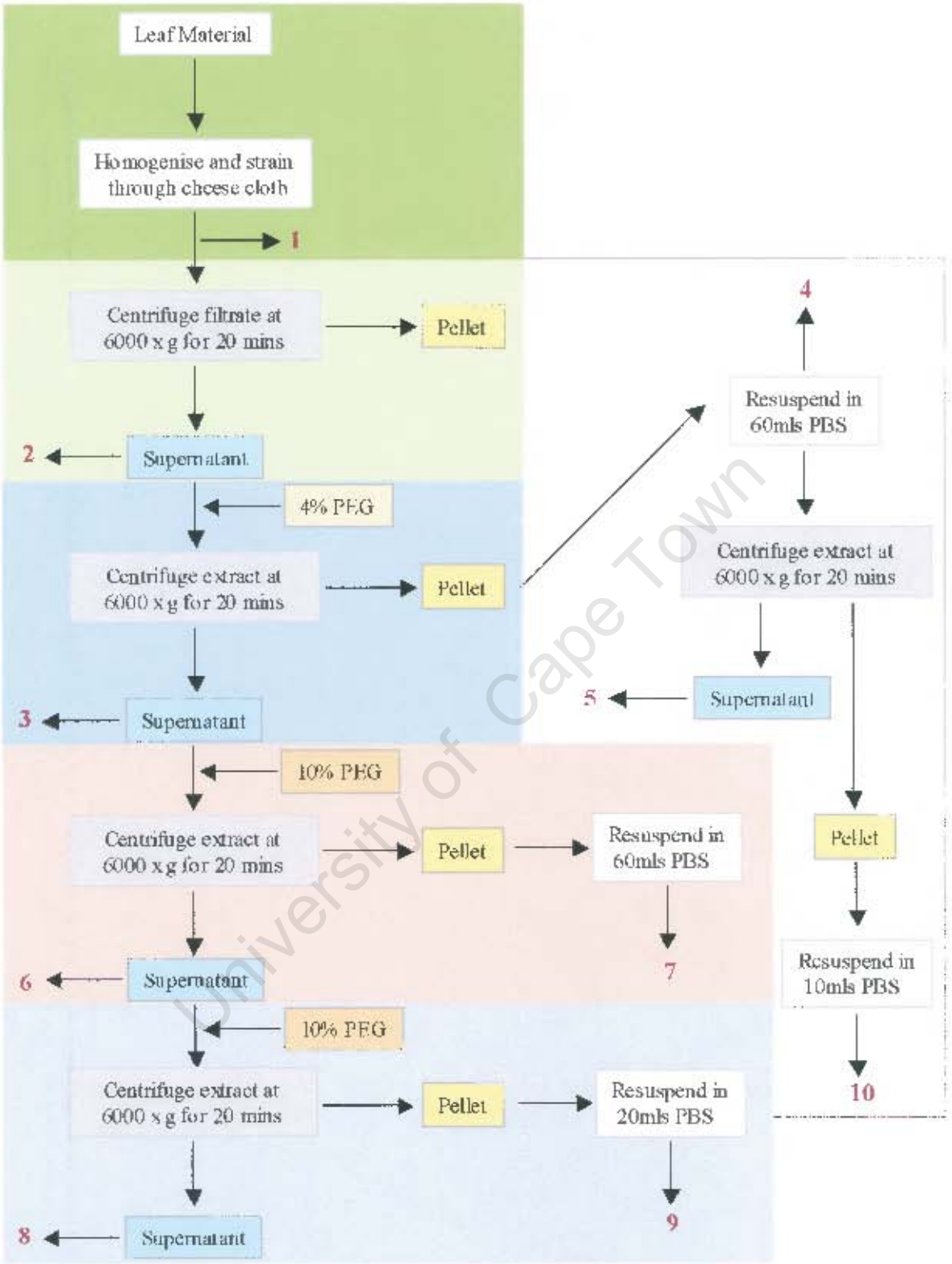

FIGURE 4.3: Schematic diagram showing the PEG treatment of the plant extract and the collection of samples (1-10)

4.2.7 Transmission electron microscopy of HPV-16 $L1_{TMV}$

A protocol similar to that outlined in Chapter 3 (section 3.2.7) was followed. In brief, concentrated plant protein extracts and infected leaf sap were negatively stained with 2% uranyl acetate and viewed with a JEOL 200CX transmission electron microscope. The plant extract was viewed either by immunotrapping the particles with J4 (binds linear epitope aa 261-280) and V5 (binds conformational epitope) monoclonal antibodies at a dilution of 1:50 on carbon coated copper grids or untrapped. In the case of immunogold labelling, the extract was immunotrapped with guinea pig anti HPV-16 L1 (1:100) onto carbon coated copper grids. The trapped particles were probed with Mab V5 (1:50 dilution) and secondary gold-labelled (10nm) anti-mouse (1:100). The extracts were stained with 2% uranyl acetate and viewed using a JEOL 200CX transmission electron microscope at 120kV.

4.2.8 Immunisation of rabbits with plant extracts from transient expression HPV-16 $L1_{TMV}$ and serum analysis

Concentrated *N. benthamiana* sap extract (~65 fold concentrated) from the transient expression of HPV-16 $L1_{TMV}$ was injected into three white New Zealand rabbits. For each rabbit the plant extract (with approximately 0.4µg of L1 antigen) was diluted to 500µl in PBS and injected 1:1 with Freund's incomplete adjuvant. The plant-produced antigen was administered to two subcutaneous sites and an intramuscular site at days 1, 21, 67 and 87. The rabbit serum at a dilution of 1:50 dilution was analysed for VLP specific antibodies by ELISA against baculovirus-produced HPV-16 $L1_{Bac}$ VLPs (100µl per well coated at1µg/ml concentration) and by western blots (see Chapter 3, section 3.2.8).

4.3 RESULTS

4.3.1 Analysis of *N. benthamiana* with *in vitro* transcripts of pBSG-L1 and pBSG1057 constructs

Transient expression of the foreign proteins in plants using plant viruses has been suggested to achieve higher yield than from transgenic plant expression. Therefore, in an attempt to achieve higher yields of plant-produced HPV-16 L1, the transient expression of HPV-16 L1 was

investigated using a TMV-based vector (pBSG1057) in *N. benthamiana* plants which allow for a systemic TMV infection.

The TMV infection produced by pBSG1057 (expressing GFP) was monitored on the inoculated plants using a hand-held UV lamp. GFP fluorescence was observed as spots on the infected leaves 3dpi, and systemic infection was generally observed after 8dpi. TMV infection symptoms - a slight mosaic pattern on the leaves and curling of the top infected leaves (Figure 4.4) - were evident 10-14 days post inoculation (dpi). The leaf curling was used as a marker for systemic infection for pBSG-L1 infections.

A B C

FIGURE 4.4: Leaf curling (indicated by the white arrows) and mosaic symptoms on *N. bentamiana* plants as a result of recombinant TMV infection. **A**: uninfected plant; **B**: infected with recombinant TMV expressing GFP (inoculated with in vitro transcripts of pBSG1057); **C**: infected with recombinant TMV expressing HPV-16 $L1_{TMV}$ (inoculated with in vitro transcripts of L1-pBSG).

The infection by recombinant TMV expressing HPV-16 $L1_{TMV}$ was confirmed by RT-PCR of total RNA extracted from the top leaves. A 500bp RT-PCR product was observed for the TMV coat protein (CP) and the L1 (Figure 4.5).

In order to determine if the L1 protein was being produced, leaf sap of infected *N. benthamiana* plants (top leaves) was analysed by western blot analysis. Although mRNA was detected for both HPV-16 $L1_{TMV}$ and TMV CP only TMV CP could be detected by western blot (Figure 4.6). This led to speculation that the expression of HPV-16 $L1_{TMV}$ was low and that concentrating the plant material would be necessary, as it was for the transgenic plants in Chapter 3.

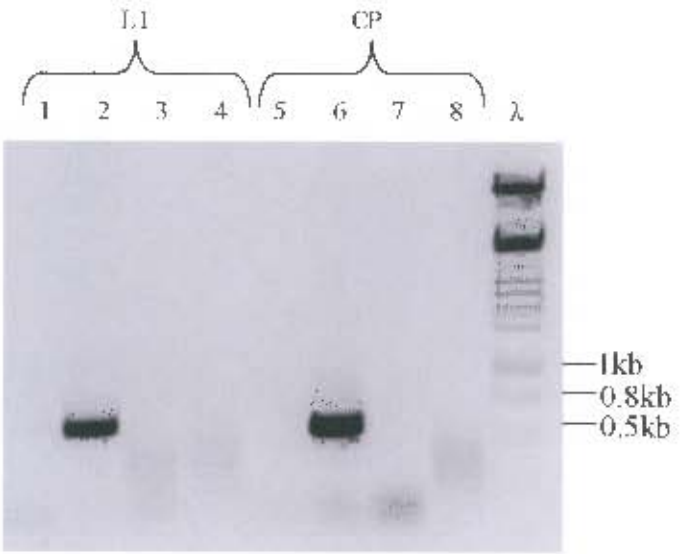

FIGURE 4.5: RT-PCR analysis of total mRNA extracted from *N. benthamiana* plants infected with recombinant pBSG-L1. Lanes 1-4 and 5-8 represent RT-PCR products for HPV-16 $L1_{TMV}$ and TMV CP respectively. Lanes **1** and **5**: water blank; **2** and **6** RT-PCR product from RNA extracted from pBSG-L1 inoculated plants; **3** and **7**: RT-PCR of RNA extracted from uninfected plant material; **4** and **8** are water RT-PCR controls. RT-PCR product of 500bp in lanes 2 and 6 indicate the transient expression of HPV-16 $L1_{TMV}$ and TMV CP respectively.

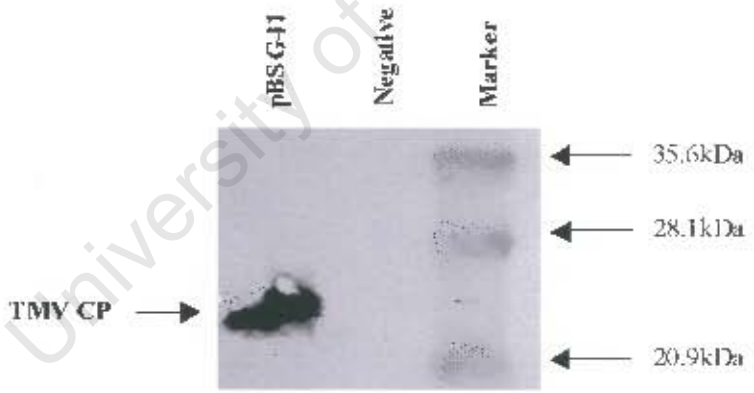

FIGURE 4.6: Western blot analysis of leaf sap from *N. benthamiana* infected with *in vitro* synthesised transcripts of L1-pBSG showing TMV coat protein detected using anti-TMV polyclonal rabbit serum

4.3.2 PEG precipitation of TMV particles from crude plant material and concentration of the crude extract

Since the infected *N. benthamiana* plant material from the transient expression of HPV-16 $L1_{TMV}$ protein needed to be concentrated, it was important to establish a protocol to remove the TMV from the crude extract. A precipitation experiment was designed to investigate the effects of

precipitation of TMV and possibly the HPV-16 $L1_{TMV}$ with PEG at 4%, 14% and 24% (w/v) concentrations. The precipitates (pellets from centrifugation) were resuspended in $1/10^{th}$ of the supernatant volume with PBS.

The protocol illustrated in Figure 4.3 was followed and samples collected at various stages of the precipitation and resuspension were analysed by western blot analysis for the TMV coat protein and the HPV-16 $L1_{TMV}$ protein. Samples 3 (4% PEG), 6 (14% PEG) and 8 (24% PEG) were viscous and hence could not be analysed by SDS-PAGE gels, therefore their content could not be analysed. Nonetheless since the main objective of this experiment was to look at precipitation of the TMV and HPV-16 $L1_{TMV}$, analysis of samples 1, 2, 4, 5, 7, 9 and 10 would be sufficient.

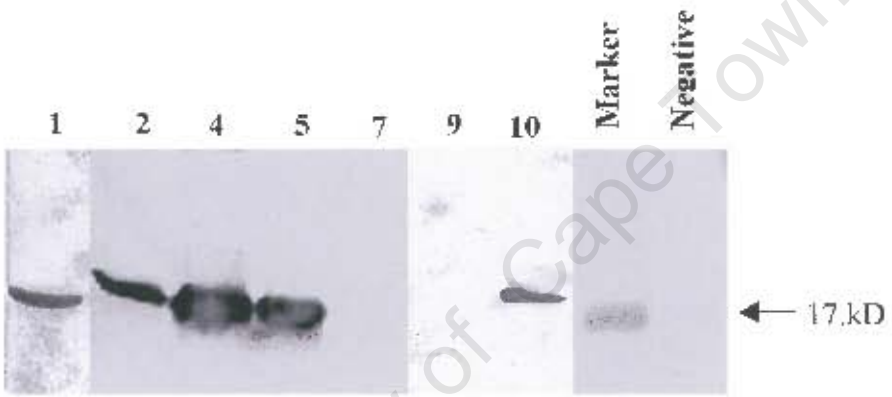

FIGURE 4.7: Western blot analysis for TMV (using anti-TMV polyclonal antibody) in various samples as a result of PEG precipitation. Samples 1, 2, 4, 5 and 10 are positive for TMV CP. Samples 7 (precipitate as a result of 14% PEG) and 9 (precipitate as a result of 24% PEG) are negative for TMV CP suggesting that 4% PEG is sufficient for complete precipitation of TMV particles.

Samples 1, 2, 4, 5 and 10 were found to have TMV in them suggesting that 4% PEG was sufficient to precipitate all the TMV particles (Figure 4.7). The HPV-16 $L1_{TMV}$ protein was difficult to detect by western blot and the SDS PAGE gels were unable to resolve the samples 4 and 7, in both cases smears were observed on the western blots with Mab H16:J4 (data not shown) suggesting that a proportion of HPV-16 $L1_{TMV}$ precipitates with 4% PEG treatment. Since binding of Mab H16:J4 to the unresolved HPV-16 $L1_{TMV}$ on the western blot analysis of sample 4 was observed, it was speculated that not all the HPV-16 $L1_{TMV}$ precipitates with 4% PEG and hence the protocol for concentrating the HPV-16 $L1_{Tr}$ in Chapter 3 was modified to accommodate the 4% PEG treatment to precipitate the TMV from the plant extract from transient expression of HPV-16 $L1_{TMV}$.

4.3.3 Analysis of the antigenicity of HPV-16 $L1_{TMV}$ protein

In an attempt to detect and isolate the transiently expressed HPV-16 $L1_{TMV}$, twenty-five *N. benthamiana* plants were inoculated with freshly *in vitro* synthesised RNA transcript. The TMV infection symptoms were evident and the plants were harvested 14dpi. The antigenicity characterisation of the HPV-16 $L1_{Tr}$ (Chapter 3) and HPV16-16 $L1_{TMV}$ were carried out together: therefore, the negative control was the non-transgenic plant concentrate and for positive control was the baculovirus-derived HPV-16 $L1_{Bac}$. The leaf material (80g) was homogenised in high salt PBS (0.5M NaCl), strained through cheesecloth and most of the chloroplasts centrifuged out at 6000 x g. The supernatant was concentrated 65-fold by a combination of ultracentrifugation and use of a YM-30 Centricon (Amicon Separations) centrifugal filter device.

The HPV-16 $L1_{TMV}$ protein in the concentrated plant extract was captured on ELISA plates using anti-HPV-16 L1 guinea pig serum, and was probed with the a panel of characterised Mabs (see chapter 1, section 1.5.1.4, Table 1.3). The binding of the conformation-specific antibodies, H16:V5, H16:U4 and H16:E70 to the concentrate (Figure 4.8) indicated the formation of higher order L1 structures such as capsomers or VLPs.

A significant population of the transiently expressed HPV-16_{TMV} protein apparently folds correctly since all the three conformation and type specific Mabs (H16:V5, H16:E70 and H16:U4) bind the antigen. The binding of neutralising Mabs H16:V5 and H16:E70 further suggests the potential of the transiently expressed HPV-16 $L1_{TMV}$ protein to elicit a neutralising immune response. The high binding to Mab H16:I23 is surprising in comparison to that of the HPV-16 $L1_{Tr}$ and HPV-16 $L1\Delta C483_{Tr}$ (Figure 3.4, Chapter 3) but suggestive of a large proportion of denatured / unassembled particles since H16:I23 has almost half the binding affinity for intact VLPs as for free protein (Christensen *et al.*, 1996a). The higher binding of Mab H16:D9 supports the suggestion of denatured / unassembled particles of HPV-16_{TMV}.

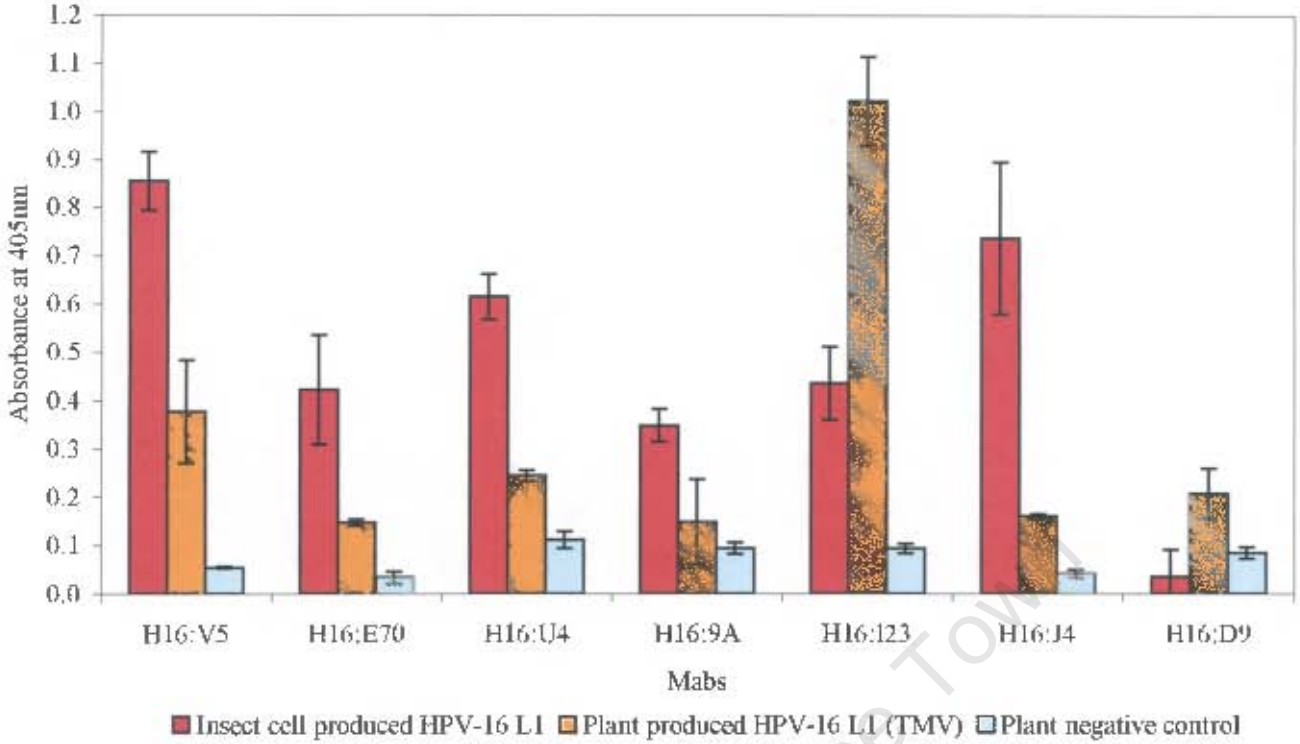

FIGURE 4.8: Monoclonal antibody characterisation by capture ELISA of the HPV-16 $L1_{TMV}$ transiently expressed in *N. benthamiana* and HPV-16 $L1_{Bac}$ expressed in insect cells by recombinant baculovirus.

4.3.4 Estimation of the HPV-16 $L1_{TMV}$ and $L1\Delta C483_{TMV}$ protein yield

The estimation of the yield of HPV-16_{TMV} was worked out in exactly the same manner as that for HPV-16 $L1_{Ty}$ and HPV-16 $L1\Delta C483_{Ty}$ in Chapter 3 (section 3.3.3). The HPV-16 $L1_{Bac}$ at a dilution of 0.13 ng/µl was used for antigenicity determination of HPV-16 $L1_{TMV}$. Therefore, based on the absorbance and dilution, an approximate concentration of HPV-16 $L1_{TMV}$ capsomers / VLPs was worked out (Table 4.1) from the binding of Mabs H16:V5, H16:E70 and H16:U4 and liner epitope recognising Mab H16:J4 to the capsomers to the HPV-16 $L1_{TMV}$.

The yield based on Mabs H16:V5, H16:E70 and H16:J4 are very similar with Mab H16:U4 indicating a slighter higher yield.

Table 3.1: Summary of calculated concentrations of HPV16 $L1_{TMV}$ capsomers based on the binding of the various Mabs to HPV-16 $L1_{Bac}$.

	Concentration of capsomers per kilogram of fresh transgenic leaf material			
Antigen	**H16:V5**	**H16:E70**	**H16:U4**	**H16:J4**
HPV-16 $L1_{TMV}$	27.7 µg	21.9 µg	36.4 µg	23.9 µg

4.3.5 Electron microscopy of the plant extracts

In the leaf sap taken from top leaves a small population of HPV-16 L1 VLPs were observed, (Figure 4.9) together with predominant aggregates of pentamers. TMV rods of two clearly different size classes were observed, 300nm and 370 nm. The presence of wild type 300nm TMV rods is indicative of recombination in the TMV population where in some cases the HPV-16 $L1_{TMV}$ gene was deleted somewhere in the cycle of replication and movement of the virus within the plants. Rabindran & Dawson (2001) reported similar recombination when they used TMV to express a smaller gene, GFP (30kDa) in *N. benthamiana*.

The concentrate was also viewed by immunotrapping the particles with Mabs H16:J4 and H16:V5. Most of the particles observed in this case were pentameric capsomers (10nm) with a few exceptions of higher ordered structures (Figure 4.10). The VLPs observed in Figure 4.10 are very similar in size and structure to those produced in insect cell by recombinant baculovirus (Figure 3.7, Chapter 3). Some of the VLPs in Figure 4.10 have possibly encapsidated some nucleic acid since the centres of the particles are not heavily stained. A similar observation was made for the VLPs from the transgenic expression of HPV-16_{Tr} in Chapter 3 (Figure 3.6).

To confirm that capsomers and VLPs did result from transient expression of HPV-16 L1 using a TMV vector, the putative HPV-16 $L1_{TMV}$ particles in the plant concentrate were immunotrapped using the anti HPV-16 guinea pig serum and probed with H16:V5 and gold-labelled secondary anti-mouse antibodies (10nm gold particles). Gold labelling of the capsomers was observed (Figure 4.11) in the concentrated plant extract; no binding was observed in the negative control, thereby confirming the expression of HPV-16 $L1_{TMV}$ capsomers by recombinant TMV based vector.

