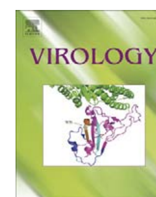
View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Virology 388 (2009) 344–353

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro



Enhanced papillomavirus-like particle production in insect cells

Tilo Senger [a], Lysann Schädlich [a], Lutz Gissmann [b], Martin Müller [c,*]

[a] Department of Genome Modifications and Carcinogenesis, German Cancer Research Centre, D-69120 Heidelberg, Germany
[b] Department of Botany and Microbiology (honorary member), King Saud University, Riyadh, Saudi Arabia
[c] Research Group Tumorvirus-Specific Vaccination Strategies, German Cancer Research Centre, D-69120 Heidelberg, Germany

ARTICLE INFO

Article history:
Received 27 February 2009
Accepted 1 April 2009
Available online 1 May 2009

Keywords:
Papillomavirus
Virus-like particle
Baculovirus
MultiBac
Assembly

ABSTRACT

Human papillomavirus (HPV) L1 self-assembles into virus-like particles (VLPs), which are the basis for the two commercially available prophylactic vaccines. For one of them (Cervarix®) HPV 16 and 18 VLPs are being produced in insect cells using the baculovirus expression system. However, due to low yield, production of VLPs remains challenging for certain other PV types. Here we report that employment of a modified baculovirus-based (MultiBac) expression system (Berger, I., Fitzgerald, D. J., and Richmond, T. J. (2004). Baculovirus expression system for heterologous multiprotein complexes. Nat. Biotechnol. 22(12), 1583–7) permits substantially improved VLP production of several PV types up to 40-fold. Highest VLP yields were achieved when two copies of the L1 gene were expressed from independently controlled cassettes. We have evaluated the production of HPV 57 L1 VLPs by the MultiBac system in more detail. Whereas the level of the HPV 57 L1 protein was only slightly increased in comparison to the standard protocol we monitored a strongly enhanced yield of HPV 57 VLPs. Our results imply that a critical concentration of L1 within the producer cell is required for efficient VLPs assembly. We show evidence that in addition a dominant negative factor in conventionally produced recombinant baculoviruses contributes to differences in VLP yield. This phenomenon might be attributable to the absence of the viral cysteine protease V-CATH in the modified baculovirus system. We anticipate that use of the MultiBac expression system will facilitate capsid production for papillomaviruses and thereby enable the generation of vaccines against infections by many of the as yet untargeted HPV types.

© 2009 Elsevier Inc. All rights reserved.

Introduction

Papillomaviruses (PVs) are a heterogeneous group of double-stranded DNA viruses, which consists of more than 200 known types (zur Hausen, 1999). PVs are host- and tissue-specific, with different types featuring a characteristic differential biology. Cutaneous human PVs (HPVs) primarily infect the skin, causing skin warts or epithelial tumours whereas genital warts and anogenital cancer attribute to infections by mucosal HPV types. Most prominently, virtually all cervical cancer cases are etiologically linked to infections with so-called high-risk HPVs with infections by types 16 and 18 alone accounting for approximately 70% of them (Bosch et al., 2002; Clifford et al., 2003; Munoz et al., 2003). Recently, two commercial vaccines preventing *de novo* infections with HPV 16 and 18 have been licensed (Lowy and Schiller, 2006). The vaccines that prevent CIN lesions induced by the vaccine HPV types (Harper et al., 2004; Koutsky et al., 2002; Mao et al., 2006; Villa et al., 2005) are based on the viral major capsid protein L1, which has the intrinsic ability to self-assemble into highly immunogenic virus-like particles (VLPs) with a diameter of 55 nm (Baker et al., 1991). For large-scale VLP production, both companies apply recombinant *in vitro* expression systems: Gardasil® (Merck and Co., Inc.) and Cervarix® (GlaxoSmithKline Biologicals) are produced in yeast and baculovirus-infected insect cells, respectively.

Applying baculovirus as vectors for recombinant protein expression has proven to be safe, easily manageable, and straightforward to scale up (Cory and Hails, 1997; Ikonomou et al., 2003). Moreover, with Cervarix®, a biopharmaceutical produced in baculovirus-infected insect cells has been widely approved for the first time. Thus far, VLPs of a variety of different HPV types have been generated successfully in insect cells (Christensen et al., 1996; Roden et al., 1996; Suzich et al., 1995; Volpers et al., 1994). However in our attempt to generate VLPs of cutaneous HPV types we have experienced a comparatively low yield when compared to production of particles from several different HPV 16 L1 constructs. Traditionally, recombinant *Autographa californica* nuclear polyhedrosis virus (AcNPV) is being generated by cotransfecting into insect cells the linearized AcNPV genome with a transfer plasmid carrying the gene of interest in an expression cassette controlled by a late viral promoter. Upon recombination of the expression cassette into the AcNPV genome, progeny virus could be harvested and utilised for productive infections. In recent years several improvements to baculovirus vector-based expression systems have been reported. Flexibility has been added by the availability of shuttle vectors allowing the

* Corresponding author. Fax: +49 6221 42 4932.
E-mail address: martin.mueller@dkfz-heidelberg.de (M. Müller).

