

TITLE

"MODIFIED PAPILLOMA VIRUS L2 PROTEIN AND
VLPs FORMED THEREFROM"

FIELD OF INVENTION

THIS INVENTION relates to a modified papilloma virus L2 protein and VLPs formed therefrom, in particular, antigens and vaccines containing said VLPs that may be effective in treatment of infections caused by such viruses.

PRIOR ART

Papilloma viruses (PV) infect both humans and animals (see for review "Papilloma Virus Infections in Animals" by J. P. Sundberg which is described in Papilloma Viruses and Human Disease, edited by K. Syrjanen, L. Gissmann and L. G. Koss, Springer-Verlag 1987). Human papilloma viruses are a family of small DNA viruses which induce benign hyperproliferative lesions of the cutaneous and mucosal epithelia. Of the 70 different virus types which have been identified, more than 20 are associated with anogenital lesions (de Villiers, 1989. J. Virol. 63 4898-4903).

In particular, HPV16 is associated with pre-malignant and malignant diseases of the genito-urinary tract, and in particular, with carcinoma of the cervix (Durst *et al.*, 1983. P.N.A.S. 80 3812-3815; Gissmann *et al.*, 1984. J. Invest. Dermatol. 83 265-285). The detection of antibodies against HPV16 fusion proteins (Jenison *et al.*, 1990. J. Virol. 65 1208-1218; Köchel *et al.*, 1991. Int. J. Cancer 48 682-688) and synthetic HPV16L1 peptides (Dillner *et al.*, 1990. Int. J. Cancer 45 529-535) in the serum of patients with HPV16 infection confirms that there are B epitopes within the capsid proteins of HPV, though few patients have HPV 16L1-specific antibodies identified by these techniques. There is no system for PV propagation *in vitro*, and human genital lesions associated with HPV16 infection contain few PV particles and low levels of viral structural proteins. Thus further studies on papilloma viruses have been limited.

PV capsids comprise two virally encoded structural proteins, designated L1 and L2, which are assembled onto a DNA-protein complex (Galloway *et al.*, 1989. *Adv. Virus Res.* 37 125-171). A single virus capsid is a T=7d icosahedron composed of 72 pentameric capsomeres, each of which contains five molecules of the major capsid protein, L1 (Baker *et al.*, 1991. *Biophys. J.* 60 1445-1456; Finch *et al.*, 1965. *J. Mol. Biol.* 13 1-12). The minor capsid protein, L2, is present at approximately 1/10 the abundance of L1 (Doorbar *et al.*, 1987. *J. Virol.* 61 2793-2799) and has an unknown structural role. L1 protein is directed to the nucleus by a C-terminal nuclear localization signal (Zhou *et al.*, 1991. *Virology* 185 625-632); virus assembly occurs in the nucleus (Orth *et al.*, 1977. *J. Virol.* 24 108-120; Pfister *et al.*, 1987. *Papilloma viruses: particles, genome organisation and proteins*, p. 1-18 /n K. Syrjanen, L. Gissmann, and L. G. Koss (ed.), *Papilloma viruses and human disease*. Springer-Verlag KG, Berlin). Recombinant L1 protein self-assembles into particles resembling virus capsids (Zhou *et al.*, 1993. *J. Gen. Virol.* 74 763-768), but assembly is enhanced in the presence of L2 protein, which may be required for assembly of infectious virions (Hagensee *et al.*, 1993. *J. Virol.* 67 315-322; Zhou *et al.*, 1991. *Virology* 185 251-257).

Recombinant PV L1 protein and the combination of recombinant PV L1 and PV L2 proteins have formed the basis of vaccines for the prevention and treatment of papilloma virus infections and used as antigens for the detection of papilloma virus (International Patent Application Publication No. WO93/02184). The presence of the PV L2 protein increases the immunogenicity of the vaccine (Zhou *et al.*, 1991. *Virology* 185 251-257).

Subsequent to International Publication No. WO93/02184 and the published research by Zhou and others (Zhou *et al.*, 1991, *Virology* 185 251-257), other workers have developed expression systems for the expression of human papilloma virus VLPs.



International Patent Application Publication No. WO94/20137 is directed to the expression of the L1 protein of human papilloma virus and the production of VLPs in Sf-9 insect cells using a baculovirus expression system.

As well, the formation of capsids following expression of L1 and L2 in mammalian cells and insect cells were disclosed in two research articles (Hagensee *et al.*, 1993, J. Virology 67 315 and Kirnbauer *et al.*, 1992, PNAS 89 12180). Two other International Patent Application Publications, namely WO94/00152 and WO94/05792, were directed to recombinant papilloma virus L1 proteins and their use as vaccines and for diagnostic purposes.

One problem, however, with VLP formation which includes PV L2 protein is the incorporation of DNA into the capsid. The inclusion of DNA in the capsid of papilloma virus VLP is not desirable for the vaccines comprising these VLPs. Indeed, in some countries, there are legislative requirements limiting the amount of DNA allowed in a vaccine shot. The level of 10 picograms of DNA per shot has been typically used as an upper limit. This requirement may be due in part to fears of infection from introducing foreign DNA into a healthy individual. The concern with vaccines comprising native L2 protein is that amounts of DNA exceeding this level may be included.