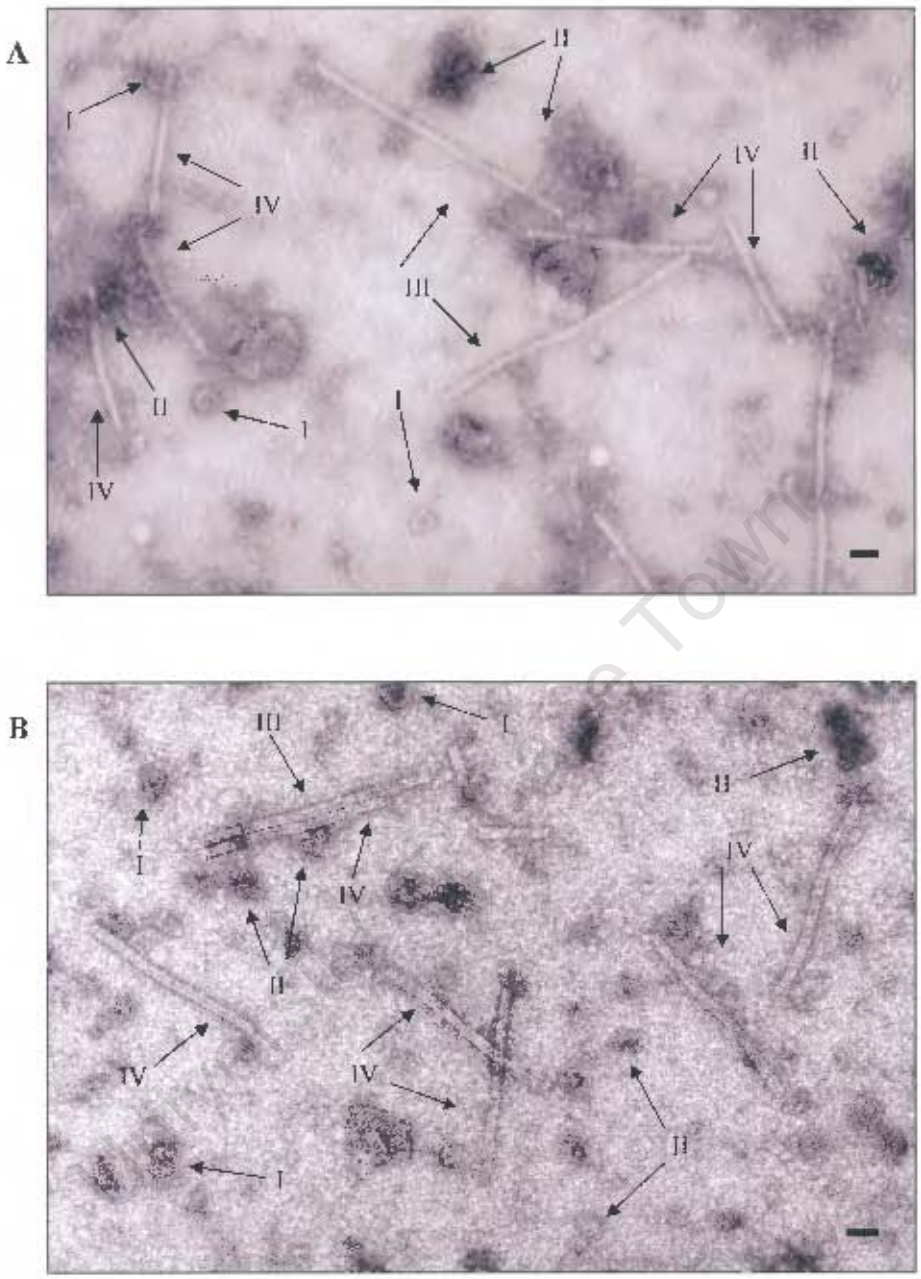

FIGURE 4.9: Electron micrographs of crude leaf extracts of top leaves (**Bar = 50nm**). TMV rods of two distinct lengths, 30nm diameter by 300 (**IV**) and 370nm (**III**) length are observed in micrographs **A** and **B**. The 300nm TMV rods represent recombined TMV where the HPV-16 L1 $_{TMV}$ gene has been deleted and 370nm TMV rods represent the recombinant TMV expressing the HPV-16 L1$_{TMV}$. Also observed in the micrographs are HPV-16 L1 $_{TMV}$ VLPs (**I**) and aggregates of pentamers (**II**) resulting from unassembled / broken down particles.

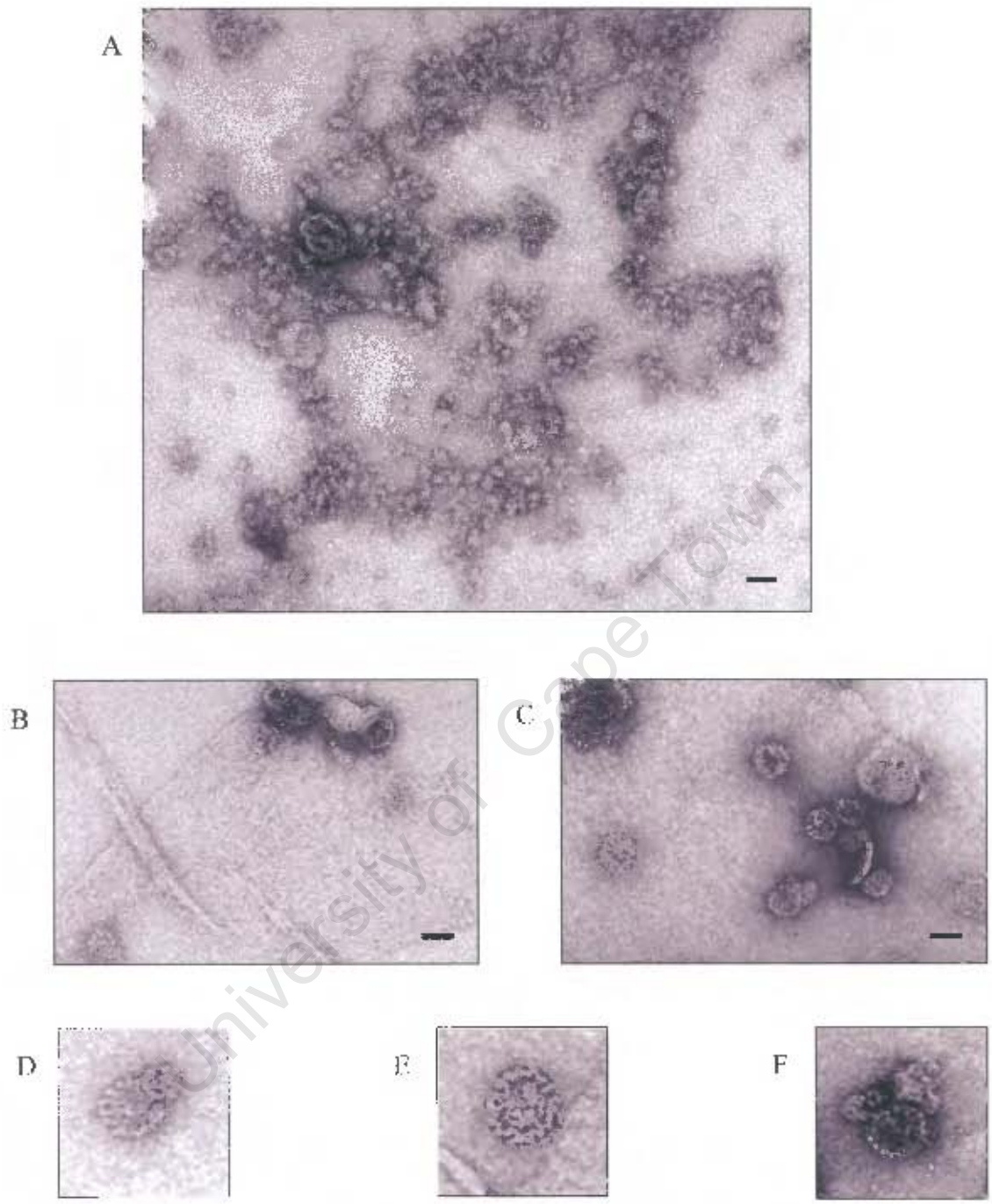

FIGURE 4.10: Electron micrographs of HPV-16 $L1_{TMV}$ VLPS and capsomers in concentrated plant extract (Bar = 50nm). Micrograph **A** shows predominantly capsomers trapped with Mab H16:J4 and micrographs **B** and **C** show VLPs trapped with Mab H16:V5. **D**, **E** and **F** show individual particles HPV-16 $L1_{TMV}$ VLPs where particles D and possibly E contain encapsidated nucleic acid.

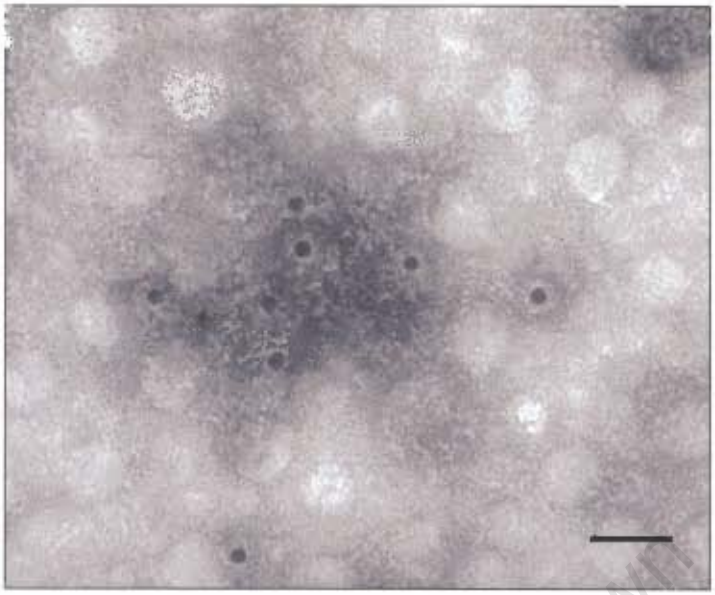

FIGURE 4.11: Electron micrograph of immunogold labelled capsomers of HPV-16 $L1_{TMV}$ that were trapped onto copper grids with anti HPV-16 L1 guinea pig serum (**Bar = 50nm**). The trapped capsomers were probed with Mab H16:V5, which was detected using secondary gold labelled goat anti-mouse antibodies. The gold particles are approximately 10nm in diameter.

4.3.6 Analysis of the immune response to plant-derived HPV-16 $L1_{TMV}$

The rabbit sera, at a dilution of 1:50, were analysed for VLP specific antibodies by indirect ELISA using baculovirus-produced HPV-16 $L1_{Bac}$ VLPs. A weak response was observed for one of the three rabbits (ID 5); however, the pre-immune serum was found cross-react with the baculovirus-produced VLPs. The response in rabbit ID 6, if any, was limited to that observed in the sera from day 67 whereas rabbit ID 4 was a non-responder. Rabbit ID 5 seems to have shown an increase in immune response to the crude plant extract containing HPV-16 $L1_{TMV}$ as a result of immunisation on days 1 and 67.

Sera from day 21 was checked by western blotting against denatured HPV-16 $L1_{Bac}$ VLPs produced in insect cells (data not shown, results similar to those shown in Figure 3.8 were obtained): two bands similar to those for the control with Mab H16:J4 were observed for sera from rabbit ID 5 and 6 binding to the denatured HPV-16 $L1_{Bac}$ protein. Serum from the prebleed bound proteins in the denatured HPV-16 $L1_{Bac}$ preparation, but not at the position of L1,

suggesting that the VLPs were not absolutely pure and that probably the bands are due to rabbit antibodies binding to denatured insect cell or baculovirus proteins.

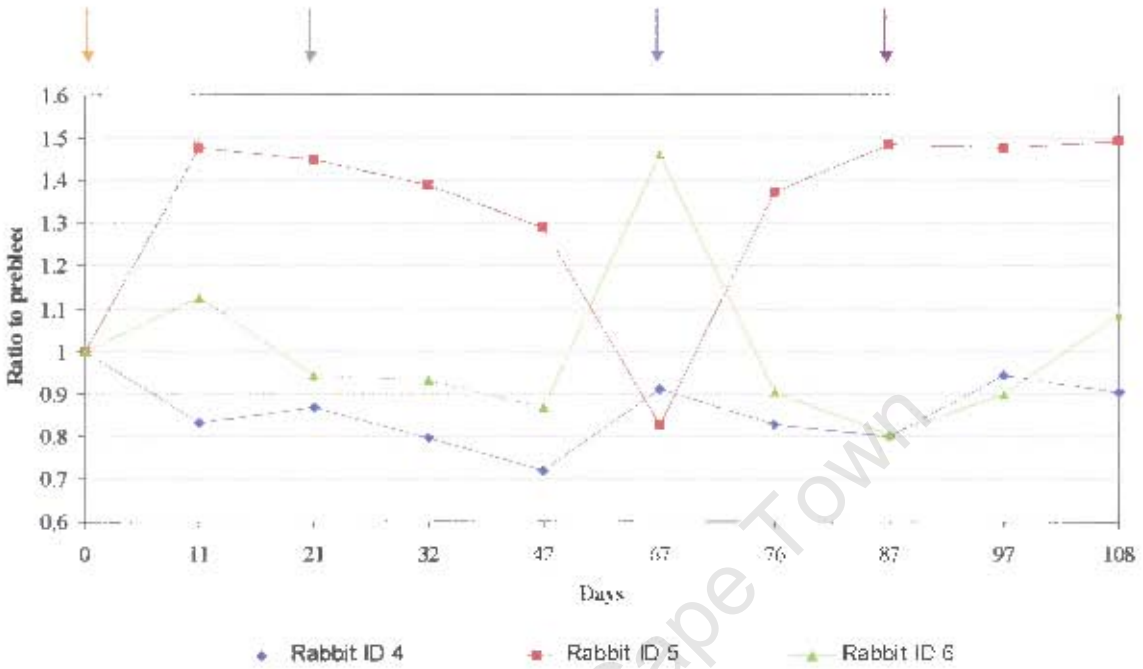

FIGURE 4.12: Analysis of sera from New Zealand white rabbits immunised with concentrated plant extract from the transient expression of HPV-16 $L1_{TMV}$. The rabbit sera were tested against recombinant baculovirus-produced HPV-16 $L1_{Bac}$ VLPs, 100µl of 1µg/ml coated per well onto ELISA plates. The rabbits were immunised on days **1**, **21**, **67** and **87**.

4.4 DISCUSSION

Plants that have been used traditionally as sources of food, fibre and fuel are now being considered as alternative factories for pharmaceutical products of interest, with low expenses, lack of contamination with animal pathogens and ease of manipulation being the chief attractions (Dixon & Arntzen, 1997). Plant viruses have shown great potential for the production of large amounts of protein in a short period of time. The use of plant virus vectors in normal plants instead of using transgenic plants for the production of foreign proteins presents several advantages, although both systems are of great value under different circumstances (Dixon & Arntzen, 1997; Scholthof, 1999). Since plant are infected after they have been grown to a certain stage with the viral vector toxicity caused by the expressed protein are reduced. Using plant

viruses for foreign protein production allows great versatility in theory, because it allows for quick turnaround time for expression and test of expression levels of various constructs and their antigenicity (Awram *et al.*, 2002). The growing interest in plant viruses as an expression system has led to the development of various vectors based on TMV, *Cow pea mosaic virus*, *Tomato bushy stunt virus*, *Alfalfa mosaic virus* and *Plum pox virus* vectors.

This chapter reports the initial attempts of using a TMV-based vector for the expression of HPV-16 L1 in *N. benthamiana*. The initial results from this study show that the HPV-16 $L1_{TMV}$ was successfully expressed transiently in *N. benthamiana* plants; however expression was at levels that were only detected when the infected plant material was concentrated. The binding of the transiently expressed HPV-16 $L1_{TMV}$ to conformation specific neutralising Mabs, H16:V5 and H16:E70 is indicative that the antigen has potential to eliciting a neutralising immune response. The detailed antibody characterisation of the HPV-16 $L1_{TMV}$ clearly indicates that antigenicity of the plant-produced L1 was very similar if not identical to that produced in insect cells by recombinant baculovirus (Figure 4.8). The data also indicates that there was a significant amount of unassembled L1, based on the binding of H16:D9, which is not known to bind intact particles, and H16:I23, which has half the binding affinity for intact VLPs compared to denatured L1 protein (Christensen *et al.*, 1996a). The EM data showed that there were an appreciable number of capsomers compared to the numbers of VLPs (Figure 4.10) and the validity of these capsomers is confirmed by the immunogold labelling with Mab H16:V5. Capsomers have been shown capable of eliciting neutralising antibodies (Fligge *et al.*, 2001; Giroglou *et al.*, 2001; Unckell *et al.*, 1997) and immunisation with pentameric L1 protein of COPV fused to GST protected beagles against COPV challenge (Yuan *et al.*, 2001). The VLPs (Figure 4.9 and 4.10) are similar in morphology to those that baculovirus produced (Figure 3.7, Chapter 3).

Gerber *et al.* (2001) demonstrated that intramuscular immunisation of mice with 0.3μg of HPV-16 L1 VLPs produced in insect cells by recombinant baculovirus elicited a strong anti HPV-16 L1 immune response. However, the immune response observed in the rabbit experiments was low (Figure 4.12, rabbit ID 5). The response was confirmed by western blot analysis where two distinct bands (data not shown), monomer and possibly trimer. These results are very similar to those describe in Chapter 3 (section 3.3.5, Figure 3.8). The weak immune response could possibly be attributed to the concentration of HPV-16 $L1_{TMV}$ in the plant extracts. The anti HPV-16 $L1_{TMV}$ immune response could potentially therefore be improved by using a plant extract that

has a higher concentration of $L1_{TMV}$ antigen: this can be done by developing a bulk extraction protocol from a larger source of infected plant material. This is very similar to the finding for the L1 produced in transgenic plants (chapter 3).

It is important to note that deletion of the foreign gene by the plant virus vectors is the main disadvantage of this system. The deletion of the foreign gene in TMV vectors has been reported (Rabindran & Dawson, 2001). Similar recombination was observed (Figure 4.9) when analysing the crude leaf sap of the top infected leaves from pBSG-L1 infection and initial. One way of getting round this would be to try inoculate the plants with very dilute infected leaf inoculum or by inoculating with a homogenous population isolated from infecting *N. tabacum* cv Xanthi where viral infection is not systemic and hence results in local lesions.

There are only three reports of complete viral proteins expressed by plant viral vectors. These are the use of TMV for production of FMDV VP1 protein (Wigdorovitz *et al.*, 1999), the expression of VP60 protein of the rabbit hemorrhagic disease virus and rotavirus VP6 protein by potyvirus based vectors (Fernandez-Fernandez *et al.*, 2001; O'Brien *et al.*, 2000), and the use of *Tomato bushy stunt virus* for the expression of HIV gp24 (Zhang *et al.*, 2000). Other than these, most of the plant virus expression has been limited to expression of epitopes either fused to the coat protein or to adjuvants. Wigdorovitz *et al.* (1999) reported an expression of 50-150µg per gram of fresh harvested leaves for the transient expression of FMDV VP1 protein by TMV. A yield of 6-80µg per gram of leaf tissue was achieved for the expression of a HVR1 R9 mimotope of hepatitis C virus fused to the C-terminus of the cholera toxin B-subunit using a TMV vector (Nemchinov *et al.*, 2000). The product yield achieved from the transient expression of HPV-16 $L1_{TMV}$ (20-30µg/kg of fresh leaf material) was almost 10 fold that from the transgenic expression of HPV-16 $L1_{Tr}$ reported in Chapter 3, yet in comparison to the expression of FMDV VP1 and HVRI R9 CTB fusion protein by TMV, the expression of the HPV-16_{TMV} is extremely low (1000 fold less). Other than the recombination observed as a result of the unstable vector, there is no obvious explanation for the lower yield. The recombination observed in the initial work with L1ΔC483 gene in *N. benthamiana* could possibly be as a result of the L1 protein accumulating in the cytoplasm. Optimising the L1 sequence towards TMV preferred codons or even plant codons might help overcome the TMV recombination problem.

In summary, this chapter describes the initial successful transient expression of HPV L1 protein in plants. The protein is capable both of assembling into vaccine-appropriate structures such as pentamers and VLPs and of the correct antigenicity. Further development of this expression system could provide a considerably cheaper means of producing HPV vaccines compared to the presently used yeast and baculovirus systems.

Chapter 5

Chimaeric HPV-16 L1 particles presenting the common neutralising epitope for the L2 minor capsid protein of HPV types 6 and 16

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ABSTRACT

Both the *Human papillomavirus* (HPV) major (L1) and minor capsid (L2) proteins have been well investigated as potential vaccine candidates. The L1 protein alone can fold into virus-like particles (VLPs) that are highly immunogenic. In this chapter the potential of using HPV-16 L1 subunits to display a well-characterised HPV-16 L2 epitope (LVEETSFIDAGAP), which was a common-neutralising epitope for HPV types 6 and 16, in various regions of the L1 structure is investigated. The L2 sequence was introduced by PCR -mediated replacement of 13 codons into sequences coding for L1 surface loops D-E (ChiΔC-L2) and E-F (ChiΔA-L2), an internal loop C-D (ChiΔH-L2), into the h4 helix (ChiΔF-L2) and between the h4 and β-J structural regions (ChiΔE-L2). The chimaeric protein product was characterised using a panel of monoclonal antibodies (Mabs) that bind to conformational and linear epitopes, as well with as a polyclonal antiserum raised to the L2 epitope. All five chimaeras reacted with the polyclonal anti-L2 serum. ChiΔA-L2, ChiΔE-L2 and ChiΔF-L2 reacted with all the L1 antibodies; ChiΔC-L2 did not bind H16:V5 and H16:E70, and ChiΔH-L2 did not bind any conformation-dependent Mab. The chimaeric particles elicited high titre anti-L1 immune responses in Balb/c mice. Of the five chimaeras tested only ChiΔH-L2 did not elicit an L2 response, while ChiΔF-L2 elicited the highest L2 response. This study provides support for the use of PV particles as vectors to deliver various epitopes in a number of locations internal to the L1 protein, and for the potential of using chimaeric PV particles as multivalent vaccines.

5.1 INTRODUCTION

Numerous serological studies have shown that there is a strong immune response to the L1 protein in humans after infection with genital HPV types. The response is long-term but type-specific (Carter *et al.*, 1996, 2000; Kirnbauer *et al.*, 1994; Wang *et al.*, 1997). Most of the neutralising epitopes previously identified are non-linear and conformation-dependent, and their surface location and amino acid (aa) composition have been partially characterised by monoclonal antibody (Mab) binding and immunisation studies. In HPV-16, the L1 residues 50, 266 and 282 are considered to be vital for the binding of the neutralising Mabs H16:V5 and H16:E70 (White *et al.*, 1999). Linear epitopes that are identified by Mabs H16:J4 (aa 261-280) and H16:I23 (aa 111-130) show weak

cross-neutralising activity for HPV types 18, 31,33,45, 55 and 59 in addition to HPV-16 (Combita *et al.*, 2002).

Population biology studies on co-existence of different serotypes of microorganisms have suggested that vaccination against a particular serotype maybe ineffective because other pathogenic serotypes may emerge as result (Lipsitch, 1997; May & Nowak, 1995; Nowak *et al.*, 1995). Antagonism between HPV types was first suggested by Evans & Bond (1992) who found a decreased risk for the development of cervical squamous intraepithelial lesion in women with a history of anogenital warts. Silins *et al.* (1999) reported on protection by HPV-6 against HPV-16 carcinogenesis. Therefore it is critical to address serotype antagonism such as the one between HPV-16 and HPV-6 from an effective vaccine perspective.