0042-6822/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2009.04.004

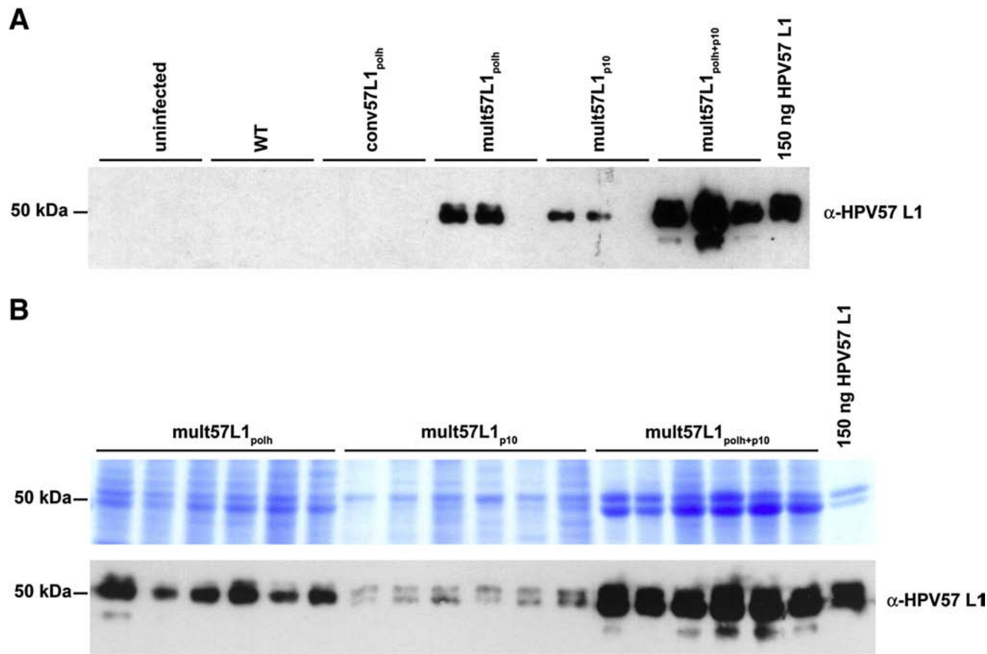


Fig. 1. Expression of HPV 57 L1 using the MultiBac expression system substantially increases VLP yield with high reproducibility. (A) Purified VLPs from infected insect cells. TN High Five cells were infected with a conventionally generated baculovirus transgenic for HPV 57 L1 (conv57L1$_{polh}$), or with MultiBac-based virus carrying HPV 57 L1 in the polh-controlled cassette (mult57L1$_{polh}$), in the p10-controlled cassette (mult57L1$_{p10}$) or in both cassettes simultaneously (mult57L1$_{polh+p10}$). Three days post-infection cells were lysed and capsids were purified by a CsCl density gradient centrifugation. Equal volumes of the three gradient peak fractions per infection were loaded on an SDS-PAGE gel for immunoblotting using an L1-specific MAb. As controls, mock capsid purifications were carried out after WT AcMNPV infection and after no infection and fractions corresponding to the peak after infection with mult57L1$_{polh+p10}$ were loaded. (B) To demonstrate reproducibility, six independent virus stocks were generated for each of the viruses mult57L1$_{polh}$, mult57L1$_{p10}$, and mult57L1$_{polh+p10}$. Independent TN High Five cell infections followed by capsid purification were carried out. Three peak fractions per CsCl gradient were pooled and loaded on SDS-PAGE gels, which were either Coomassie-stained and used for densitometric quantification (top) or immunoblotted using an L1-specific MAb (bottom).

introduction of two genes into independently controlled expression cassettes (Harris and Polayes, 1997). Moreover, the incorporation of the recombinant expression cassette into the baculovirus genome by site- and orientation-specific Tn7-mediated transposition has improved and greatly simplified the progeny virus selection procedure (Airenne et al., 2003; Luckow et al., 1993). On the other hand, protein yield could be enhanced significantly by the deletion of the genes encoding for the AcNPV enzymes chitinase (chiA) and v-cathepsin (V-CATH), which are involved in liquefaction of the host cell during AcNPV pathogenesis, (Hom and Volkman, 2000; Kaba et al., 2004; Slack et al., 1995; Suzuki et al., 1997). These improvements have been combined in a recently described variant baculovirus expression system named MultiBac (Berger et al., 2004).

Here we report that application of the novel baculovirus expression system permits substantially improved VLP production of several PV types by multiple folds. We also provide a mechanistic analysis of the superior VLP recovery rate achieved with this expression system implying that a critical concentration of full-length L1 in the producer cell is required for efficient VLP assembly.

Results

MultiBac expression system enables improved yield of HPV 57 VLPs

The production of VLPs of certain HPV types, such as HPV type 16, has been carried out successfully in insect cells by applying the baculovirus expression system (Kirnbauer et al., 1993). However, employment of conventional baculovirus-based expression systems has proven to be arduous for certain other HPV types due to very low yields. In our hands, one such problematic PV has been HPV 57, a wart-associated cutaneous α-papillomavirus.

A recently developed baculovirus-based expression system named MultiBac allows for the synchronous expression of heterologous proteins from two expression cassettes driven by the polyhedrin

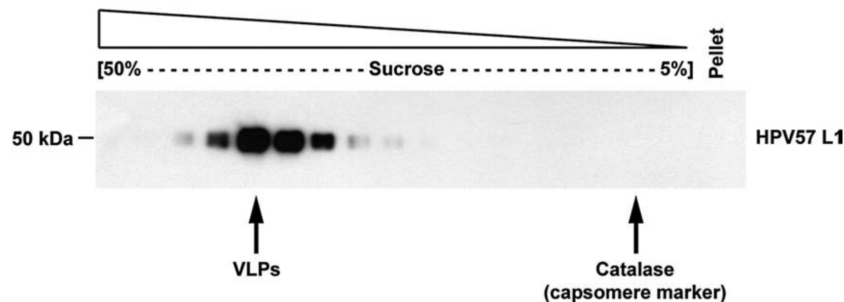


Fig. 2. HPV 57 L1 produced with the MultiBac expression system assembles into properly folded VLPs. Particles produced after infection with Multibac-based AcNPV recombinant for HPV 57 L1 in both expression cassettes were analysed by centrifugation through a linear sucrose gradient. The gradient was fractionated and samples of each fraction were immunoblotted probing with L1-specific MAb. As calibration markers HPV 16 VLPs and catalase, a marker for capsomeres, were used.

Table 1
MultiBac expression system permits efficient VLP production of various PV constructs and it enables production of mutant HPV constructs.

