Positively charged sequences of human papillomavirus type 16 capsid proteins are sufficient to mediate gene transfer into target cells via the heparan sulfate receptor

Latifa Bousarghin, Antoine Touzé, Alba-Lucia Combita-Rojas and Pierre Coursaget

Using synthetic peptides we have shown that positively charged sequences present at the C terminus of the L1 protein and the N and C termini of the L2 protein of human papillomavirus type 16 (HPV-16) bind to both DNA and heparan sulfate receptors. Moreover, these short amino acid sequences are sufficient to mediate gene transfer in COS-7 cells. The L1 proteins of other HPVs were shown to contain one or two DNA- and heparin-binding sequences that have the capacity to transfer genes. These DNA-binding sequences also recognized the enhancing packaging sequence of bovine papillomavirus type 1. The results suggest that the L2 protein could participate in DNA packaging during maturation of virions.

INTRODUCTION

Papillomaviruses are non-enveloped DNA tumour viruses that persistently infect squamous epithelia. Their viral capsids are 50–55 nm in diameter and contain 72 pentamers of the major structural L1 protein (capsomers) centred on the vertices of a T=7 icosahedral lattice (Baker et al., 1991). The minor structural protein L2 is present at a ratio of about 1/30 of L1 (Kirnbauer et al., 1993). The L1 protein can self-assemble into virus-like particles (VLPs) in the presence or absence of the L2 protein (for a review see Schiller & Roden, 1995).

The use of artificial virus vectors consisting of DNA packaged in vitro into or associated with recombinant papillomavirus VLPs was recently described (Kawana et al., 1998; Stauffer et al., 1998; Touzé & Coursaget, 1998; Rossi et al., 2000; Bousarghin et al., 2002). Papillomaviruses are difficult to propagate in vitro, and thus the production of these pseudovirions offers a model to study human papillomavirus (HPV) cell entry. The pseudovirions obtained have the ability to transfer the plasmid DNA into cells where the reporter gene is then expressed. Entry of these artificial gene delivery vehicles into cells is dependent on the interaction between VLPs and the cell surface. Two putative cell receptors have been identified for HPVs. The first is α-6 integrin (Evander et al., 1997; McMillan et al., 1999) and the second comprises the cell-surface glycosaminoglycans (GAGs) (Joyce et al., 1999; Giroglou et al., 2001; Combita et al., 2001), which interact with the C-terminal portion of HPV L1 (Joyce et al., 1999).

Translocation of L1 and L2 proteins to the nucleus is mediated by nuclear localization signals (NLS) containing, as do the majority of NLS, a high proportion of positively charged residues (K and R). The prototype NLS is the SV40 large T antigen NLS (KKKRK). HPV-16 L1 contains two NLS. One has six basic amino acids (aa 499–504). The other is a bipartite NLS (aa 484–486, 499–500) (Zhou et al., 1991). The L2 protein contains two NLS corresponding to the arginine- and lysine-rich sequences located at the N and C termini of the protein (Sun et al., 1995). Two DNA-binding sequences have been identified in the L1 and L2 proteins of HPV-16. The first DNA-binding sequence was identified at the N-terminal end of L2 (Zhou et al., 1994). The second was identified at the C terminus of the L1 protein of HPV-11 and HPV-16 (Li et al., 1997; El Mehdaoui et al., 2000; Touzé et al., 2000). In addition, the nine C-terminal amino acids of the L2 protein have been shown to be necessary for infection of bovine papillomavirus type 1 (BPV-1) pseudovirions (Roden et al., 2001). The DNA-binding activity of these two domains is sequence-independent. However, it has recently been shown that DNA binding to BPV-1, HPV-6 and HPV-16 VLPs is enhanced by the presence of a 120 bp DNA sequence located in the BPV-1 E1 gene (Zhao et al., 1999; El Mehdaoui et al., 2000).

We have previously demonstrated that HPV L1 VLPs obtained by self-assembly of L1 protein lacking the last nine C-terminal amino acids had lost their capacity to bind DNA and transfer genes into target cells (Touzé et al., 2000). Moreover, addition of this sequence at the N terminus of the major capsid protein of an RNA virus, the rabbit papillomavirus type 1, has also been shown to enhance gene transfer into mammalian cells (Coursaget, 2001).
Haemorrhagic disease virus (RHDV), allowed the production of chimeric VLPs that could encapsidate DNA and transfer reporter genes into cells (El Mehdaoui et al., 2000). These experiments suggest a central role for this short sequence in DNA binding, interacting with the cell surface of target cells and facilitating the nuclear import of complexes owing to the presence of an NLS. In order to verify the role of this positively charged sequence, we investigated the possibility of using a synthetic peptide corresponding to the 14 C-terminal amino acids of the HPV-16 L1 protein. To this end, we synthesized a peptide containing this sequence (VCM-β) using the method described by Zhoa et al. (1999), and a 7-1 kbp plasmid encoding luciferase (pCMV-Luc; Ozyme). The peptides were synthesized by Sigma Genosys or Neosystem.