With the crystal structure of many viruses having being solved, there exists great potential in methodically designing chimaeric vaccines and using well characterised virions to deliver and present immunodominant epitopes. In this regard, plant viruses have been extensively used for the presentation of antigenic epitopes such as from HIV gp41, VP2 of canine parvovirus and mink enteritis virus, malarial B-cell epitope, outer membrane F protein peptide of *Pseudomonas aeruginosa* and the D2 peptide of fibronectin-binding protein B of *Staphylococcus aureus* (Table 1.6: Chapter 1, Section 1.6.2.8), on the virus capsid surface. Due to the recent development of the recombinant technology to express subunit vaccines, the use of human viruses for delivery and presentation of epitopes has been limited. However, with the rapid expansion in technology to solve crystal structures of viruses, there has been a drive to use this crystal structure data for multivalent / chimaeric vaccine development. Most of the chimaeric work using human viruses has been with polyomavirus (Jenkins *et al.*, 1990; Tang *et al.*, 1997), hepatitis B virus (Bisht *et al.*, 2002; Netter *et al.*, 2001; Niikura *et al.*, 2002; Pumpens *et al.*, 2002; Yim *et al.*, 1996), HIV (Deml *et al.*, 1997a, 1997b; Tang *et al.*, 1997; Wagner *et al.*, 1996) and more recently, human papillomavirus (Liu *et al.*, 2000, 2002; Muller *et al.*, 1997; Peng *et al.*, 1998; Schafer *et al.*, 1999; Slupetzky *et al.*, 2001).

There are a number of studies describing the use of papillomavirus-like particles to deliver / display foreign epitopes (Greenstone *et al.*, 1998; Liu *et al.*, 2000, 2002; Muller *et al.*, 1997; Nieland *et al.*, 1999; Slupetzky *et al.*, 2001). L1 C-terminal fusion chimaeras have worked well in presenting the epitopes of the HPV 16 E7 protein (Jochmus *et al.*, 1999; Muller *et al.*, 1997), HIV-1 p18 and HIV-1 gp120 CTL epitopes (Liu *et al.*, 2000, 2002) and HIV-1 gp41 epitopes (Slupetzky *et al.*, 2001), in HPV types 11, 16 and BPV-1. Attempts have been made to insert epitopes into the core sequences of

the PV L1 with an end result of capsomers rather than VLPs being formed (Slupetzky *et al.*, 2001). There is sufficient data in the literature to suggest that capsomers are immunogenic and capable of eliciting a neutralising immune response (Fligge *et al.*, 2001; Rose *et al.*, 1998 Yuan *et al.*, 2001); capsomers have moreover been shown to bind neutralising antibodies raised to intact VLPs (Chen *et al.*, 2000b).

A common-neutralising epitope for human papillomavirus types 6 and 16 is present between aa 108-120 (LVEETSFIDAGAP) of the HPV-16 minor capsid protein, L2 (Kawana *et al.*, 1998). Sera of mice immunised with this peptide cross-reacted with L1/L2 capsids of HPV types 6, 11 and 18 (Kawana *et al.*, 1998, 1999, 2001a). Preincubation of monkey COS-1 cells with the synthetic L2 epitope reduces the susceptibility of COS-1 cells to infection with HPV-16 L1/L2 pseudovirions (Kawana *et al.*, 2001b).

Based on the reports of cross-reactivity of the neutralising L2 epitope characterised by Kawana *et al.* (Kawana *et al.*, 1998, 1999, 2001a) and the evidence that VLPs can be used to deliver foreign epitopes (Jenkins *et al.*, 1990; Jochmus *et al.*, 1999; Liu *et al.*, 2000, 2002; Muller *et al.*, 1997; Netter *et al.*, 2001; Niikura *et al.*, 2002; Roy, 1996; Schafer *et al.*, 1999; Slupetzky *et al.*, 2001), it was decided to investigate the possibility of making chimaeric vaccine candidates with the L2 epitope displayed on HPV-16 L1 particles, which hopefully maintained the neutralising Mab H16:V5 and H16:E70–binding epitopes. Five HPV-16 L1 chimaeras were constructed that displayed the L2 epitope as replacement sequences, expressed them in insect cells using a baculovirus expression system, and characterised the chimaeric products with a panel of Mabs. This study opens various avenues for multivalent vaccine development using PV particles.

5.2 MATERIALS AND METHODS

5.2.1 Synthesis of chimaeric constructs and expression in insect cells by recombinant baculovirus

Chimaeric constructs were prepared by PCR mutagenesis of the 39 bp regions to be replaced, with the primers being designed with the 3' ends coding for sections of the L2 peptide (Table 5.1). The HPV 16 L1 504[ΔA266T] gene (see Chapter 2 for details on mutagenesis of residue 266) cloned in

the pSK plasmid (pSK 504[ΔA266T]) vector (Stratagene Cloning Systems) was used as a template. The mutagenesis was carried out using a two-step PCR system. First, a chimaeric template was generated using the Expand Long Template PCR System (Roche) that is suited for the accurate amplification of large DNA templates. The PCR mixture (50µl) was digested with *Dpn*I restriction endonuclease (to digest methylated template DNA); 5µl of this was used in the second step using *Pfu* DNA polymerase (Promega). DH5α *E. coli* competent cells were transformed with the amplified DNA and the chimaeric product was sequenced. The PCR-based strategy for the replacement of the epitope into the L1 gene is summarised in Figure 5.1.

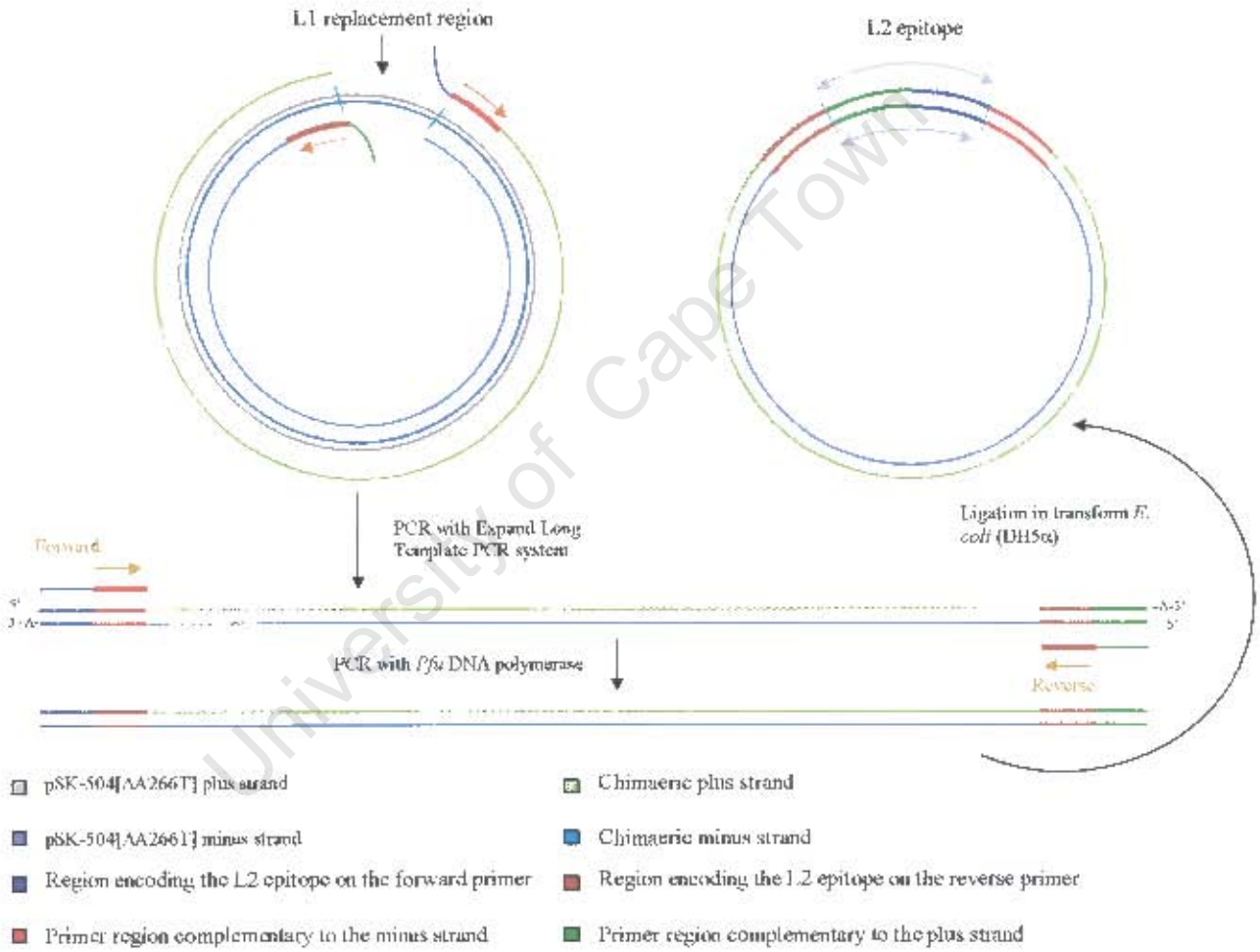

FIGURE 5.1: Schematic representation of the PCR strategy to replace the L2 epitope into the L1 sequence. Primers encoding part of the L2 epitope were used to create a chimaera template using Expand long template PCR system and the original plasmid DNA was digested with *Dpn*I. The amplified template was further PCR amplified using *Pfu* DNA polymerase and the competent DH5α were transformed with the *Pfu*-amplified chimaera.

Chimaeric construct	Nucleotide position of L2	Residue position of L2	Region	Primers used to insert the L2 epitope -LVEETSFIDAGAP *5' – TTA GTG GAA GAA ACT AGT TTT ATT GAT GCT GGT GCA CCA – 3'* *3' – AAT CAC CTT CTT TGA TCA AAA TAA CTA CGA CCA CGT GGT – 5'*
ChiΔA-L2	522 - 558	174 – 186	Loop E - F	Forward 5'- *TATTGATGCTGGTGCA*CCACCATTAGAGTTAATAAACACAGTTATTCAGG - 3' Reverse 5' - *AAACTAGTTTCTTCCACTAA*GGATCCTTTGCCCCAGTGTTC - 3'
ChiΔC-L2	393-429	131-143	Loop D - E	Forward 5'- *TTTTATTGATGCTGGTGCACCA*AGAGAATGTATATCTATGGATTACAAACAAACAC - 3' Reverse 5' - *CTAGTTTCTTCCACTAA*TTCTGTGTCATCCAATTTATTTAATAAAGGATGGCCACTAATG - 3'
ChiΔE-L2	1293-1329	431-443	Coil between h4 and β-J	Forward 5'- *TATTGATGCTGGTGCACC*ATACACTTTTTGGGAAGTAAATTTAAAGGAAAAG - 3' Reverse 5' - *AAACTAGTTTCTTCCACTAA*TTTTTGACAAGCAATTGCCTGGG - 3'
ChiΔF-L2	1242-1278	414-426	h4 helix	Forward 5'- *TTTTATTGATGCTGGTGCACCA*GCTTGTCAAAAACATACACCTCCAGCACCT - 3' Reverse 5' - *CTAGTTTCTTCCACTAA*TGTGCCTCCTGGGGGAGGTTGTAGACC - 3'
ChiΔH-L2	243-279	81-93	Loop C - D	Forward 5' - *TATTGATGCTGGTGCACCA*GATACACAGCGGCTGGTTTGGGC - 3' Reverse 5' - *AAACTAGTTTCTTCCACTAA*GGGGTCAGGTAAATGTATTCTAAATACCCTG - 3'

TABLE 5.1: Summary of chimaeric constructs, the regions of L2 display and the primers used in their construction. The L2 epitope coding region on the primers is indicated by the underlined text.

The L1 chimaeric constructs were expressed in insect cells using the Bac-to-Bac® baculovirus expression system (Life Technologies). The chimaeras were cloned into pFastbac1 vector (*Sal*I / *Xba*I restriction sites). The DNA from these clones was used to transfect DH10bac *E coli* cells to prepare bacmid clones. The bacmid DNA was transfected into *sf21* insect cells using Cellfectin (Life Technologies). The modified Bac-to-Bac® protocol described in Chapter 2 was followed to amplify the recombinant virus and to infect the *sf21*insect cells for expression of the chimaeric VLPs.

5.2.2 Preparation of chimaeric HPV-16 L1 particles

The infected insect cells were spun down at ~3 000 x g, resuspended in high salt phosphate buffered saline (PBS with 0.5M NaCl) and sonicated 4 times at 5-second intervals. The sonicated material was overlaid onto a 40% sucrose cushion and pelleted at 114 000 x g for 3 hrs. The pellet was resuspended in CsCl buffer (PBS with 0.4g/ml CsCl) with sonication (4 times at 5 second intervals). The suspension was centrifuged at 114 000 X g at 10°C for 24 hrs in a Beckman SW50.1 rotor. 500 µl fractions were collected and analysed by indirect ELISA using Mab H16:J4 (binds linear epitope 261 – 280; (Christensen *et al.*, 1996a). Fractions that bound Mab H16:J4 were pooled and dialysed against PBS at 4°C overnight.

5.2.3 Expression and purification of HPV-16 L2 protein in *E. coli*

The HPV-16 L2 gene was amplified by PCR from a HPV 16 L2-E2-E7 construct (provided by John Schiller, NIH) and cloned into the pProEx^{TM} HT Prokaryotic Expression Vector (Life Technologies). Competent DH5α *E coli* cells were transformed and protein expression was induced as per manufacturer's recommendations. The induced cells were pelleted and lysed (50mM Tris-HCl, pH 8.5, 5mM 2-mecaptoethanol, 1mM PMSF, 10µg/µl lysozyme) on ice for half an hour. Triton-X 100 (1% final concentration) was added and incubated at 37°C, the nucleic acid was digested using 5µl DNAse I (1mg/ml) and 5µl RNAse A (10mg/ml) per 1 ml lysis mix at room temperature for 30 min. The L2 protein (insoluble) was pelleted at 12 000 x g for 5 minutes and resuspended in a 50mM Tris-HCl pH8.0, 5mM 2-mercaptoethanol, 10mM EDTA pH8.0, 0.5% Triton-X 100 buffer. The L2 protein was pelleted at 12 000 X g and resuspended in PBS.

5.2.4 Western blot analysis of the chimaeric protein product

The chimaeric protein was denatured for 10 minutes at 100°C in SDS disruption solution (containing reducing agent β-mercaptoethanol). The denatured chimaeric protein was resolved on a 10% SDS PAGE gel. The resolved gel was transferred onto nitrocellulose membrane by semi-dry electrophoresis (BioRad) for 25mins at 25V. The membrane was blocked using 1% non-fat milk for 2 hrs and incubated with primary antibody at a dilution of 1:1000 overnight at 4°C. The membrane was washed with PBS/0.05% Tween-20 and probed with alkaline phosphatase-labelled secondary antibody diluted 1:2000, for 1 hr at room temperature. Reaction was detected colorimetrically using 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) and 4-nitro blue tetrazolium chloride (NBT) substrates.

5.2.5 Antibody characterisation of chimaeric particles by ELISA

A dilution series of each of the chimaeric protein products was tested against Mab H16:J4, and dilutions that gave similar absorbance by ELISA were selected in order to normalise the concentrations. The normalised dilutions were used for further antibody characterisation by ELISA. The chimaeric protein was coated onto ELISA plates overnight at 4°C. The plates were washed 4 times with PBS and blocked with 1% non-fat milk. The monoclonal and polyclonal antibodies (see Table 1.3, Chapter 1) were diluted at 1:500 in PBS/milk and allowed to bind to the chimaeric proteins for 2 hrs at room temperature followed by 4 stringent washes. The bound primary antibody was probed with the relevant alkaline phosphatase labelled secondary antibody (goat anti-rabbit and goat anti-mouse). The binding was detected using p-nitrophenyl phosphate (Sigma) and the ELISA plate absorbance was read at 405nm.

5.2.6 Transmission electron microscopy of chimaeric particles

The chimaeric particles were viewed either after direct adsorption onto carbon coated copper grids, or after immunotrapping with preadsorbed H16: J4 Mab (binds linear epitope aa 261-280) at a dilution of 1:50. The grids were stained with 2% uranyl acetate and viewed using a JEOL 200CX transmission electron microscope.

5.2.7 Immunisation of mice with chimaeric protein particles

Six groups of 5 mice were used for this study. The 8-week-old female BALB/c mice were immunised with chimaeric protein (100μg) at 3 subcutaneous sites with Freund's incomplete adjuvant (1:1). The immunisations were done at 0, 2 and 4 weeks, and the sera (200μl) collected at weeks 0 (pre-immunisation), 1, 3 and 5.

5.2.8 Serum analysis for immune response to HPV-16 L1

ELISA plates were coated overnight at 4°C with 100μl of baculovirus-produced native HPV-16 L1 VLPs at a concentration of 1μg/ml. The plates were washed 3 times with PBS, blocked with 1% non-fat milk (300μl) in PBS and incubated at room temperature for 2 hours. After washing the ELISA plates were probed with serum (that had been cross absorbed with a sonicated mixture of *Sf*21 cells infected with GUS expression baculovirus) 100μl diluted in 1% non-fat milk, and incubated for 2 hours. Anti-mouse–HRP conjugate (DAKO; 100μl diluted 1:2000 in 1% non-fat milk) was added to the ELISA plates after washing four times with PBS. The plates were incubated for an hour at 37°C and washed four times with PBS. 100μl per well of 3,3',5,5'-tetramethyl-benzidine substrate (TMB Microwell Peroxidase Substrate System, KPL) was added, and plates allowed to react in the dark for 15 minutes. The reaction was stopped with addition of 100 μl of 0.5M H_2SO_4 / well and the optical density were read at 450nm.

5.2.9 Serum analysis for immune response to HPV-16 L2 protein

The L2 protein was denatured for 10 minutes at 100°C in SDS disruption solution and resolved by SDS-PAGE on a 10% gel. The resolved proteins were transferred onto nitrocellulose membrane by semi-dry electrophoresis (BioRad) for 45mins at 17V. The membrane was blocked using 1% non-fat milk protein for 2 hrs and incubated overnight at 4°C with L2 polyclonal antibody (anti-peptide aa 108-120) at a dilution of 1:1000 for the positive control, and 1:100 for the mouse sera, to detect the L2 epitope immune response. The membrane was washed with PBS/Tween and probed with alkaline phosphatase labelled secondary antibody at 1:5000 for 1 hr at room temperature. This was detected by a colorimetric reaction using NBT and BCIP.

5.2.10 Structural modelling of chimaeric particles

The chimaeric proteins were modelled using SWISS-MODEL which is a fully automated protein structure homology-modelling server, accessible via the ExPASy web server (Guex & Peitsch, 1997; Peitsch, 1996; http://www.expasy.org/swissmod/SWISS-MODEL.html). The SWISS-MODEL software aligns the sequence using BLASTP2, SIM and ProModII, the comparative modelling is done by ProMod and ProMod II, energy minimisation using Gromos96. The atomic structure of HPV-16 L1 (PDB ID 1L0T) was used as a template for all the chimaeric structure modelling.

The SWISS-MODELed proteins were assembled into particles using VIPER oligomer generator and analysed using VIPER to assess the inter subunit contacts (Reddy *et al.*, 2001; http://mmtsb.scripps.edu/viper/oligomer_new4.html). VIPER analysis identifies and tabulates the contacting residues at the various subunit interfaces, in the quaternary structure based on simple distance criteria where two residues are considered to be in contact, if the distance between the CB-atom of side chain atoms falls within the distance limits obtained based on the structures available in the PDB file. These contacts are classified based on the nature of the contacting residues i.e polar, hydrophobic, acidic or basic. The VIPER generated VLPs were edited in MolMol software (Koradi *et al.*, 1996) and the structural images were drawn and rendered using PyMOL software (DeLano Scientific, Inc).

5.3 RESULTS

5.3.1 Synthesis and expression of chimaeric constructs

The solving of the crystal structure of the mutant HPV-16 L1 (L1ΔN10) by Chen *et al.* (2000) has opened up various avenues for the design of novel chimaeric HPV vaccines. Five regions of the L1 structure identified as having potential for displaying the L2 epitope were selected for this study (Table 5.1). The neutralising L2 epitope sequence was introduced by a PCR-based strategy (Figure 5.1), involving replacing 13 codons in the L1 gene sequences (Table 5.1) coding for surface loops D-E (ChiΔC-L2) and E-F (ChiΔA-L2), an internal loop C-D (ChiΔH-L2), the h4 helix (ChiΔF-L2) and the region between the h4 helix and β-J sheet (ChiΔE-L2). The C-D loop is not surface-exposed in VLPs and would only putatively be exposed in pentameric capsomers. Multiple sequence alignment shows that the C-D loop and the h4 helix are highly conserved between PV types (Figure 5.3).

Secondary structure prediction data indicated that the h4 helix would be destroyed by substitution of the L2 epitope into this region. Models illustrating the display of the L2 epitope at various positions are shown in Figure 5.3 based on the solved atomic structure of HPV-16 L1 (Modis *et al.*, 2002). In brief, the L2 sequence in ChiΔA-L2 is potentially displayed on the outer rim of the pentamer head, ChiΔC-L2 on the pentamer head, ChiΔE-L2 and ChiΔF-L2 on the C-terminal arm and ChiΔH-L2 on an internal loop at the base of the pentamer.

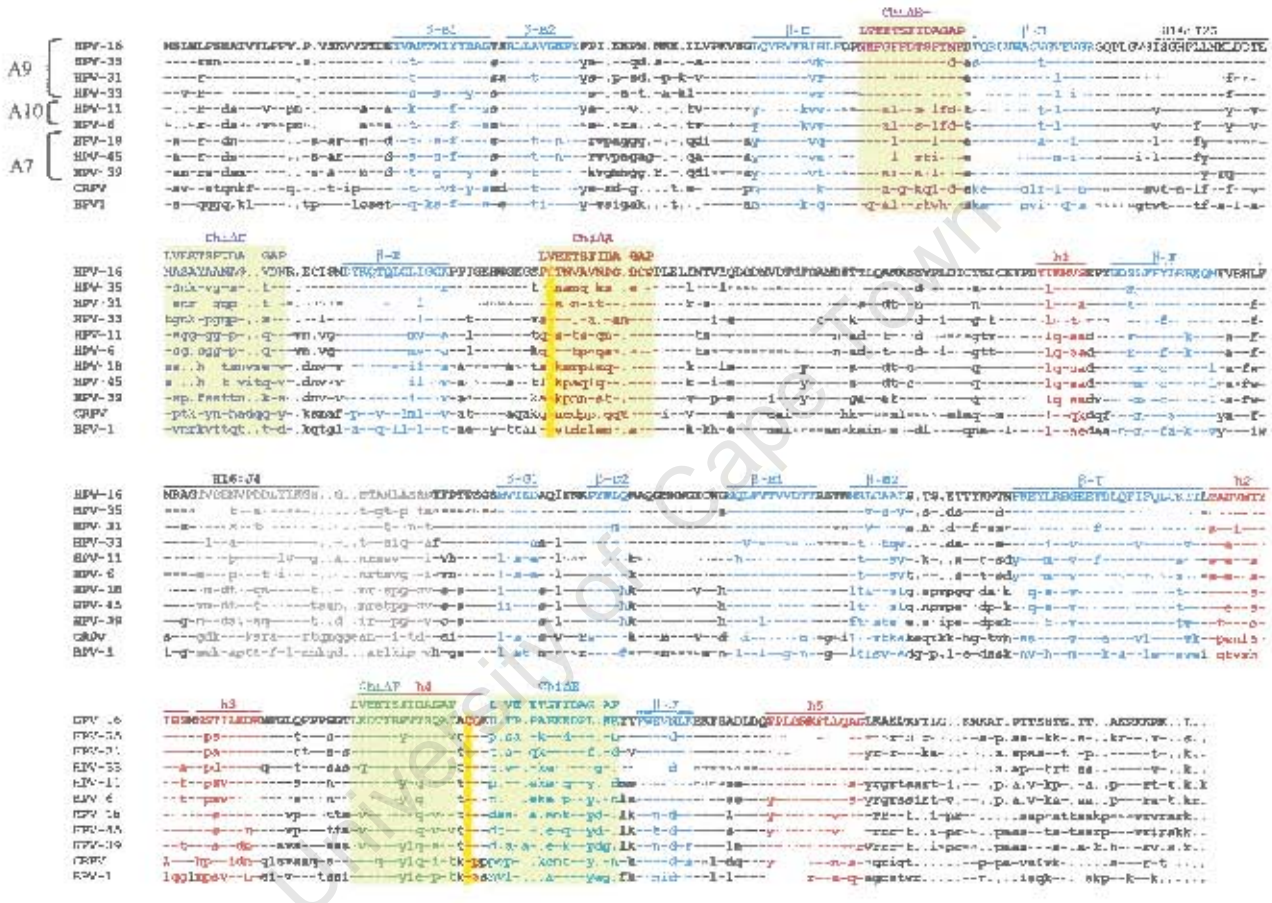

FIGURE 5.2: Sequence alignments of major capsid protein of nine HPV types from groups from groups A7, A9 and A10 and BPV-1 and CRPV. Alignments conducted using the DNA software analysis program DNAMAN (Lynnon BioSoft, Quebec, Canada) The secondary structural elements identified from the crystal structure of N-terminal truncated HPV-16 L1 (ΔN10L1) are shown about the sequence; β-sheets are represented in blue and α-helices in red. The conserved cysteine residues 175 and 428 are highlighted in yellow. The regions where the L2 epitope were replaced with the HPV-16 L1 sequences are highlighted for each chimaera.