SUMMARY OF THE INVENTION

Thus it is an object of the present invention to provide a virus-like particle that incorporates a substantially minimal amount of DNA.

A further object is to provide a vaccine which overcomes the aforementioned problem.

The invention, therefore, in one aspect, includes a method for production of one or more papilloma virus-like particles (VLPs) which incorporates a substantially minimal amount of DNA including the steps of:-



- (1) constructing a recombinant DNA molecule which encodes a papilloma virus L2 protein that binds a substantially minimal amount of DNA; and
- (2) introducing said recombinant DNA molecule into a suitable host cell so that a papilloma virus L1 protein and said papilloma virus L2 protein is expressed and said VLPs are formed therefrom.

The term "a substantially minimal amount of DNA" covers the situation where essentially no DNA is bound by the PV L2 protein or where there is 10 picograms of DNA or less per vaccine shot or other DNA limit set by legislation.

It will be appreciated that a second aspect of the invention lies in the recombinant DNA molecule which encodes said papilloma virus L2 protein.

Further, another aspect of the invention resides in said papilloma virus L2 protein. The L2 protein is preferably modified so that any one or more of the 1-12 amino acid residues adjacent the N-terminal end of the L2 protein are different compared to the wild type L2 protein.

Most preferably the invention includes within its scope L2 mutant(s) shown hereinafter in FIG. 4.

In another aspect, the present invention resides in the novel papilloma virus VLP formed from the said papilloma virus L1 and L2 proteins. The L2 protein forming the VLP preferably has a minimal number of amino acid modifications

The invention, in another aspect, includes a vaccine containing the papilloma virus VLPs with or without a suitable adjuvant.

In relation to step (1), the recombinant DNA molecules are suitably constructed from a source of papilloma virus genome whereby the L2 gene may be amplified by PCR amplification using suitably designed primers. The recombinant DNA molecules are

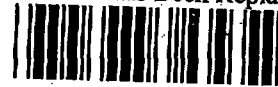


preferably amplified from a suitable plasmid containing the PV genome or part thereof. The preferable genome is HPV16 genome.

Preferably primers include those that change one or more of the bases 1-36 from the 5' end of the L2 gene. Changes are preferably by deletion or substitution. A list of the most preferable primers for amplification are listed below (see FIGS. 6, 7 and 8). The L1 and L2 genes may be transcribed from any mammalian or viral promoter with a mammalian or viral polyadenylation signal. Preferably the L1 and L2 genes are transcribed from any vaccinia virus promoter which may be an early promoter or a late promoter as considered appropriate. A list of such promoters is given in Davidson & Moss, 1989, J. Mol. Biol. 210 749-769 and 1989, J. Mol. Biol. 210 771-784. A suitable promoter from which to initiate transcription of the L2 gene is the vaccinia virus late promoter 4b.

The L1 and L2 genes may be encoded on separate vectors or on the same vector. Suitable vectors include plasmids, cosmids and recombinant viruses. Preferably the recombinant DNA molecules are contained in one or more recombinant viruses which may transfect those cells. Suitable viruses that may be used for this purpose include baculovirus, vaccinia, sindbis virus, SV40, Sendai virus adenovirus, retrovirus and poxviruses. Suitable host cells may include host cells that are compatible with the above viruses and these include insect cells such as *Spodoptera frugiperda*, CHO cells, chicken embryo fibroblasts, BHK cells, human SW13 cells, drosophila, mosquito cells derived from *Aedes albopictus* or monkey epithelial cells. It will also be appreciated that other eukaryote cells may comprise yeast cells or other mammalian cells.

Suitable expression systems include prokaryotic expression systems including *E. coli* and any plasmid or cosmid expression vector or eukaryotic systems including host cells described above in combination with a recombinant virus vector or alternatively, yeast cells and yeast plasmids.



The VLPs may be obtained from the transfected cells by any suitable means of purification. A preferable method of production and purification of papilloma virus-like particles is provided in WO93/02184. The VLPs may be combined with any suitable adjuvant such as ISCOMS, alum, Freund's Incomplete or Complete Adjuvant, Quil A and other saponins or any other adjuvant as described for example in Vanselow, 1987, S. Vet. Bull. 57 881-896.

Reference may now be made to various preferred embodiments of the invention as illustrated. In these preferred embodiments, it should be noted that the specific papilloma viruses, VLPs and specific constructs of DNA recombinant molecules are given by way of example.

EXPERIMENTAL

1. *CONSTRUCTION OF A MODIFIED PAPILLOMA VIRUS L2 PROTEIN*

Materials and Methods

Plasmid construction: For expression in VV, the open reading frame corresponding to the 474-amino-acid HPV16L2 coding region was amplified by PCR from a plasmid containing the HPV16 genome. The 5' primer introduced a *Bam*HI site upstream from the L2 open reading frame ATG and the 3' primer introduced a *Sma*I site beyond the termination codon. The amplified L2 fragment was recovered by elution from an agarose gel, cut with *Bam*HI and *Sma*I, and ligated into RK19 (Kent, 1988. Ph.D. thesis. University of Cambridge, Cambridge, England), creating RK19/16L2, in which L2 expression is driven by the VV late promoter 4b. The HPV16L2 and 4b promoter were then transferred to the VV expression vector pSX3 (Zhou *et al.*, 1991. Virology 185 251-257) for rVV construction.