HPV-16 and -31 VLPs were expressed in SF21 cells infected with recombinant baculoviruses encoding the HPV-16 and -31 L1 open reading frames, respectively, and purified according to previously described procedures (El Mehdaoui et al., 2000; Combita et al., 2001; Bousarghin et al., 2002). In addition, HPV-16 L1Δ9 VLPs composed of the L1 protein lacking the nine C-terminal amino acids were used (Touze et al., 2000).

**METHODS**

**Plasmids, peptides and VLPs.** Three different plasmids were used: a 7-2 kbp plasmid encoding β-galactosidase (pCMV-β; Ozyme), a 7-3 kbp plasmid (pCMV-PEβ) (El Mehdaoui et al., 2000) obtained by insertion of the 120 bp BPV-1 packaging enhancing sequence (PES) described by Zhao et al. (1999), and a 7-1 kbp plasmid encoding luciferase (pCMV-Luc; Ozyme). The peptides were synthesized by Sigma Genosys or Neosystem.

**VLP/DNA and peptide/DNA complex formation.** VLP/DNA complexes were obtained according to a previously described method (Combita et al., 2001; Touze et al., 2001; Bousarghin et al., 2002). Ten µg VLPs and 1 µg DNA were mixed in 40 µl 50 mM NaCl, pH 5, and incubated for 30 min at room temperature before transfection. Peptide/DNA complexes were obtained according to the same method. One µg plasmid DNA and an amount of peptide corresponding to a DNA to peptide charge ratio (+/-) of 1/10 were mixed in 40 µl 50 mM NaCl, pH 5, and incubated for 30 min at room temperature. This charge ratio corresponded to peptide/DNA mass ratios ranging from 8-6 to 15-6 for L1 peptides, except for peptide HPV-16 L1 Cta (peptide/DNA mass ratio of 20), 6-2 to 6-4 for L2 peptides and SV40 large T NLS and 17-4 to 100 for other peptides. Charge ratios take into account histidines, since DNA/peptide complexes were obtained at pH 5.

**DNA retardation assay and protection of DNA from DNase.** DNA binding was investigated by agarose gel retardation assays. One µg plasmid was incubated for 30 min at 25 °C in 50 mM NaCl, pH 5, with an amount of peptide corresponding to DNA/peptide charge ratios ranging from 1/1 to 1/10. Complexes were electrophoresed through a 1 % agarose gel in 1 X TAE buffer.

To evaluate the amount of protected plasmid DNA, the complexes were treated with Benzonase (Merck) as previously described (Touze & Coursaget, 1998; El Mehdaoui et al., 2000). The amount of DNA was quantified using Molecular Analyst Software (Bio-Rad) and results expressed as the percentage of Benzonase-protected DNA.

**Detection of DNA binding by ELISA.** The DNA-binding activity of the peptides was detected by ELISA as previously described (Touze et al., 2000), with some modifications. The digoxigenin-labelled PES probe (120 bp) was obtained by PCR amplification of the PES sequence from the plasmid pBPV-CMV-β (El Mehdaoui et al., 2000) in the presence of digoxigenin-labelled dUTP (Roche Diagnostics). PCR was performed using the following oligonucleotide primers: upper, 5′-CCGGGAATTCGAGTAGGACATCACTGAGGAGGA-3′, and lower, 5′-CCGGGAATTCCTCGCTGCTTACATTTTGTTTGC-3′. A PES-negative DNA probe was obtained by PCR amplification of a 290 bp fragment corresponding to the cDNA of E1/E4 HPV-16 mRNA in the presence of digoxigenin-dUTP, using the following primers: upper, 5′-CTTAAGATCCGACAGATGCTATGCTTACATCTGTG-3′, and lower, 5′-CTTAAGATCCGACAGATGCTATGCTTACATCTGTG-3′. A 96-well plate (Maxisorp, Nunc) was seeded with 1 µg peptide per well diluted in PBS, pH 7-6, and incubated overnight at 4 °C. BSA was used as negative control. After a blocking step with 5 % BSA in PBS for 1-5 h at 37 °C, equimolar amounts of digoxigenin-labelled DNA probes (0-5 ng PES− probe, 0-2 ng PES+ probe) diluted in PBS were added. After 1 h incubation at room temperature and four washes, bound DNA was revealed by anti-digoxigenin antibodies covalently linked to horseradish peroxidase (Roche Diagnostics) diluted 1 : 1000 in PBS. After 1 h at 37 °C and four washes, bound antibodies were revealed using 2,2′-azino-bis(3-ethylbenzthiazoline-sulfonic acid) and H2O2. Absorbance was read at 405 nm and the results shown are the means of four determinations.