		Conventional	Multibac		
		polh	polh	p10	polh/p10
WT L1 proteins	HPV2	≤0.05 mg	0.2–0.4 mg	0.1–0.2 mg	0.8–1.5 mg
	HPV3	0.1 mg	N/A	N/A	2.0 mg
	HPV6[a]	<det	N/A	N/A	0.7 mg
	HPV10	0.1 mg	N/A	N/A	4.0 mg
	HPV11[a]	≤0.15 mg	N/A	N/A	1.2 mg
	HPV18[a]	≤0.15 mg	N/A	0.9 mg	1.3 mg
	HPV27	≤0.05 mg	0.15–0.4 mg	0.1–0.3 mg	0.8–1.2 mg
	HPV57	≤0.15 mg	0.6–1.2 mg	0.3–0.8 mg	1.5–2.8 mg
	HPV77	0.5 mg	N/A	N/A	4.0 mg
	BPV5	<det	N/A	1.2 mg	2.5 mg
	BPV6	<det	N/A	1.2 mg	3.2 mg
Mutant L1 proteins	HPV2 C172S, C422S L1	≤0.05 mg	0.1–0.2 mg	N/A	0.3–0.5 mg
	HPV27 C173S, C424S L1	≤0.05 mg	0.1–0.15 mg	N/A	0.2–0.4 mg
	HPV57 C173S, C4283S L1	≤0.05 mg	0.1–0.2 mg	N/A	0.2–0.35 mg
	HPV16 L1E7 (1–60[a])	≤0.05 mg	3.4 mg	5.1 mg	4.3 mg

The yield for the different baculovirus stocks infecting 10^8 High Five insect cells at an MOI of 2 is shown.
N/A: not analysed.
<det: below detection limit.
[a] For these types, MultiBac-based viruses were generated with codon-modified L1 genes.

(polh) and the p10 late viral promoters (Berger et al., 2004). We produced MultiBac-based baculoviruses expressing full-length HPV 57 L1 from either of the two cassettes alone or concomitantly (mult57L1$_{polh}$, mult57L1$_{p10}$, mult57L1$_{polh+p10}$) as well as a conventionally generated baculovirus recombinant for HPV 57 L1 (conv57L1$_{polh}$) and carried out side-by-side infections of TN High Five cells followed by capsid purification by CsCl gradient purification. Whereas negligible amounts of HPV 57 L1 VLPs were detected in the gradient fractions after infection with conv57L1$_{polh}$, the HPV 57 L1 VLP yield was substantially increased when the MultiBac-derived viruses had been used for the infection (Fig. 1A). Strikingly, the L1 VLP yield obtained after infection with mult57L1$_{polh+p10}$ was considerably higher than the combined yield after infection with mult57L1$_{polh}$ and mult57L1$_{p10}$.

To determine the variation of the three MultiBac-derived baculovirus vectors, six independent stocks were generated of each of the three recombinant viruses mult57L1$_{polh}$, mult57L1$_{p10}$, and mult57L1$_{polh+p10}$. SDS-PAGE analysis of the samples collected after independent infections with these virus stocks and subsequent capsid purification demonstrated reproducible yields for all three constructs with variations of only 1.5 to 2.4 fold (Fig. 1B).

To verify that the HPV 57 particles produced after infection with MultiBac-derived AcNPVs are properly assembled, we characterized them by sedimentation analysis. As exemplified only for particles obtained after infection with mult57L1$_{polh+p10}$, their migration pattern was virtually identical with the pattern exhibited by HPV 16 L1 VLPs obtained by applying the conventional expression system (Fig. 2). Almost all of the L1 was found in fractions corresponding to VLPs indicative of the high capsid selectivity of the CsCl gradient purification. These data demonstrate that substantially increased yields of *bona fide* HPV 57 VLPs can be produced with the MultiBac expression system.

MultiBac expression system permits efficient particle production of various PV types

To determine whether the MultiBac expression system also allows for enhanced VLP production of PVs other than HPV 57, we generated an extended panel of recombinant baculoviruses. Viruses carrying the full-length L1 gene of HPV types 2, 3, 10, 27, 77 or BPV types 5 or 6 were either based on the conventional or on the MultiBac system. Productive infections with each virus stock followed by capsid purification were performed and the VLP content in the yield was quantitated following SDS-PAGE analysis and Coomassie-staining. For each of the above PV, the VLP yield was increased between 8 and 40 fold when the MultiBac expression system was used compared to application of a conventional system (Table 1). Noticeably, in our hands, VLP production for some of these PVs, such as BPV 5 and 6, failed entirely when conventional baculovirus-based expression systems were applied. Consistent with the findings for HPV 57, the VLP yield for all tested PVs was highest when L1 was expressed from both, the polh and the p10 cassette concomitantly. We also aimed to increase the VLP recovery rate for HPV 6, 11, and 18, for which production used to be unsatisfactory in our laboratory. Employment of the Multibac expression system improved VLP yield considerably for all three types (Table 1). However, only a non-stringent comparison between the different expression systems is feasible as these HPV L1s were introduced into the MultiBac-based recombinant AcNPVs as codon-modified L1 genes. To illustrate the integrity of the produced capsids, they were further purified by heparin affinity chromatography and analysed by electron microscopy (Fig. 3).

In addition to the production of VLPs using wild-type L1 proteins we also expressed genes encoding for modified L1 proteins using the MultiBac expression system. For the production of HPV 2, 27, and 57 capsomeres, we replaced two highly conserved cysteine residues of L1 involved in intercapsomeric disulfide bond formation with serine residues (Li et al., 1998; Sapp et al., 1998), and generated the respective recombinant baculoviruses either conventionally or with the MultiBac system. Additionally, AcNPVs transducing an HPV 16 L1E7$_{1–60}$ fusion protein resulting in formation of chimeric VLPs (cVLPs) (Muller et al., 1997) were prepared. In our hands, production of particles composed of either of these proteins has proven rather inefficient so far. However, employment of the MultiBac expression system resulted in up to 0.5 mg of capsomeres and 4.3 mg of cVLPs per standard purification (Table 1) underscoring the efficacy of the MultiBac system also for the production of mutant L1-based PV particles.