To create the simplified vector pUC18/4b16L2 and facilitate transfer of mutant L2 genes between vectors used for VV

expression, a Klenow-blunted *MluI-EcoRI* fragment carrying the VV 4b promoter and the whole HPV16L2 open reading frame was cleaved from RK19/16L2 and inserted into pUC18. This plasmid was used as the DNA template for PCR amplifications with primers designed to create C-terminal truncations of L2. Mutants $\Delta 374$, $\Delta 384$, $\Delta 394$, $\Delta 404$, and $\Delta 414$, C-terminally truncated to residues 374, 384, 394, 404, and 414 of the L2 protein, respectively, were created by using a common 5' primer (M13RSP) (Zhou *et al.*, 1991. Virology 185 625-632) and a panel of 3' primers introducing stop codons (TAA) at codons 374, 384, 394, 404, and 414. To create N-terminal truncation and point mutations, we used a panel of 5' primers. N-terminal deletions ($\Delta 1-2$, $\Delta 1-3$, $\Delta 1-4$, $\Delta 1-5$, $\Delta 1-6$, $\Delta 1-7$, $\Delta 1-8$, $\Delta 1-9$, $\Delta 1-10$, $\Delta 1-11$, $\Delta 1-12$, $\Delta 1-13$, $\Delta 1-14$, $\Delta 1-15$, $\Delta 1-20$, $\Delta 1-40$, $\Delta 1-60$, $\Delta 1-80$, and $\Delta 1-100$) were created by using the set of primers which introduced ATG codons at positions corresponding to amino acids 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 40, 60, 80, and 100, respectively. For N-terminal point mutations (designated H3P; K4P; 2,4,5,N; R5P; S6N; S6P; A7P; K8P; R9P; and 8,9N), amino acids 3, 4, 5, 6, 7, 8, and 9 were changed to either proline (Pro) or asparagine (Asn) by the mismatched-primer method (Zhou *et al.*, 1991. Virology 185 625-632). All mutations were confirmed by direct sequencing of the expression plasmids.

Cells and virus. CV-1 cells were maintained in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal or newborn bovine serum (CSL, Melbourne, Australia). Plaque-purified isolates of rVVs were propagated in CV-1 cells grown in Dulbecco's modified Eagle's medium supplemented with 2.5% fetal bovine serum (CSL, Melbourne, Australia).

rVV construction. We used previously described methods (Zhou *et al.*, 1991. Virology 185 251-257) for rVV construction. Briefly, plasmids including the HPV16L2 gene with various mutations driven from the VV late promoter 4b, the

Escherichia coli gpt gene (Coupar *et al.*, 1988. Gene 68 1-10; Falkner *et al.*, 1988. J. Virol. 62 1849-1854) as a selectable marker, and flanking fragments of the VV B24R gene (Kotwal *et al.*, 1989. J. Virol. 63 600-606; Smith *et al.*, 1989. J. Gen. Virol. 70 233-2343) or thymidine kinase (TK) gene were transfected into VV WR strain-infected (0.05 PFU per cell) CV-1 cells by calcium phosphate precipitation. Virus plaques were purified twice in CV-1 cells in the presence of mycophenolic acid at a concentration of 25 μ g/ml.

Immunoprecipitation of L1 and L2 proteins. CV-1 cells were infected with HPV16L1 rVV or HPV16L2 rVV at a multiplicity of infection of about 20 PFU per cell. At 48 h, 5×10^5 infected cells were lysed with RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris [pH 8.0]). Lysed cells were centrifuged briefly at 12,000 $\times g$, and the supernatant was used for immunoprecipitation. Immunoprecipitation were carried out with a 1:20 dilution of monoclonal anti-HPV16L1 antibody (McLean *et al.*, 1990. J. Clin. Pathol. 43 488-492) or a 1:2,000 dilution of rabbit anti-HPV16L2 antibody (provided by D. A. Galloway). The precipitated L1 or L2 protein was collected with protein A-Sepharose beads and washed four times in RIPA buffer. Proteins were removed from protein A-Sepharose beads by boiling in polyacrylamide gel electrophoresis (PAGE) sample buffer and separated by SDS-PAGE for analysis.

Southwestern assays. The Southwestern (DNA-protein) assays were based on previously published procedures (McCall *et al.*, 1991. J. Invest. Dermatol. 97 111-114; Moreland *et al.*, 1991. J. Virol. 65 1168-1176). Immunoprecipitated HPV16L1 and HPV16L2 proteins were separated on a SDS-10% polyacrylamide gel and transferred to a nitrocellulose filter by electroblotting. Filters were blocked with blocking buffer (10 mM Tris [pH 7.5], 5% nonfat skim milk, 10% glycerol, 2.5% Nonidet P-40, 0.1 mM dithiothreitol [DTT], 150 mM NaCl) at 4°C for 12 h. The filters were then washed with

binding buffer (10 mM Tris [pH 7.5], 40 mM NaCl, 1 mM EDTA, 1 mM DTT, 8% glycerol, 0.125% skim milk). ^{32}P -labelled probes were added to binding buffer, and incubation was continued for 4 h at 4°C. The filters were washed with five changes of binding buffer. After being air dried, they were wrapped and exposed to X-ray films. They were subsequently reprobbed with anti-HPV16L1 or anti-HPV16L2 anti-serum and ^{125}I -protein A to confirm protein transfer.