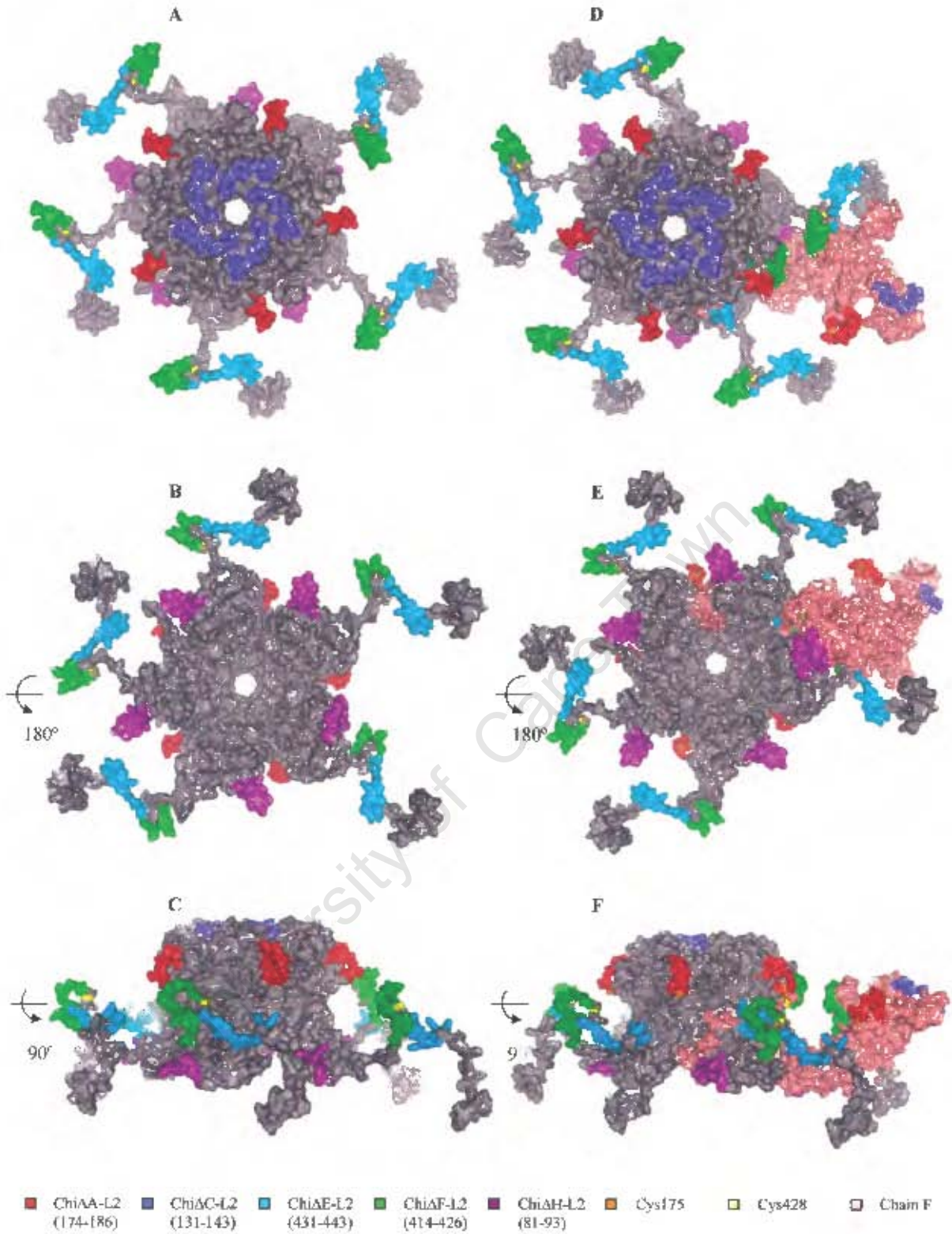

FIGURE 5.3: Structural models of pentamers HPV-16 L1 highlighting the regions where the L2 epitope was replaced with HPV-16 L1 sequence and the conserved cysteines (175, 428). The models are based on the atomic model of HPV-16 L1 by Modis *et al.* (2002) and drawn using the PyMOL structural software (DeLano Scientific). Models **D, E** and **F** show the interaction of a subunit from a neighbouring pentamer. Models **A** and **D** show the top surface of the pentamer; **B** and **E** show the pentamers rotated at 180° along the X axis; **C** and **F** show the rotation of 90° along the X axis.

The chimaeric constructs were successfully expressed in insect cells using recombinant baculoviruses, and purified using CsCl gradient centrifugation. Fractions were analysed by ELISA using H16:J4 Mab, since the epitope that binds this Mab was not altered in any construct. Positive fractions were pooled and dialysed for further analysis. The sizes of the chimaeric L1-L2 protein products - predicted as 55KDa - were confirmed by western blot analysis using H16:J4 Mab (Fig 5.4).

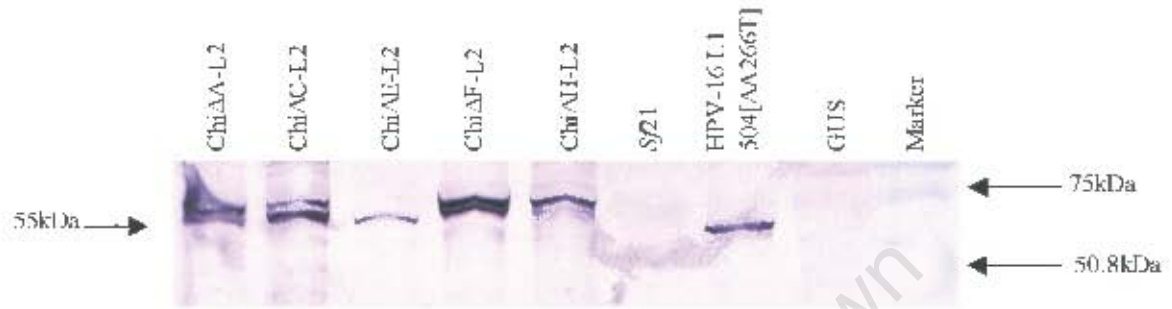

FIGURE 5.4: Western blot analysis of chimaeric protein products using H16:J4 Mab. A ~55kDa protein product was observed similar to that of HPV-16 L1 504[AA266T].

5.3.2 Antigenic characterisation of the chimaeric L1 protein products using Mabs

The ELISA binding results for purified chimaeric proteins with a panel of characterised Mabs (Christensen *et al.*, 1996a, 2001; Roden *et al.*, 1997; White *et al.*, 1999) are summarised in Figure 5.5. All the chimaeric particles/proteins bound the antibodies specific for the linear epitopes (H16:J4, aa 261-280; H16:D9, unknown; and H16:I23, 111-130). All the conformationally dependent "neutralising" Mabs (H16:V5, H16:E70, H16:U4 and H16:9A) reacted with particles of chimaeras ChiΔA-L2, ChiΔF-L2 and ChiΔF-L2. Of the conformationally dependent Mabs, particles of chimaera ChiΔC-L2 recognised only H16:U4 and H16:9A, indicating the disruption of the V5 and E70 "neutralising" epitopes. Particles of ChiΔH-L2 showed very weak binding to conformation-dependent Mabs, and strong binding to H16:D9, which predominantly binds denatured HPV-16 L1 VLPs (Christensen *et al.*, 1996a). These data indicated that the protein aggregation products that resulted from ChiΔH-L2 were either unstable and/or misassembled.

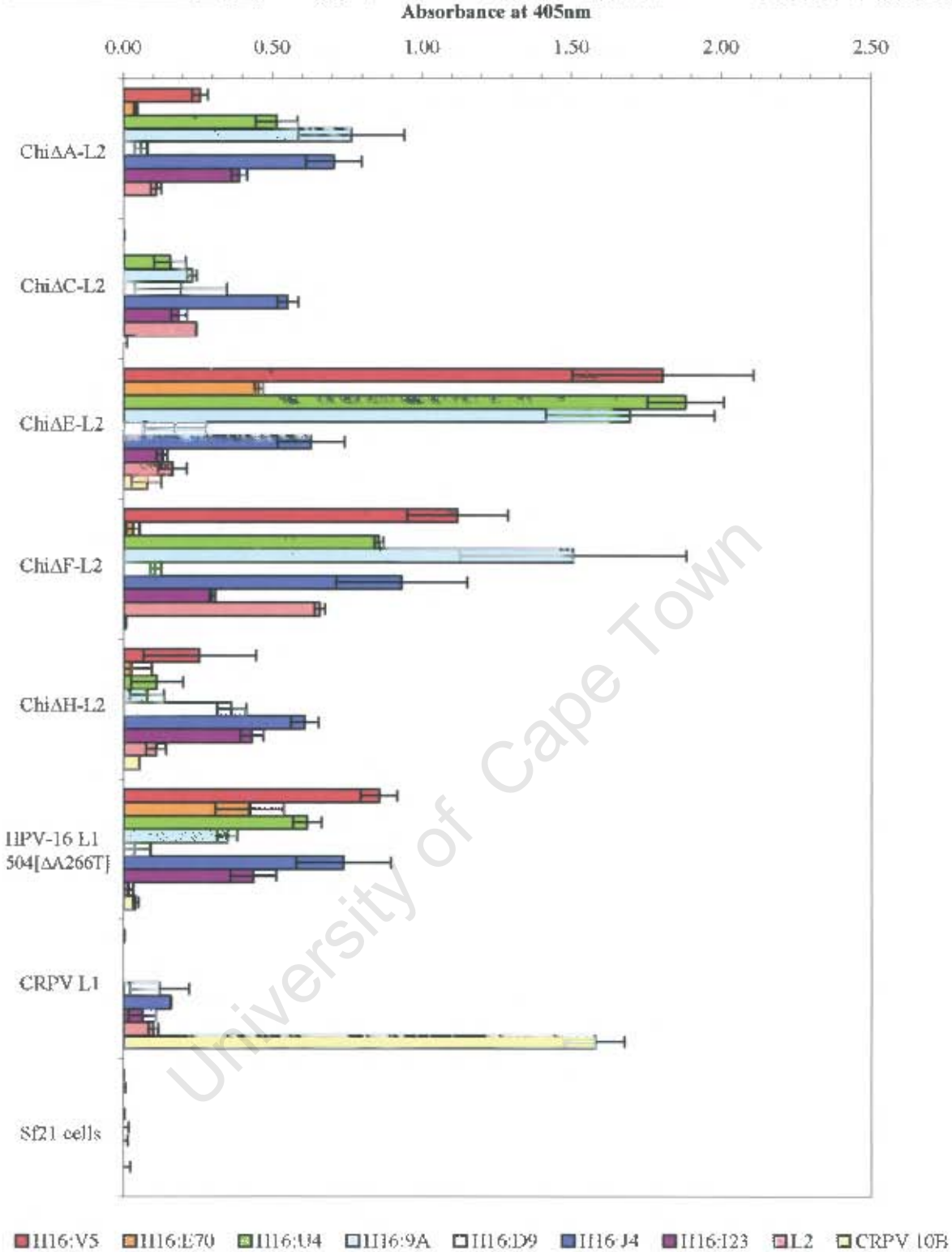

FIGURE 5.5: Antibody-binding characterisation of the chimaeric L1 and HPV-16 L1 504[ΔA266T] purified gene products using a panel of Mabs and a polyclonal anti L2 epitope antibodies. CRPV L1 was used as a negative control.

The polyclonal antiserum (provided by Dr N Christensen, Penn State University) raised in rabbits to the L2 epitope peptide (aa 108-120) bound all the chimaeric particles (Figure 5.5), indicating that all the HPV-16 chimaeras displayed the L2 epitope. ChiΔF-L2 had the highest binding affinity, indicating that the L2 epitope in the L1 sequence region aa 414-426 is perhaps the most highly exposed of all the constructs.

5.3.3 Transmission electron microscopy of chimaeric particles

Chimaeric particles made from constructs ChiΔC-L2 and ChiΔE-L2 formed recognisable VLPs and capsomers, whereas the rest of the chimaeras apparently only formed capsomers and amorphous aggregates (Figure 5.5). In the case of ChiΔF-L2 and ChiΔH-L2, particles that resembled VLPs were observed on only two micrographs: therefore, it is possible that these are potentially artefacts rather than true VLPs.

5.3.4 Immunisation of BALB/c mice with chimaeric particles and analysis of immune response

The immune response to the linear L2 epitope was analysed by western blotting. The strongest response was obtained from mice immunised with ChiΔF-L2 (Figure 5.7). The C-terminal arm of the L1 is exposed on the surface of the HPV virion, near the outer rim of the pentamer and hence thought to be highly immunogenic. ChiΔC-L2 and ChiΔE-L2 had a slightly higher L2-epitope response than ChiΔA-L2. This correlates well with L2 antibody binding, where ChiΔF-L2 had the highest affinity followed by ChiΔE-L2, ChiΔC-L2 and ChiΔA-L2 (Figure 5.5). The immune response from the L2 epitope in ChiΔH-L2 was barely detected, suggesting that the L2 epitope was not well presented on this chimaera. This could potentially be attributed to the C-D loop being an internal loop in HPV-16 L1 particles (Figure 5.2).

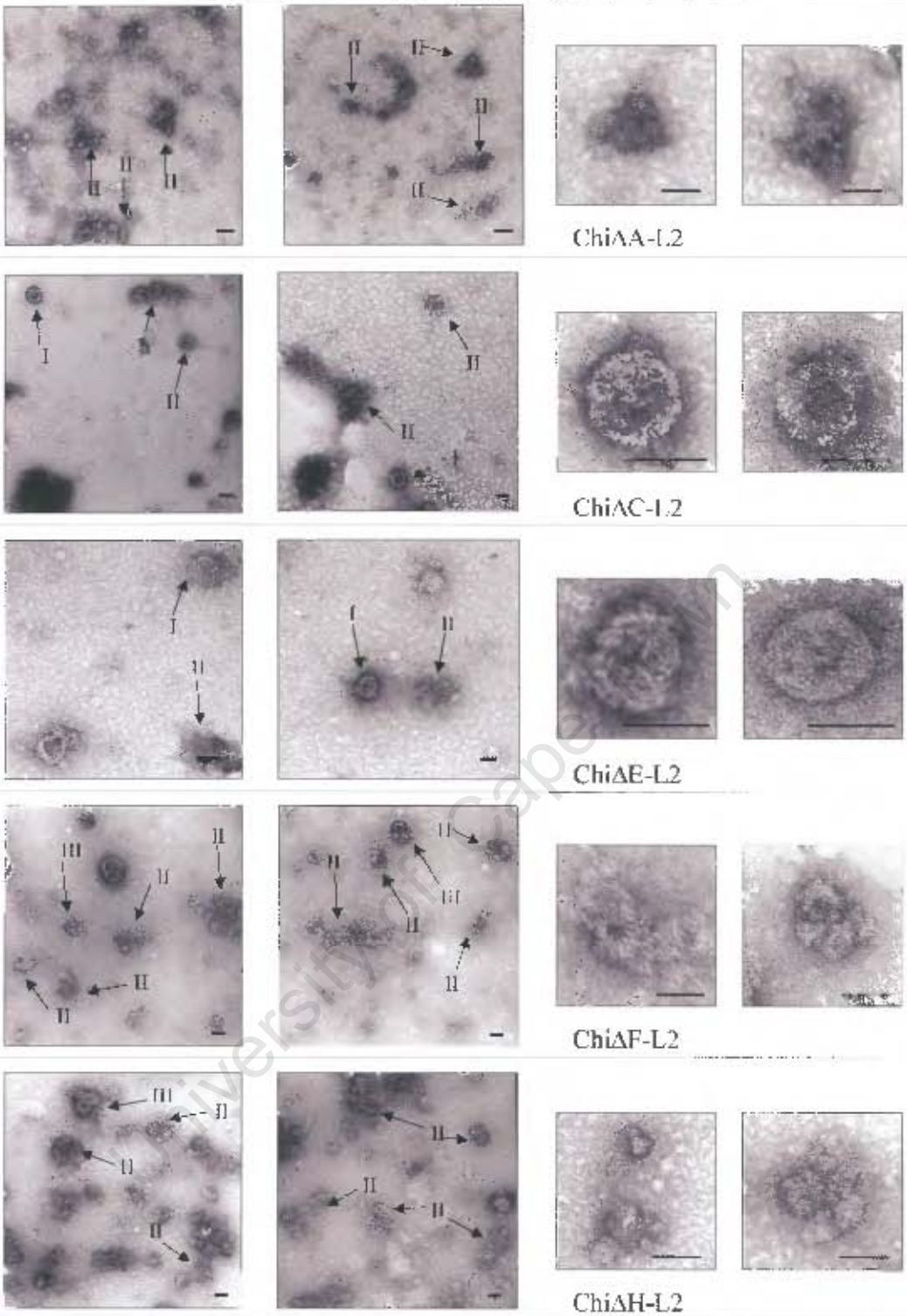

FIGURE 5.6: Electron microscopy of chimaeric particles trapped with Mab H16:J4 onto carbon-coated copper grids and negatively stained with 2% uranyl acetate (**Bar = 50nm**). Enlargements on the right show individual particles for ChiΔC-L2 and ChiΔE-L2; particles in state of disassembly for ChiΔF-L2; pentameric aggregates for ChiΔA-L2 and ChiΔH-L2.
I: VLPs
II: Aggregates of pentamers
III: Possibly pentamers

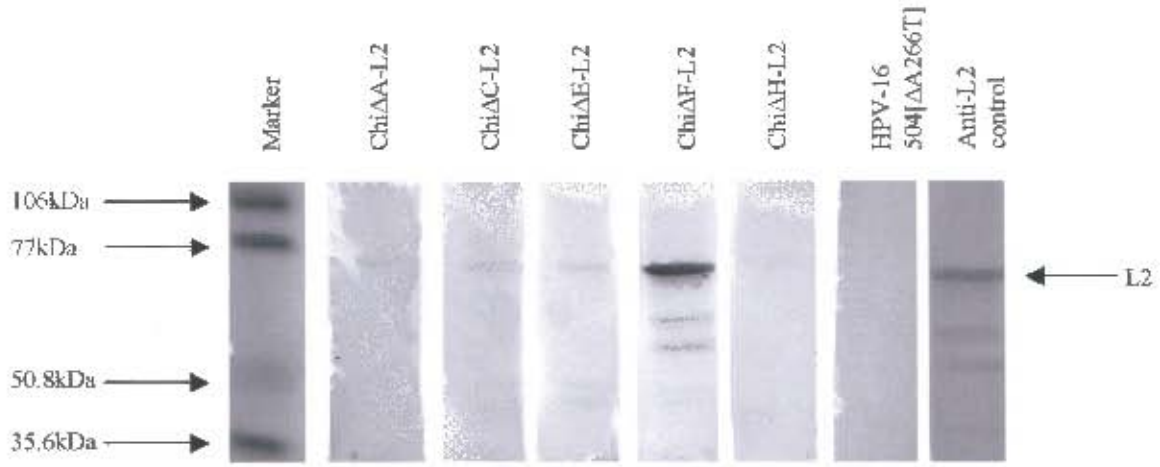

FIGURE 5.7: Western blot analysis of sera from Balb/c mice immunised with the various chimaeric proteins and HPV-16 L1 504[ΔA266T] VLPs against HPV-16 L2 protein expressed in E. coli.

The results above indicated that four of the chimaeras were capable of binding one or more conformation-specific neutralising Mabs. It was important first to determine if these particles could induce an HPV-16 L1 immune response, and second, if the L2 epitope displayed by the chimaeras was immunogenic.

Analysis of sera from mice immunised with the chimaeric particles indicated that there was a strong immune response (titres ≥102400) to HPV-16 L1 VLPs: end point titration data are shown in Figure 5.8. Particles of ChiΔE-L2 induced the strongest immune response (Figure 5.8), similar to that elicited with native HPV-16 L1 VLPs (titres ≥ 819200), followed by ChiΔF-L2 (titres ≥ 204800), then ChiΔA-L2, ChiΔC-L2 and ChiΔH-L2 with similar end point titres (≥102400). This data correlates well with the antibody characterisation data (Figure 5.5).

It is interesting to note that the L1-specific immune response to ChiΔC-L2 is much lower than that of ChiΔE-L2, yet both of these assemble recognisable VLPs. It is speculated that this is due to the structural alteration of the immunodominant region (V5 and E70 epitopes).

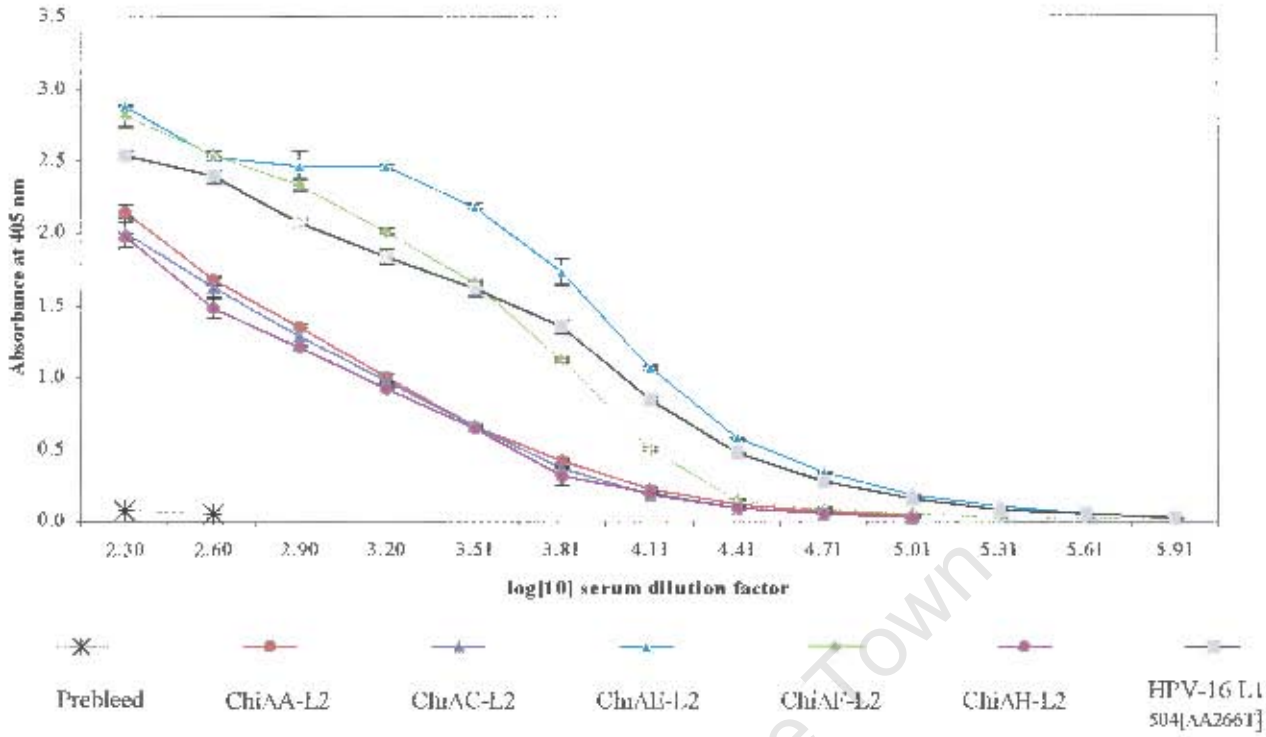

FIGURE 5.8: End point titrations of sera from Balb/c mice immunised with chimaeric proteins and HPV-16 L1 504[ΔA266T].