Increased VLP yield and L1 expression levels do not directly correlate

The VLP yield was most favourable with MultiBac-based baculoviruses that induced L1 expression from the polh- and the p10-controlled multiple cloning sites concomitantly. To address the question whether simply different expression levels of the L1 protein correlate with the improved VLP yield, we infected TN High Five cells with conv57L1$_{polh}$, mult57L1$_{polh}$, mult57L1$_{p10}$, and mult57L1$_{polh+p10}$. Three days post-infection, the HPV 57 L1 content in the cell lysates was determined by immunoblotting. We observed that L1 expression

levels were similar in the MultiBac-based virus-infected cells, whereas lower amounts of L1 protein were detectable in the cells infected with conv57L1$_{polh}$ (Fig. 4A). In addition, two L1 protein species of different sizes (55 and 45 kDa) were detected in the conv57L1$_{polh}$-infected cells, possibly a result of proteolytic degradation.

Quantification of total HPV 57 L1 protein levels three days post-infection and of HPV 57 VLPs obtained after subsequent capsid purification indicates that slight differences in L1 concentrations correlate with pronounced disparities in VLP yield (Fig. 4B). We also analysed the L1 expression in TN High Five cells infected with the same set of recombinant AcNPVs by immunofluorescence microscopy using an HPV 57 L1-specific MAb (T. Senger unpublished). Consistent with the biochemical analyses of L1 expression levels (Fig. 4A) we detected the highest amounts of HPV 57 L1 in cells infected with double-recombinant MultiBac (single-recombinant MultiBac: intermediate amounts of L1, conventionally generated: lowest amounts of L1) viruses (Fig. 4C). However, as evident by both assays, HPV 57 L1 expression levels correlate disproportionately with the increase in VLP recovery rate upon employment of MultiBac-based viruses suggesting that superior VLP yields are not solely attributable to elevated L1 expression.

It has been reported that L1 transcript quality and quantity can have a major impact on recombinant VLP production (Neeper et al., 1996). To evaluate whether the differences of expressed L1 amounts upon infection with conventional or MultiBac-derived AcNPVs are due to differences in the transcript levels, we isolated poly(A)$^{+}$-RNA from the TN High Five cells three days post-infection and determined the amount of L1 transcripts using a radiolabelled HPV 57 L1-specific probe. Surprisingly, two distinct L1-specific transcript species of different sizes were identified in cells expressing L1 from a polh-controlled MultiBac-based cassette (Fig. 5). In comparison, a considerably longer transcript species of much lower abundance was detected in cells in which L1 expression was driven from a p10-controlled cassette transduced by MultiBac virus. As expected, all

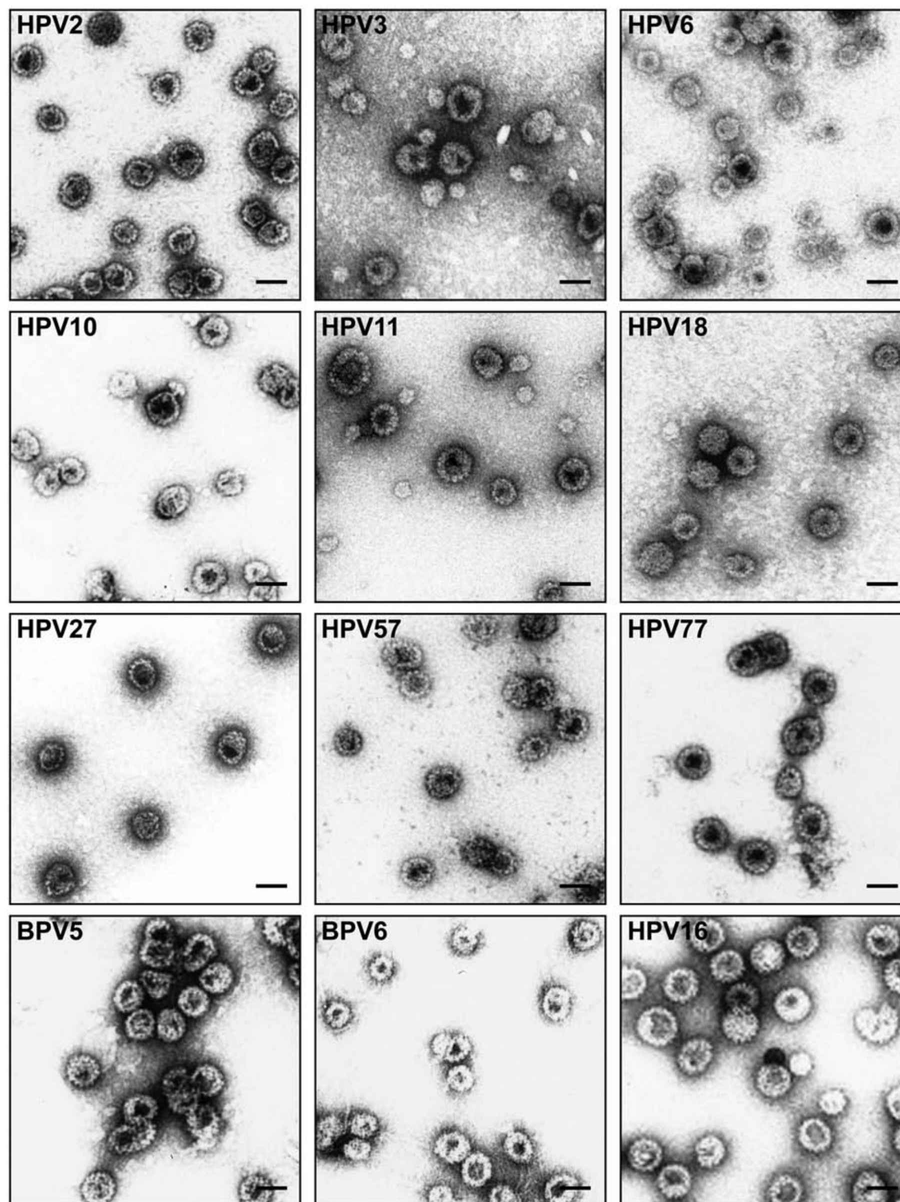


Fig. 3. MultiBac expression system permits VLP production of various PV types. VLPs from HPV 2, HPV 3, HPV 6, HPV 10, HPV 11, HPV 18, HPV 27, HPV 57, HPV 77, BPV 5, and BPV 6 were produced upon infection with MultiBac-based AcNPV recombinant for the respective L1 gene in both multiple cloning sites. VLPs were purified by CsCl gradient centrifugation and heparin affinity chromatography. Samples were analysed by electron microscopy. As control, conventionally generated baculovirus inducing HPV 16 L1 expression was used for a parallel infection followed by the same purification protocol. Bars indicate 50 nm in all panels.

