

# Efficient Production of Papillomavirus Gene Delivery Vectors in Defined In Vitro Reactions

Carla Cerqueira,<sup>1</sup> Cynthia D. Thompson,<sup>1</sup> Patricia M. Day,<sup>1</sup> Yuk-Ying S. Pang,<sup>1</sup> Douglas R. Lowy,<sup>1</sup> and John T. Schiller<sup>1</sup>

<sup>1</sup>Laboratory of Cellular Oncology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

**Papillomavirus capsids can package a wide variety of nonviral DNA plasmids and deliver the packaged genetic material to cells, making them attractive candidates for targeted gene delivery vehicles. However, the papillomavirus vectors generated by current methods are unlikely to be suitable for clinical applications. We have developed a chemically defined, cell-free, papillomavirus-based vector production system that allows the incorporation of purified plasmid DNA (pseudogenome) into high-titer papillomavirus L1/L2 capsids. We investigated the incorporation of several DNA forms into a variety of different papillomavirus types, including human and animal types. Our results show that papillomavirus capsids can package and transduce linear or circular DNA under defined conditions. Packaging and transduction efficiencies were surprisingly variable across capsid types, DNA forms, and assembly reaction conditions. The pseudoviruses produced by these methods are sensitive to the same entry inhibitors as cell-derived pseudovirions, including neutralizing antibodies and heparin. The papillomavirus vector production systems developed in this study generated as high as  $10^{11}$  infectious units/mg of L1. The pseudoviruses were infectious both in vitro and in vivo and should be compatible with good manufacturing practice (GMP) requirements.**

## INTRODUCTION

Papillomaviruses (PVs) are small non-enveloped, circular double-stranded DNA (dsDNA) viruses whose icosahedral capsid is composed of only two proteins, L1, the major capsid protein, and L2, the minor capsid protein. L1 assembles into pentameric capsomers, 72 of which assemble into a T = 7 icosahedron. Most of L2 is located internally but is nonetheless essential for infection.<sup>1,2</sup> It also supports capsid assembly and stabilization and, depending on the PV type, is also required for DNA packaging.<sup>3–11</sup>

The simplicity of PV capsids, together with their ability to package plasmids up to 8 kb in length that are entirely devoid of PV sequences,<sup>12</sup> makes them attractive candidates for gene delivery vectors. PVs have a unique tissue tropism and mode of infection. They are epitheliotropic and, in normal tissues, are strictly tropic for disrupted mucosal and/or cutaneous epithelia,<sup>13</sup> where they must first bind to

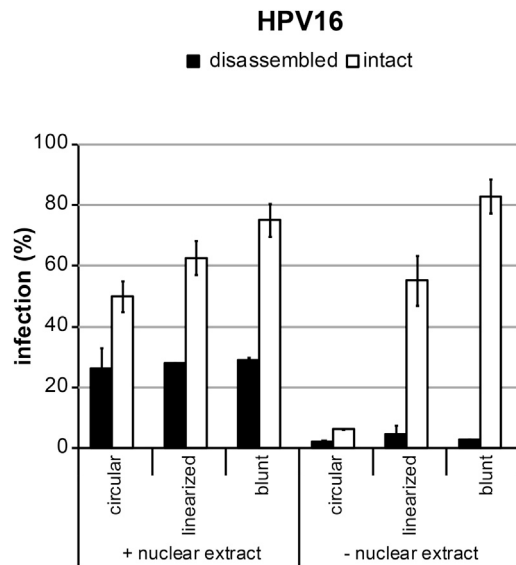
specifically modified forms of heparan sulfate proteoglycans (HSPGs) on the underlying basement membrane.<sup>14,15</sup> PV pseudovirions (PsVs) preferentially transduce epithelial cells and have shown promise as vectors for efficient gene transfer and genetic immunization at cervicovaginal and other mucosal sites in preclinical models. They are particularly adept at inducing long-lived antigen-specific CD8 tissue-resident memory cells in epithelium transduced by the vectors.<sup>16</sup> In addition, it was recently shown that human PV (HPV) PsVs have a strong and unexpected tropism for cancer cells,<sup>17</sup> presumably because the surface of many cancer cells, particularly carcinomas and melanomas, evolve to express the types of HSPG modifications that are normally found only on the basement membrane. The ability to bind and infect a wide variety of human tumor types, coupled with their inability to bind or infect normal tissue surfaces, make HPV PsVs good candidates for targeted gene delivery into tumor cells for cancer therapy applications.

Efficient intracellular generation of PV PsVs has relied on both the SV40 origin of replication (*ori*) being included in the target DNA and their production in cells expressing the SV40 T-antigen (generally 293TT cells)<sup>12,18,19</sup> to drive intracellular production of a high number of copies of the pseudogenome. In this production system, a fraction of the PsVs encapsidate cellular DNA fragments instead of the target pseudogenome. Next-generation sequencing of DNA extracted from these PsV preparations has determined that very small amounts of the T-antigen gene are incorporated in purified preparations of the particles (Chris Buck and Mike Tisza, personal communication). Because T-antigen is a well-established oncoprotein, this cell culture-dependent PsV production system would likely be unsuitable for generating PV vectors for clinical applications. In this study, we describe an efficient cell-free in vitro production method for generating high-titer stocks of PV PsVs from virus-like particles (VLPs) and bacterially derived plasmids. This production system is

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**Correspondence:** John T. Schiller, Laboratory of Cellular Oncology, National Cancer Institute, National Institutes of Health, 9000 Rockville Pike, Building 37, Room 4112B, Bethesda, MD 20892, USA.

**E-mail:** [schillej@mail.nih.gov](mailto:schillej@mail.nih.gov)



**Figure 1. HPV16 Can Package Linear DNA**

Intact or disassembled HPV16 VLPs were reassembled with a GFP reporter plasmid with (+) or without (–) nuclear extract as indicated. Three types of GFP reporter plasmids were used: circular supercoiled, linearized, or blunt (lacking the potential to be re-circularized). After reassembly and nuclease treatment, the resultant PsVs were used to infect HeLa cells. The number of infected cells (GFP-positive) was determined 72 hr p.i. by flow cytometric analysis. Shown is a representative experiment. The error bars represent the deviation between duplicates.

T-antigen-independent and is therefore more likely to meet good manufacturing practice (GMP) production requirements.

We recently reported that HPV16 L1/L2 VLPs are capable of packaging circular plasmids, provided they are shorter than 8 kb in length, in a cell-free *in vitro* reaction to generate infectious PsVs, but only when a mammalian cell nuclear extract was included in the reaction.<sup>20</sup> In that study, we focused on packaging supercoiled circular plasmids because this is the conformation of the viral genome in authentic virions, and the primary goal of that study was to investigate the basic mechanism of preferential viral genome encapsidation. We proposed that our technology could be used to package a wide range of expression vector plasmids and, therefore, that HPV16 PsVs produced by this method could be used as vectors for clinical gene delivery. However, for gene delivery applications, it would be advantageous to have vectors based on other PV types as well. As demonstrated for the commercial HPV vaccines, Cervarix and Gardasil, we would expect that the delivery of one type of PsV would induce type-restricted neutralizing antibodies that would prevent subsequent gene transfer with the same PsV type. Additionally, Cervarix and Gardasil vaccinees would not be candidates for genetic transfer using an HPV16-based vehicle. Therefore, we decided to extend our studies to phylogenetically diverse human and animal PV types. We also examined the generation of infectious PsV in *in vitro* reactions using alternative conformations of the pseudogenome because it was possible that more relaxed plasmid conformations than supercoiled

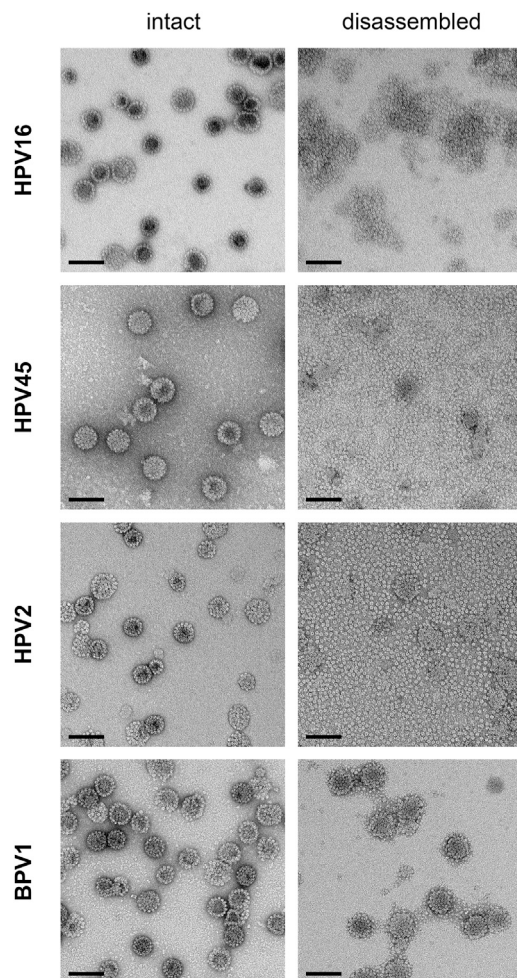
closed circles might be more efficiently packaged. We also examined the requirement for a nuclear extract under each experimental condition because it would be preferable to omit the extract in a GMP production scheme if feasible. Finally, we used the *in vivo* PsV mouse cervicovaginal challenge model to compare PsVs produced by standard intracellular procedures with those produced under the most efficient sets of defined cell-free reaction conditions developed in this study.