5.3.5 Structural modelling of chimaeric protein

The chimaeric proteins were modelled to address the antigenicity results, especially for ChiΔC-L2 and ChiΔH-L2. The modelling was carried out using the Web – based SWISS-MODEL which uses BLASTP2 to align the similarities of target sequence with sequences of known structure (atomic structure of HPV-16, 1L01.pdb) and ProModII to generate the models which are subjected to energy minimisation using GROMOS96. The modelled proteins together with the reference HPV-16 L1 were subjected to VIPER analysis and assembly to determine the intersubunit contacts.

The predicted contact differences between the HPV-16 L1 and the chimaeric proteins are summarised in Appendix D (Tables D.1-D.5) and the diagrams of the modelled and assembled chimeric and HPV-16 L1 particles are shown in Figures 5.9-5.13. The subunit numbering for the contacts is shown in Figure D.1 (Appendix D). The HPV-16 L1 sequence (see chapter 2) was used as a reference sequence where the residue 266 is an alanine. Therefore, for all the chimaeric models, the difference as a result of residue 266 is shown in the contact tables in Figures 5.9-5.13

and annotated with a symbol (*). The L2 epitope in all the modelled chimaeras was surface exposed with the greatest exposure seen for ChiΔE-L2 and ChiΔF-L2 (Figures 5.11-5.12).

The effect of loss of the disulphide bond in ChiΔA-L2 where the cysteine 175 is replaced with valine is shown in the Figure 5.9. The most obvious change in the predicted interactions of the L2 epitope replaced in the E-F loop (residues 163-242) of ChiΔA-L2 is with the h4 helix (residues 414-429) and the H-I loop (residues 348-360).

The L2 epitope in the D-E loop (residues 110-149) is not predicted to have any impact on the residues 50, 266 or 282, which remain accessible according to the model of ChiΔC-L2 in Figure 5.10. Therefore it is difficult to address the loss in binding of ChiΔC-L2 to Mabs H16:V5 and H16:E70. The predicted contact data for ChiΔC-L2 shows that residues S280 and T283 have the interactions with D142 changed from polar-acidic to polar-hydrophobic; this could potentially affect the binding of the Mabs H16:V5 and H16:E70 to residue S282.

No major differences were predicted for ChiΔE-L2 (Table D.3, Appendix D), where the coil between the h4 helix and β-J sheet has major interactions with the E-F loop (residues 163-232) and h1 helix (residues 234-239).

In ChiΔF-L2 (Table C.4), the major interactions of the h4 helix (414-429) are with the B-C loop (residues 50-67), and apart from the loss of the interaction of the L426:C175 (H-H) interaction between the A1:F1 subunits (Table D.3, Appendix D), there is no gross conformational change predicted.

The model of ChiΔH-L2 (Figure 2.13) where the C-D loop (79-94) interactions only occur between subunits of the neighbouring pentavalent pentamer, shows is a slight change predicted in the interaction with the h3 helix (residues 395-402), but gross changes in C-D:C-D loop interactions (Figure D.2, Appendix III for more illustrations of this interaction). Since none of the key Mab H16:V5 and H16:E70 binding epitopes are affected in the modelled structure of ChiΔH-L2 it is not possible to directly address the antigenicity result data for ChiΔH-L2 where no conformation-specific Mabs bound the protein product however, the trace log of the SWISS MODELing (Table D.6, Appendix D) does indicate a bad loop modelling.

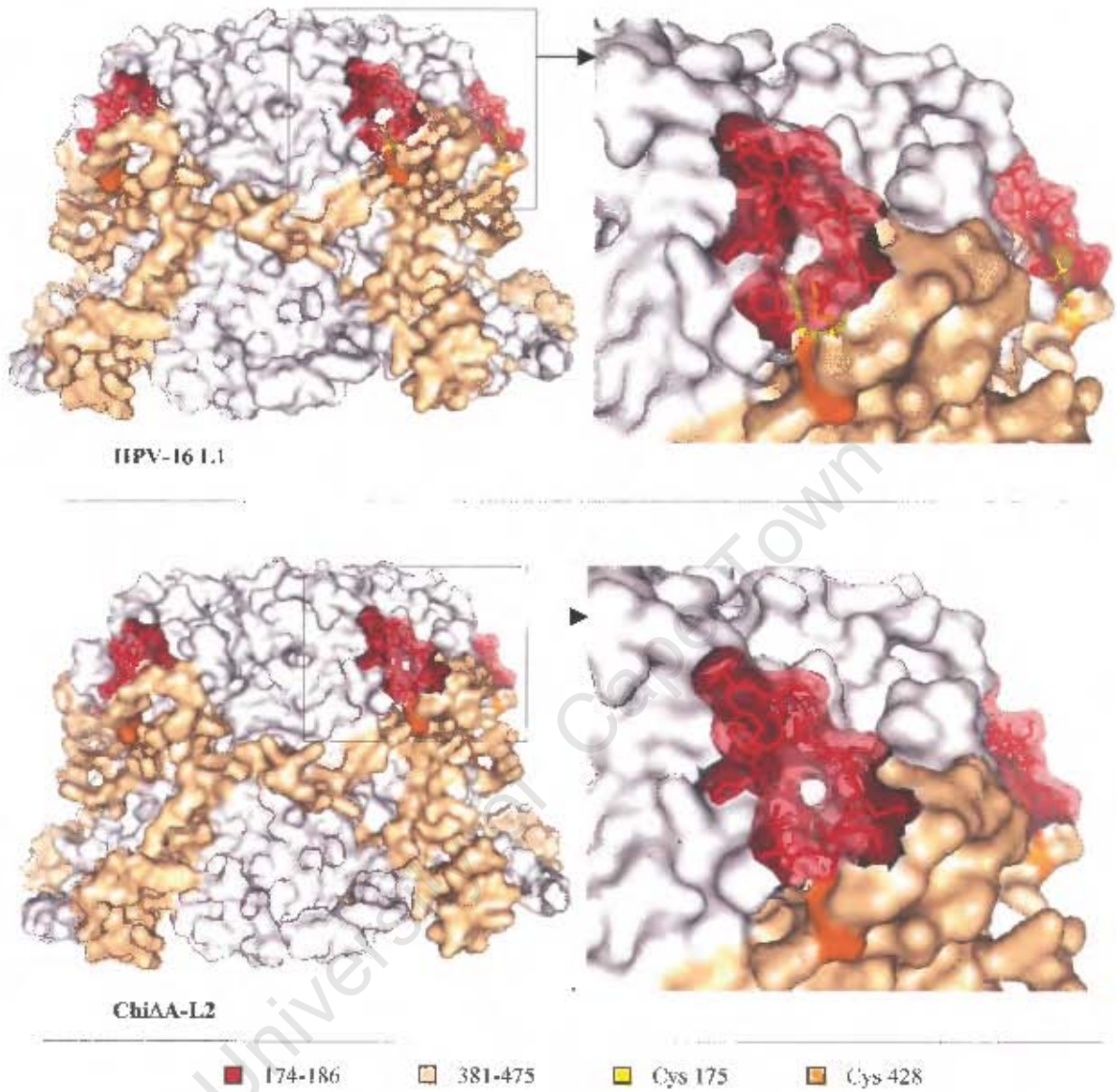

FIGURE 5.9: Structural diagrams of ChiAA-L2 and HPV-16 L1 protein modelled using SWISS-MODEL (Guex & Peitsch, 1997, Peitsch, 1996) and assembled using VIPER (Reddy *et al.*, 2001) oligomer generator version 4 highlighting the residues 174-186. The contacts of the assembled particle were calculated using VIPER analysis tool (see appendix D for subunit numbering and contacts).

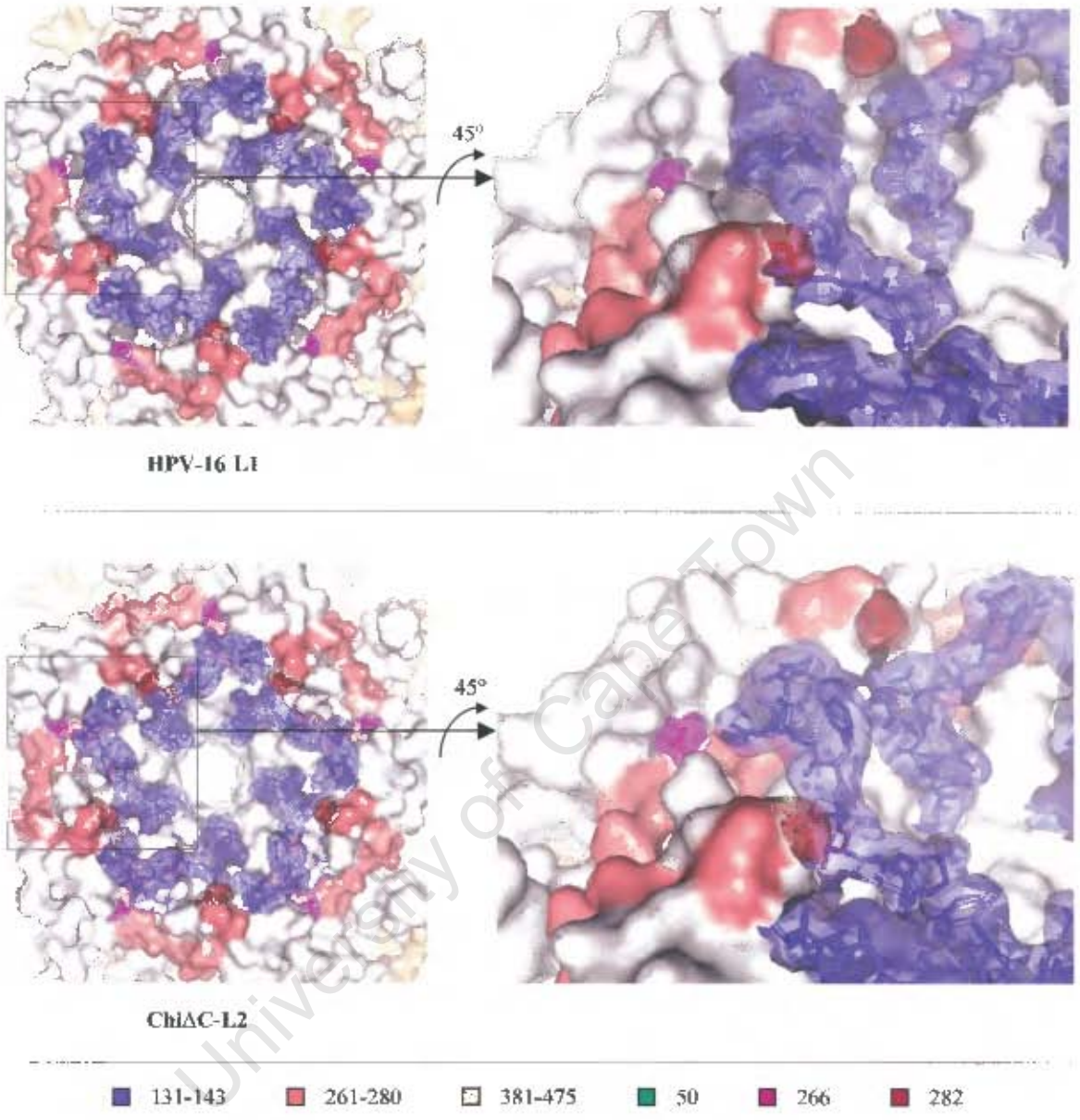

FIGURE 5.10: Structural diagrams of ChiΔC-L2 and HPV-16 L1 protein modelled using SWISS-MODEL (Guex & Peitsch, 1997, Peitsch, 1996) and assembled using VIPER (Reddy *et al.*, 2001) oligomer generator version 4 highlighting the residues 131-143 and the F-G (261-280) loops. The contacts of the assembled particles were calculated using VIPER analysis tool (see appendix D for subunit numbering and contacts).

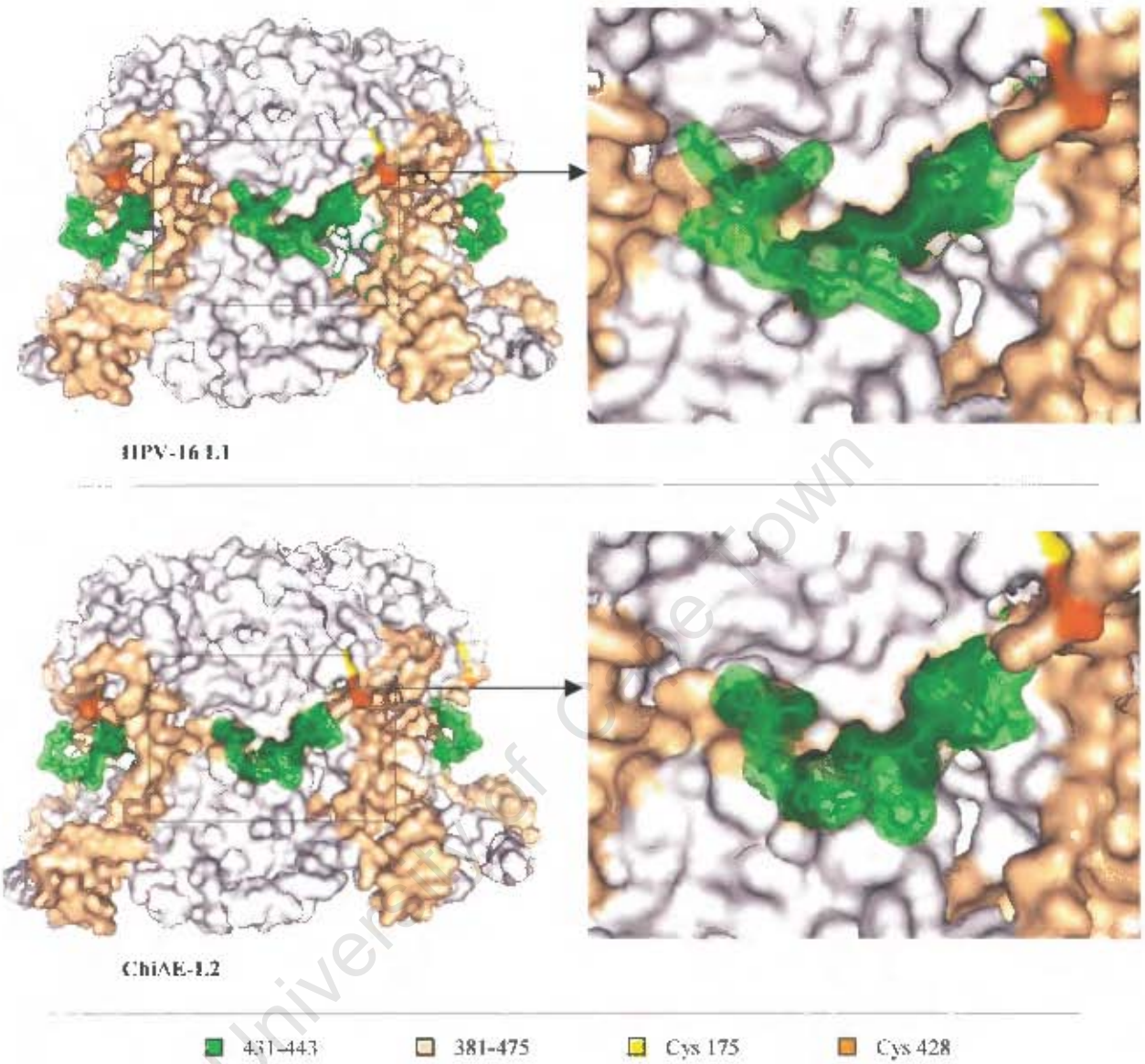

FIGURE 5.11: Structural diagrams of ChiΔE-L2 and HPV-16 L1 protein modelled using SWISS-MODEL (Guex & Peitsch, 1997, Peitsch, 1996) and assembled using VIPER (Reddy *et al.*, 2001) oligomer generator version 4 highlighting the residues 431-443. The contacts of the assembled particles were calculated using VIPER analysis tool (see appendix D for subunit numbering and contacts).

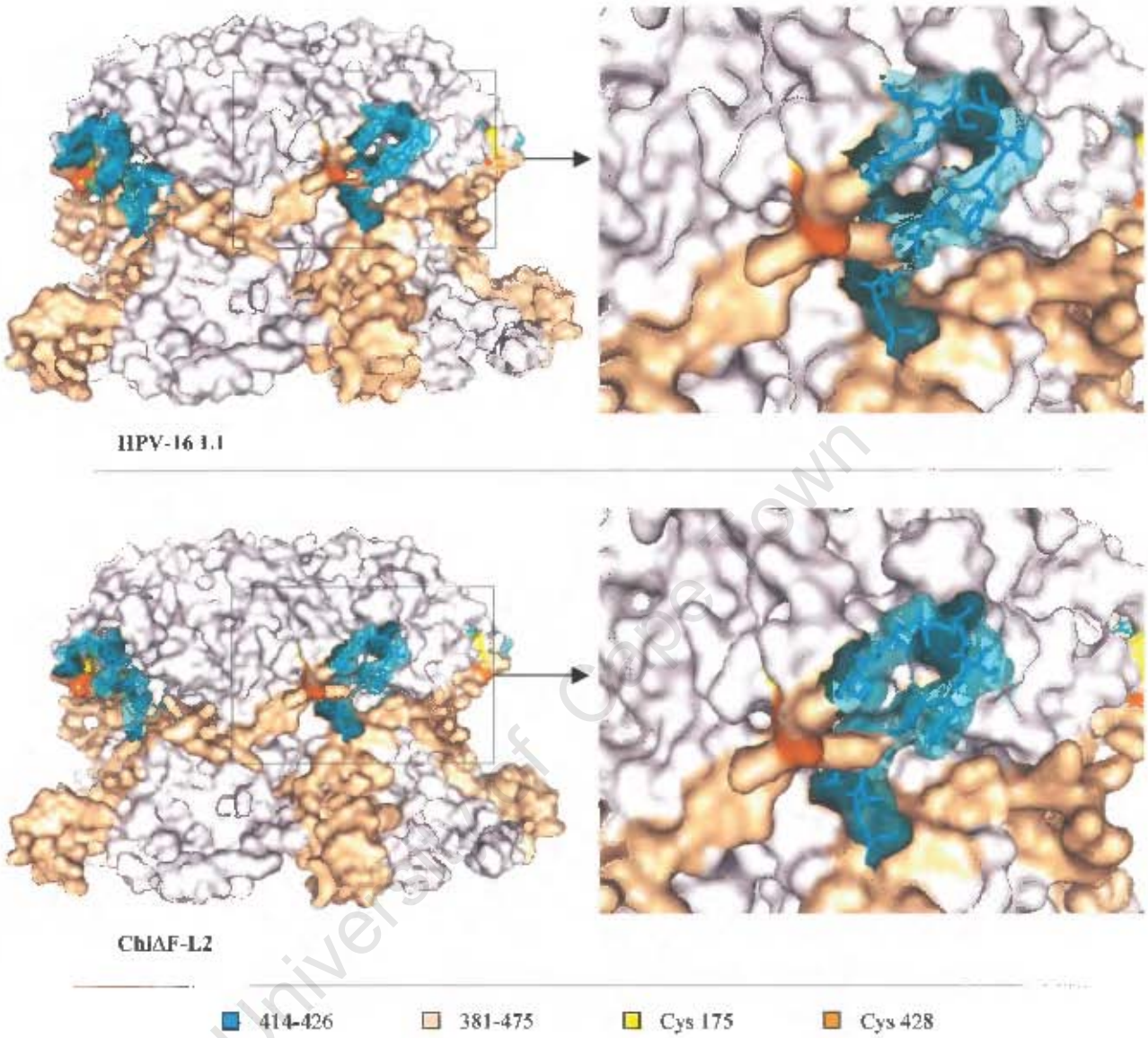

FIGURE 5.12: Structural diagrams of ChiΔF-L2 and HPV-16 L1 protein modelled using SWISS-MODEL (Guex & Peitsch, 1997, Peitsch, 1996) and assembled using VIPER (Reddy *et al.*, 2001) oligomer generator version 4 highlighting the residues 414-426. The contacts of the assembled particles were calculated using VIPER analysis tool (see appendix D for subunit numbering and contacts).

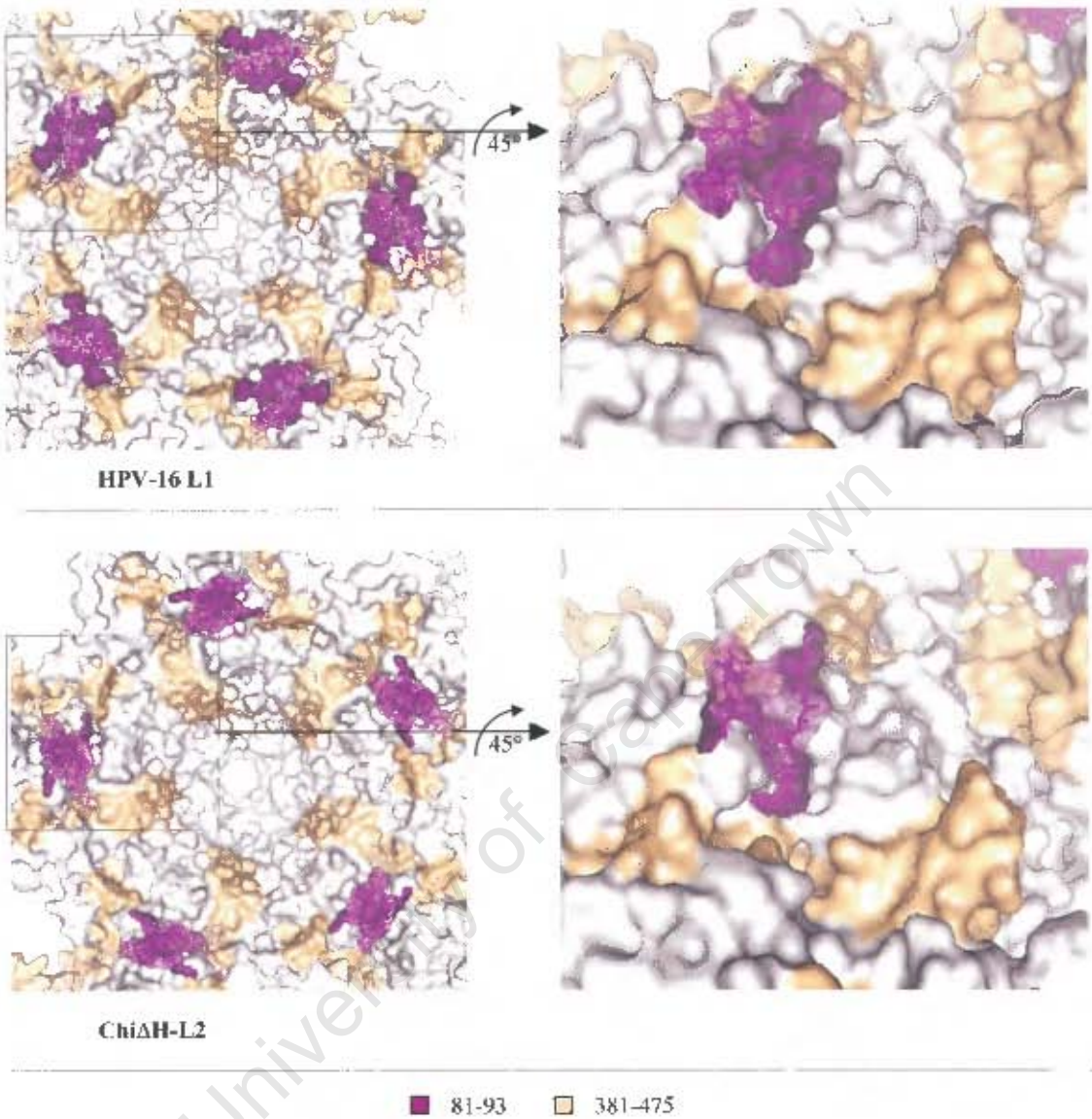

FIGURE 5.13: Structural diagrams of ChiΔH-L2 and HPV-16 L1 protein modelled using SWISS-MODEL (Guex & Peitsch, 1997, Peitsch, 1996) and assembled using VIPER (Reddy *et al.*, 2001) oligomer generator version 4 highlighting the residues 81-93. The contacts of the assembled particles were calculated using VIPER analysis tool (see appendix D for subunit numbering and contacts).