**Transfection experiments.** COS-7 cells grown in monolayers in Dulbecco’s Minimal Essential Medium (D-MEM)/Glutamax (Invitrogen) supplemented with 10 % foetal calf serum (FCS), 100 IU penicillin ml−1 and 100 µg streptomycin ml−1 were seeded in 96-well plates (Nunc). After washing with serum-free medium, pCMV-Luc/peptides or pCMV-Luc/VLP complexes diluted in 50 µl culture medium were added to each well. After 1 h incubation at 37 °C, the complexes were removed and 50 µl D-MEM/Glutamax supplemented with 10 % FCS was added. Cells were then incubated for 48 h at 37 °C. Luciferase gene expression was measured by luminescence assay (luciferase reporter gene assay with constant light signal; Roche Molecular Biochemical). The luminescence was integrated over 0 s (Victor2, Wallac, Perkin Elmer) and results were expressed as counts per second (c.p.s.) per well. At that time, each well contained approximately 6 × 106 cells. For experiments using pCMV-β/peptide complexes, in situ β-galactosidase activity was determined according to a previously described procedure (Müller et al., 1993).

To evaluate the effects of GAGs on transfection by L1 and L2 peptides, peptide/DNA complexes and 5 µg heparin ml−1 were added simultaneously to the cells according to the protocol described by Mislick & Baldeschweiler (1996). After 48 h incubation at 37 °C, luciferase gene expression was measured and results were expressed as percentages of inhibition of gene transfer compared with the gene transfer with peptide/DNA complexes alone.

The role of GAGs was also investigated by delsulvation of cells. COS-7 cells were seeded into 96-well plates and the medium was supplemented with 50 mM sodium chloride after 12 h incubation at 37 °C. After an additional 48 h incubation period, cells were washed twice with 200 µl PBS, and 200 µl D-MEM/Glutamax medium was added. Peptide/DNA complexes were then added to the cells and luciferase gene expression was measured after 48 h incubation at 37 °C. Results were expressed as percentage inhibition of gene transfer compared with gene transfer obtained with untreated cells.

**Detection of heparin binding.** The interaction between peptides and heparin was tested by using a competition assay derived from the heparin-binding assays described by Giroglou et al. (2001) for HPV-33 VLPs. Microtitre plates (Maxisorp, Nunc) were coated with 20 ng per well of either heparin–BSA (Sigma) or BSA. The plates
were incubated at 4 °C overnight. After four washings with PBS containing 0.1 % Tween 20, non-specific binding sites were blocked by incubation for 1 h at 37 °C with PBS plus 1 % FCS.

After washing, 200 ng of peptides or BSA diluted in PBS per well was added. Following incubation at 37 °C for 60 min and four washes, HPV-31 VLPs (200 ng per well) were added and incubated at 37 °C for 60 min. After washing, mouse anti-HPV-31 VLP antibodies diluted 1:1000 in PBS, 0 % Tween 20 and 10 % FCS were added and incubated at 45 °C for 60 min. Bound antibodies were detected with mouse anti-IgG antibodies covalently linked to horseradish peroxidase. After 1 h incubation at 45 °C and four washings, 100 μl 2 M H2SO4 and the absorbance was read at 492 nm with an automated plate reader. The absorbance of the control well was subtracted from values for test wells. Results are expressed as the percentage reduction of HPV-31 VLP binding to heparin.