## RESULTS

### Some PV Capsids Can Package Plasmids Independent of Nuclear Components

We started the study by employing our previously described HPV16 PsV production reaction<sup>20</sup> but compared linearized with supercoiled circular GFP reporter plasmid (GFP plasmid) as the packaging substrate. For the packaging reaction, we disassembled HPV16 L1/L2 VLPs using low salt and DTT or left them intact and mixed the resulting capsid proteins with the supercoiled circular or linearized DNA in the presence or absence of nuclear extract. To linearize the plasmid, we simply cut it with a restriction enzyme at a single site that did not disrupt the GFP gene or its promoter and heat-inactivated the enzyme. After reassembly, all unpackaged plasmid DNA was digested for 6 hr with nucleases. Successful packaging of the plasmid was functionally evaluated by quantifying infection of HeLa cells, as determined by the percentage of green fluorescent cells using flow cytometry. For the supercoiled circular DNA, results were consistent with those reported previously<sup>20</sup> (Figure 1). Packaging of the circular plasmid into infectious PsVs required the presence of nuclear extract for both disassembled and intact capsid proteins (Figure 1). A higher number of infectious PsVs were generated when intact particles were used. For the linearized plasmid, we observed superior PsV formation with both disassembled and intact particles in the presence of nuclear extract. Surprisingly, there was also substantial PsV assembly using the linear plasmid and intact particles, even in the absence of nuclear extract (Figure 1). This plasmid had been linearized using a restriction enzyme that produces overlapping ends; this might have permitted its re-circularization during the packaging reaction. Therefore, in addition, we repeated the assembly reactions using a linearized plasmid with blunt ends, made by deletion of a short sequence outside of the GFP expression cassette. The infection results using this plasmid preparation were comparable with those with the linearized plasmid under all combinations of assembly state and nuclear extract addition (Figure 1). We also tested a relaxed plasmid in which we introduced single-stranded nicks. The results were similar to those using the supercoiled circular plasmid, with the generation of infectious PsVs only in the presence of nuclear extract (data not shown).

We next investigated the generation of infectious PsV in our cell-free assembly reactions for 20 additional PV types. The PsV production reactions were performed with disassembled and intact particles using circular, linearized, or blunt DNA in the presence or absence of nuclear extract for HPV16, 31, 33, 52, and 58 ( $\alpha$ 9 types); HPV18, 39, 45, 59, and 68 ( $\alpha$ 7 types); HPV2 ( $\alpha$ 4 type); HPV26 ( $\alpha$ 5 type); HPV6 ( $\alpha$ 10 type); HPV5 and 8 ( $\beta$ 1 types); and HPV38 ( $\beta$ 2 type) and also the animal types bovine PV (BPV)1, MusPV1, MmPV1



**Figure 2. Disassembly of Different PV VLPs**

HPV16, HPV45, or HPV2 particles were disassembled in 100 mM NaCl, 20 mM Tris (pH 8.2), 2 mM DTT, and 0.01% Tween 80 for 3 hr at 37°C. BPV1 particles were disassembled in 50 mM NaCl, 20 mM Tris (pH 8.2), 2 mM DTT, and 0.01% Tween 80 for 3 hr at 37°C. Samples were analyzed by transmission electron microscopy. Scale bars represent 100 nm.

(formerly RhPV1), and SfPV1 (formerly CRPV1). Disassembly of all PV types using the conditions established for HPV16 was confirmed by transmission electron microscopy (EM) (Figure 2; data not shown). The only exception was BPV1, which resisted disassembly even in 50 mM NaCl and 2 mM DTT and exhibited somewhat expanded capsids after treatment at best (Figure 2). BPV1 has more L1 disulfide bonds than most other PV capsids,<sup>12,21</sup> which probably explains its greater resistance to disassembly. We tested several additional disassembly conditions for BPV1, including higher DTT concentrations and longer disassembly incubations (up to 16 hr), but none of the tested conditions led to complete disassembly (data not shown). Nonetheless, we analyzed BPV1 packaging under our standard destabilization conditions, and BPV capsids thus generated are, for simplicity, referred to as “disassembled” in Table 1 and elsewhere.

To standardize the results of the initial survey of cell-free *in vitro* PsV production across PV types, we infected HeLa cells with an equivalent amount of total L1 for all types and defined categories to define the observed levels of infectivity. These intervals were defined as not infectious (–) to indicate infection less than 7%, low infectivity (+) to indicate infection greater than 7% but less than 35%, intermediate infectivity (++) to indicate infection greater than 35% but less than 65%, and high infectivity (+++) to indicate infection greater than 65% (Table 1).

For the  $\alpha 9$  types, generating infectious PsV with circular DNA using either disassembled or intact particles required the presence of nuclear extract. The exception was HPV58, which resulted in low amounts of PsVs with circular DNA in the absence of nuclear extract. As had been true for HPV16, linearized or blunt DNAs were generally better substrates for packaging into intact particles than circular DNAs. The packaging of linearized DNA into intact particles was nuclear extract-independent for all tested  $\alpha 9$  types. Another exception among the  $\alpha 9$  group was that HPV58 generated similar infectious PsV titers with disassembled and intact VLPs, whereas infectivity was higher with intact particles for other types.

The PsV production pattern for  $\alpha 7$  types differed notably from that of the  $\alpha 9$  types. Infection was very high for most representatives, except for HPV18. HPV18 PsV production had a pattern very similar to the one described for HPV16. All other  $\alpha 7$  types tested, HPV39, 45, 59, and 68, appeared to efficiently package all forms of the pseudogenome when disassembled. Disassembled VLPs generated more PsV than intact particles, in contrast to most of the  $\alpha 9$  types tested. Also, the generation of PsV from circular DNA and disassembled particles of the latter  $\alpha 7$  types was not dependent on nuclear extract because high infection rates were observed with previously disassembled particles regardless of the presence of nuclear extract. For the intact particles, although packaging was improved with nuclear extract, there was also substantial packaging without it. In general, linear DNA seemed to be a better substrate than circular DNA for generating  $\alpha 7$ -type PsVs.

VLPs of HPV26, an  $\alpha 5$  type, could also efficiently generate PsVs. Disassembled particles packaged all of the pseudogenome forms tested in the presence or absence of nuclear extract, although circular DNA packaging was better in the presence of nuclear extract. With intact particles, packaging of circular or linear DNA occurred in the presence of nuclear extract. In the absence of nuclear extract, only linear DNA could be packaged, with blunt DNA being slightly superior to linearized DNA.

The VLPs of HPV2, an  $\alpha 4$  type, HPV6, an  $\alpha 10$  type, and HPV40, an  $\alpha 8$  type, were inefficient at generating PsV in the *in vitro* reactions. Low levels of infection were observed under a limited number of reaction conditions; for instance, when linear or blunt DNA was added to disassembled or non-disassembled VLPs in the presence of nuclear extract.

**Table 1. Infection by Papillomaviral Vectors**

Clade	Virus Type	Disassembled						Intact					
		+ Nuclear Extract			– Nuclear Extract			+ Nuclear Extract			– Nuclear Extract		
		Circular	Linearized	Blunt	Circular	Linearized	Blunt	Circular	Linearized	Blunt	Circular	Linearized	Blunt
$\alpha 9$	16	+	++	++	–	+	+	++	+++	+++	–	++	++
	31	+	+	+	–	–	–	+	+	++	–	+	++
	33	+	–	+	–	–	–	+	++	++	–	+	+
	52	–	–	–	–	–	–	+	+	+	–	+	+
	58	+++	+++	+++	+	+	++	++	+++	+++	+	+	++
$\alpha 7$	18	+	+	++	–	–	–	++	++	++	–	+	+
	39	++	++	++	++	++	++	++	++	+++	+	++	+++
	45	++	+++	+++	+++	+++	+++	++	+++	+++	+	++	++
	59	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+++
	68	++	++	++	++	++	++	+	++	+++	+	++	++
$\alpha 5$	26	++	++	++	+	+	++	+	++	+++	–	+	++
$\alpha 4$	2	–	+	+	–	–	–	–	+	+	–	–	+
$\alpha 10$	6	+	+	+	–	–	–	–	+	+	–	+	+
$\alpha 8$	40	+	–	+	+	+	+	+	+	+	–	+	+
$\beta 1$	5	–	+	+	–	–	–	–	–	+	–	–	–
	8	+	+	++	+	+	+	+	+	+	–	–	+
$\beta 2$	38	+	+	+	–	–	–	+	+	+	–	+	+
	MusPV1	++	++	++	+	+	+	+	+	+	–	+	+
	BPV1	+	+	+	–	–	–	–	+	+	–	+	+
	SfPV1	–	–	–	–	–	–	–	–	–	–	–	–
	MmPV1	–	–	+	–	–	–	–	+	+	–	+	+

The indicated papillomavirus types were either disassembled or left intact. VLPs were then incubated with a GFP plasmid that was either circular, linearized, or blunt in the presence (+) or absence (–) of nuclear extract for 20 hr at 37°C. After nuclease treatment, HeLa cells were infected with the resultant preparations, and the percentage of infected cells (GFP-positive) was analyzed 72 hr p.i. by flow cytometry and categorized as follows: –, infection < 7%; +, infection 7.1%–35%; ++, infection 35.1%–65%; +++, infection > 65%. Percentages were evaluated based on the average of at least three independent experiments.