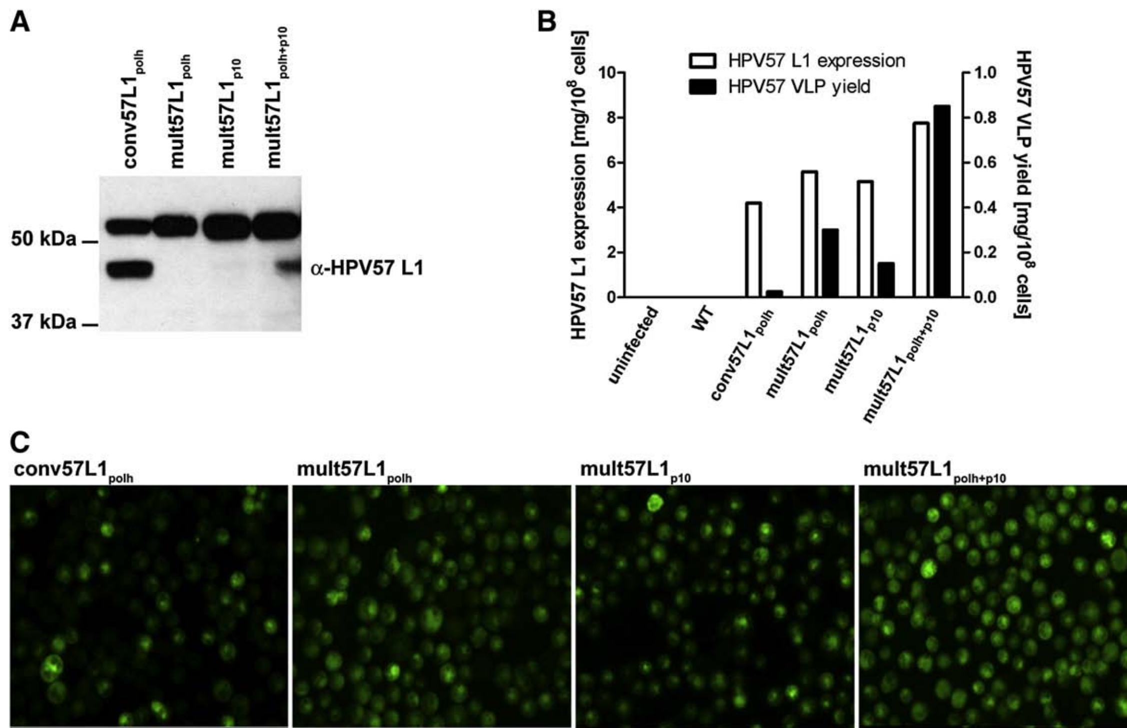


Fig. 4. A slight increase in HPV 57 L1 expression levels strongly enhances VLP yield. (A) TN High Five cells were infected with a conventionally generated baculovirus transgenic for HPV 57 L1 (conv57L1$_{polh}$), or with MultiBac-based virus carrying HPV 57 L1 in the polh-controlled cassette alone (mult57L1$_{polh}$), in the p10-controlled cassette alone (mult57L1$_{p10}$) or in both cassettes simultaneously (mult57L1$_{polh+p10}$). Three days post-infection L1 expression was determined in cell lysates by western blotting. (B) Quantification of HPV 57 L1 expressed three days post-infection and of HPV 57 VLPs after subsequent capsid purification. Protein amounts were quantified by densitometric means after SDS-PAGE analysis. (C) Detection of HPV 57 L1 in infected insect cells by immunofluorescence. TN High Five cells were infected with conv57L1$_{polh}$, mult57L1$_{polh}$, mult57L1$_{p10}$, or mult57L1$_{polh+p10}$. Three days post-infection, cells were immunostained using the HPV 57 L1-specific MAb TS57-1 and Fluor 488-conjugated goat anti-mouse IgG.

three transcript species were present in the mult57L1$_{polh+p10}$-infected cells. Importantly, the presence of a single highly abundant L1 transcript in cells infected with conv57L1$_{polh}$ indicates that the observed two protein species are likely due to posttranscriptional events. Whereas we did not further analyse the structure of the different transcripts we conclude from these data that differences in L1 expression are not caused by different promoter activities and/or RNA stability.

Conventional baculovirus confers a functionally dominant negative factor

The wild-type baculovirus genome encodes for a cysteine protease, V-CATH, which is involved in the destruction of host insect cell tissue in the course of *in vivo* replication of AcNPV (Slack et al., 1995). An essential factor required for V-CATH activation is represented by the chitinase chiA (Hom and Volkman, 2000). Both genes have been inactivated in the baculovirus genome utilised in the MultiBac expression system (Berger et al., 2004). We assumed that conventional AcNPVs carry a dominant negative factor for production of PV L1. To verify this hypothesis, we produced conventional or MultiBac-based AcNP viruses, both recombinant for the Green Fluorescent Protein (GFP) under the control of the polh promoter (convGFP$_{polh}$, multGFP$_{polh}$) for coinfection studies. First, we ascertained by immunofluorescence microscopy that TN High Five cells can be coinfected with at least two different AcNPVs leading to ectopic expression of GFP and HPV 57 L1 within the same cell as exemplified here for convGFP$_{polh}$ and mult57L1$_{polh}$ coinfection (Fig. 6A). Next, we infected TN High Five cells either with conv57L1$_{polh}$ or mult57L1$_{polh}$ alone or together with convGFP$_{polh}$ or multGFP$_{polh}$ and quantified the GFP and HPV 57 L1 amounts in the cell lysates and the amount of VLPs in the CsCl gradient fractions after capsid purification. Results are shown in Fig. 6B. As described above, expression levels of the HPV 57 L1 protein differed only slightly upon infection with conv57L1$_{polh}$ or mult57L1$_{polh}$ alone, whereas a 13-fold higher VLP yield was achieved after infection with the mult57L1$_{polh}$. However, when mult57L1$_{polh}$ was coinfected with convGFP$_{polh}$, HPV 57 VLP recovery rate declined to levels comparable to those obtained after conv57L1$_{polh}$ single infection whereas levels of L1 protein were only slightly affected. In