RESULTS AND DISCUSSION

DNA binding and gene transfer with HPV-16 C-terminal synthetic peptide

HPV-16 L1 Ctb synthetic peptide (SSTSTTAKRKKRK, nt 492–505) was mixed with pCMV-β DNA (72 kbp) and the DNA/peptide interaction was measured by determining the amount of peptide required to retard the migration of the plasmid DNA during agarose gel electrophoresis. As shown in Fig. 1(A), the electrophoretic mobility of DNA was reduced for charge ratios (~+/+) of 1/3 to 1/5 and was completely abolished for a charge ratio of 1/10. Retardation was not observed when using the positively charged SV40 large T NLS peptide (PPKKKRKVA) at the same charge ratios. We further hypothesized that the formation of such complexes might prevent DNA degradation by nuclease. L1 peptide/pCMV-β DNA complexes were therefore treated for 1 h at 37 °C with Benzonase. Two antigenic peptides, HPV-16 E4 Ag (KPSPWAPKKHRLS) and HPV-16 L1 Ag (cVEGVPDDIYKKGSG), with a charge ratio of 1/10 corresponding to a mass ratio of DNA/peptide of 1/100, and SV40 large T NLS peptide were used as control peptides with varying proportions of positively charged amino acids. Seventy-two per cent DNA protection was observed with HPV-16 L1 Ctb peptide (Fig. 2) compared with 55 % with HPV-16 VLPs. No protection was observed with HPV-16 E4 Ag peptide (Fig. 2, lane 6) and only 23 % with the SV40 large T NLS peptide (not shown). The ability of the HPV-16 L1 Ctb peptide to deliver DNA into cells was then investigated with a β-galactosidase reporter plasmid in COS-7 cells (Fig. 1B). Transfections were performed with 1 μg DNA per well. The highest level of β-galactosidase expression was observed for a DNA/peptide charge ratio of 1/10. This peptide is one of the few examples of a natural peptide capable of interacting with plasmid DNA and carrying it across cellular barriers, as observed with the HIV-1 VpR C-terminal sequence (Kichler et al., 2000). Moreover, pre-incubation of COS-7 cells with HPV-16 L1 Ctb peptide induced 60 % inhibition of the gene transfer obtained with HPV-31 pseudovirions, compared with an absence of inhibition (0 %) observed after pre-incubation with the SV40 large T NLS peptide. This result suggests that the peptide binds to the HPV-31 cell-surface receptor. An alternative explanation could be that the excess of peptide binds to the DNA associated with the VLPs, resulting in a decrease in gene transfer due to the lower capacity of the peptide to transfer the gene compared with that obtained with the VLPs. In order to rule out this hypothesis, COS-7 cells were pre-incubated with HPV-16 L1 Ctb peptide followed by the addition of plasmid DNA to the cell culture medium. The absence of gene transfer (not shown) thus supports the hypothesis that the peptide binds to the cell receptor of HPV-31.

Gene transfer to COS-7 cells using pCMV-Luc DNA was also investigated under varying conditions of pH and molarity used for the binding of the DNA to the peptides. A gene transfer of 500 ± 52 c.p.s. was observed when DNA/peptide complexes were prepared at pH 5 in presence of 50 mM NaCl. Gene transfer decreased to 235 ± 63 c.p.s. at

Fig. 1. Formation of peptide/DNA complexes. (A) Agarose gel shift assay. (B) β-Galactosidase expression. The charge ratios of pCMV-β and HPV-16 L1 peptide were 1/1, 1/2, 1/3, 1/5, 1/10 and 1/20. BSA was used as a negative control.

Fig. 2. Benzonase protection of plasmid DNA complexed to positively charged synthetic peptides. Lane 1, DNA with Benzonase (positive control); lane 2, DNA alone (negative control); lane 3, HPV-16 L2 Nt; lane 4, HPV-16 L2 Ct; lane 5, HPV-16 L1 Ctb; lane 6, HPV-16 E4 Ag.
pH 7.5, 50 mM NaCl, and to 225 ± 49 c.p.s. at pH 7.5, 150 mM NaCl. At pH 7.2, with 50 mM Tris/HCl, 100 mM NaCl, gene transfer of 210 ± 35 c.p.s. was observed. The results obtained indicate that HPV-16 L1 Ctb peptide binds to DNA under conditions resembling the inside of the cell.

**DNA binding and gene transfer with positively charged peptides derived from the L1 or L2 proteins of HPVs**

There are two positively charged sequences at the C terminus of HPV L1 proteins and one such sequence at the N- and C-terminal ends of HPV L2 proteins. In order to investigate whether the gene transfer properties observed for the HPV-16 L1 Ctb peptide are features shared by similar sequences observed in HPV L1 and L2, various synthetic peptides corresponding to positively charged sequences observed in HPV L1 and L2 were investigated (Table 1).

These peptides were tested for DNA binding by gel retardation assay and for gene delivery to COS-7 cells using pCMV-Luc DNA as reporter plasmid (7-1 kbp). The results indicated that all the peptides bound to DNA at a 1/10 charge ratio (Table 1), with the exception of the control peptides HPV-16 E4 Ag, SV40 large T NLS, HPV-16 L1 Cta, HPV-31 L1 Ctb and HPV-58 L1 Ctb peptides. A mobility shift was only observed at a 1/40 ratio for these five peptides.