Three cutaneous  $\beta$  HPVs were also examined. For the two  $\beta$ 1s, production was low in general, although HPV8 produced more PsV than HPV5 under most reaction conditions, which parallels the low yield of HPV5 PsVs in our standard intracellular production system. For HPV5, we could observe very low infection only for linearized or blunt DNA in the presence of nuclear extract. Generation of infectious PsV by HPV38 (a  $\beta$ 2 type) was also poor under the best of conditions.

For the animal types, infectivity was generally very low or absent under the various reactions conditions (Table 1). The notable exception was that disassembled MusPV1 VLPs were reasonably efficient at generating PsVs from all three forms of DNA when mixed with nuclear extract. For BPV1, infectivity was very low and only observed with a limited subset of conditions. Because MmPV1 and SfPV1 generated few or no infectious PsVs under any of the experimental conditions when assayed on HeLa cells, we also tried infecting 293TT cells (data not shown). There was no infection with MmPV1, and, for SfPV1, only low infection was observed with linear or blunt DNA in intact particles. The results for MmPV1 were not surprising because this virus is also deficient in generated PsVs in our standard cell culture production system. In contrast, SfPV1 generates substantial titers of PsVs in the cell culture system.

Taken together, these results show that PV can package circular and linear DNA under cell-free conditions. Unexpectedly, many virus types could generate infectious PsVs in the absence of nuclear components, but the DNA form that was best packaged as a pseudogenome varied among the PV types. Members of the  $\alpha$ 7 clade were exceptionally proficient at generating infectious PsVs under these cell-free reaction conditions.

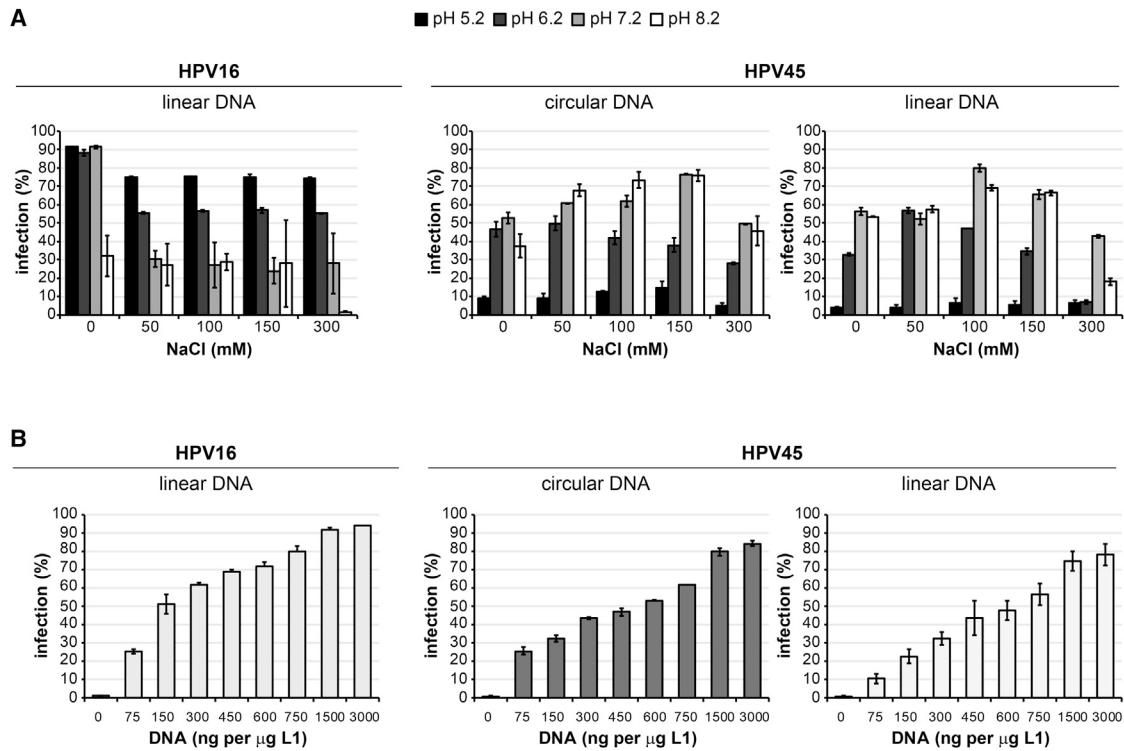
#### Optimization of Cell-free Production of PV Vectors

The results presented above showed that the capsid proteins from 18 of the 21 PVs tested could generate at least some infectious PsVs under conditions using linear DNA as the pseudogenome substrate in the absence of a nuclear extract, and 9 of 21 types could also package circular DNA in the absence of nuclear extract. To devise the simplest possible cell-free production scheme possible, we focused on optimization of the in vitro-produced (IVP) PsVs without nuclear extract. We chose HPV16 as a prototype of a virus that preferentially packages linear DNA from intact particles and HPV45 as an example of a virus that efficiently packages both circular and linear DNA from disassembled particles. In part, these types were selected because they can produce highly concentrated VLP stocks for use as the starting material for the reactions. For the initial optimization, we tested different pH levels and NaCl concentrations in the reassembly reaction. We used intact HPV16 VLPs and disassembled HPV45 VLPs and mixed them with either linear or circular DNA in pH 5.2, 6.2, 7.2, or 8.2 buffer in combination with NaCl concentrations ranging from 0–300 mM NaCl (Figure 3). As with the previous experiments, we used HeLa infection as a measure of PsV production. To control for any effect the different salt and pH conditions could have directly on PsV infection, we also infected cells under each condition with

standard PsVs derived via intracellular production. None of the tested conditions affected infection with control PsVs (data not shown), indicating that any observed effects under the experimental conditions could be attributable to the efficiency of PsV production. Additionally, nuclease treatment was also unaffected under all pH and NaCl conditions (data not shown), indicating that differences in infectivity were not the result of inhibition of DNA digestion leading to capsid-independent plasmid transfection. At 600 mM NaCl, PsV production was greatly reduced, and no infection was observed at higher salt concentrations (data not shown). PsV production for HPV16 was similar at NaCl concentrations ranging from 50–300 mM but was increased with decreasing pH. When no salt was added to the buffer, production was notably higher than with salt and essentially the same at pH 5.2, 6.2, and 7.2 (Figure 3A) but reduced at pH 8.2. The trend toward increased PsV production at lower pH and NaCl concentrations suggests that ionic interactions between the pseudogenome and the capsid interior may be important in the packaging reaction. It is important to note that input VLPs are produced in the presence of 500 mM salt and diluted for the reassembly reaction; therefore, the amount of NaCl added to the reaction buffer can vary depending on the L1 concentration of the VLP preparation. Based on these considerations, we decided to use pH 5.2 with no added salt as our standard reaction condition for HPV16 PsV production. This would assure good packaging even if substantial amounts of salt were added when more dilute VLP preparations were used in the reactions.

For HPV45, there are almost no infectious PsVs generated at pH 5.2 when either circular or linear DNA is used, perhaps reflecting an inhibition of capsomer assembly at low pH (Figure 3A). At pH 6.2, substantial infectivity was observed, but the infectivity was generally better at pH 7.2 and 8.2 for both conformations of plasmids tested (Figure 3). At pH 7.2 and 8.2, PsV production tended to peak at 100–150 mM NaCl, with concentrations of 600 mM NaCl or higher preventing assembly (data not shown). We decided to use pH 7.2 and 150 mM NaCl for both circular and linear pseudogenomes because these conditions were the closest to physiological conditions and gave high infectivity.