Binding specificity assay. DNA sequences binding specifically to L2 were selected from a pool of double-stranded, 76-mer oligonucleotides (R76) containing a central stretch of 26 random base pairs, flanked by two unique sequences of 25 bp each (Sorger *et al.*, 1986. J. Mol. Biol. 191 639-658). The sequences of these oligonucleotides were:-

R76

5'-CAGGTCAGATCAGCGGATCCTGTCTG (N)₂₆

GAGGCGAATTCAGTGCATGTGCAGC-3'

Forward primer 5'-GCTGCACATGCACTGAATTCGCCTC-3'

Back primer 5'-CAGGTCAGATCAGCGGATCCTGTCTG-3'

Random oligonucleotide-binding assays were performed essentially as described previously (Treacy *et al.*, 1991. Nature 350 577-584). Briefly, L1 and L2 proteins purified by immunoprecipitation were electrophoresed on an SDS-PAGE gel (10% polyacrylamide) and transferred to a nitrocellulose filter. Filters were incubated for 4 h at 4°C in buffer A (20 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid [HEPES]-HCl [pH 7.9], 1 mM DTT, 10% glycerol, 0.01% Nonidet P-40) containing 50 mM KCl and 5% skim milk. The ^{32}P -labelled pool of random oligonucleotides was prepared by primed synthesis with the forward primer annealed to the random 76-mer oligonucleotide template, added to the filter, and incubated overnight at 4°C. Washes were performed at 4°C in buffer A adjusted to 100 mM KCl (two 10-min washes) and then in buffer A adjusted to 200 mM KCl (one 10-min wash). Filters were autoradiographed, the area

of the filter corresponding to bound DNA was excised, and the DNA was eluted by heating at 100°C in water. Eluted DNA was amplified by 30 cycles of PCR with forward and backward primers and purified on 2% agarose gels. For subsequent rounds of binding, the 76-bp product was labelled by 18 cycles of PCR in the presence of ³²P-labelled nucleotide, as described previously (Sorger *et al.*, 1986. J. Mol. Biol. 191 639-658). DNA from the fifth round of selection which remained bound to L2 after washing in buffer A was eluted and amplified as described above, cloned into pUC18, and sequenced.

Immuno-DNA binding assay. Extracts from HPV16L2 rVV-infected cells were prepared and immunoprecipitated as described above. Immune complexes attached to protein A-Sepharose beads were incubated with *Bam*HI-*Pst*I-digested HPV16 DNA for 2 h at 4°C in DNA-binding buffer (10 mM Tris [pH 7.4], 100 mM NaCl, 1 mM MgCl₂, 1 mM EDTA, 8% glycerol, 1 mM DTT, 5% skim milk). Following incubation, the beads were washed five times with the same buffer at room temperature. Protein-DNA complexes were eluted in 1% SDS-5 mM EDTA at 65°C. DNA was extracted with phenol twice and precipitated with ethanol. Samples were run on a 1.5% agarose gel and blotted onto nylon membranes. The DNA bound to the membranes were detected by Southern blotting with ³²P-labelled HPV16 DNA digested with *Pst*I-*Bam*HI.

DNA sequencing. Dideoxy DNA sequencing was performed with the Sequenase version 2.0 kit (U.S. Biochemicals, Cleveland, Ohio). To denature double-stranded DNA for sequencing, we incubated 2 µg of DNA for 30 min at 37°C in 200 mM sodium hydroxide. DNA was ethanol precipitated and sequencing carried out as specified by the manufacturer.

RESULTS

HPV16L2 protein binds DNA. To investigate the DNA-binding activity of PV structural proteins, we expressed HPV16L1 or HPV16L2 in eukaryotic cells by using rVV. L1 and L2 proteins were

purified from cell lysates by immunoprecipitation, separated by SDS-PAGE (10% polyacrylamide), and transferred to nitrocellulose filters. Binding of these proteins to DNA was investigated by Southwestern blotting with ^{32}P -labelled PV genomic DNA and bacteriophage λ DNA in a buffer containing 40 mM NaCl. HPV16L2 protein bound both HPV genomic DNA and λ DNA (FIG. 1B, lanes 2 to 5). HPV16L1 protein, in contrast, failed to bind any labelled DNA (FIG. 1A, lanes 2 to 5), although the purified L1 protein was detectable on the filters by using an anti-HPV16L1 monoclonal antibody (FIG. 1A lanes 1 and 6). Further experiments in buffers containing up to 150 mM NaCl showed no binding of DNA to HPV16L1 (data not shown). These results suggest that HPV16L2 protein, but not HPV16L1 protein, contains DNA-binding sequences and that the recognition of DNA by HPV16L2 may not be sequence specific.

HPV16L2 N terminus is important for DNA binding. To identify the protein sequence responsible for binding of L2 to DNA, we first checked for DNA-binding motifs in the predicted amino acid sequence of HPV16L2. HPV16L2 has two highly charged regions, rich in lysine and arginine. The first makes up the N terminus of the protein (MRHKRS AKRTKR) from amino acids 1 to 12; the second lies within the C terminus (RKRRKR) from amino acids 456 to 461.