The complexes formed between DNA-binding peptides and pCMV-Luc plasmid DNA were used to transfect COS-7 cells, and gene transfer was measured by the luciferase activity determined 2 days after transfection (Fig. 3). The cut-off value for gene transfer positivity was set up at 25 c.p.s. (mean value observed with DNA alone ± 3 SD). No gene transfer was observed with the two control peptides (HPV-16 L1 Ag, HPV-16 E4 Ag), nor with the positively charged peptide corresponding to the SV40 large T NLS. Using an HPV-16 L1 Ctb scramble peptide (HPV-16 L1 CtbSc), a gene transfer of 118 c.p.s. was observed, compared with 413 c.p.s. with the reference peptide. The reduction in gene transfer with the scramble peptide suggested that gene transfer is a sequence-specific-dependent phenomenon. The residual capacity of this scramble peptide to transfer the gene could be attributed to a generic charge effect. This is in agreement with the fact that similarly charged peptides such as HPV-31 Ctb and HPV-58 Ctb peptides have no or low capacity to transfer genes.

Using the L1 positively charged peptides, the highest level of gene transfer was observed with the sequence located at the C-terminal end of the L1 protein (L1 Ctb peptides) compared with the level observed with the sequence located upstream (L1 Cta) for types 16 and 45. Among the efficient peptides, the highest level of transfer was observed with the upstream sequence for types 31, 33 and 58. It should be noted that no gene transfer was observed with HPV-16 L1 Cta peptide (22 c.p.s.) and very low levels with HPV-31 L1 Ctb and HPV-58 L1 Ctb peptides (89 and 27 c.p.s., respectively).

These results indicate the presence of two L1 sequences that bind DNA and play a role in gene transfer for HPV-33 and -45 L1 VLPs, and show that these properties are linked to the last amino acids of the L1 protein for HPV-16, and the first

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**Table 1. DNA and heparin binding of HPV L1 or L2 positively charge peptides**

<table>
<thead>
<tr>
<th>Origin</th>
<th>Sequence</th>
<th>Position (aa)</th>
<th>DNA binding (µg peptide)*</th>
<th>Heparin binding (% inhibition)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV-16 L1 Ag</td>
<td>cVGENVPDPDL1IKGSG</td>
<td>267–281</td>
<td>&gt; 40</td>
<td>12</td>
</tr>
<tr>
<td>HPV-16 E4 Ag</td>
<td>KPSPWAPKKHRLS</td>
<td>33–46</td>
<td>&gt; 40</td>
<td>18</td>
</tr>
<tr>
<td>SV40 large T NLS</td>
<td>PPKKKRKVA</td>
<td>125–133</td>
<td>&gt; 40</td>
<td>30</td>
</tr>
<tr>
<td>HPV-16 L1 Cta</td>
<td>GLKAPKFGLGRKATPTT</td>
<td>473–491</td>
<td>&gt; 40</td>
<td>24</td>
</tr>
<tr>
<td>HPV-16 L1 Ctb</td>
<td>STSTTAKKKRKRL</td>
<td>492–505</td>
<td>8</td>
<td>97</td>
</tr>
<tr>
<td>HPV-16 L1 CtbSc</td>
<td>KSRSRKTAKSTRL</td>
<td></td>
<td></td>
<td>44</td>
</tr>
<tr>
<td>HPV-31 L1 Cta</td>
<td>GYARPKFKACKR</td>
<td>474–486</td>
<td>15</td>
<td>100</td>
</tr>
<tr>
<td>HPV-31 L1 Ctb</td>
<td>TTTPAKRRKTKK</td>
<td>493–504</td>
<td>&gt; 40</td>
<td>25</td>
</tr>
<tr>
<td>HPV-33 L1 Cta</td>
<td>GLKAPFKLKR</td>
<td>472–481</td>
<td>15</td>
<td>80</td>
</tr>
<tr>
<td>HPV-33 L1 Ctb</td>
<td>RTSSAKKKVKK</td>
<td>488–499</td>
<td>12</td>
<td>74</td>
</tr>
<tr>
<td>HPV-45 L1 Cta</td>
<td>GLRRPTIGPRKR</td>
<td>477–489</td>
<td>15</td>
<td>82</td>
</tr>
<tr>
<td>HPV-45 L1 Ctb</td>
<td>RPAKRVRIRSKK</td>
<td>502–513</td>
<td>15</td>
<td>100</td>
</tr>
<tr>
<td>HPV-58 L1 Cta</td>
<td>GLKAPRKLKR</td>
<td>472–481</td>
<td>10</td>
<td>73</td>
</tr>
<tr>
<td>HPV-58 L1 Ctb</td>
<td>RAPSTKRKKVKK</td>
<td>487–498</td>
<td>&gt; 40</td>
<td>40</td>
</tr>
<tr>
<td>HPV-16 L2 Nt</td>
<td>MRHKRSARKTCKRA</td>
<td>1–13</td>
<td>3</td>
<td>90</td>
</tr>
<tr>
<td>HPV-16 L2 Ct</td>
<td>MLRKKRKRL</td>
<td>454–462</td>
<td>6</td>
<td>93</td>
</tr>
</tbody>
</table>