Having established standard conditions for generating IVP HPV16 and HPV45 PsVs, we next tested the effect of pseudogenome concentration on PsV production. In the previous experiments, 150 ng DNA/1  $\mu$ g L1 was routinely used, corresponding to an approximate 1:1 ratio of assembled capsids to DNA molecules. We tested a range from 75–30,000 ng of linearized GFP plasmid for HPV16 and linearized or circular plasmid for HPV45 by using the standardized conditions noted above. After the assembly reaction, samples were nuclease-treated, and PsV production was evaluated by analyzing the infection of HeLa cells. For both virus types, we observed a clear dose-dependent increase in infection with increasing DNA concentration (Figure 3B), although the increase in infectivity was not directly proportional to the amount of DNA and tended to plateau at the high amounts. We chose 450 ng DNA per 1  $\mu$ g L1 as the standard for further studies because PsV production was relatively



**Figure 3. Optimization of In Vitro Reassembly**

(A) Reassembly of intact HPV16 or disassembled HPV45 was assessed. Reassembly reactions were performed at the indicated pH and NaCl concentrations for 20 hr at 37°C with 150 ng of GFP plasmid (either circular or linearized plasmid as indicated). All samples were nuclease-treated prior to infection of HeLa cells. Infection was scored 72 hr p.i. Shown is a representative experiment. The error bars show the deviation between duplicates. (B) Intact HPV16 was incubated at pH 5.2 with the indicated amounts of linearized GFP plasmid (linear DNA). Disassembled HPV45 was incubated at pH 7.2 and 150 mM NaCl with the indicated amounts of circular or linearized GFP plasmid. Reactions were incubated 20 hr at 37°C and then nuclease-treated prior to infection. Shown are representative experiments. The error bars show the deviation between duplicates.

efficient using these conditions, and adding more plasmid DNA per 1  $\mu$ g L1 would be impractical in larger-scale production.

Based on these findings, we attempted to produce highly concentrated stocks of PV vectors that could transduce a luciferase and GFP expression plasmid (pCLucf). For HPV16, we incubated intact HPV16 VLPs in citrate buffer (pH 5.2), 0.02% Tween 80, and 450 ng/ $\mu$ g L1 of either linearized or circular luciferase (Luc)/GFP plasmid for 48 hr at 37°C. After 48 hr, samples were nuclease-treated for 3 hr with 0.2% BAL-31 and 0.2% benzonase at 37°C in buffer containing 10 mM MgCl<sub>2</sub> and 0.5 M NaCl. For HPV45, the dilution required for standard disassembly led to a larger volume; therefore, we adjusted the disassembly protocol. HPV45 particles were disassembled in 200 mM NaCl, 20 mM Tris (pH 8.2), 2 mM DTT, and 0.01% Tween 80 for 6 hr at 37°C. We confirmed that particles disassembled under these conditions (data not shown). After disassembly, HPV45 capsid proteins were reassembled in buffer containing 100 mM Tris (pH 7.2), 150 mM NaCl, 10 mM CaCl<sub>2</sub>, 0.02% Tween 80, and 450 ng/ $\mu$ g L1 of linearized or circular pCLucf according to the concentrations determined previously. For reassembly, the reaction was incubated for 48 hr at 37°C and nuclease-treated as for

HPV16. We then titered our virus stocks in 293TT cells and examined the particles by EM.

We observed that many of the HPV45 particles had larger than normal diameters, suggesting that these expanded particles were rather loosely assembled immature PsVs (Figure S1A, –GSSG). To improve the quality of the particles, we added 5 mM L-glutathione oxidized (GSSG) to our reassembly protocol because GSSG has been shown to improve capsid maturation.<sup>12</sup> We incubated the reassembly mix for 30 hr without GSSG to allow capsid assembly and then added GSSG to a final concentration of 5 mM for a further 15 hr to allow for maturation. This addition resulted not only in tighter and more uniform particles but also in a higher titer stock for HPV45, especially with linear DNA (Figures S1A and S1B, +GSSG). The addition of GSSG did not affect HPV16 titers or capsid morphology, which is not surprising because the capsids had not undergone a disassembly step (Figure S1A). Nevertheless, we included the GSSG step during HPV16 production to maximize capsid stability for in vivo infection studies (discussed below). To partially purify the PsVs and increase their concentration, we centrifuged them over an Optiprep cushion. After centrifugation, the titers for both HPV16 and

**Table 2. Titer of IVP PsV Stocks**

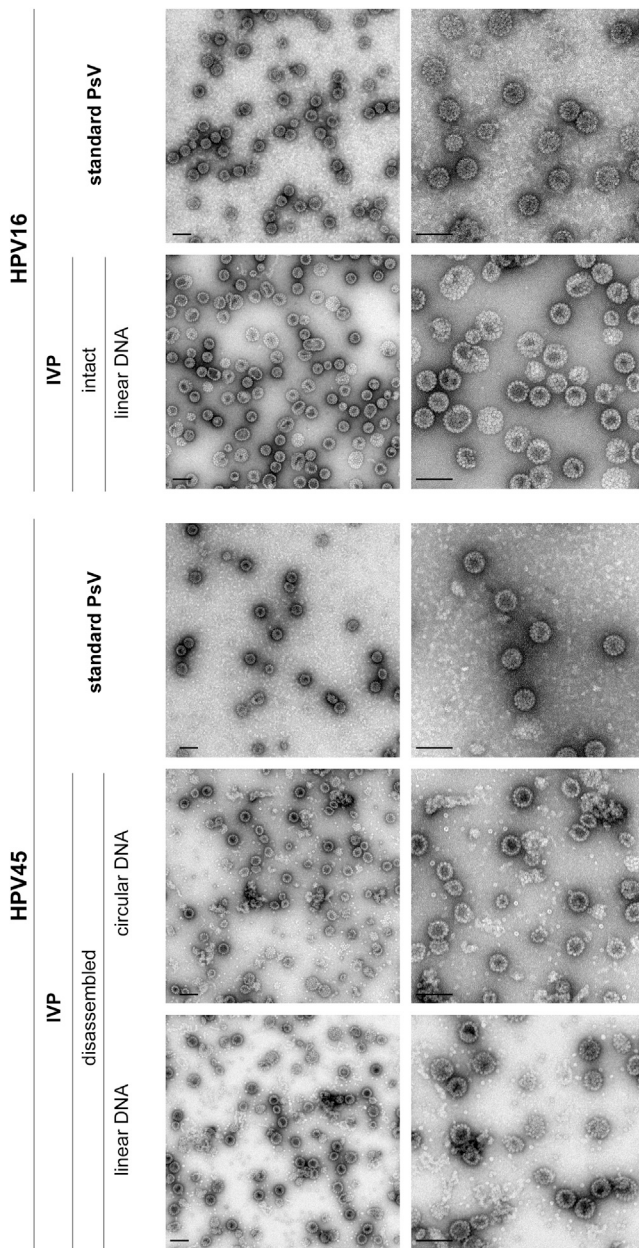
		Preparation Type	Disassembled?	DNA	Titer (IU/mg L1)		Genome Copies (Copies/mg L1)	
					Mean	SD	Mean	SD
$\alpha 9$	HPV16	standard	–	circular	6E+09	8E+09	2E+11	2E+11
		IVP	N	linear	5E+09	2E+09	6E+11	3E+11
	HPV58	standard	–	circular	3E+10	5E+10	8E+11	7E+11
		IVP	Y	circular	8E+09	7E+09	9E+11	6E+11
		IVP	Y	linear	8E+10	7E+10	2E+12	2E+12
		IVP	N	linear	1E+10	2E+10	1E+11	2E+11
$\alpha 7$	HPV39	standard	–	circular	3E+10	5E+10	2E+11	3E+11
		IVP	Y	circular	5E+08	5E+08	3E+11	3E+11
		IVP	Y	linear	4E+09	7E+08	3E+11	4E+11
	HPV45	standard	–	circular	6E+10	4E+10	2E+12	1E+12
		IVP	Y	circular	3E+10	3E+10	9E+11	2E+12
		IVP	Y	linear	5E+11	4E+11	2E+12	1E+12
HPV59	standard	–	circular	3E+09	1E+09	1E+11	1E+11	
	IVP	Y	circular	2E+09	2E+09	7E+10	4E+10	
	IVP	Y	linear	1E+11	2E+11	7E+11	1E+12	
	HPV68	standard	–	circular	2E+10	3E+10	3E+11	1E+11
		IVP	Y	circular	2E+09	2E+09	7E+11	6E+11
		IVP	Y	linear	1E+11	1E+11	4E+11	2E+11
$\alpha 5$	HPV26	standard	–	circular	6E+10	5E+10	4E+11	3E+11
		IVP	Y	circular	2E+09	2E+09	1E+11	7E+10
		IVP	Y	linear	9E+10	3E+10	2E+12	2E+12
		IVP	N	linear	9E+08	9E+08	5E+10	3E+10
MusPV1	standard	–	circular	2E+10	2E+10	6E+11	8E+11	
	IVP	Y	circular	1E+08	1E+08	9E+10	1E+11	
	IVP	Y	linear	1E+09	1E+09	3E+11	3E+11	

Depicted are the viral titers per milligram of L1 for the different PsV preparations. The number of genome per plasmid copies per milligram of L1 for each virus type is also shown. The values represent the mean results from at least three independent experiments  $\pm$  SD. N, no; Y, yes.