5.4 DISCUSSION

The main goal of this study was to further investigate the potential of HPV-16 VLPs for delivery or presentation of foreign epitopes in a multivalent vaccine and hence production of a L1 / L2 multivalent HPV vaccine. With the data from the crystal structure study of truncated HPV-16 L1 (10 aa N-terminal deletion; Chen *et al.*, 2000) it was possible to logically identify regions with potential for display of foreign epitopes (Figures 5.2 and 5.2). Kawana *et al.* (1999) identified a region in the HPV-16 L2 minor capsid protein (aa 108-120) that was capable of reducing susceptibility of the COS-1 cells to HPV-16 pseudovirion infection (Kawana *et al.*, 2001b). This L2 epitope was selected for incorporation into the putative multivalent vaccine candidates.

The main factor taken into account when considering putative regions to display the L2 epitope was the preservation of the Mab H16:V5 and Mab H16:E70 binding epitope: (Chen *et al.*, 2000; Roden *et al.*, 1997; White *et al.*, 1999). White *et al.* (1999) mapped the binding of these neutralising Mabs on HPV-16 L1 VLPs to residues Phe-50, Ala-266 and Ser-282. The H16:V5 Mab has been shown to completely block the binding of 70% of human sera to HPV-16 virions (Roden *et al.*, 1997); hence, the H16:V5 binding site is considered a major immunodominant epitope and is used as a marker for the efficacy of HPV-16 vaccines. Based on the data in Chapter 2, where the mutation of the alanine to a threonine at residue 266 reduces the binding affinity of Mab H16:E70, it was thought appropriate to use the construct HPV-16 L1 504[ΔA266T] for the chimaeric, as the activity of the immunodominant region would be potentially decreased, thereby possibly increasing the antigenicity of the L2 epitope.

Electron microscopy of chimaeric L1 protein aggregates (Figure 5.5) purified by isopycnic CsCl gradient fractionation from recombinant baculovirus-infected insect cells indicated that ChiΔC-L2 and ChiΔE-L2 appeared to retain the ability to assemble into recognisable VLPs as well as capsomers, while the other three constructs only formed capsomers and irregular aggregates. This indicates that substitutions of the L2 peptide sequence in the D-E loop which is located on the pentamer head (Figure 5.10) and C-terminal (region between h4 and β-J sheet) arm (Figure 5.11) are better tolerated at a structural level than the other substitutions in terms of complete assembly, but that none of the substitutions affected the first-stage pentameric capsomer assembly.

ChiΔC-L2 has potential of inducing a neutralising L1 response since it binds the neutralising H16:U4, H16:J4, H16:I23 and H16:9A Mabs, the latter targeting the N terminal region of HPV-16 L1 (Christensen *et al.*, 2001). Substitution in the D-E loop does not affect the gross structure (Figure 5.6) but there is definitely an effect on the binding of Mab H16:V5 and H16:E70 (Figure 5.5). The modelled ChiΔC-L2 (Figure 5.10, Table D.2) indicates that residue 266 and 282 are surface exposed and the L2 epitope has no evident interactions with them. One possible explanation for the loss in binding of the Mabs H16:V5 and H16:E70 to ChiΔC-L2 is due to the possible affect as a result of the change in interactions of residues 280 and 283 to residue 142 from polar-acidic to polar-hydrophobic. Alternatively, the SWISS-MODEL generated model for the ChiΔC-L2 is incorrect.

The low binding affinity of conformation-specific Mabs to particles of ChiΔH-L2 could be attributed to the alteration of the L1 structure by the substitution of the L2-epitope into the C-D loop. The C-D loop is highly conserved among PV types (Figure 5.2); therefore, substitution of the L2 epitope into the loop could well have the effect of preventing the formation of VLPs, as shown by the EM results (Figure 5.6). The contacts from the modelled ChiΔH-L2 (Figure 5.13) indicate a gross change in contacts between C-D loop (residues 79-94) which forms important contacts between subunits in pentavalent pentamers and adjacent subunits in hexavalent pentamers (Modis *et al.*, 2002). The C-D loop from one pentamer interacts directly with the C-D loop from a neighbouring pentamer in an opposing manner (Figures 5.13 and D.2). Nonetheless, it seems to allow for pentamer formation as seen by the EM (Figure 5.6); however, there seems to be a general change in conformation of the pentamer since the conformation-specific Mabs do not bind ChiΔH-L2 particles. The only logical conclusion that can be drawn is that the intra-C-D loop interaction changes to an extent that that alters overall structure of the pentamer.

Substitution of the L2 epitope into h4 helix clearly destroys the helix in ChiΔF-L2 particles, yet the epitope remains surface exposed (Figure 5.12). The EM data (Figure 5.6) indicates pentamers and a possibility of VLPs that are unstable. Hence, it is possible to assume that the h4 helix is required for the stability of the particles.

The L2 epitope in ChiΔA-L2 is probably displayed on the outer rim of the pentamer (Figure 5.9), which interacts with the C-terminal arms from neighbouring pentamers. This interaction is potentially weakened or perhaps lost in this construct, as the cysteine at residue 175 that forms a

disulphide bond with residue 428 is replaced by a valine (Figures 5.2 and 5.9). Mutagenesis experiments performed by Fligge *et al.* (2001), Li *et al.* (1998) and Sapp *et al.* (1998) have identified Cys-175 and Cys-428 - both conserved among all PV types - as being likely to form disulphide bonds. The atomic structure of HPV-16 L1 model by Modis *et al.* (2002) indicates that the Cys-175 forms a disulphide bond with the Cys-428 from an invading C-terminal arm. Therefore the substitution of the cysteine to a valine at residue 175 could potentially interfere on the formation of the inter-pentameric disulphide bond, which could prevent assembly into VLPs. Based on this data, chimaera ChiΔA-L2 to was expected to form only capsomers, which is what was observed (Figure 5.5).

Overall, the most immunogenic chimaera was ChiΔF-L2, where the L2-epitope was presented in place of the h4 helix: this region (aa 414-426) is highly exposed in the atomic structure of HPV-16 L1 proposed by Modis *et al.* (2002), and therefore potentially highly immunogenic. Chimaera ChiΔF-L2 bound all the neutralising Mabs and induced a strong L1 and L2-epitope response. It is suspected that the h4 helix in native VLPs forms important interactions, and that altering this region probably prevents the formation of VLPs. Chen *et al.* (2001) showed that deletion of the region aa 408-431 resulted in pentamer formation only, and these observations support our finding that ChiΔF-L2 is only seen to form capsomers. Particles of ChiΔE-L2 would ideally be expected to be equally immunogenic in regard to the L2-epitope as ChiΔF-L2, but this is not the case. In the atomic structure of HPV-16 L1, the residues 431 and 432 are less accessible due to the disulphide bond between Cys-175 and Cys-428 and the region 433-443 is overall less accessible than the h4 helix region. Therefore ChiΔE-L2, even though was capable of assembling into VLPs, induced a relatively weak L2 response. The antibody characterisation data indicated that particles of ChiΔF-L2 had the highest binding affinity to the L2-epitope antibody (Figures 5.5, 5.7) and these particles induced a strong L1 immune response (titres ≥ 204800) in BALB/c mice, though lower than that of ChiΔE-L2 (≥ 819200). The stronger L1 immune response induced by particles of ChiΔE-L2 could be attributed to the formation of VLPs, which are probably more immunogenic than capsomers.

Protein modelling is a powerful method of predicting conformational changes as a result of mutations and insertions based on existing structural data; however, modelling of loops is not a trivial task. This chapter used a very basic modelling approach to analyse the chimaeric proteins products which proved not to be very successful at addressing a variety of structural questions.

Nonetheless, it provide fair amount of insight: however the modelling of these chimaeras could be immensely improved by using more versatile and powerful structural softwares for homology modelling such as WHATIF (Vriend, 1990) and Modeller (Sali *et al.*, 1995; Sanchez & Sali, 1997).

In summary, this chapter reports the successful investigation of the use of HPV-16 L1 to present the HPV-16 L2 epitope LVEETSFIDAGAP (Kawana *et al.*, 1999), which cross neutralises HPV types 6 and 16 (Kawana *et al.*, 2001a). Of all the chimaeras tested, chimaera ChiΔF-L2 (substitution into the h4 helix region; 414-426) was found to be the most effective at presenting the L2 epitope while maintaining the "neutralising" Mab H16:V5 and H16:E70 binding. H16:V5 Mabs have been shown to block more than 70% of patient (HPV-16 infected) sera from binding HPV-16 pseudovirion (Roden *et al.*, 1997); thus maintaining the V5 epitope is critical for eliciting a neutralising HPV-16 response.

Recent studies have shown that sera raised to two linear epitopes on HPV-16 were capable of neutralising HPV types 16, 18, 31, 33, 45, 55 and 59 (Combita *et al.*, 2002). All of the chimaeras detailed in this chapter bind Mabs raised to these two epitopes. Therefore, the investigation into the use of PV to display foreign epitopes opens avenues to the synthesis of novel multivalent vaccines, where the epitopes could be displayed in a variety of regions, and multiple epitopes could also potentially be displayed on the particle, in different surface locations.

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Mrs Debbie de Villiers for the analysis of mice sera

Chapter 6

Conclusion

Cervical cancer continues to have a devastating impact on women's health around the world, especially in developing countries, where it is the leading cause of cancer-related mortality (zur Hausen 2002). A vaccine would clearly offer a cost effective long-term strategy to reduce this cervical cancer burden, particularly for developing countries where effective screening programmes are not well established, or are simply not available. Vaccines would prevent the sexually transmitted HPV infection, which causes premalignant cervical lesions in women that can progress to cancer. The encouraging experimental results from testing vaccine preparations in animal models of papillomavirus-related disease have prompted both commercial and public institutions to pursue the clinical development of HPV vaccine candidates. The first success 'proof of concept' phase II trial by Merck has shown protection from HPV infection in volunteers immunised with VLP-based HPV-16 vaccine prepared in *S. cerevisiae* (Koutsky *et al.*, 2002).

The majority of prophylactic HPV vaccine development has focused on the capsid proteins, which have the property of self-assembling into virus-like particles. HPV-16 has been the focus of study for HPV vaccines as it is the most prevalent HPV type found in histologically confirmed invasive cervical cancers (Clifford *et al.*, 2003; Steller, 2002). Antibody-mediated neutralisation of infectious HPV virions has been shown to be genotype-restricted. The neutralising conformation-specific Mabs H16:V5 and H16:E70 only bind and neutralise HPV-16 virions (Christensen *et al.*, 1996a; Roden *et al.*, 1997; White *et al.*, 1999). Wang *et al.* (1997) showed that Mab H16:V5 was able to block more than 75% of HPV-16 infected patient sera from binding to HPV-16 virions. Therefore Mab H16:V5 has become a benchmark in candidate HPV-16 major capsid protein-based vaccines. This clearly demonstrates that HPV-16 has an immunodominant epitope and HPV-16 L1 variants have been shown to have decreased H16:E70 binding (Roden *et al.*, 1997; White *et al.*, 1999). This could represent a problem if vaccination with a particular HPV-16 variant resulted selection of HPVs which "escape" neutralisation.

The South African HPV-16 L1, when mutated at residue 266 from an alanine to a tyrosine (504[ΔA266T]) as described in chapter 2, had reduced affinity for Mab H16:E70. However, this gene sequence was found to be highest expressing by Touze *et al.* (1998) when expressed by recombinant baculovirus in insect cells. Therefore it is important to analyse HPV-16 variants for the correct antigenicity prior to large-scale expression. Results in chapter 2 demonstrate that the h5 helix is important for maintain the correct conformation of the HPV-16 L1 particles. Further, deletion of 9 resides from the N-terminus or the deletion of the NLS (21 residues) from the C-terminus or mutation of the conserved cysteine at residue 428 to a glycine does not affect the antigenicity of the resulting particles, especially to the neutralising conformation-specific Mabs H16:V5 and H16:E70. The deletion study described in chapter 2 indicates that there is great potential for chimaeric HPV (or any PV) particles expressing / presenting foreign epitopes on the N- and C-termini (L1ΔC483, M-ΔN10 and M-ΔN10ΔC483) of the HPV-16 L1. Epitopes can be fused to the C-terminal deletions as far as the end of the h5 helix (residue 475 in HPV-16 L1) without major structural impact (Jochmus *et al.*, 1999; Liu *et al.*, 2000, 2002; Muller *et al.*, 1997; Slupetzky *et al.*, 2001). The mixed population of particles (55 and 30nm) resulting from the deletion mutant M-ΔN10 are the subject of a great structural debate on the role of the β-hairpin in virion assembly to T=1 and T=7 particles, unfortunately this debate can not be addressed within the scope of this thesis. No other deletants or mutants, other than the ΔC483, were found to be suitable for further expression in plant-based systems.

The efficacy of a HPV-16 VLP vaccine produced in *S. cerevisiae* was recently tested in a controlled clinical trial, where complete protection against HPV-16 infection was achieved in vaccinated women (Koutsky *et al.*, 2002). This clearly provides support for large-scale commercial production and application of the candidate HPV-16 vaccine. However, Sanders & Taira (2003) estimated the cost of vaccinating an individual, in a 3 injection protocol with a booster required every 10 years, to be US$ 100 per injection. This is an extremely costly undertaking for any health care system, or for individuals in developing countries. Therefore, there is great emphasis for the development of cheaper vaccine alternatives for developing countries. Plants provide a great alternative to traditional vaccine development systems as they eliminate the risk of contamination with animal pathogens, provide a heat-stable environment, and enable oral delivery. These reasons, in addition to the simplistic plant growth requirements, make plants an inexpensive means for expression of vaccines or pharmaceutical antigens.

The potential for production of plant-based HPV-16 vaccines by two different expression systems was investigated in chapters 3 and 4. To date there have been not been any reports on the successful expression of any HPV capsid proteins in plants. The expression of the HPV-16 L1 protein in plants described in Chapters 3 and 4 are the first two reports on the successful expression in plants. The transgenic plant expressed HPV-16 $L1_{Tr}$ and $L1\Delta C483_{Tr}$ (chapter 3) and transiently expressed HPV-16 $L1_{TMV}$ (chapter 4) formed VLPs and maintained their antigenicity. The binding of the neutralising conformation-specific Mabs H16:V5 and H16:E70 to the plant-produced L1 antigen provided further evidence that these plant-produced antigens have the potential to elicit a neutralising immune response. However, the immunogenicity data gained from immunising rabbits with partially purified extracts are not encouraging. These results are reinforced by the experience of Dinapoli *et al.* (2002) who found that transgenic potato-produced HPV-11 VLPs were only useful as a booster. Nonetheless, the situation could almost certainly be improved by using a higher concentration of plant-produced HPV-16 L1 antigen during immunisation.

One of the major limitations of expression of recombinant antigens in transgenic plants is the low yield but higher yields can be achieved by transient expression using plant virus vectors. The yield of HPV-16 L1 expressed transiently in *N. benthamiana* using TMV (chapter 4) was almost 10 fold greater than that from the transgenic expression (chapter 3). The main concern using plant virus vectors is their stability during expression. Recombination that resulted in the deletion of the HPV-16 L1 gene was observed during expression using the TMV vector pBSG-L1 (chapter 4), which possibly contributed to a lower recombinant protein yield compared to those achieved by other researchers using similar expression systems. The insert size for HPV-16 L1 (~1.5kb) is in fact near the upper limit for TMV expression (Dr K. E Palmer, Large Scale Biology Corporation; personal communication) Smaller insert size (<1kb) seems to be more stable in TMV vectors; unfortunately data from the deletion study in chapter 2 indicates that such a gene size for HPV-16 L1 (or other PVs) would probably result in the loss of antigenicity, if indeed any particles formed. However, using other more insert tolerant plant virus vectors such as the PVX vector may prove to be more useful. The data from chapters 3 and 4 clearly sets the basis for further development of plant-based HPV-16 vaccines, which have great potential for use in developing countries. It important to note that the field of plant-based subunit vaccine development is relatively new compared to the production systems for traditional vaccines, and with the rapid advances in plant molecular biology, the plant-based vaccine development is bound to progress faster.

A recent study by Clifford *et al.* (2003) investigated the contribution made by different HPV types to invasive cervical cancer, of the 10 058 cases (8550 for squamous cell carcinoma and 1508 for adeno and adenosquamous-carcinoma. In squamous cell carcinoma, HPV-16 was the predominant type (46-63%) followed by HPV-18 (10-14%), 45 (2-8%), 31 (2-7%) and 33 (3-5%) in all regions except Asia where HPV types 58 (6%) and 52 (4%) were more frequently identified whereas in adenosquamous carcinoma HPV-18 was the predominant type in every region (37-41%), followed by 16 (26-36%) and 45 (5-7%). Therefore, for an effective prevention of cervical cancer, it is imperative to address the impact of a vaccine that confers type specific protection in individuals with multiple HPV genotype infection. Serum antibody responses to HPV capsids has been established to be type specific, except for HPV-6 and –11 that contain both type specific epitopes and epitopes shared between HPV-6 and –11 (Christensen *et al.*, 1994, 1996a). A study by Silins *et al.* (1999) described the existence of an antagonistic interference between HPV-6 and HPV-16, where HPV-6 conferred some protection against HPV-16 cervical carcinogenesis. Hence, it would be ideal for a HPV candidate vaccine to offer protection against a broad range of HPV serotypes.

Chapter 5 describes various novel HPV-16 L1 chimaeras expressing the common neutralising L2 epitope of HPV-6 and –16. Presentation of the L2 epitope in place of the h4 helix (ChiΔF-L2) and the coil between the h4 and β-J sheet (ChiΔE-L2) elicited the greatest anti-L1 immune response. Presentation of the L2 epitope in the E-F loop (ChiΔA-L2) resulted in the replacement of the cysteine at residue 175 by a valine; and even though the chimaeric product from of ChiΔA-L2 retained the antigenicity to bind neutralising conformation-specific Mabs H16:V5 and H16:E70, it was unable to assemble into VLPs. This is identical to the observation in chapter 2, with the mutation of the cysteine 428 to a glycine. Presentation of the L2 epitope on the D-E loop (ChiΔC-L2) and the coil between the h4 and β-J sheet (ChiΔE-L2) did not affect the assembly of the chimaeras into VLPs, but ChiΔC-L2 VLPs were unable to bind Mabs H16:V5 and H16:E70. ChiΔF-L2 retained its antigenicity, induced a strong L1 and L2-epitope response, but formed predominantly capsomers. The C-D loop is evidently critical for maintaining the correct conformation of particles, since the presentation of the L2 epitope in this region completely destroyed the ability of the ChiΔH-L2 protein product (aggregates of what looks like pentamers) to bind any of the conformation-specific Mabs. Therefore the data presented in chapter 5 indicates that of the various regions explored for the L2 epitope presentation, the h4 helix proved to be the most suitable.

The results from the deletion study described in chapter 2 and the chimaeric study in chapter 5 open avenues for the development of multivalent HPV vaccines and for using HPV as a vector for delivery of other important epitopes. Once the plant-based expression systems have been optimised for HPV-16 L1 production, these chimaeric vaccines could easily be produced in bulk relatively cheaply. Further, with the various reports on the encapsidation of nucleic acid (Fligge *et al.*, 2001; Kawana *et al.*, 1998; Stauffer *et al.*, 1998; Touze & Coursaget, 1998; Unckell *et al.*, 1997; Zhao *et al.*, 1998) into VLPs and the encapsidation examples in chapter 2, 3 and 4, the potential for multivalent chimaeric HPV VLP / DNA vaccine is enormous.