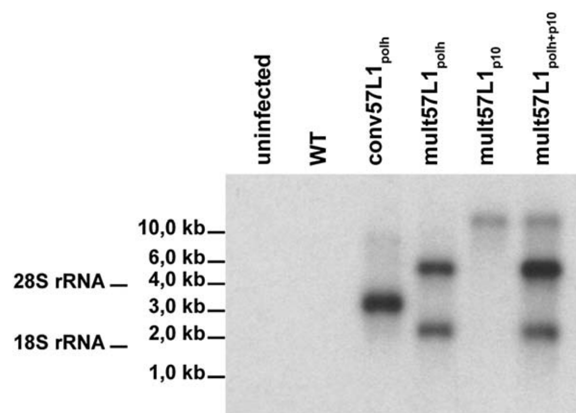


Fig. 5. HPV 57 L1-specific transcript levels in insect cells infected with baculoviruses generated with the conventional or the MultiBac system. TN High Five cells were infected with WT AcMNPV, with a conventionally generated baculovirus transgenic for HPV 57 L1 (conv57L1$_{polh}$), or with MultiBac-based virus carrying HPV 57 L1 in the polh-controlled cassette alone (mult57L1$_{polh}$), in the p10-controlled cassette alone (mult57L1$_{p10}$) or in both cassettes simultaneously (mult57L1$_{polh+p10}$). Three days post-infection cellular poly(A)$^+$-RNA was isolated, size-fractionated on an agarose gel, Northern blotted, and probed for HPV 57 L1 transcripts using a radiolabelled probe. Specificity of the applied probe was verified by reverse transcription of the poly(A)$^+$-RNA, HPV 57 L1 transcript-specific PCR amplification and sequencing of the amplified cDNA.

contrast, upon coinfection with mult57L1$_{polh}$ and multGFP$_{polh}$, HPV 57 VLP yield was not decreased. Conversely, coinfection with conv57L1$_{polh}$ and convGFP$_{polh}$ resulted in low VLP yield, yet, coinfection with conv57L1$_{polh}$ and multGFP$_{polh}$ surprisingly rescued the VLP yield. In terms of GFP expression, highest levels were detected in cells infected with the two different MultiBac-based viruses (i.e. multGFP$_{polh}$ and mult57L1$_{polh}$), intermediate levels were observed upon coinfection of one MultiBac-derived and one conventionally generated virus (i.e. conv57L1$_{polh}$ and convGFP$_{polh}$), and low level GFP expression was detected when the two conventionally produced viruses (i.e. multGFP$_{polh}$ and conv57L1$_{polh}$ or convGFP$_{polh}$ and mult57L1$_{polh}$) were used for coinfection. These data illustrate, that a dominant negative factor is conferred by conventionally generated baculoviruses leading to a decrease in HPV 57 L1 expression levels and, most notably, VLP yield.

To investigate whether the negative effect on HPV 57 L1 VLP yield upon infection with conv57L1$_{polh}$ can be rescued by an increased MOI, we infected TN High Five cells with conv57L1$_{polh}$ or mult57L1$_{polh}$ at MOIs between 0.01 and 20 and analysed protein amount and VLP yields. A second L1 species of reduced size after infection with conv57L1$_{polh}$ occurred independently of MOI (Fig. 7). In contrast, only the full-length L1 protein was detected in cells infected with mult57L1$_{polh}$. For both AcNPVs, increased MOIs lead to elevated L1 expression, yet, quantification of full-length L1 revealed higher L1 expression levels after infection with mult57L1$_{polh}$ (Figs. 7A and B). For this virus, highest VLP yield was achieved at MOIs between one and two and decreased with higher MOIs. No such prominent optimal MOI was observed for conv57L1$_{polh}$. Here, VLP yield increased only slightly with elevated MOIs peaking at an MOI of 10 consistent with the hypothesis of a dominant negative factor conferred by the