HPV45 were determined and compared with standard PsV stocks produced in 293TT cells (Table 2). For HPV16, our IVP PV vector production generated virus titers very similar to the titers obtained for our standard HPV16 PsVs. For HPV45, we could obtain particles that were very similar or slightly higher in titer than the standard PsV production (Table 2).

Given these encouraging results, we decided to extend PsV stock production to other PV types that demonstrated high infectivity in our preliminary experiments, specifically HPV58, 39, 59, 68, 26, and MusPV1 (Table 1). For all virus types, we used disassembled VLPs with circular or linearized DNA, and for HPV58 and 26, we also used intact VLPs with linearized DNA because it was not completely clear from the preliminary studies which conditions would produce the highest titers. When intact particles were used, the protocol described for HPV16 was used, and for disassembled particles, the protocol described for HPV45 was applied.

For HPV58, when linear DNA was used, high titers very similar to standard PsV production were obtained for both VLPs that were disassembled or left intact. When circular DNA was used as the packaging substrate, titers were about 10-fold lower. For the  $\alpha 7$  types, we only used disassembled particles. HPV59 IVP PsVs had either a similar titer or, in the case of linear DNA, even higher titers as standard preparations, but HPV39 titers were about 10- to 100-fold lower. For HPV68, packaging of linear DNA resulted in infectivity similar to the standard produced PsV, whereas, for circular DNA, infectivity was reduced about 10-fold. Production of in vitro HPV26 vectors also led to high titers, with the highest titers obtained for disassembled particles with linear DNA. In the case of circular DNA and disassembled particles, infectivity was reduced about 10-fold compared with standard PsVs and about 100-fold lower for linear DNA with intact particles. For MusPV1, only disassembled particles were used, and we obtained 10-fold fewer infectious particles than standard



**Figure 4. Electron Microscopy of HPV16 and HPV45 IVP Vectors**

Intact HPV16 particles were assembled at pH 5.2 with a linearized Luc/GFP plasmid for 30 hr at 37°C. HPV45 was disassembled and then reassembled at pH 7.2 with 150 mM NaCl, 10 mM CaCl<sub>2</sub>, and 0.02% Tween 80 with linearized or circular Luc/GFP for 30 hr at 37°C. Both HPV16 and HPV45 were then incubated for a further 15 hr with 5 mM GSSG. After nuclease treatment, samples were centrifuged over an Optiprep cushion, and the virus-containing fraction was analyzed by electron microscopy. HPV16 or HPV45 PsVs produced by the standard cell-based protocol were included for comparison. Scale bars represent 100 nm.

PsVs when linear DNA was used and about 100-fold fewer with circular DNA. It is important to note that, even when the titers obtained with the IVP PsVs were lower compared with the standard

production method, they were still relatively high, at least 10<sup>8</sup> infectious units/mg of L1.

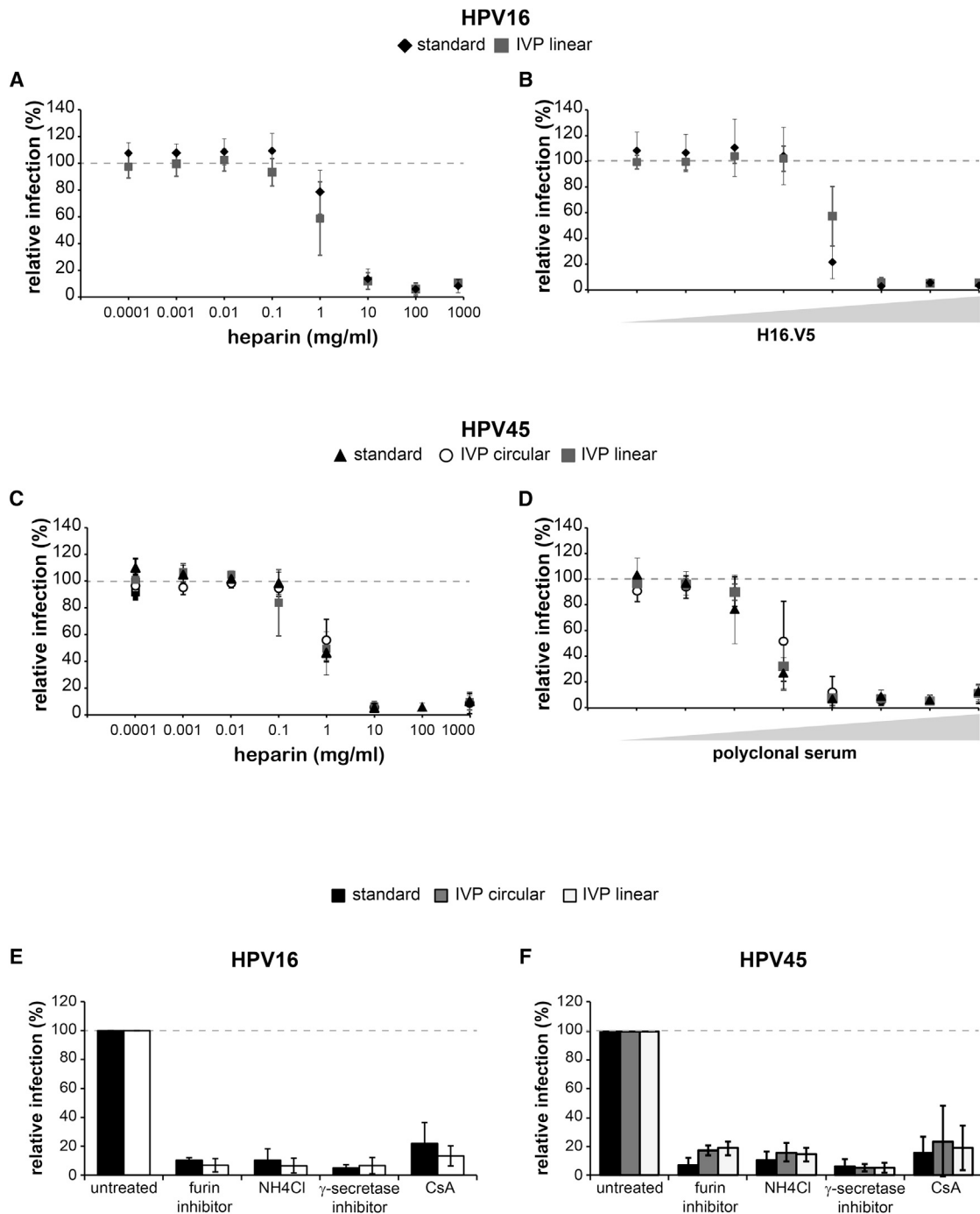
#### Characterization of IVP PsVs

Having obtained high titer stocks of virus using our cell-free production system, it was important to further characterize the particles in comparison with standard PsVs produced in 293TT cells. First, we determined the number of plasmid copies present for the stocks (Table 2). For most types, at least 10<sup>11</sup> copies were incorporated per 1 mg L1. The genome copies did not completely correlate with infectivity. For instance, HPV39 genome copies were similar for IVP circular, IVP linear, and standard preparations, whereas titers varied almost 100-fold. As part of the characterization, we also analyzed particle morphology and sensitivity to biochemical inhibitors of infection. We again concentrated on HPV16 and HPV45 as examples of assembly reactions based on intact and disassembled capsid proteins, respectively. Initially we characterized the particles in the final preparations by EM (Figure 4). For HPV16, most of the particles had a uniform VLP morphology although some particles were slightly expanded. For HPV45, the morphology was more variable. Although there were many well assembled particles, there were also a considerable number of expanded and partially assembled particles in the preparation (Figure 4). We also analyzed the high-titer viral stocks for the other PV types (Figure S2), and the results were similar to the ones obtained for HPV16 or HPV45. Generally, if the particles had been disassembled prior addition to the production reaction, then the resultant IVP stock had more expanded and partially assembled particles than those produced from intact particles (Figure S2).

The susceptibility of our IVP PsVs and control PsVs to heparin, neutralizing antibodies, and entry inhibitors was compared (Figure 5). HPV16 and HPV45 PsVs were incubated with serial dilutions of heparin for 1 hr on ice and subsequently tested for 293TT cell infection. IVP HPV16 with linear DNA and IVP HPV45 containing either linear or circular DNA were found to be susceptible to heparin inhibition to the same extent as the control standard PsVs (Figures 5A and 5C). This suggests that the IVP virus also uses HSPGs for attachment to immortalized cells in vitro. We also incubated the same virus preparations with neutralizing antibodies and measured the capacity of the antibodies to prevent 293TT infection. For HPV16, a well characterized monoclonal neutralizing antibody, H16.V5,<sup>22–26</sup> was used; for HPV45, we used a rabbit polyclonal serum raised against HPV45 L1 VLPs. In both instances, the IVP particles were neutralized to the same extent as the standard PsV preparations (Figures 5B and 5D). These findings indicate that the major neutralizing epitopes recognized by these reagents are retained on the IVP PsVs.