To determine whether either charged region was involved in DNA binding to L2, we made a series of deletion mutants with mutations in HPV16L2. Constructs encoding various C-terminal or N-terminal mutations of HPV16L2 protein were inserted into plasmid pSX3, which had been previously shown to efficiently direct the synthesis of the HPV16L1 proteins in rVVs (Zhou *et al.*, 1991. Virology 185 625-632). CV-1 cells were infected with rVV containing each mutant L2 gene, and cell lysate was analyzed by immunoprecipitation with a rabbit anti-HPV16L2 antibody. The expected relative size of each mutated protein was confirmed by comparing the electrophoretic mobility of truncated proteins with wild-

type L2 protein (data not shown).

The deletion mutants were examined for binding to ^{32}P -labelled HPV16 genomic DNA by the Southwestern procedure under conditions associated with binding of native L2 protein. All C-terminal deletion mutants tested, including $\Delta 374$, which had the longest deletion, bound HPV DNA in proportion to the amount of immunoreactive L2 protein present (FIG. 2, lane $\Delta 374$). To delineate the contribution of the N terminus of L2 to DNA binding, we constructed N-terminal deletion mutants of L2. Three mutants, in which the first 60, 80 and 100 amino acids of L2 were deleted, each failed to bind HPV DNA (FIG. 2, lanes $\Delta 1-60$ through $\Delta 1-100$). To further characterize the N-terminal DNA-binding region, we tested a series of smaller deletions between amino acids 1 and 15 for their DNA-binding activity. Each of these, including the smallest deletion (which was missing only the arginine residue at position 2), failed to bind DNA (FIG. 3A, lanes $\Delta 1-2$ to $\Delta 1-15$). These results suggested that critical DNA-binding sequences were in the N terminus of L2 and that binding was dependent on charged amino acids including the arginine at position 2.

L2 protein uses an arginine-rich motif for DNA binding. The amino acid sequence of the N terminus for HPV16L2, starting from position 1, is MRHKRSAKRTKR (one-letter code with charged amino acids underlined). The role of the N terminus of L2 protein in DNA binding was further assessed by site-specific mutagenesis. Ten substitution mutants with mutations of the first 9 amino acids were expressed by rVV, and their DNA-binding activities were examined by Southwestern blotting with ^{32}P -labelled HPV16 genomic DNA. Similar amounts of the various mutant L2 proteins constructed were available for DNA binding, as determined by analysis of immunoreactive L2 protein on the blots, with the exception of K8P (FIG. 3B, lower panel). Mutation of some charged amino acid residues (Lys-4, Lys-8, Arg-9) to Pro or Asn (K4P and 8,9N) abolished DNA binding (FIG. 3B, lanes

K4P and 8,9N), while substitution of Arg-5 with Pro (R5P) reduced binding activity (lane R5P). In contrast, substitution of Arg-9 with Pro (R9P) had no effect on DNA binding (lane R9P). Mutation of the neutral amino acids between the Lys-Arg clusters had less effect on DNA-binding activity. Mutations termed H3P, S6P, and A7P, in which substitution of His-3, Ser-6, and Ala-7 for Pro had been produced, showed binding of DNA comparable to that with wild-type L2 (lanes H3P, S6P, and A7P); in contrast, changing Ser-6 to Asn (S6N) abolished DNA binding (lane S6N). These results suggest that the four charged amino acid clusters are important for DNA binding. In each of these charged amino acid clusters, retention of at least one charged amino acid appears necessary for DNA binding. A flexible secondary structure might also be important for L2-DNA interaction because substitution of Ser-6 with Pro and of Arg-5 and Arg-9 did not abolish the DNA binding, whereas the substitution of Ser-6 with Asn removed the L2 DNA-binding function. A summary of L2-DNA interaction results is given in FIG. 4.

L2-DNA interaction has no DNA sequence specificity. We used a library of oligonucleotides and bound these to purified HPV16L2 protein to select for any high-affinity target DNA sequences. The oligonucleotides were random at 26 positions and were flanked by primer and cloning sequences. After incubation of L2 with a pool of these oligonucleotides, oligonucleotides bound to L2 protein were eluted and amplified by PCR for subsequent rounds of selection. Selection and amplification were carried out six times. DNA clones recovered after the selection rounds 5 and 6 were sequenced. Probability theory predicts that among 52 random 26-mer sequences, any trinucleotide would be found in 20 clones (95% confidence interval, 15 to 26) and any specified sequence of 4 nucleotides would be found in 5 of the 26-mers (95% confidence interval, 0 to 9). Among 52 26-mer clones were observed that the most commonly observed trinucleotide (GGG) was present in 24