Appendix A

Papillomavirus types

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TABLE A.1: Characterised *Human papillomavirus* types

Type	Group	L1 gene GenBank accession no.	Site of infection/source
HPV-1a	E1	NC_001356	Plantar warts
HPV-2a	A4	NC_001352	Common warts
HPV-3	A2	NC_001588	Flat warts
HPV-4	B2	NC_001457	Common warts
HPV-5	B1	NC_001531	Benign warts, laryngeal papillomatosis
HPV-6	A10	AY0115008	Genital warts, laryngeal papillomatosis
HPV-6b	A10	NC_001355	Genital warts, laryngeal papillomatosis
HPV-7	A8	NC_001595	'Butchers' warts, oral papillomas of HIV patients
HPV-8	B1	NC_001532	Benign and malignant EV lesions
HPV-9	B1	NC_001596	Epidermodysplasia verruciformis lesions
HPV-10	A2	NC_001576	Flat warts
HPV-11	A10	NC_001525	Laryngeal papillomas, genital warts
HPV-12	B1	NC_001577	EV lesions
HPV-13	A10	NC_001349	Oral focal epithelial hyperplasia
HPV-14	B1	NC_001578	Epidermodysplasia verruciformis lesions
HPV-15	B1	NC_001579	Epidermodysplasia verruciformis lesions
HPV-16	A9	NC_001526	Anogenital intraepithelial neoplasia and caners
HPV-17	B1	NC_001580	Epidermodysplasia verruciformis lesions
HPV-18	A7	NC_ 01357	Anogenital intraepithelial neoplasia and caners
HPV-19	B1	NC_001571	Epidermodysplasia verruciformis lesions
HPV-20	B1	NC_001679	Epidermodysplasia verruciformis lesions
HPV-21	B1	NC_001680	Epidermodysplasia verruciformis lesions
HPV-22	B1	NC_001681	Epidermodysplasia verruciformis lesions
HPV-23	B1	NC_001682	Epidermodysplasia verruciformis lesions
HPV-24	B1	NC_001683	Epidermodysplasia verruciformis lesions
HPV-25	B1	NC_001582	Epidermodysplasia verruciformis lesions
HPV-26	A5	NC_001583	Common warts under immunosuppression
HPV-27	A4	NC_001584	Common warts
HPV-28	A2	NC_001684	Flat warts
HPV-29	A2	NC_001685	Common warts
HPV-30	A6	NC_001585	Laryngeal carcinomas
HPV-31	A9	NC_001527	Anogenital intraepithelial neoplasia and caners
HPV-32	A1	NC_001586	Oral focal epithelial hyperplasia, oral papillomas
HPV-33	A9	NC_001528	Anogenital intraepithelial neoplasia and cancers
HPV-34	A11	NC_001587	Anogenital intraepithelial neoplasia
HPV-35	A9	NC_001529	Anogenital neoplasia and cancers
HPV-36	B1	NC_001686	Actinic keratosis, EV lesions
HPV-37	B1	NC_001687	Keraroacanthoma[#]
HPV-38	B1	NC_001688	Melanoma[#]
HPV-39	A7	NC_001535	Anogenital intraepithelial neoplasia and cancers
HPV-40	A8	NC_001589	Anogenital intraepithelial neoplasia
HPV-41	E	X56147	Cutaneous squamous cell carcinomas
HPV-42	A1	NC_001534	Anogenital intraepithelial neoplasia
HPV-43[‡]	A8	U12504	Anogenital intraepithelial neoplasia
HPV-44	A10	NC_001689	Anogenital intraepithelial neoplasia
HPV-45	A7	NC_001590	Anogenital intraepithelial neoplasia and cancers
HPV-47	B1	NC_001530	Epidermodysplasia verruciformis lesion
HPV-48	B2	NC_001690	Cutaneous squamous cell carcinoma
HPV-49	B1	NC_001591	Flat wart under immunosuppression

HPV-50	B2	NC_001691	Epidermodysplasia verruciformis lesion
HPV-51	A5	NC_001533	Anogenital intraepithelial neoplasia and cancers
HPV-52	A9	NC_001592	Anogenital intraepithelial neoplasia and cancers
HPV-53	A6	NC_001593	Anogenital intraepithelial neoplasia
HPV-54	A1	NC_001676	Anogenital intraepithelial neoplasia
HPV-55	A10	NC_001692	Anogenital intraepithelial neoplasia
HPV-56	A6	NC_001594	Anogenital intraepithelial neoplasia and cancers
HPV-57	A4	NC_001353	Oral papillomas and inverted maximmary sinus papillomas
HPV-58	A9	NC_001443	Anogenital intraepithelial neoplasia and cancers
HPV-59	A7	NC_001635	Anogenital intraepithelial neoplasia
HPV-60	B2	NC_001693	Epidermoid cysts
HPV-61	A3	NC_001694	Anogenital intraepithelial neoplasia
HPV-62‡	A3	U12499	Anogenital intraepithelial neoplasia
HPV-63	E1	NC_001458	Myrmecia wart
HPV-64‡	A11	U12495	Anogenital intraepithelial neoplasia
HPV-65	B2	NC_001459	Pigmented wart
HPV-66	A6	NC_001695	Cervical carcinoma
HPV-67‡	A9	U12499	Anogenital intraepithelial neoplasia
HPV-68‡	A7	M73258	Anogenital intraepithelial neoplasia
HPV-69	A5	NC_002171	Anogenital intraepithelial neoplasia and cancers
HPV-70	A7	NC_001711	Vulvar papilloma
HPV-71	A2	NC_002644	Anogenital intraepithelial neoplasia
HPV-72	A3	X94164	Oral papilloma (HIV patients)
HPV-73	A11	X94165	Oral papilloma (HIV patients)
HPV-74	A10	U40822	Anogenital intraepithelial neoplasia
HPV-75	B1	Y15173	Common warts in organ allograft recipient
HPV-76	B1	Y15174	Common warts in organ allograft recipient
HPV-77	A2	Y15175	Common warts in organ allograft recipient
HPV-82	A5	NC_002172	Anogenital intraepithelial neoplasia
HPV-83	A3	NC_000856	Anogenital intraepithelial neoplasia
HPV-84	A3	NC_002676	Anogenital intraepithelial neoplasia
HPV-86	A3	NC_003115	Anogenital intraepithelial neoplasia (Terai & Burk, 2001)
HPV-87#	A3	NC_002627	Anogenital intraepithelial neoplasia (Menzo et al., 2001)
HPV-89#	A3	NC_004103	Anogenital intraepithelial neoplasia (Terai & Burk, 2002)
HPV-90#	A8	NC_004104	Anogenital intraepithelial neoplasia (Terai & Burk, 2002)
HPV-91#	A8	NC_004085	Anogenital intraepithelial neoplasia (Terai & Burk, 2002)

individual isolates recently sequenced
‡ partial sequences

TABLE A.2: Abbreviations of animal papillomaviruses

PV	
BPV	Bovine papillomavirus
COPV	Canine oral papillomavirus
CRPV	Cottontail rabbit papillomavirus
ROPV	Rabbit oral papillomavirus
DPV	Deer papillomavirus
EPC	Equinus papillomavirus
ECPV	Equus caballus papillomavirus
EEPV	European elk papillomavirus
FPV	Fringilla coelebs papillomavirus
MuRPV	Multimammate rat papillomavirus
OvPV	Ovine papillomavirus
PhSpPV	Phocoena spinipinnis papillomavirus
PsErPV	Psittacus erithacus papillomavirus
RdPV	Reindeer papillomavirus
RhMPV	Rhesus monkey papillomavirus

TABLE A.3: Characterised animal papillomavirus types

Host	Designation	Group	L1 gene GenBank accession no.
Domestic cattle (*Bos taurus*)	BPV-1	C	NC_001522
	BPV-2	C	NC_001521
	BPV-3	C	NC_004197
	BPV-5	C	NC_004195
Common chimpanzee (*Pan troglodytes*)	CCPV	A10	NC_001838
Domestic dogs (*Canis familiaris*)	COPV	E	NC_001619
Cottontail rabbit (*Sylvilagus floridanus*)	CRPV	E	NC_001541
Deer (*Odocoileus virginianus*)	DPV	C	NC_001523
Equine (various)	EPV	C	NC_004194
Horse (*Equus caballus*)	ECPV	C	NC_003748
European elk (*Alces alces*)	EEPV	C	NC_001524
Chaffinch (*Fringilla coelebs*)	FPV	S	NC_004068
Multimammate rat (*Mastomys natalensis*)	MuRPV	S	NC_001605
Domestic sheep (*Ovis domesticus*)	OPV-1	C	NC_001789
	OPV-2	C	NC_001790
Pygmy chimpanzee (*Pan paniscus*)	PCPV	A10	X62844
Burmeister's porpoise (*Phocoena spinipinnis*)	PhSpPV	S	NC_003348
Grey parrots (*Psittacus erithacus*)	PsErPV	S	NC_003973
Rabbit (*Sylvilagus*)	ROPV	E	NC_002232
Reindeer (*Rangifer tarandus*)	RdPV	C	NC_004196
Rhesus macaque (*Macaca mulatta*)	RhMPV	A12	NC_001678

Appendix B

Cellular proteins and their functions

Contents

TABLE B:1: Abbreviations of cellular proteins and their function 167

TABLE B:1: Abbreviations of cellular proteins and their function

Protein	Function
AP-1	Transcriptional regulator of HPV LCR. Interacts with E7.
BAK	Pro-apoptotic protein which is destroyed by E6.
cdk	cyclin dependent kinase
E6AP	Forms a complex (ubiquitin ligase) with E6 that then targets p53 degradation.
E2F	Cellular transcription factor that forms a complex with pRb. E7 proteins from high-risk viruses disrupt this complex.
GM-CSF	Granulocyte / macrophage colony stimulating factor. Functions as a cytokine to regulate growth, gene expression and differentiation
Keratin	Keratin filaments associate with E4 protein in some epithelial cells.
MAR	Matrix attachment region
NF1	Nuclear factor 1 (transcription factor) binds HPV LCR.
Oct-1	Transcriptional regulator of HPV LCR.
$p21^{CIP1}$	Modulator of p53-mediated growth arrest. Binds to E7.
$p27^{KIP1}$	Cyclin-dependent kinase inhibitor binds to E7.
p34cdc2	Protein kinase that phosphorylates E1. See also PKA, PKC, CKI, CKII and DNA-dependent protein kinase.
p53	A tumor suppressor protein whose transcription and degradation are affected by E6. Transcription affected by E7.
ppRb	Hypophosphorylated pRb.
pRb	Retinoblastoma protein, an important "pocket protein" bound by E7
SP1	Transcriptional factor/enhancer. Binding sites in the LCR.
TFIID	Transcriptional factor II D
TNFα	Tumor necrosis factor alpha is transcriptional repressor of HPV LCR in weakly tumorigenic cell lines
Tyk2	Tyrosine kinase 2. Essential for signal transduction processes of IFN-α receptor
YY1	"Yin and yang" 1, a transcriptional factor that interacts with the HPV LCR

Appendix C

Maps of non-commercial vectors

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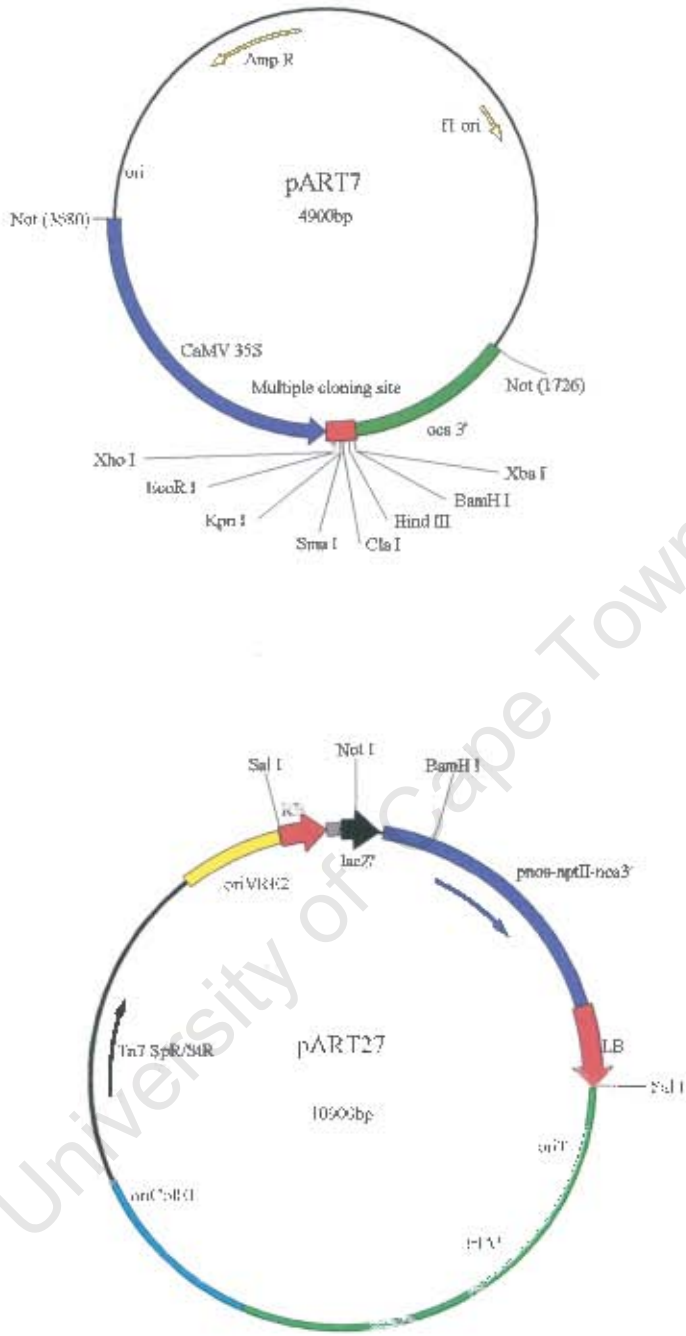

Figure C.1: Vector map of pART7 and binary vector pART27

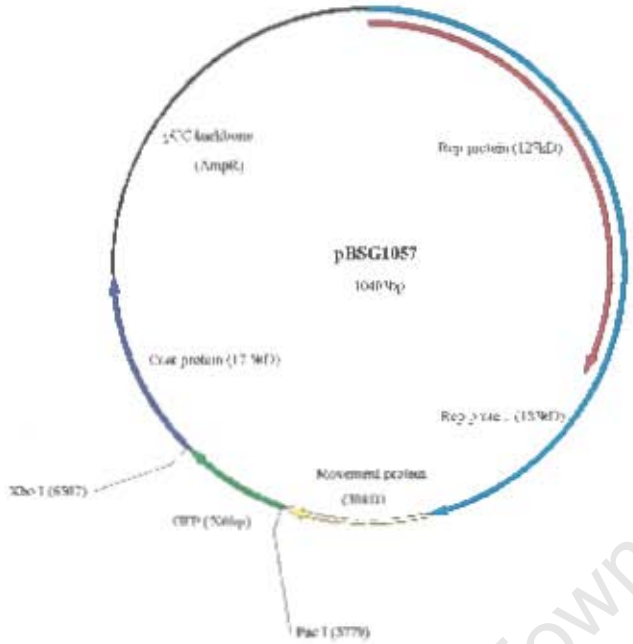

Figure C.2: Vector map of TMV vector pBSG1057

Appendix D

Protein modelling of chimaeras

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TABLE D.5: Differences between calculated subunit contacts for ChiΔH-L2 and HPV-16 L1. 177

TABLE D.6: Trace log of the SWISS MODELLed ChiΔH-L2 chimaera. 177

FIGURE D.2: Illustrations of the differences in the C-D:C-D loop interaction between HPV-16 L1 and ChiΔH-L2. Purple colour represent the amino acid region 81-93. 179

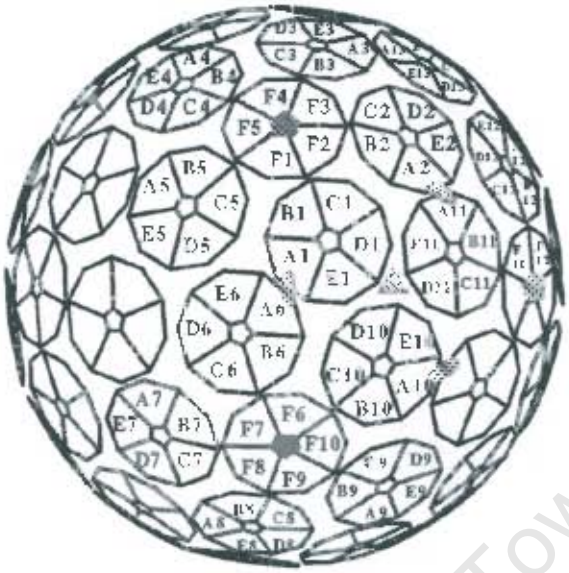

FIGURE D.1: Organisation of papillomavirus subunits and their designated numbers for inter and intrasubunit interactions (http://mmtsb.scripps.edu/viper). The 2 (oval), 3(triangle) and 5 (pentagon) fold axis are shown in grey coloured shapes.

TABLE D.1: Differences between calculated subunit contacts for ChiΔA-L2 and HPV-16 L1.
Contacts calculated using VIPER analysis tool (Reddy *et al.*, 2001) and the differences between the contacts of HPV-16 L1 and ChiΔA-L2 were checked using WordDiff software (www.softinterface.com).

HPV-16 L1	ChiΔA-L2
A_1: B_1 QUASI_5-FOLD	**A_1:B_1 QUASI_5-FOLD**
D:182_I:348 (D-H)	D:182_I:348 (A-H)
G:183_A:346 (G-H)	A:183_I:348 (P-H)
G:183_I:348 (H-H)	A:183_K:361 (H-B)
G:183_K:361 (H-B)	G:184_K:361 (H-B)
G:185_L:344 (P-H)	G:184_Y:363 (H-P)
G:185_Y:363 (H-P)	A:185_Y:363 (H-P)
G:185_R:365 (H-B)	A:185_R:365 (H-B)
	*G:266_R:361 (H-A)
	*T:266_T:358 (P-P)
	*A:266_K:361 (P-B)
A_1: B_1 QUASI_5-FOLD	**A_1:B_1 QUASI_5-FOLD**
L:344_G:185 (H-P)	A:346_I:183 (H-H)
A:346_G:183 (H-H)	I:348_G:182 (H-A)
_:348_G:182 (H-A)	I:348_A:183 (H-H)
K:361_G:183 (B-H)	K:361_A:183 (B-H)
*K:361_A:266 (B-H)	K:361_G:184 (B-H)
Y:363_G:185 (P-H)	*K:361_T:266 (B-P)
R:365_G:185 (B-H)	Y:363_G:184 (P-H)
	Y:363_A:185 (P-H)
	R:365_A:185 (B-H)
A_2: F_1 QUASI_3-FOLD	**A_2:F_1 QUASI_3-FOLD**
Q:424_N:177 (P-P)	Q:424_E:177 (P-N)
Q:424_V:178 (P-H)	Q:424_T:178 (P-P)
I:426_G:175 (H-H)	A:425_S:177 (H-A)
T:426_N:177 (P-P)	I:426_V:175 (H-H)
C:428_G:175 (H-H)	I:426_E:176 (H-A)
	T:426_E:177 (P-A)
	A:427_E:176 (H-A)
	G:428_V:175 (H-H)
B_1: C_1 QUASI_5-FOLD	**B_1:C_1 QUASI_5-FOLD**
P:182_I:348 (P-H)	D:182_I:348 (A-H)
G:183_A:346 (G-H)	A:183_I:348 (P-H)
G:183_I:348 (G-H)	A:183_K:361 (H-B)
G:183_K:361 (P-B)	G:184_K:361 (H-B)
G:185_L:344 (P-H)	G:184_Y:363 (H-P)
G:185_Y:363 (P-P)	A:185_Y:363 (H-P)
G:185_R:365 (P-B)	A:185_R:365 (H-B)
	*T:266_T:358 (P-P)
	*T:266_K:361 (P-B)

HPV-16 L1	ChiΔA-L2
C_1: D_1 QUASI_5-FOLD	**C_1:D_1 QUASI_5-FOLD**
P:182_I:348 (P-H)	D:182_I:348 (A-H)
G:183_A:346 (G-H)	A:183_I:348 (H-H)
G:183_I:348 (H-H)	A:183_K:361 (H-B)
G:183_K:361 (H-B)	G:184_K:361 (H-B)
G:185_L:344 (P-H)	G:184_Y:363 (H-P)
G:185_Y:363 (P-P)	A:185_Y:363 (H-P)
G:185_R:365 (H-B)	A:185_R:365 (H-B)
C:185_Y:363 (H-P)	A:185_Y:363 (H-P)
*R:266_K:361 (H-B)	*T:266_T:358 (P-P)
	*T:266_K:361 (P-B)
	Y:276_H:120 (P-H)
C_1: D_10 QUASI_2-FOLD	**C_1:D_10 QUASI_2-FOLD**
	L:176__:120 (A-H)
D_1: B_1 QUASI_5-FOLD	**D_1:B_1 QUASI_5-FOLD**
P:182_I:348 (P-H)	D:182_I:348 (A-H)
G:183_A:346 (G-H)	A:183_I:348 (H-H)
G:183__:348 (G-H)	A:183_K:361 (H-B)
G:183_K:361 (H-B)	G:184_K:361 (H-B)
D:184_K:361 (A-B)	G:184_Y:363 (H-P)
C:185_L:344 (P-H)	A:185_Y:363 (H-P)
C:185_Y:363 (H-P)	A:185_R:365 (H-B)
C:185_R:365 (H-B)	*T:266_T:358 (P-P)
*A:266_T:358 (H-P)	
F_1:F_2 ICOS._5-FOLD	**F_1:F_2 ICOS_5-FOLD**
P:182_I:348 (P-H)	D:182_I:348 (A-H)
G:183_A:346 (G-H)	A:183_I:348 (H-H)
G:183_I:348 (G-H)	A:183_K:361 (H-B)
G:183_K:361 (A-B)	G:184_K:361 (H-B)
C:185_L:344 (P-H)	G:184_Y:363 (H-P)
	A:185_Y:363 (H-P)
	*T:266_T:358 (P-P)
	*T:266_K:361 (P-B)

H->Hydrophobic(nonpolar)	Purple-> Difference in HPV-16 L1
P -> Polar	Plum-> Only in HPV-16 L1
A -> Acidic	Red-> Difference in ChiΔA-L2
B -> Basic	Green-> Only in ChiΔA-L2

TABLE D.2: Differences between calculated subunit contacts for ChiΔC-L2 and HPV-16 L1. Contacts calculated using VIPER analysis tool (Reddy *et al.*, 2001) and the differences between the contacts of HPV-16 L1 and ChiΔC-L2 were checked using WordDiff software (www.softinterface.com).

HPV-16 L1	ChiΔC-L2
A_1: B_1 QUASI_5-FOLD	**A_1:B_1 QUASI_5-FOLD**
N:131_K:125 (P-B)	L:131_K:125 (H-B)
N:131_D:128 (P-A)	L:131_D:128 (H-A)
N:131_H:259 (P-B)	L:131_I:147 (H-H)
N:131_F:261 (P-H)	L:131_H:259 (H-B)
A:132_S:145 (H-A)	L:131_F:261 (H-H)
Y:135_L:132 (P-H)	V:132_K:125 (H-B)
Y:135_S:145 (P-A)	V:132_E:145 (H-A)
V:141_Y:355 (H-P)	V:132_I:147 (H-H)
V:141_N:357 (H-P)	E:134_E:145 (A-A)
D:142_Y:355 (A-P)	T:135_E:145 (P-A)
N:143_N:357 (P-P)	D:139_N:357 (A-P)
Q:279_D:142 (P-A)	G:141_N:357 (H-P)
S:280_D:142 (P-A)	N:142_Y:355 (H-P)
	P:143_N:357 (H-P)
	G:265_E:362 (H-A)
	*T:266_T:358 (P-P)
	*T:266_K:361 (P-B)
	S:280_A:142 (P-H)
	T:283_A:142 (P-H)
A_1: E_1 QUASI_5-FOLD	**A_1:E_1 QUASI_5-FOLD**
L:131_K:125 (H-B)	L:131_K:125 (H-B)
D:128_N:131 (A-P)	D:128_L:131 (A-H)
D:142_Q:279 (A-Q)	A:142_S:280 (H-P)
D:142_S:280 (A-P)	A:142_T:283 (H-P)
D:142_T:283 (A-P)	E:145_V:132 (A-H)
E:145_A:132 (A-H)	E:145_E:134 (A-A)
E:145_Y:135 (A-P)	T:145_T:135 (A-P)
Q:140_Y:135 (H-P)	T:147_V:132 (H-H)
I:147_A:132 (H-H)	K:259_L:131 (B-H)
K:259_N:131 (B-P)	R:261_L:131 (H-H)
R:261_N:131 (H-P)	Y:355_Q:141 (P-H)
Y:355_V:141 (P-H)	Y:355_A:142 (P-P)
Y:355_D:142 (P-A)	Y:355_P:143 (P-H)
Y:355_N:143 (P-P)	N:357_D:139 (P-A)
N:357_Q:140 (P-H)	N:357_Q:141 (P-H)
N:357_A:139 (P-H)	N:357_P:143 (P-H)
N:357_S:140 (P-H)	*K:361_*T:266 (B-P)
N:357_V:141 (P-H)	
N:357_N:143 (P-P)	
*K:361_A:266 (B-H)	
B_1: C_1 QUASI_5-FOLD	**B_1:C_1 QUASI_5-FOLD**
N:131_K:125 (P-B)	L:131_K:125 (H-B)
N:131_D:128 (P-A)	L:131_D:128 (H-A)
N:131_H:259 (P-B)	I:131_I:147 (H-H)
N:131_F:261 (P-H)	I:131_H:259 (H-B)
A:132_E:145 (H-A)	L:131_F:261 (H-H)
Y:135_L:132 (P-H)	V:132_K:125 (H-B)
Y:135_E:145 (P-A)	V:132_E:145 (H-A)
Y:135_Q:140 (P-H)	V:132_I:147 (H-H)
A:139_N:357 (H-P)	E:134_E:145 (A-A)
V:141_Y:355 (H-P)	T:135_E:145 (P-A)
V:141_N:357 (H-P)	D:139_N:357 (A-P)
D:142_Y:355 (A-P)	G:141_N:357 (H-P)
N:143_N:357 (P-P)	N:142_Y:355 (H-P)
Q:279_D:142 (H-A)	P:143_N:357 (H-P)
S:280_D:142 (P-A)	*T:266_T:358 (P-P)
T:283_D:142 (P-A)	*T:266_K:361 (P-B)
	S:280_A:142 (P-H)
	T:283_A:142 (P-H)