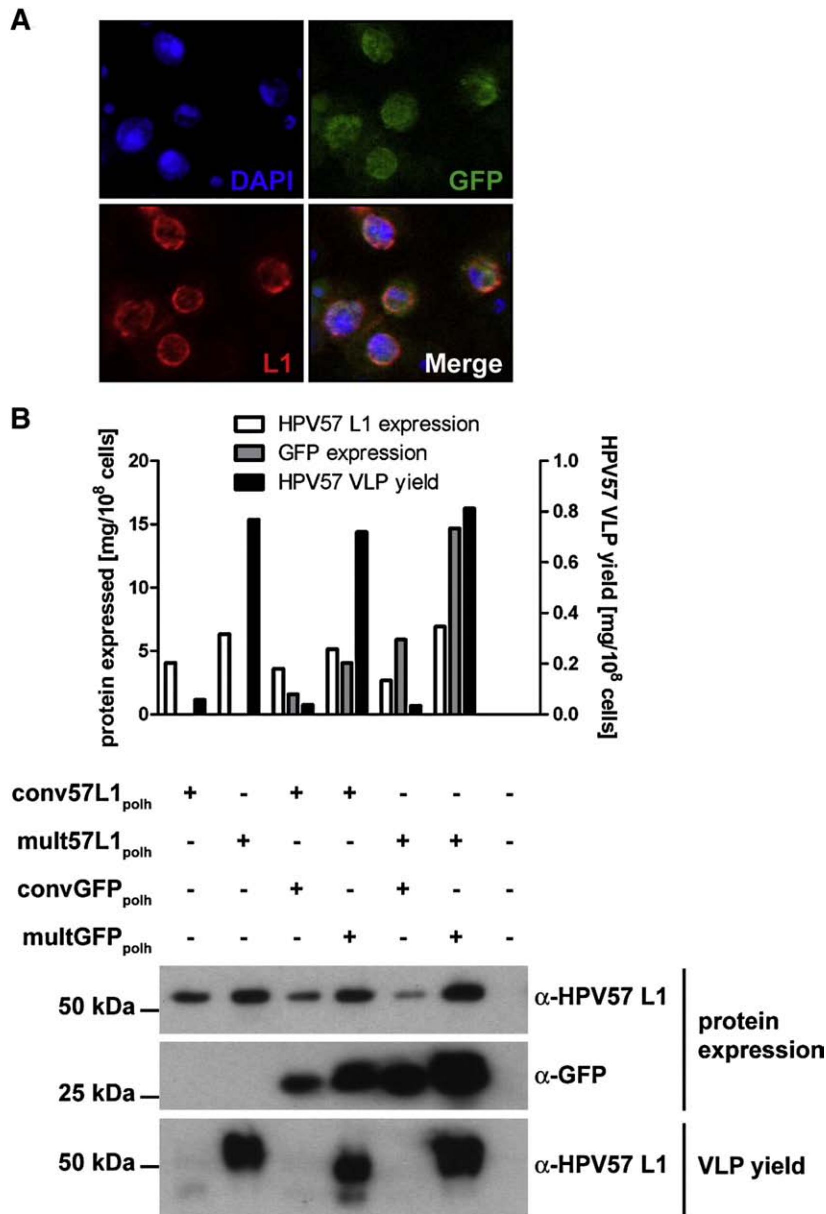


Fig. 6. Conventionally produced AcNPV confers a dominant negative effect on the stability of the recombinant protein upon coinfection. (A) Detection of double infected cells by immunofluorescent staining. TN High Five cells were coinfected with convGFP$_{polh}$ and mult57L1$_{polh}$. Three days after the infection, cells were stained with the HPV 57 L1-specific MAb TS57-1 and Cy3-conjugated goat anti-mouse IgG and DAPI. (B) TN High Five cells were infected either with conv57L1$_{polh}$ or with mult57L1$_{polh}$ alone, or together with convGFP$_{polh}$ or multGFP$_{polh}$. The amounts of GFP and HPV 57 L1 in the cell lysates and the amount of VLPs in the peak fractions after capsid purification were evaluated by SDS-PAGE followed by immunoblotting or by Coomassie-staining and densitometric quantification of HPV 57 L1 and GFP.

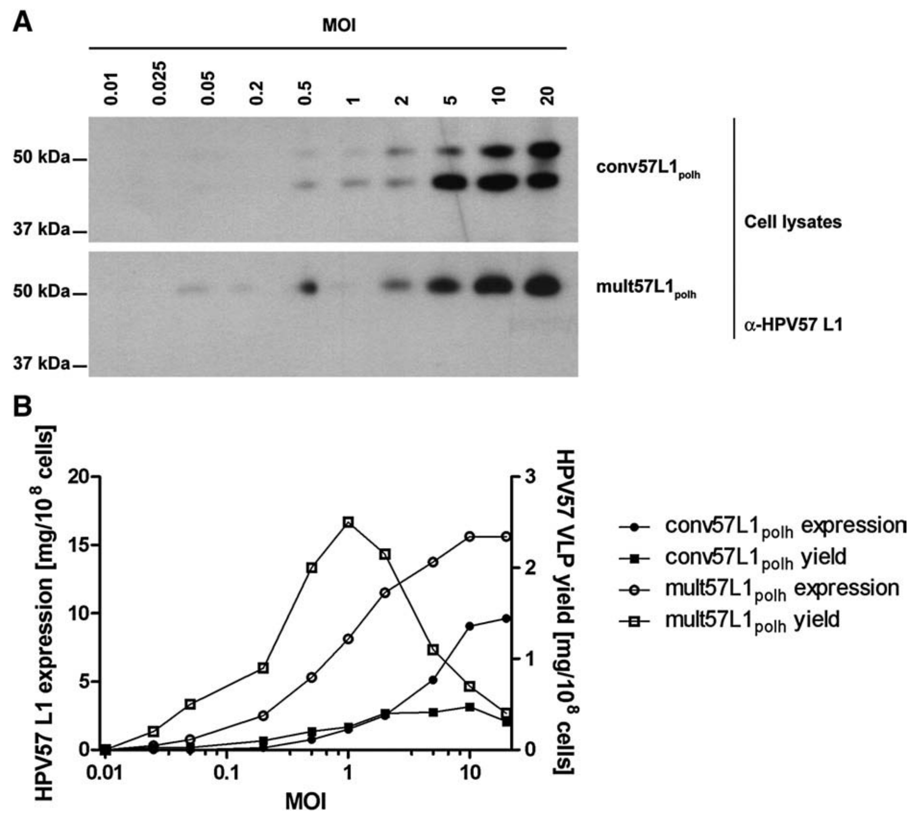


Fig. 7. Dominant negative effect conferred by conventionally produced AcNPV is independent of MOI. (A) Detection of HPV 57 L1 expressed in TN High Five cells upon infection $conv57L1_{polh}$ or $mult57L1_{polh}$ at different MOI. Three days after the infection, cells were lysed and equal volumes of the lysates were immunoblotted probing with L1-specific MAb. (B) Quantification of HPV 57 L1 expression and HPV 57 VLP yield after infection with different MOI. The amounts of HPV 57 L1 in the cell lysates and the amount of VLPs in the peak fractions after capsid purification were evaluated by SDS-PAGE followed by immunoblotting or by Coomassie-staining and densitometric quantification. The respective values are plotted over the MOI applied for $conv57L1_{polh}$ and $mult57L1_{polh}$.

conventionally generated AcNPV which cannot be compensated for by a higher virus dose.