*Minimal amount required to achieved complete DNA shift with 1 µg plasmid DNA.
†Inhibition of HPV-31 VLP binding to heparin (data are the mean of two experiments).
Gene transfer with HPV-16 L1 or L2 peptides

C-terminal sequence of HPV-31 and -58. Gene transfer with the HPV-16 L1 Ctb peptide was 574 c.p.s. compared with 2200 c.p.s. with HPV-16 VLPs (Fig. 3), and gene transfer with the HPV-31 L1 Cta peptide was 1048 c.p.s. compared with 4345 c.p.s. with HPV-31 VLPs (not shown). Thus, the level of gene transfer obtained with L1 peptides was 24–26 % of that observed with the corresponding VLPs.

L2-derived peptides were also tested to verify the role of L2 in DNA binding and gene transfer. The results showed that the N- and C-terminal L2 peptides bound to and protected DNA from Benzonase degradation (65 % and 88 %, respectively) (Fig. 2). COS-7 transfection was observed with both peptides with luciferase activity of 860 and 1115 c.p.s., respectively (Table 1 and Fig. 3). These findings indicate the presence of two DNA binding sequences on HPV-16 L2 protein, contrary to the findings of Zhou et al. (1999). Roden et al. (2001) recently observed that the nine C-terminal amino acids of L2 are necessary for BPV infection. Our findings suggest that this could be due to the presence of a strong DNA-binding sequence at the C terminus of the protein. This raises the question of which DNA-binding sequence detected within the L1 and L2 proteins interacts with the PES identified on the BPV-1 genome between nucleotides 1506 and 1625 (Zhao et al., 1999). This PES was also recognized by HPV-6b VLPs despite the phylogenetic distance between these two papillomavirus types. Zhao et al. (1999) have therefore suggested that other papillomaviruses may use the same packaging sequence that we confirmed using HPV-16 VLPs (El Mehdaoui et al., 2000). It has been shown that the presence of L2 in HPV VLPs dramatically increase their gene transfer efficiency (Roden et al., 1996; Unckell et al., 1997; Kawana et al., 1998) and that packaging with BPV-1 L1 + L2 VLPs is increased in the presence of a papillomavirus DNA sequence (Zhao et al., 1999), suggesting that specific DNA binding to L2 is necessary to obtain efficient packaging. We therefore investigated gene transfer into COS-7 cells, using L1 and L2 synthetic peptides complexed with a pCMV-β plasmid and a pPES-CMV-β plasmid (El Mehdaoui et al., 2000). No transfection was observed with HPV-16 L1 Cta and HPV-31 L1 Ctb peptides, whichever plasmid was used. However, when using the two HPV-16 L2 peptides or the HPV-16 L1 Ctb, HPV-31 L1 Cta and HPV-45 L1 Cta peptides, a two- to threefold significant increase in the level of gene transfer was observed (Table 2) when the plasmid contained the BPV PES, in comparison with the levels obtained with the pCMV-β plasmid (P = 10⁻³, 10⁻⁵, <10⁻³, 7 × 10⁻⁴ and 2 × 10⁻⁴, respectively). No increase in gene transfer was observed when using Lipofectamine and pCMV-PES-β (438±3 cells per well) compared with the use of pCMV-β (470±2 cells per well). This suggested that the increase observed in gene transfer with L1 or L2 peptides is not due to differences in gene transfer efficiency of the two plasmids used, nor to a post-entry effect of PES on luciferase expression.

To confirm the higher binding of positively charged sequences of L1 and L2 proteins with PES sequences compared with random DNA as suggested by transfection experiments, binding of the HPV-16 L1 Ctb, HPV-16 L2 Ct and SV40 large T peptides to probes containing PES or not was also investigated by ELISA. Absence of DNA binding with PES-positive and PES-negative DNA sequences was observed for the SV40 Large T peptide (A = 0.007 ± 0.001 and 0.014 ± 0.007, respectively). Increased DNA-binding activity to DNA sequences containing the PES sequence of BPV1 was observed for both the HPV 16 L1 Ctb peptide (0.390 ± 0.040 versus 0.030 ± 0.020) and the HPV 16 L2 Ct peptide (0.440 ± 0.040 versus 0.030 ± 0.020).