clones (twice in 5, three times in 1), whereas the most common series of 4bp (GGGG) was observed in 8 clones. Thus there was no evidence that any short nucleotide sequence was represented among these clones more frequently than would be expected by chance alone. Further, no more complex conserved nucleotide patterns were observed by using standard sequence alignment programs. These results suggested that high-affinity binding between L2 and DNA is a DNA sequence-independent process. Two additional experiments confirmed this observation. First, extract from cells infected with HPV16L2 rVV was immunoprecipitated with anti-L2 antibody, and the precipitated protein-antibody complexes, attached to Sepharose beads, were allowed to bind to a mixture of restriction fragments from HPV16 genomic DNA. After unbound DNA was washed away, bound DNA fragments were resolved on an agarose gel and detected by Southern blotting with ^{32}P -labelled HPV16 DNA. An HPV16L2-containing extract, bound to Sepharose beads with anti-L2 antibody, retained each of the HPV16 DNA fragments (FIG. 5, lanes 3, 6, and 7), whereas none of these fragments were bound by an L1 rVV-infected cell extract bound to the bead with anti-L1 antibody (data not shown) or by wild-type VV-infected cell extract bound to the beads with L2-specific antibodies (FIG. 5, lane 2). Second, a fixed amount of L2 protein was incubated with ^{32}P -labelled HPV DNA fragments, in the presence of a 100-fold (lane 4) or 1,000-fold (lane 5) excess of unlabelled λ DNA, and L2, together with any bound DNA, was immunoprecipitated from the mixture by antibody to L2. Phage λ DNA was able to prevent binding of HPV DNA. We conclude that L2 does not interact with DNA in a DNA sequence-specific manner by each of these criteria.

2. PRODUCTION OF VIRUS-LIKE PARTICLES WITH MODIFIED PAPILLOMA VIRUS L2 PROTEIN

Virus-like particles formed with the modified papilloma virus L2 protein can be produced by the procedures outlined in

WO93/02184. A suitable method for the production of virus-like particles is given below by way of example.

CV-1 cells were grown under standard cell culture conditions at 37°C in an atmosphere of 5% CO₂ to a 80% confluency in Dulbecco's modified Eagle's medium (Gibco or CSL) supplemented with 10% foetal calf serum (CSL) in a tissue culture flask. Cells were then infected with 1 to 2 pfu/cell of recombinant vaccinia virus 16L1 (pSX16L₁) and recombinant vaccinia virus 16L₂ Δ7 (Rkgpt19 Δ1-7) in Dulbecco's modified Eagle's medium supplemented with 2.5% foetal calf serum. Mycophenolic acid was added to the medium at a final concentration of 25 µg/ml. The cell culture was incubated for a further 48-60 hours. Cells were then scraped from the tissue culture flask and pelleted by centrifugation 1,500 x g at 4°C for 10 mins. The pellet was dissolved and the cells were resuspended in 20 ml of phosphate buffered saline (pH 7.4) containing 2 mM PMSF (protease inhibitor). The cell suspension was stored at 4°C or frozen at -20°C when the purification procedure was interrupted.

The cell suspension was then placed in a Wheaton glass Dounce homogeniser and kept on ice for 10 mins. Cells were disrupted by 50 strokes of the Dounce homogeniser. A small sample of the resuspension was checked by microscopy to determine whether the cells were disrupted. Homogenization was continued until all the cells were disrupted.

The lysate was centrifuged at 1500 x g for 10 mins at 4°C. The cloudy supernatant was discarded and the pellet was resuspended by aspiration in 20 ml of phosphate buffered saline (pH 7.4) containing 2 mM PMSF. The resuspended material was then sonicated (Vibra Cell Sonicator from Sonics Materials Inc. USA, setting 80) for 30 sec on ice in order to release viral particles from the nuclei. The sonicate was diluted to 60 ml with phosphate buffered (pH 7.4) saline containing 2 mM PMSF.

In each of four 38 ml ultra clear centrifuge tubes, 15 ml

of resuspended ice cold sonicate was layered on top of 23 ml of ice cold 20% w/v sucrose in phosphate buffered saline (pH 7.4). The sonicate was centrifuged at 95000 x g (rotor midpoint) for 2 hrs at 4°C in a SW-28 rotor. The supernatant and sucrose were discarded and the pellet was washed with phosphate buffered saline (pH 7.4). The pellet was resuspended in 10 ml of phosphate buffered saline (pH 7.4) containing 2 mM PMSF by sonication (Vibra Cell Sonicator from Sonics Materials Inc USA, setting 80) for 60 sec on ice. The resuspension was diluted to 20 ml with phosphate buffered saline (pH 7.4) containing 2 mM PMSF. Cesium chloride was added at 0.481 g/ml to a final volume of 23 ml. The resuspension was centrifuged at 220000 x g (mid-tube) for 18 hours at 21°C in a SW-41 rotor. After centrifugation, two bands were observed. An upper band was observed approximately 1 cm below the meniscus whereas a lower band was noted approximately 1 cm below the upper band. Both bands contained virus-like particles. Both bands were removed by aspiration. The virus-like particles in the upper band were often not as well formed as the virus-like particles from the lower band. The bands were dialysed against 5 litres of phosphate buffered saline (pH 7.4) for 2 hrs at room temperature or up to 24 hrs at 4°C. The virus-like particle preparations were stored at -20°C.