To evaluate sensitivity to inhibitors of cellular entry, 293TT cells were infected with the IVP or standard particles in the presence of a furin inhibitor (decanoyl-RVKKR-chloromethylketone [dec-RVKKR-cmk]), a neutralizing lysosomotropic agent (NH<sub>4</sub>Cl), an inhibitor of  $\gamma$ -secretase (compound XXI), or a cyclophilin inhibitor (cyclosporin A [CsA]). These inhibitors are well known to inhibit HPV16 and HPV45 entry into cells.<sup>27–32</sup> All compounds inhibited IVP and





**Figure 5. PsVs Prepared by the IVP Method Have Similar Neutralization and Biochemical Inhibition Profiles as Standard PsVs**

(A and B) Standard or HPV16 IVP PsVs containing a packaged Luc/GFP plasmid were pre-incubated with dilutions of heparin (A) or H16.V5 antibody (B) for 1 hr on ice prior to infection of 293TT cells. The percentage of infected cells (GFP-positive) was determined 72 hr p.i. by flow cytometry. Shown are the mean values for at least three independent experiments  $\pm$  SD normalized to untreated virus. (C and D) Standard or IVP HPV45 PsVs were pre-incubated with dilutions of heparin (C) or polyclonal HPV45 serum (D) for 1 hr on ice. Infection and analysis were performed as for (A) and (B). (E and F) 293TT cells were infected with standard or IVP PsVs in the presence of 10  $\mu$ M furin inhibitor (dec-RVKR-cmk), 20 mM NH<sub>4</sub>Cl, 300 nM  $\gamma$ -secretase inhibitor (compound XXI), or 10  $\mu$ M CsA, or left untreated. The percentage of infected cells (GFP-positive) was determined 72 hr p.i. by flow cytometry. (E) Infection with HPV16. (F) Infection with HPV45. Shown are the mean values for at least three independent experiments  $\pm$  SD normalized for untreated cells. The physical state of the packaged plasmid is indicated as either circular or linear.

standard PsV infection of 293TT cells to the same extent (Figures 5E and 5F), suggesting that the two production systems generate PsVs that utilize the same cellular entry pathway. We confirmed that the inhibitors also inhibited infection into HeLa cells (data not shown). The above analyses were extended to include HPV58, 39, and 26 and MusPV1 (Figure S3). For all of these types, we observed the same heparin and inhibitor sensitivity of the IVP particles and the standard PsVs as reported previously.<sup>33,34</sup> These types were also sensitive to a furin inhibitor, NH<sub>4</sub>Cl, compound XXI, and CsA to the same extent as the standard virus (Figure S3; data not shown).

As a final characterization, the *in vivo* infectivity of HPV16 and HPV45 was evaluated using our previously described mouse cervicovaginal challenge model.<sup>13</sup> The cervicovaginal epithelium was disrupted by intravaginal instillation of nonoxynol-9, and the mice were infected intravaginally with IVP HPV16 or HPV45 or the standard PsV for each type. The same 293TT cell infectious units were delivered for all preparations. Infection of the vaginal tract was measured by luciferase expression on days 1–7 after infection (Figure 6). We analyzed the infectivity of three different HPV16 and HPV45 IVP stocks in different experiments but also the three HPV45 stocks in the same experiment (Figure 6F). The results were very consistent for the HPV16 preparations (Figures 6A–6C). The kinetics of infection with the IVP PsVs were similar to the standard PsVs. A slight delay was observed on day 1 for the IVP particles. Cervicovaginal infectivity of the IVP particles was in general two to five times lower compared with the standard PsVs (Figures 6A–6C). For HPV45, the results were more variable (Figures 6D–6F). Although the kinetics of cervicovaginal infection were very similar for the IVP and standard PsVs, one HPV45 IVP preparation performed almost as well as the standard virus (Figures 5D and 5F, IVP circular or linear 1), whereas the others had approximately 10–20 times lower *in vivo* infectivity (Figures 6E and 6F). We also tested HPV58 and HPV26 (Figures 6G and 6H) in the cervicovaginal model. For HPV58, PsVs generated from disassembled VLPs and the circular genome had the best *in vivo* infection ratio, about five to eight times less infectious than standard PsVs. PsVs generated from disassembled VLPs and linear DNA or intact VLPs and linear DNA were 30–100 times less infectious *in vivo* than the standard virus. For HPV26, the virus stocks were more dilute, necessitating the use of fewer infectious units per mouse, leading to an overall lower signal (Figure 6H). Under these conditions, PsVs generated from disassembled particles with either circular or linear DNA only had about 1.5–2 times less infectivity than the standard preparations. PsVs generated from intact particles were about three times less infectious than standard PsVs in the cervicovaginal challenge model. These results show that, although we used the same infectious units per mouse (based on *in vitro* infection), different PsVs, unexpectedly, have different *in vivo* infectivity efficiencies.

To address the possibility that the delivered linear DNA can re-circularize during infection, we performed an experiment to determine which form of the plasmid leads to reporter gene expression *in vivo*. The luciferase gene was split from its promoter by linearizing the

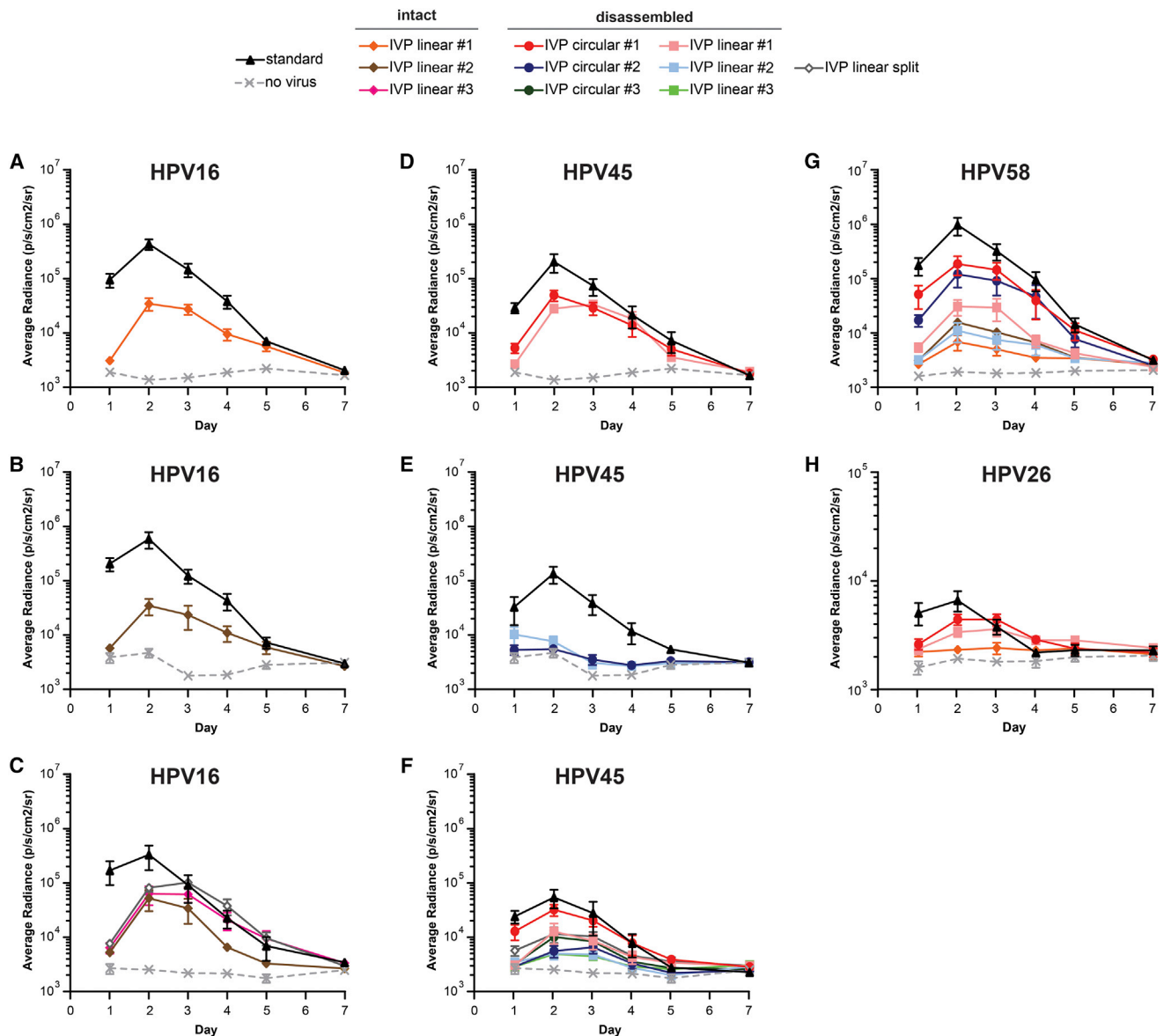
plasmid at a restriction site located between the two sequences. Virus stocks were prepared using this linearized DNA (referred to as “IVP linear split” virus), and mice were infected as described above. We assume that the luciferase gene would only be efficiently expressed if the plasmid had re-circularized. Luciferase expression was observed in the vaginal tract of mice infected with HPV16 and HPV45 carrying this split plasmid (Figures 6C and 6F, IVP linear split) with kinetics and levels of expression similar to the other IVP PsVs. These results indicate that most of the transduced plasmid assumes a circular conformation, which might be less prone to integrate into the host cell DNA than linear forms.