These findings indicated that gene transfer was enhanced in the presence of the BPV PES with all peptides that had the capacity to bind to DNA. This confirmed that the PES is not papillomavirus type-specific and suggested that both L1 and L2 proteins recognized BPV PES. In addition, the results suggested that the very low level of gene transfer observed...
with HPV-16 L1 Cta and HPV-31 L1 Ctb peptides in the presence of pCMV-Luc plasmid was not a consequence of the fact that these peptides have a higher affinity for papillomavirus DNA.

### Binding of positively charged peptides derived from HPV L1 or L2 proteins to heparin and cell GAGs

As L1 and L2 positively charged sequences are able to transfer genes, it could be hypothesized that such peptides bind to DNA and to cells and that the NLS activity of such peptides sends the plasmid DNA to the nucleus where the reporter gene is expressed. GAGs have been proposed as cell receptors for HPVs (Joyce et al., 1999; Combita et al., 2001; Giroglou et al., 2001) and a sequence has been identified at the C terminus of the HPV-11 L1 protein that interacts with this cell receptor (Joyce et al., 1999). This sequence was included in the HPV-16 L1 Ctb peptide.

The interaction between peptides and heparin was tested using a competition assay measuring the binding of HPV-31 VLPs to heparin-coated plates. The results were expressed as percentage inhibition of HPV-31 VLP binding to heparin. Inhibition greater than 50 % was considered to indicate binding of the peptide to heparin. To rule out the hypothesis that these findings were due to binding between peptides and VLPs, the interaction between HPV-31 VLPs and HPV-16 L1 Ctb, HPV-31 L1 Cta, HPV-45 L1 Ctb and HPV16 E4 Ag peptides was investigated by ELISA. No binding ($A < 0.05$) of HPV-31 VLPs to any of the peptides investigated was observed. These results indicated that all peptides inhibited binding of VLPs to heparin with the exception of HPV-16 E4 Ag, HPV-16 L1 Cta, HPV-31 L1 Ctb and HPV-58 L1 Ctb (Table 1). It should be noted that peptides that did not deliver genes into cells did not bind to heparin, nor did they bind efficiently to DNA. In addition, the absence of binding of HPV-16 L1a9 VLPs to heparin (data not shown) confirmed and extended the results of Joyce et al. (1999) with HPV-11, suggesting that binding to heparin is mediated by the L1 protein 15 C-terminal amino acids.

To test whether GAGs could competitively inhibit gene delivery, COS-7 cells were transfected with all the DNA/peptide complexes that transferred genes in the presence of heparin. Following exposure to DNA/peptide complexes and heparin, cells were washed, incubated in fresh culture medium and assayed for luciferase expression 48 h later. Gene transfer was inhibited by 67–90 % when heparin was added to the DNA/peptide complexes, depending on the peptide used (Fig. 4). Inhibition of gene transfer was not observed after pre-incubation of the complexes with BSA in place of heparin. The role of proteoglycans in binding of HPV to cells was also assessed by inhibition of their sulfation with sodium chlorate. For this purpose, COS-7 cells were grown in the presence of sodium chlorate for 12 h and then transfected with HPV-16 L1 Ctb, HPV-33 L1 Ctb, HPV-45 L1 Ctb and L2 N- and C-terminal peptides. Luciferase gene expression was inhibited by pretreatment with sodium chlorate in a dose-dependent manner (data not shown). Gene transfer was dramatically decreased compared with untreated cells, with a decrease of 69 % for HPV-16 L1 Ctb peptide and 70 % for HPV-45 L1 Ctb (Fig. 4). These findings suggested that L1-derived peptides use heparan sulfate to enter cells and deliver genes into cells, as do VLPs.

The results obtained are in agreement with those of gene transfer using HPV-16 L1a9 VLPs made of L1 protein lacking the last nine amino acids (Touzé et al., 2000), indicating that these amino acids are essential for DNA binding and gene transfer. These findings are in agreement with those of Giroglou et al. (2001) indicating that HPV-33 VLPs made of L1 protein lacking the last seven amino acids still bind to target cells since we identified a second positively charged sequence able to bind heparan sulfate at positions 472–481 of the C terminus of HPV-33 L1 protein. However, they are not in agreement with the fact that HPV-33 VLPs made of L1 protein lacking the 22 last amino acids still bind to target cells, since this deletion partially deletes the second DNA- and heparin-binding sequence. However, it should be noted that the VLPs used by Giroglou et al. (2001) were composed of L1 and L2 proteins and that binding to heparin could be due to the presence of the two DNA- and heparin-binding sequences identified at the N and C termini of L2.