(a) *Detection of L1 and L2 proteins*

Samples of cesium chloride purified preparations of virus like particles were diluted (1:10) in 5x reducing buffer (0.05 M (final concentration) Tris-Cl (pH 6.8), 10% glycerol, 10% sodium dodecyl sulphate (SDS), 10% 2- β -mercaptoethanol (0.05%) and made up with water to 100%). The diluted samples were loaded on to SDS-PAGE (10% polyacrylamide) gel and electrophoresed. Standard procedures and conditions were followed (Towbin *et al.*, 1979, Virology 175 1-9).

Molecular weight determination of proteins: Following electrophoresis, the gel was stained for 1-24 hours with coomassie

blue stain (coomassie brilliant blue (1 g/l), 40% methanol, 10% acetic acid and 50% water). The gel was destained in methanol-acetic acid solution (40% methanol, 10% acetic acid and 50% water) for 1-24 hours and dried.

Identification of L1 and L2 proteins: On a separate but identical SDS-PAGE gel the protein species contained therein were analysed by western blotting to determine whether the L2 protein was present on the virus like particles. Standard western blotting techniques were employed (Harlow and Lane, 1988, Immunoblotting (Chapter 12) In: Antibody - A laboratory manual, Harlow and Lane (Eds.), Cold Spring Harbour Laboratory Press).

Proteins from the SDS-PAGE gel were transferred to a nitrocellulose filter paper. The nitrocellulose filter paper was cut into strips and blocked with phosphate buffered saline (pH 7.4) containing 5% skim milk powder at 4°C overnight. The primary antibody was incubated with the nitrocellulose paper strips in phosphate buffered saline (pH 7.4) containing 5% milk powder for 1.5 hours at room temperature with agitation. The primary antibody included monoclonal mouse anti-HPV16L1 antibody and polyclonal rabbit anti-HPV16L1 antibody for the detection of HPV16L1 protein and polyclonal rabbit anti-HPV16L2 antibody for the detection of HPV16L2 protein. Following incubation with the primary antibody the nitrocellulose paper strips were washed three times (10 mins per wash) in phosphate buffered saline (pH 7.4) containing 0.05% tween 20. A second antibody was incubated with the nitrocellulose paper strips in phosphate buffered saline (pH 7.4) containing 5% skim milk powder for 1 hr at room temperature with agitation. The second antibody was either horse radish peroxidase anti-rabbit or horse radish peroxidase anti-mouse immunoglobulin. Three washes as described above were repeated. The nitrocellulose paper strips were rinsed for 30 sec to 1 min with phosphate buffer (pH 7.4). The nitrocellulose paper strips were developed after placing them in a solution

containing 18 ml DAB (Di aminobenadine), 27 ml of phosphate buffer (pH 7.6), 3 ml 0.3% cobalt chloride and 30 μ l of 30% hydrogen peroxide for 10-90 sec or as soon as the band appears. The nitrocellulose paper strips were rinsed with water and dried.

Total protein determination: Three 5 μ l samples from each cesium chloride gradient purified preparation was analysed for the total amount of protein present by BCA protein assay reagent as described by the manufacturer (Pierce).

(b) *Detection of virus-like particle formation*

A sample (approximately 0.05 ml) of a virus-like particle preparation that had been purified by centrifugation on a cesium chloride gradient and dialysed against 0.1 M to 0.5 M Tris-HCl for at least 2 hrs but preferably 24 hrs at 4°C to remove the cesium chloride from the preparation was placed onto a formvar coated EM grid and negatively stained with either 1% or 2% ammonium molybdate (pH 6.5). The grids containing the samples were examined using a Hitachi H-800 transmission electron microscope.

FIGURE LEGENDS**FIG. 1**

Characterization of HPV16L1 (A) and HPV16L2 (B) DNA-binding activity. HPV16L1 and HPV16L2 proteins, from rVV-infected CV-1 cells, were immunoprecipitated by L1- or L2-specific antibodies, separated by SDS-PAGE, and transferred to nitrocellulose. Proteins were renatured in blocking buffer containing DTT and incubated with ^{32}P -labelled DNA from HPV16 (lane 2), HPV6b (lane 3), HPV11 (lane 4), or phage λ (lane 5). Lane C is the immunoprecipitate from wt VV-infected cells. Unbound DNA was removed, and DNA-binding proteins were detected by autoradiography. The positions of the L1 and L2 proteins, determined by immunoblotting, are shown (lanes 1 and 6).

FIG. 2

Definition of the HPV16L2 DNA-binding region. L2 proteins were separated by SDS-PAGE, transferred to a nitrocellulose filter, and probed with ^{32}P -labelled HPV16 DNA (A) or L2-specific antiserum (B). C-terminal amino acid deletions are designated as follows: amino acids 374 to 474 as $\Delta 374$, 384 to 474 as $\Delta 384$, 394 to 474 as $\Delta 394$, 404 to 474 as $\Delta 404$, and 414 to 474 as $\Delta 414$. Removal of amino acids up to 100 from the C-terminal end had no effect on DNA binding. N-terminal amino acid deletions were designated as follows: amino acids 1 to 60 as $\Delta 1-60$, 1 to 80 as $\Delta 1-80$, and 1 to 100 as $\Delta 1-100$. Removal of any N-terminal sequence diminished DNA binding markedly. The L2 bands are indicated by arrows.