HPV-16 L1	ChiΔC-L2
C_1: D_1 QUASI_5-FOLD	**C_1:D_1 QUASI_5-FOLD**
N:131_K:125 (P-B)	L:131_K:125 (H-B)
N:131_D:128 (P-A)	L:131_D:128 (H-A)
N:131_H:259 (P-B)	L:131_I:147 (H-H)
N:131_F:261 (P-H)	I:131_H:259 (H-B)
A:132_S:145 (H-A)	I:131_F:261 (H-H)
Y:135_L:132 (P-H)	V:132_E:145 (H-A)
Y:135_S:145 (P-A)	V:132_I:147 (H-H)
V:141_Y:355 (H-P)	S:134_E:145 (P-A)
A:139_N:357 (H-P)	T:135_E:145 (P-A)
S:140_N:357 (H-P)	S:139_N:357 (A-P)
V:141_Y:355 (H-P)	G:141_Y:355 (P-A)
V:141_N:357 (H-P)	G:141_N:357 (H-P)
D:142_Y:355 (A-P)	A:142_Y:355 (H-P)
N:143_N:357 (P-P)	P:143_Y:355 (H-P)
A:266_K:361 (H-B)	P:143_N:357 (H-P)
Q:279_D:142 (H-A)	*T:266_T:358 (P-P)
S:280_D:142 (P-A)	*T:266_K:361 (P-B)
T:283_D:142 (P-A)	S:280_A:142 (P-H)
	T:283_A:142 (P-H)
D_1: E_1 QUASI_5-FOLD	**D_1:E_1 QUASI_5-FOLD**
N:131_K:125 (P-B)	L:131_K:125 (H-B)
N:131_D:128 (P-A)	L:131_D:128 (H-A)
N:131_H:259 (P-B)	L:131_H:259 (H-B)
N:131_F:261 (P-H)	L:131_F:261 (H-H)
A:132_E:145 (H-A)	V:132_E:145 (H-A)
Y:135_L:132 (P-H)	V:132_I:147 (H-H)
Y:135_E:145 (P-A)	T:135_E:145 (P-A)
V:141_Y:355 (H-P)	S:141_N:357 (H-P)
T:142_Y:355 (A-P)	A:142_Y:355 (H-P)
N:143_N:357 (P-P)	P:143_Y:355 (H-P)
*A:266_T:358 (H-P)	P:143_N:357 (H-P)
G:279_D:142 (H-A)	*T:266_T:358 (P-P)
S:280_D:142 (P-A)	S:280_A:142 (P-H)
T:283_D:142 (P-A)	T:283_A:142 (P-H)
F_1:F_2 ICOS_5-FOLD	**F_1:F_2 ICOS_5-FOLD**
N:131_K:125 (P-B)	L:131_N:195 (H-P)
N:131_D:128 (P-A)	L:131_D:128 (H-A)
N:131_H:259 (P-B)	L:131_I:147 (H-H)
N:131_F:261 (P-H)	I:131_H:259 (H-B)
A:132_E:145 (H-A)	L:131_F:261 (H-H)
Y:135_L:132 (P-H)	V:132_K:125 (H-B)
Y:135_E:145 (P-A)	V:132_E:145 (H-A)
Y:135_Q:140 (P-H)	V:132_I:147 (H-H)
A:139_N:357 (H-P)	E:134_E:145 (A-A)
V:141_Y:355 (H-P)	T:135_E:145 (P-A)
V:141_N:357 (H-P)	S:139_N:357 (A-P)
D:142_Y:355 (A-P)	G:141_N:357 (H-P)
N:143_N:357 (P-P)	A:142_Y:355 (H-P)
Q:279_D:142 (H-A)	P:143_Y:355 (H-P)
S:280_D:142 (P-A)	P:143_N:357 (H-P)
T:283_D:142 (P-A)	*T:266_T:358 (P-P)
	*T:266_K:361 (P-B)
	S:280_A:142 (P-H)
	T:283_A:142 (P-H)

H->Hydrophobic(nonpolar)	Purple-> Difference in HPV-16 L1
P -> Polar	Blue-> Only in HPV-16 L1
A -> Acidic	Red-> Difference in ChiΔC-L2
B -> Basic	Green-> Only in ChiΔC-L2

TABLE D.3: Differences between calculated subunit contacts for ChiΔE-L2 and HPV-16 L1.
Contacts calculated using VIPER analysis tool (Reddy *et al.*, 2001) and the differences between the contacts of HPV-16 L1 and ChiΔE-L2 were checked using WordDiff software (www.softinterface.com).

HPV-16 L1	ChiΔE-L2
A_1: B_1 QUASI_5-FOLD	**A_1:B_1 QUASI_5-FOLD**
	G:265_B:382 (H-A)
	*T:266_T:358 (P-P)
	*T:266_K:361 (P-B)
A_1: E_1 QUASI_5-FOLD	**A_1:E_1 QUASI_5-FOLD**
*K:361 A:266 (B-H)	*T:266_T:358 (P-P)
	*K:361 T:266 (B-P)
A_1: F_1 QUASI_2-FOLD	**A_1:F_1 QUASI_2-FOLD**
R:41_P:433 (B-H)	K:41_S:436 (B-A)
V:49 R:41 (H-B)	E:433 R:41 (A-B)
N:431 V:49 (B-H)	
P:433_R:41 (H-B)	
A_2: F_1 QUASI_3-FOLD	**A_2:F_1 QUASI_3-FOLD**
H:431 E:189 (B-A)	L:431_E:189 (H-A)
T:432 E:189 (P-A)	V:432_E:189 (H-A)
P:434_T:191 (H-H)	V:432_T:191 (H-H)
P:434_N:192 (H-P)	E:434_E:189 (A-A)
A:435_N:192 (H-P)	E:434_T:191 (A-H)
A:435_K:206 (H-B)	E:434_N:192 (A-P)
P:436_T:165 (H-H)	T:435_N:192 (P-P)
P:436_N:192 (H-P)	T:435_K:206 (P-B)
P:436 V:194 (H-H)	S:436_T:165 (P-H)
P:436_K:206 (H-B)	S:436_V:194 (P-H)
E:438_K:162 (A-B)	I:436_K:162 (H-B)
T:438_P:163 (A-H)	I:438 E:163 (H-E)
E:438_V:194 (A-H)	I:438 V:194 (H-H)
B_1: C_1 QUASI_5-FOLD	**B_1:C_1 QUASI_5-FOLD**
	*T:266_T:358 (P-P)
	*T:266_K:361 (P-B)
C_1: D_1 QUASI_5-FOLD	**C_1:D_1 QUASI_5-FOLD**
*A:266 K:361 (H-B)	*T:266_T:358 (P-P)
	*T:266_K:361 (P-B)
C_1: D_10 QUASI_2-FOLD	**C_1:D_10 QUASI_2-FOLD**
_:165_P:440 (H-H)	I:165_A:440 (H-H)
G:166_P:440 (H-H)	G:166_A:440 (H-H)
E:167_P:440 (A-H)	E:167_A:440 (A-H)
E:167_L:441 (A-H)	E:167_G:441 (A-H)
E:167_K:442 (A-B)	E:167_A:442 (A-H)
K:168 K:442 (H-B)	E:167_E:443 (A-H)
W:169_K:442 (H-B)	W:169_A:442 (H-H)
L:190_K:442 (H-B)	W:169_E:443 (H-H)
T:191_K:442 (H-B)	L:190_A:442 (H-H)
N:192_E:443 (H-B)	Y:231_G:441 (P-H)
D:202_K:442 (A-B)	Y:231_P:443 (P-H)
G:206_K:442 (H-B)	D:232_A:440 (A-H)
A:207_K:442 (H-B)	D:233_A:440 (A-H)
A:207_K:443 (H-B)	D:233_G:441 (A-H)
Y:231_L:441 (P-H)	Y:234_A:440 (P-H)
Y:231_K:443 (P-B)	I:235_L:438 (H-H)
D:232_L:441 (A-H)	M:237_A:440 (H-H)
D:233_P:440 (A-H)	A:440_T:358 (H-P)
Y:234_L:441 (P-H)	
T:235_K:438 (H-A)	
T:235_P:440 (H-H)	
N:236_P:440 (B-H)	
M:237_P:440 (H-H)	
S:239_K:437 (P-B)	
P:440_I:235 (H-H)	
T:441_L:438 (H-H)	

HPV-16 L1	ChiΔE-L2
B_1: D_10 ICOS_3-FOLD	**D_1:D_10 ICOS_3-FOLD**
T:39 A:435 (P-H)	I:39_T:435 (P-P)
T:39_P:436 (P-H)	T:39_A:436 (P-P)
L:40_A:435 (H-H)	I:40_P:437 (H-H)
S:40_P:436 (P-H)	S:40_T:435 (P-P)
R:41_P:436 (B-H)	R:41_S:436 (B-P)
R:41_K:437 (B-B)	R:41_E:437 (B-H)
L:42_A:435 (H-H)	K:41_L:438 (B-H)
L:42_P:436 (H-H)	R:41_A:442 (B-H)
L:43_P:436 (H-H)	L:42_T:435 (H-P)
K:109_T:438 (B-A)	L:42_S:436 (H-P)
K:109_E:439 (B-A)	L:43_S:436 (H-P)
G:110_E:438 (H-A)	G:108_L:438 (H-H)
Q:111_K:442 (P-B)	G:110_T:438 (H-H)
Q:111_K:443 (P-B)	Q:111_A:442 (P-H)
D:112_L:441 (A-H)	Q:111_P:443 (P-H)
D:112_K:443 (A-B)	P:112_G:441 (H-H)
E:369_E:438 (A-A)	P:112_P:443 (H-H)
E:369_K:442 (A-B)	D:367_K:442 (A-B)
Y:370_E:438 (P-A)	N:368_L:438 (H-H)
D:371_E:438 (A-A)	A:368_P:443 (H-H)
D:371_E:438 (A-A)	D:367_S:436 (A-P)
	D:367_P:437 (A-H)
	D:371_L:438 (A-H)
D_1: E_1 QUASI_5-FOLD	**D_1:E_1 QUASI_5-FOLD**
*A:266 T:358 (H-P)	*T:266_T:358 (P-P)
F_1:F_2 ICOS_5-FOLD	**F_1:F_2 ICOS_5-FOLD**
	*T:266_T:358 (P-P)
	*T:266_K:361 (P-B)

H-> Hydrophobic(nonpolar) — Purple-> Difference in HPV-16 L1
P -> Polar — Blue-> Only in HPV 16 L1
A -> Acidic — Red-> Difference in ChiΔE-L2
B -> Basic — Green-> Only in ChiΔE-L2

TABLE D.4: Differences between calculated subunit contacts for ChiΔF-L2 and HPV-16 L1. Contacts calculated using VIPER analysis tool (Reddy *et al.*, 2001) and the differences between the contacts of HPV-16 L1 and ChiΔF-L2 were checked using WordDiff software (www.softinterface.com).

HPV-16 L1	ChiΔF-L2
A_1:A_2 ICOS_5-FOLD	**A_1:A_2 ICOS_5-FOLD**
A_1: B_1 QUASI_5-FOLD	**A_1:B_1 QUASI_5-FOLD**
	T:266_T:356 (P-P)
A_1: E_1 QUASI_5-FOLD	**A_1:E_1 QUASI_5-FOLD**
*K:361_A:266 (R-H)	*K:361_T:266 (R-P)
A_1: F_1 QUASI_2-FOLD	**A_1:F_1 QUASI_2-FOLD**
A:44_D:416 (H-A)	A:44_E:416 (H-A)
N:57_V:421 (P-H)	N:57_T:421 (P-P)
K:59_V:421 (R-H)	N:57_D:422 (P-A)
K:59_T:422 (R-P)	N:58_T:421 (P-P)
I:60_V:418 (H-H)	K:59_I:421 (R-H)
P:63_T:417 (H-P)	K:59_D:422 (R-A)
P:63_V:418 (H-P)	I:60_T:418 (H-P)
D:416_L:45 (A-H)	P:63_T:418 (H-P)
D:416_A:44 (A-H)	P:416_T:45 (A-P)
T:417_P:63 (P-H)	E:416_A:44 (A-H)
V:418_I:60 (P-H)	I:418_I:60 (P-H)
V:418_P:63 (P-H)	I:418_P:63 (P-H)
V:421_K:59 (H-R)	I:421_K:59 (H-R)
I:422_N:57 (P-P)	D:422_N:57 (A-P)
T:422_K:59 (P-R)	D:422_N:58 (A-P)
Q:424_N:177 (P-P)	D:422_K:59 (A-R)
Q:424_V:178 (P-T)	A:428_K:59 (H-R)
T:426_G:175 (P-H)	D:426_N:177 (A-P)
T:426_N:177 (P-P)	

HPV-16 L1	ChiΔF-L2
B_1: C_1 QUASI_5-FOLD	**B_1:C_1 QUASI_5-FOLD**
C_1: D_1 QUASI_5-FOLD	**C_1:D_1 QUASI_5-FOLD**
*A:266_K:361 (H-R)	*A:266_T:359 (P-P)
	*T:266_K:361 (P-R)
D_1: D_10 ICOS_3-FOLD	**D_1:D_10 ICOS_3-FOLD**
D_1: E_1 QUASI_5-FOLD	**D_1:E_1 QUASI_5-FOLD**
	*T:266_T:356 (P-P)
F_1:F_2 ICOS_5-FOLD	**F_1:F_2 ICOS_5-FOLD**

H->Hydrophobic(nonpolar)	Purple-> Difference in HPV-16 L1
P -> Polar	Blue-> Only in HPV-16 L1
A -> Acidic	Red-> Difference in ChiΔF-L2
B -> Basic	Green-> Only in ChiΔF-L2

TABLE D.5: Differences between calculated subunit contacts for ChiΔH-L2 and HPV-16 L1. Contacts calculated using VIPER analysis tool (Reddy *et al.*, 2001) and the differences between the contacts of HPV-16 L1 and ChiΔH-L2 were checked using WordDiff software (www.softinterface.com).

HPV-16 L1	ChiΔH-L2
A_1: B_1 QUASI_5-FOLD	**A_1:B_1 QUASI_5-FOLD**
	G:366_K:362 (A-A)
	*T:265_T:390 (P-P)
	*T:268_K:361 (P-B)
A_1: E_1 QUASI_5-FOLD	**A_1:E_1 QUASI_5-FOLD**
*K:361_A:266 (B-H)	T:84_*T:265 (P-H)
	*K:361_T:266 (B-P)
A_1: F_1 QUASI_2-FOLD	**A_1:F_1 QUASI_2-FOLD**
N:81_G:84 (P-H)	N:81_G:84 (P-A)
K:82_D:79 (B-A)	D:79_T:84 (A-A)
K:82_K:82 (B-B)	D:81_E:84 (H-A)
E:83_E:404 (H-H)	D:81_T:85 (H-P)
G:84_N:81 (H-P)	I:81_S:86 (H-P)
G:84_R:97 (H-B)	V:82_V:82 (H-H)
D:87_N:82 (A-P)	V:82_F:87 (H-H)
T:88_Y:91 (P-P)	V:82_D:80 (H-A)
T:88_S:92 (P-P)	E:83_T:85 (A-P)
Y:91_T:88 (P-P)	E:83_S:86 (A-P)
N:92_D:87 (P-A)	F:83_F:87 (A-H)
N:92_T:88 (P-P)	E:83_L:89 (A-H)
R:97_G:84 (B-H)	E:84_D:79 (A-A)
W:402_G:84 (H-H)	E:84_L:81 (A-H)
E:404_P:83 (H-H)	E:84_T:84 (A-A)
E:404_P:86 (H-H)	E:84_T:85 (A-P)
	E:84_S:86 (A-P)
	T:85_L:81 (P-H)
	T:85_T:83 (P-A)
	T:85_I:84 (P-A)
	S:86_L:81 (P-H)
	S:86_T:83 (P-A)
	S:86_R:97 (P-B)
	S:86_W:402 (P-H)
	S:86_F:404 (P-H)
	P:87_V:82 (H-H)
	P:87_E:83 (H-A)
	D:89_V:82 (A-H)
	D:89_D:83 (A-A)
	R:97_S:86 (B-P)
	W:402_S:86 (H-P)
	F:404_S:86 (H-P)

H->Hydrophobic(nonpolar)	Purple-> Difference in HPV-16 L1
P -> Polar	Blue-> Only in HPV-16 L1
A -> Acidic	Red-> Difference in ChiΔH-L2
B -> Basic	Green-> Only in ChiΔH-L2

TABLE D.6: Trace log of the SWISS MODELLed ChiΔH-L2 chimaera. The log highlighted in yellow shows the difficulty in modelling the C-D with the L2 epitope replacement.

```
AlignMaster output
==================================================

Length of target sequence: 505 residues

Reading user-defined template list

Extracting template sequences

Running pair-wise alignments with target sequence
Sequence identity of templates with target:

1L0T_monomer.pdb: 96.8 % identity
```

```
Looking for template groups
Global alignment overview:

Taget Sequence: |=============================================---|
1L0T_monomer.pdb |---------------------------------------------...

AlignMaster found 1 regions to model separately:
1: Using template(s)   1L0T_monomer.pdb

Creating Batch files for ProMod (if any):
Batch.1: residues 1   479 of submitted sequence.

Exiting AlignMaster

ProModII trace log for Batch.1
==============================================================

ProModII: ProMod     version 3.5 date Jul 19 1999 17:15
ProModII: SPDBV      version 3.5
ProModII: Loop       version 2.60
ProModII: LoopDB     version 2.60
ProModII: Parameters version 3.5
ProModII: Topologies version 3.5
ProModII: Loading Template: 1L0T_monomer.pdb
ProModII: 1L0T monomer.pdb : some sidechain atoms are missing -->
reconstructing them
ProModII: Loading Raw Sequence
ProModII: Aligning Raw Sequence
ProModII: Refining Raw Sequence Alignment
ProModII: C-terminal overhang trimmed for chain ' ', End at residue: 474
ProModII: adding blocking groups
ProModII: Adding Missing Sidechains
ProModII: Trying Ligating with anchor residues ASP 89 and ALA 92
ProModII: Trying Ligating with anchor residues ILE 88 and ALA 92
ProModII: Trying Ligating with anchor residues ILE 88 and PRO 93
ProModII: Trying Ligating with anchor residues PHE 87 and PRO 93
ProModII:  Number of Ligations found: 3
ProModII: all loops are bad; continuing CSP with larger segment
ProModII: Trying Ligating with anchor residues PHE 87 and ASP 94
ProModII:  Number of Ligations found: 201
ProModII: ACCEPTING loop  46: clash=   0 FF=        227.9 PP=  2.00
ProModII: Building CSP loop with anchor residues PRO 80 and GLU 83
ProModII: Building CSP loop with anchor residues ASP 79 and GLU 83
ProModII:  Number of Ligations found: 3
ProModII: all loops are bad; continuing CSP with larger segment
ProModII: Building CSP loop with anchor residues ASP 79 and GLU 84
ProModII:  Number of Ligations found: 5
ProModII: all loops are bad; continuing CSP with larger segment
ProModII: Building CSP loop with anchor residues PRO 78 and GLU 84
ProModII:  Number of Ligations found: 207
ProModII: all loops are bad; continuing CSP with larger segment
ProModII: Building CSP loop with anchor residues PRO 78 and THR 85
ProModII:  Number of Ligations found: 500
ProModII: ACCEPTING loop  314: clash=   0 FF=        443.5 PP= -5.00
ProModII: Optimizing Sidechains
ProModII: Dumping Preliminary Model
ProModII: Adding Hydrogens
ProModII: Optimizing loops and OXT (nb = 15)
ProModII: Final Total Energy:        6321.945 KJ/mol
ProModII: Removing Hydrogens
ProModII: Fixing Atom Nomenclature
ProModII: Dumping Sequence Alignment
***
```

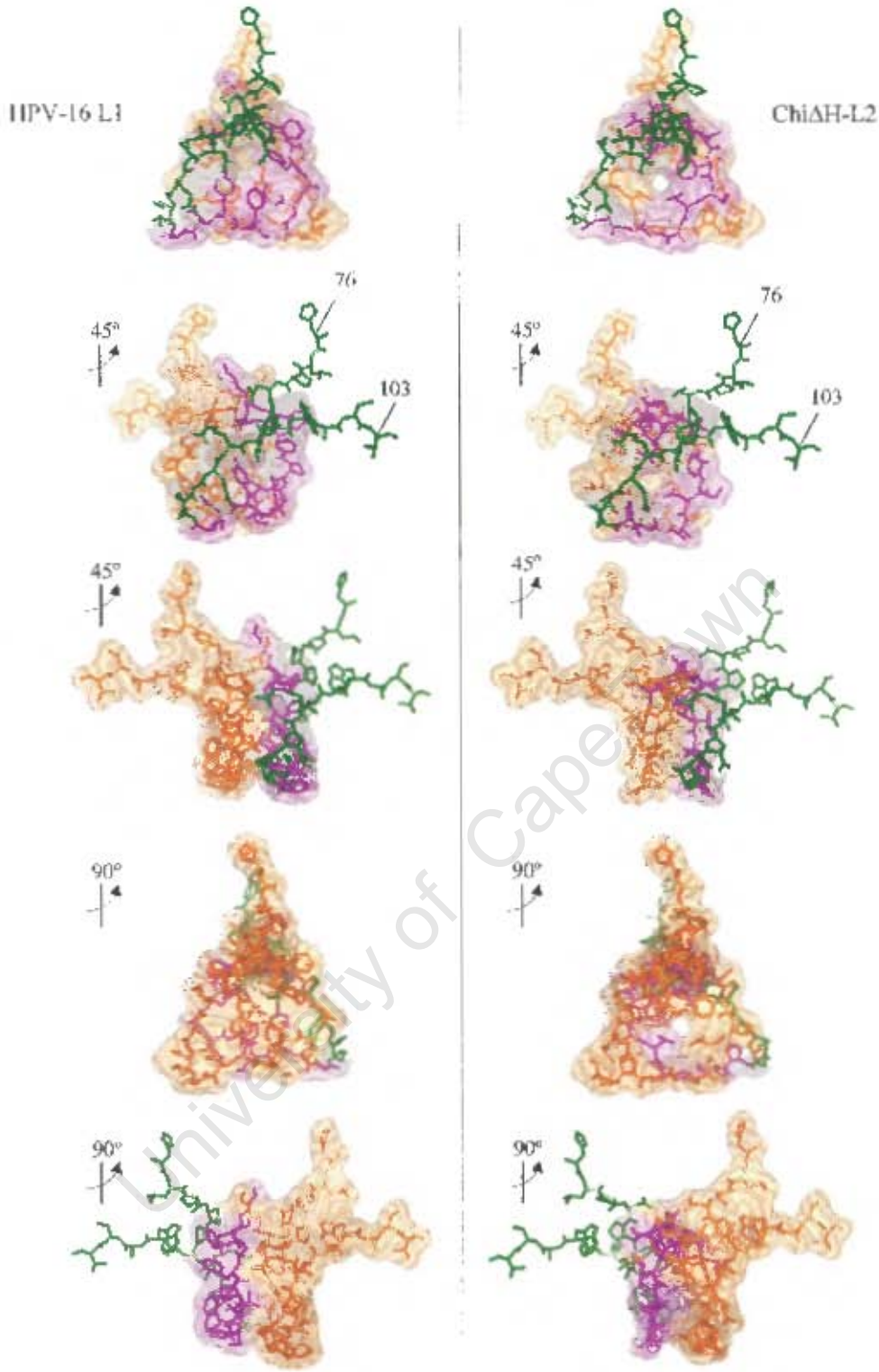

FIGURE D.2: Illustrations of the differences in the C-D:C-D loop interaction between HPV-16 L1 and ChiΔH-L2. Purple colour represent the amino acid region 81-93.

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