## DISCUSSION

In this study, we have developed strategies that could be applied to the GMP production of high-titer PV vectors. Our results show an unexpected variability across virus types in both the efficiency of packaging of different plasmid forms and capsid assembly. For example, some PVs package DNA better when left intact (e.g., HPV16), whereas others package DNA more efficiently when disassembled (e.g., HPV45). Although there are noticeable trends for specific clades, not all PVs within a given clade follow a pattern. For instance, in the  $\alpha 7$  clade, HPV39, 45, 59, and 68 behave similarly, whereas HPV18 is seemingly more similar to the  $\alpha 9$  HPV16 (Table 1). Among  $\alpha 9$  clade members, most types displayed a pattern that was similar to HPV16, but the HPV58 pattern was distinct. The molecular mechanisms and biological significance, if any, of these differences among types within and between clades are uncertain.

The strategy we have developed for IVP vector production was not designed to be physiologically relevant, so our results should not be overinterpreted in this context. HPV virion assembly in the nucleus involves packaging of covalently closed and nucleosome-bound viral genomes and, as we previously demonstrated, is promoted by nuclear factors. The results in this study (Table 1) demonstrate that the requirement of these factors for most PV types can be abrogated by the use of naked linear DNA as the packaging substrate. Possibly, the flexibility of a naked linear DNA, in comparison with a nucleosome-bound closed circular one, reduces topological constraints during the packaging reaction that normally require the activity of molecular chaperones. Interestingly, in some instances, linear DNA could even be efficiently packaged into intact capsids. Perhaps, in our production and purification system, the intact capsids of many types retain a substantial number of holes that permit entry of the flexible linear DNA molecules into the capsid interior. Closed circular DNA species may be too rigid to similarly enter the capsids. Further studies might identify physical differences in the capsid preparations of different PV types that would correlate with efficiency of PsV production using intact VLPs as substrates in our defined assembly reactions.

PV vector production under defined *in vitro* conditions have been described in the past, using circular DNA with disassembled HPV particles.<sup>35,36</sup> In those studies, the particle-to-infectivity ratio was not reported. Using this earlier pseudovirion production strategy,



**Figure 6. Kinetics of In Vivo Intravaginal Infection**

(A–H) Depo-Provera-treated BALB/c mice were nonoxynol-9-treated prior to infection.  $1 \times 10^7$  infectious units HPV16 (A–C), HPV45 (D–F), and HPV58 (G) or  $3 \times 10^6$  infectious units HPV26 (H) containing a packaged Luc/GFP plasmid were inoculated intravaginally (5 mice/group). The plasmid was either circular, linear, or linear split as indicated. Luciferase expression was monitored daily following delivery. The mean value of the average radiance  $\pm$  SEM measurement per group is shown. Three different virus preparations for each condition, as indicated, were compared with infection by standard PsV preparations. Note the different scale for the HPV26 experiment.

which has not been widely adopted by others in the field, we were only able to generate low titers of infectious particles. In addition, all of the infectivity was lost when the particles were treated with nucleases (unpublished results). Using our optimized defined in vitro production system, we obtained pseudovirus preparations that had high in vitro infections titers and low genome-to-infectious unit ratios that were often very similar to those of PsVs obtained using the widely employed cellular production method (Table 2). The similar neutralization and cell entry characteristics support the contention that our

IVP PsVs have a native or nearly native conformation (Figure 5; Figure S3). Of importance for future clinical use, we demonstrated that IVP PsVs were also infectious in vivo in our mouse cervicovaginal challenge model. There was a trend of lower infectivity with the in vitro versus the intracellularly produced PsV, but the reason for this difference remains to be determined.

Although titers and DNA packing efficiencies obtained in the IVP method were generally similar to those obtained for the standard

virus, we observed relatively high lot-to-lot variation. These variations may be linked to the different VLP and DNA preparations used in the assembly reactions. During scale-up for clinical application, further standardization of operations procedures for VLP and DNA production and biochemical characterization of the resulting PsVs would be desirable.

One potential complication concerning therapeutic DNA delivery methods is the possibility of integration of the delivered DNA into the host DNA. This would be a greater concern with linear DNA than with circular DNA because, after transfection, linear plasmids lead to higher rates of DNA integration into the host than closed circular plasmids.<sup>37,38</sup> The potential for insertional mutagenesis would make integration of the transduced DNA undesirable for most anticipated therapeutic applications of the PV vectors. However, we found that the linear plasmid re-circularized either after encapsidation or during infection. Further studies should evaluate the absolute risk of integration of the transduced DNA into host tissue and whether the rates differ for circular, linearized cohesive-end, and blunt-end DNA. It is also possible that plasmids with different sequences could have different integration rates for a given form, so the rates should be analyzed on a case-by-case basis.

The techniques developed in this study should enable the clinical evaluation of IVP vectors as agents for intravaginal vaccination or tumor treatment. Importantly, for tumor therapy applications, the development of this defined cell-free technique will enable the generation of PsVs that can transduce cytotoxic genes. Generation of this type of PsVs would be difficult with the standard PsV production methods because these would require transfection of the plasmid into the packaging cells, which would result in deleterious effects during the production process.

## MATERIALS AND METHODS

### Cell Lines, Antibodies, and Reagents

293H (Invitrogen), HeLa (ATCC), and 293TT (Christopher B. Buck, National Cancer Institute [NCI]) cells were grown in DMEM supplemented with 10% fetal bovine serum (DMEM-10).

Dec-RVCR-cmk (344930), compound XXI ((S,S)-2-[2-(3,5-difluorophenyl)-acetylamino]-N-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-benzo[e][1,4]diazepin-3-yl)-propionamide, 565790), and cyclosporin A (239835) were all purchased from Calbiochem. NH<sub>4</sub>Cl (A0171), heparin (H4784), benzonase (E1014), and GSSG (G4376) were purchased from Sigma.

H16.V5 was a kind gift from Neil Christensen.<sup>22</sup>

### VLP and PsV Production

L1L2 VLPs used for IVP were produced according to the standard protocol described by Buck and Thompson,<sup>18</sup> with the substitution of 293H instead of 293TT cells for production. Briefly, 293H cells were transfected with an L1L2 bicistronic expression plasmid. 48 hr after transfection, cells were harvested, and virus was matured for

24 hr at 37°C in Dulbecco's phosphate-buffered saline with calcium and magnesium supplemented with 9.5 mM MgCl<sub>2</sub>, 0.25% Brij58 (P5884, Sigma), 0.1% Plasmid Safe DNase (E3110K, Epicenter), 0.1% benzonase (E1014, Sigma), and 25 mM ammonium sulfate (pH 9.0). The cell lysate was then chilled, and the NaCl concentration was adjusted to 0.8 M. The cell lysate was then clarified by centrifugation for 20 min at 20,000 × g. VLPs were purified from the clarified lysate on a 27%/33%/39% Optiprep gradient.

Standard PsV production was performed as described for VLPs, but 293TT cells were utilized. A GFP-only plasmid or a firefly Luc/GFP double expression plasmid was co-transfected with the L1L2 expression plasmid. Maturation and purification was done as described for VLPs.

The following plasmids were used for L1 and L2 expression: p2sheLL, p5sheLL, p6sheLLr, p16sheLL, p18sheLL, p31sheLL, p33sheLL, p45sheLL, p52sheLL, and p58sheLL for HPV2, 5, 6, 16, 18, 31, 33, 45, 52, and 58, respectively. For the animal PV, pSheLL, pCRPVsheLL, pMusheLL, and pRhSheLL were used to produce BPV1, MmPV1, MusPV1, and SfPV1, respectively. pfwB was used as a GFP plasmid and pCLucf as a Luc/GFP plasmid. All plasmids are described at <http://home.ccr.cancer.gov/lco/plasmids.asp>.