### Table 2. Gene transfer with L1 or L2 peptides in the presence or absence of the BPV-1 packaging enhancing sequence (PES)

<table>
<thead>
<tr>
<th>Peptide</th>
<th>DNA plasmid without BPV PES (blue cells per well ± SD)</th>
<th>DNA plasmid with BPV PES (blue cells per well ± SD)</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV-16 L2 Nt</td>
<td>83 ± 16</td>
<td>235 ± 35</td>
<td>10⁻⁵</td>
</tr>
<tr>
<td>HPV-16 L2 Ct</td>
<td>58 ± 10</td>
<td>122 ± 20</td>
<td>0.01</td>
</tr>
<tr>
<td>HPV-16 L1 Cta</td>
<td>0.7 ± 1</td>
<td>0.7 ± 1</td>
<td>--</td>
</tr>
<tr>
<td>HPV-16 L1 Ctb</td>
<td>65 ± 5</td>
<td>125 ± 8</td>
<td>&lt;10⁻⁵</td>
</tr>
<tr>
<td>HPV-31 L1 Cta</td>
<td>72 ± 7</td>
<td>136 ± 13</td>
<td>7 x 10⁻⁵</td>
</tr>
<tr>
<td>HPV-31 L1 Ctb</td>
<td>3 ± 2.5</td>
<td>0.3 ± 0.6</td>
<td>0.10</td>
</tr>
<tr>
<td>HPV-45 L1 Cta</td>
<td>60 ± 10</td>
<td>141 ± 20</td>
<td>2 x 10⁻⁴</td>
</tr>
</tbody>
</table>

Data are the means of three experiments.
Structural data available concerning L1 protein (Chen et al., 2000) and immunological and DNA-binding data concerning L2 protein (Zhou et al., 1994; Roden et al., 1996; Liu et al., 1997; Shafer et al., 2002) suggest that the peptides tested in this study are inside the virions, where they would be expected to be unavailable for heparin and cell GAG binding. However, gene transfer experiments using VLPs have indicated that VLP binding to DNA, to cells and to heparin is linked to the last C-terminal amino acids of the L1 protein (Joyce et al., 1999; Touzé et al., 2000), suggesting its presence at the surface of the VLPs. The absence of heparin binding observed for HPV-16 L1a9 VLPs clearly suggests the involvement of this sequence in cell binding through its binding to GAGs.

The role of the positively charged termini of the L2 protein in viral encapsidation has been fully established (Zhou et al., 1994; Roden et al., 2001). The results of heparin binding obtained with L2 positively charged peptides raise the possibility that these sequences could also be involved in VLP and virion binding to the cell GAGs. L1 + L2 VLPs lacking either the N or C terminus of the L2 protein have been shown by immunofluorescence studies to bind to cells in the same way as wild-type VLPs (Roden et al., 2001), suggesting that these sequences do not play a central role in binding to cells. However, in the absence of data obtained with pseudovirions composed of L1 C-terminal mutants and L2 proteins (both wild-type and N-terminal mutants), it is difficult to conclude whether N-terminal positively charged sequences of the L2 protein are present on the virion surface and play a role in its binding to the cell or not.

In conclusion, using synthetic peptides we have confirmed that the DNA binding of the HPV-16 L1 protein is due to the last C-terminal amino acids. We also confirmed the binding properties of N- and C-terminal amino acids of the HPV-16 L2 protein. In addition, we determined that a second positively charged sequence located upstream from the previous L1 sequence is able to bind DNA for some HPV types (HPV-33 and -45), but not, or not significantly, for other types (HPV-16, -31 and -58). The interaction between these DNA-binding peptides and DNA induced protection from DNase degradation and was sufficient to obtain gene transfer in COS-7 cells. However, the level of gene transfer with a peptide was around 150 times less efficient than that observed with the corresponding sequence within the VLPs. It was also shown that gene transfer is mediated by binding of DNA/peptide complexes to heparan sulfate receptors, as for VLPs, confirming that positively charged DNA complexes can enter cells after binding to membrane-associated sulfated proteoglycans (Mislick & Baldeschweiler, 1996; Mounkes et al., 1999; Kichler et al., 2000). In addition, our findings are in agreement with reports that the inclusion of L2 protein in the constitution of HPV VLPs increases their gene transfer capacity (Roden et al., 1996; Unckell et al., 1997; Kawana et al., 1998; Stauffer et al., 1998; El Mehdaoui et al., 2000; Giroglou et al., 2001) and thus suggests that L2 participates in DNA encapsidation and could play a role in binding to cells.

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