FIG. 3

HPV DNA L2 protein interactions defined by using mutants with L2 N-terminal mutations. (A) N terminus-truncated L2 proteins, with deletions indicated above each lane, were separated by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with ^{32}P -labelled HPV16 DNA (upper panel). The ^{32}P -labelled DNA was removed, and the filter was reprobed with a rabbit anti-HPV16L2

antibody for quantitation of the L2 protein (lower panel). L2 bands are indicated by arrows, and molecular mass markers are indicated on the left. (B) Substitution mutants of L2 proteins, as indicated above each lane, were incubated with ^{32}P -labelled HPV16 genomic DNA (upper panel) or with rabbit anti-HPV16L2 antiserum (lower panel). L2 bands are indicated by arrows, and the molecular mass markers are shown on the left. Substitutions are coded as follows: WT, wild-type VV; H3P, His-3 to Pro; K4P, Lys-4 to Pro; 2,4,5N, Arg-2 to Asn, Lys-4 to Asn, Arg-5 to Asn; R5P, Arg-5 to Pro; S6N, Ser-6 to Asn; S6P, Ser-6 to Pro; A7P, Ala-7 to Pro; K8P, Lys-8 to Pro; R9P, Arg-9 to Pro; 8,9N, Lys-8 to Asn, Arg-9 to Asn.

FIG. 4

Binding of mutant L2 proteins to HPV DNA. For each mutant, the sequence of the protein is given (single-letter code, with conserved amino acids shown as dashes) and the binding of DNA to the protein by Southwestern blot analysis is indicated by (+ or -).

FIG. 5

DNA-binding assay for HPV16L2 proteins from rVVs. L2 protein was immunoprecipitated with anti-L2 antibody. Equal amounts of L2 protein were incubated with *Pst*I-*Bam*HI-cleaved HPV16 genomic DNA. The bound DNA fragments were eluted with 1% SDS and subjected to Southern blotting with ^{32}P -labelled HPV16 DNA (lanes 3, 6, and 7). In some experiments, a 100-fold (lane 4) or 1,000-fold (lane 5) molar excess of phage λ DNA was added to the initial incubation of HPV DNA with L2 protein. Mock assay of a control precipitate from wild-type VV-infected cells is also shown (lane 2). On the left the input DNA fragments are labelled from A to G. A linearized map of the HPV16 DNA is shown below. Restriction sites are *Pst*I (p) and *Bam*HI (b), and the corresponding fragments are labelled A to G according to size.

FIG. 6

Amino acid sequence of wild type HPV16L2 protein

FIG. 7

Deoxyribonucleic acid sequence of wild type HPV16L2 gene.

FIG. 8

Nucleotide sequence of the PCR primers used to construct HPV16L2 mutants.

C-terminal deletions

The PCR amplified 4b promoter/L2 fragments were cut with *Sma*I and cloned into pSX3 (Zhou *et al.*, 1990. J. Gen. Virol. 71 2185-2190) to create vaccinia expression plasmids.

N-terminal mutations

The restriction enzyme *Bam*HI and *Sma*I sites are underlined and start codons ATG and stop codons TAA are in bold. The amplified PCR products were digested with *Bam*HI and *Sma*I and cloned into the RK19 *Bam*HI/*Sma*I sites (Kent, 1988). The vaccinia 4b promoter and L2 mutant ORF was cloned into pSX3 (Zhou *et al.*, 1990) to produce vaccinia expressing plasmid containing various L2 mutant ORF.

**CLAIMS**

1. A papilloma virus L2 protein which does not bind DNA or binds a substantially minimal amount of DNA.
2. A papilloma virus L2 protein as claimed in Claim 1 wherein one or more of the 1-12 amino acid residues adjacent the N-terminal end of the L2 protein are modified or deleted.
3. A papilloma virus L2 protein as claimed in Claim 1 or 2 wherein the N-terminal sequence of the L2 protein comprises any one of the sequences outlined in FIG. 4.
4. An amino acid sequence comprising any one of the sequences outlined in FIG. 4.
5. A gene encoding the papilloma virus L2 protein as claimed in Claims 1, 2 or 3.
6. A DNA sequence encoding an amino acid sequence as claimed in Claim 4.
7. A method of producing one or more virus-like particles which incorporates a substantially minimal amount of DNA including the steps of:-
 - (1) constructing a recombinant DNA molecule which encodes a papilloma virus L2 protein that binds a substantially minimal amount of DNA; and
 - (2) introducing said recombinant DNA molecule into a suitable host cell so that a papilloma virus L1 protein and said papilloma virus L2 protein is expressed and said virus-like particles are formed therefrom.
8. A method of producing one or more virus-like particles as claimed in Claim 7 wherein said recombinant DNA molecule also encodes the papilloma virus L1 protein.
9. A method as claimed in Claim 7 wherein said papilloma virus L1 protein is encoded on a different DNA molecule than the recombinant DNA molecule encoding the papilloma virus L2 protein.



10. A method of producing one or more virus-like particles as claimed in Claim 9 wherein one or more DNA molecules are introduced into the host cell.
11. Virus-like particles produced by the method as claimed in any one of Claims 7-10.
12. A vaccine including virus-like particles as claimed in Claim 11.
13. A vaccine including a papilloma virus L2 protein as claimed in Claims 1, 2 or 3.