For HPV8, HPV26, HPV38, HPV39, HPV40, HPV59, and HPV68 production, we used the plasmids pVITRO-HPV8 L1L2, pVITRO-HPV26 L1L2, pVITRO-HPV38 L1L2, pVITRO-HPV39 L1L2, pVITRO-HPV40 L1L2, pVITRO-HPV59 L1L2, and pVITRO-HPV68 L1L2, respectively. All pVITRO constructs were a kind gift from Richard Roden (Department of Pathology, The Johns Hopkins University) and have been described previously by Kwak et al.<sup>33</sup>

### Plasmid Production and Linearization

All plasmids were produced in competent *Escherichia coli* DH5α (BIO-85026, Bionline) and purified using the Plasmid Plus Midi Kit (12945, QIAGEN).

pfwB was linearized using SbfI (R3642, New England Biolabs) and pCLucf with XmnI (R0194, New England Biolabs). pCLucf was digested with EcoRI (R3101, New England Biolabs) for the linear split virus used in Figure 6. Digestion was confirmed by agarose gel electrophoresis, and enzymes were heat-inactivated according to the manufacturer's instructions before use.

For blunt DNA, pfwB was digested with PmlI and SbfI. The digested fragment containing GFP was gel-purified prior to use.

### Reassembly and Nuclear Extract Preparation

Preparation of nuclear extract from 293H cells and the general reassembly reactions were performed as described previously with minor changes.<sup>20</sup> All PV VLPs except for BPV were disassembled in buffer containing 100 mM NaCl, 20 mM Tris (pH 8.2), 2 mM DTT, and 0.01% Tween 80 for 3 hr at 37°C. BPV was disassembled in buffer containing 50 mM NaCl, 20 mM Tris (pH 8.2), 2 mM DTT, and

0.01% Tween 80 for 3 hr at 37°C. For IVP, 1 µg disassembled or intact (i.e., not disassembled) VLPs were incubated for 20 hr at 37°C in buffer containing 100 mM Tris (pH 7.2), 0.02% Tween 80, 10 mM CaCl<sub>2</sub>, and 150 ng of the indicated DNA type (circular, linear, or blunt) in the presence or absence of nuclear extract from 293H cells. Samples were then nuclease-treated for 6 hr at 37°C with 0.1% benzonase (E1014, Sigma), 0.1% BAL-31 (M0213, New England Biolabs), and 10 mM MgCl<sub>2</sub>.

To test different pH and NaCl concentrations, the pH 5.2 and pH 6.2 reassembly mixture contained 100 mM citrate buffer, and the pH 7.2 and pH 8.2 buffers contained 100 mM Tris buffer. CaCl<sub>2</sub> was omitted from the buffer for the pH 5.2 and 6.2 reactions because of the formation of a calcium precipitate under these conditions. For DNA titration within the assembly reactions, the indicated amounts of DNA were added to the reassembly mix.

#### Production of High-Titer Stocks

For HPV16 and other intact particle stocks, particles were diluted in 100 mM citrate buffer (pH 5.2), 0.02% Tween 80, and 450 ng/µg of L1 of pCLucf. Samples were incubated for 30 hr at 37°C. A total of 1 µg L1 per 11 µL of reaction was used. After this time, samples were incubated for a further 15 hr with 5 mM GSSG. Particles were treated for 3 hr at 37°C with 0.2% BAL-31 and 0.2% benzonase in buffer containing 10 mM MgCl<sub>2</sub> and 0.5 M NaCl. Samples were partially purified and concentrated by cushioning on a 1-mL 39% Optiprep by centrifugation for 1 hr at 50,000 rpm in a SW55Ti rotor. The virus-containing fraction (immediately above the cushion) was collected and used for further characterization.

For HPV45 and other disassembled particles, VLPs were first disassembled for 6 hr at 37°C in 200 mM NaCl, 20 mM Tris (pH 8.2), 2 mM DTT, and 0.01% Tween 80. When disassembled, particles were reassembled in buffer containing 100 mM Tris (pH 7.2), 150 mM NaCl, 10 mM CaCl<sub>2</sub>, 0.02% Tween 80, and 450 ng/µg pCLucf. For reassembly, the disassembly mixture was diluted five times with reassembly buffer. Samples were incubated for 30 hr at 37°C and then incubated for a further 15 hr with 5 mM GSSG. Nuclease treatment, purification, and concentration were performed as for HPV16.

#### L1 Quantification

Virus samples and BSA standards were analyzed by SDS-PAGE. Gels were either stained with Coomassie (SimplyBlue SafeStain, LC6060, Thermo Fisher Scientific) or with Sypro Ruby (S12000, Thermo Fisher Scientific) for protein concentrations lower than 0.10 mg/mL. Band intensities were determined with the ImageJ 1.49v software, and L1 concentration was inferred from the BSA calibration curve.

#### Virus Titration

Titration was based on GFP expression in 293TT cells. About 24 hr before infection, 293TT cells in DMEM-10 were pre-plated in a 24-well plate at  $1 \times 10^5$  cells in 0.5 mL per well. Cells were infected

with 10-fold serial dilutions of the virus stock beginning with 1 µL. Infection, corresponding to GFP expression, was analyzed 50 hr post-infection (p.i.) by flow cytometry.

#### qPCR

Reporter plasmid copy numbers were determined by qPCR using a TaqMan assay (Thermo Fisher Scientific). Encapsidated DNA was extracted from the standard or defined virus preparation. 10 µL of each preparation was incubated at 50°C for 15 min with 90 µL of extraction buffer (20 mM Tris [pH 8.0], 20 mM DTT, 20 mM EDTA, 2.0% SDS, and 0.2% Proteinase K). DNA was purified using the QIAquick purification kit (QIAGEN) as directed by the manufacturer's instructions. qPCR was performed according to the manufacturer's instructions using forward primer 5'-CGGCATCAAGGTGAACTTCA-3', reverse primer 5'-ACCATGTGATCGCGCTTCTC-3', and probe 5'-CCAC TACCAGCAGAACA-3', with 6'-carboxyfluorescein (6FAM) as dye and minor groove binder-3' nonfluorescent quencher (MGB-NFQ) as quencher using the Applied Biosystems 7900HT fast real-time PCR system. The primers and probe were designed to amplify the GFP gene. To determine the copy number, known amounts of pCLucf plasmids were used as standards. The standards ranged from  $10^9$ – $10^5$  copies.

#### Neutralization Assays and Inhibition of Infection by Entry Inhibitors

About 24 hr prior to infection,  $4.5 \times 10^3$ /well 293TT or HeLa cells were seeded in 96-well plates.

For neutralization of infection with heparin or neutralizing antibodies, we followed a protocol described previously.<sup>39,40</sup> Briefly, PsVs were incubated with 10-fold dilutions of heparin or a neutralizing antibody (H16.V5 for HPV16 or a polyclonal serum for HPV45) and for 1 h on ice. After this time, PsV mixtures were added to the pre-plated cells. Infection, corresponding to GFP expression, was scored 72 hr p.i. by flow cytometry.

When indicated, cells were left untreated or preincubated for 30 min in DMEM-10 containing inhibitors at the following concentrations: 10 µM dec-RVKR-cmk, 20 mM NH<sub>4</sub>Cl (10 mM HEPES), 300 nM compound XXI, 10 µM CsA. Cells were infected with  $4 \times 10^4$  IU in the presence of the indicated inhibitors. Infection was scored 72 hr p.i. by flow cytometry. Inhibitors were retained during the complete infection course.

#### EM

Samples were negatively stained with 0.5% uranyl acetate for 1 s after adsorption to carbon-coated copper grids. Examination of the samples was performed with an FEI Tecnai T12 transmission electron microscope.

#### Mouse Vaginal Tract Infection

Nine- to twelve-week-old female BALB/c mice were housed in the NCI animal care facilities according to the NCI's Animal Care and Use Committee.

Infection was performed as described previously.<sup>13,15,41</sup> Mice were treated with 3 mg of Depo-Provera (Pfizer) diluted in PBS 4–5 days before pseudovirus infection. Five hours before infection, 50  $\mu$ L of 4% nonoxynol-9 (N-9, N1217, Spectrum) in 4% carboxymethylcellulose (CMC, C4888, Sigma) was instilled intravaginally. After this time, 20  $\mu$ L of the indicated infectious units of the indicated PsVs was instilled in the vaginal tract. Virus instillation was done in 2% CMC. Mice were imaged at different times following infection by intravaginal instillation of 20  $\mu$ L of firefly luciferase substrate (15 mg/mL stock, PerkinElmer, 122796). After 3 min, mouse luminescent images were acquired using a 30-s exposure at medium binning and f/1 using an IVIS 100 imager. Data were analyzed using Living Image software (PerkinElmer). Values are reported as average radiance within a region of interest drawn around the ventral area of each mouse.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at <http://dx.doi.org/10.1016/j.omtm.2017.04.005>.

## AUTHOR CONTRIBUTIONS

C.C., C.D.T., and Y.Y.S.P. conducted the experiments. C.C., C.D.T., P.M.D., Y.Y.S.P., D.R.L., and J.T.S. designed the experiments and wrote the paper.

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