As the major difference between conventionally produced and MultiBac-based viruses is the absence of V-CATH activity conferred by particles produced by the MultiBac system, it is likely that this dominant negative effect is indeed caused by V-CATH, particularly given the occurrence of truncated L1 specifically in cells infected with conventionally produced AcNPV.

Discussion

So far, the modified baculovirus expression system named MultiBac has been used primarily for the expression of heterologous multiprotein complexes (Berger et al., 2004; Bieniossek et al., 2008; Fitzgerald et al., 2006; Fitzgerald et al., 2007). We report here that employment of the MultiBac system permits enhanced VLP production of various PVs. We show that strongly increased VLP yield coincides with slightly elevated L1 expression. Our findings suggest that a dominant negative factor inherent to conventional AcNPV contributes to differences in L1 production. We conclude that the absence of V-CATH activity in MultiBac infections favours L1 yield.

We compared the VLP yield for various PV types after infection with conventionally generated or MultiBac-based AcNPVs. For HPV types 2, 3, 10, 27, 57, 77, and for BPV types 5 and 6, the yield achieved using the MultiBac system was improved up to 40-fold (Fig. 1, Table 1). With the exception of HPV 2 (Cubie et al., 1998) VLP production for any of these PVs has not yet been reported. Proper capsid structure was verified by sedimentation analysis and transmission electron microscopy (Figs. 2 and 3). For HPV types 6, 11, and 18 VLP production in insect cells has been reported previously (Roden et al., 1996). Furthermore, for these three HPV types, we introduced codon-modified L1 genes into the AcNPV genome for the generation of MultiBac-based virions whereas the conventional AcNPVs were generated using unmodified L1 genes. Hence, a direct comparison of the VLP yields upon infection with conventional and MultiBac viruses cannot be made. However, in our laboratory VLP production of HPV types 6, 11, and 18 was all but satisfactory, yet, large amounts of VLPs could be generated applying the MultiBac system. Thus, our findings suggest that the MultiBac system allows for yielding VLP production of multiple PVs.

We also expressed mutant L1 constructs that had previously proved inefficient in particle production. The enhanced yields achieved using the MultiBac system highlight its applicability for production of VLP-related particles such as the candidate prophylactic and therapeutic vaccine HPV 16 $L1E7_{1-60}$ cVLPs. A closely related cVLP-based vaccine ($L1E7_{1-55}$) has already proven safe in a phase 1/2 clinical trial (Kaufmann et al., 2007).

The MultiBac system enables expression controlled either by the polyhedrin (polh) or the p10 promoter. Both drive protein expression very late in the AcNPV life cycle with the activation of the p10 gene preceding the activation of the polh gene by few hours (Roelvink et al., 1992). We observed largely similar intracellular HPV 57 L1 levels upon polh- and p10-driven expression. Simultaneous expression from both multiple cloning sites resulted in slightly elevated HPV 57 L1 production (Fig. 4). These findings are in accordance with DiFalco et al. (1997) who reported similar results upon single and combined polh- and p10 promoter-controlled expression of insulin-like growth factor-interleukin-3 chimeras. The slight increase in HPV 57 L1 levels due to expression from both cassettes manifests itself in significantly enhanced VLP yield (Figs. 1, 4). This phenomenon is suggestive of a VLP assembly process that is sensitive for a minimal L1 concentration. Indeed, an analysis of the HPV11 capsid formation kinetics *in vitro* has

shown that it is an L1 concentration-dependent second order reaction and that a critical cooperativity of pentamers is necessary to initiate stable nucleation for further assembly (Casini et al., 2004). Hence, our findings suggest a similar capsid assembly kinetics in the *in vivo* environment.

For HPV 11, low VLP yield upon L1 expression in yeast has been attributed to a truncation of the corresponding mRNA (Neeper et al., 1996). Therefore, we analysed the HPV 57 L1 transcripts after infection with conventionally generated or MultiBac-derived AcNPVs. In our analysis no truncated HPV 57 L1 transcripts were observed. However, L1 mRNA lengths differed considerably upon infection with $conv57L1_{polh}$, $mult57L1_{polh}$, $mult57L1_{p10}$, or $mult57L1_{polh+p10}$ (Fig. 5). The mRNA of about 3 kb length for the $conv57L1_{polh}$-infected and the mRNA of about 2 kb length for $mult57L1_{polh}$-infected cell isolates were consistent with transcription initiation and termination at the annotated sites. On the other hand, the additional transcript of about 5 kb upon $mult57L1_{polh}$ infection and the single mRNA of roughly 11 kb after infection with $mult57L1_{p10}$ were not expected. The occurrence of several transcript species of unexpected sizes has been described previously for the recombinant expression of influenza virus haemagglutinin in insect cells (Possee, 1986). Such mRNA may result from run-on transcription. We hypothesize that mRNA transcripts of lengths longer than about 2 kb may be dispensable and even inhibitory for efficient VLP production. Their avoidance should be the aim of future optimization efforts. Nonetheless, the abundance of the polh- and p10-derived transcripts does not correlate closely with the corresponding L1 expression levels (Fig. 4) indicating that translation rate and protein stability have a stronger impact on VLP yield than quantitative effects on the transcript level.

Infection with conventionally generated AcNPV entails the production of a single HPV 57 L1 mRNA transcript whereas two distinct L1 protein populations occur, one of which is smaller than full-length L1 (Figs. 4 and 5). In contrast, only full-length L1 was found in lysates from MultiBac AcNPV-infected cells. Thus, the L1 protein seems to be degraded specifically in cells infected with conventional AcNPV which results in a decreased full-length L1 concentration. However, the superior performance achieved by the MultiBac-based recombinant AcNPV may not be solely accounted to elevated L1 expression or stability.

Double infection of TN High Five cells with AcNPVs recombinant for HPV 57 L1 or for GFP leads to low protein expression upon infection with two conventional virus pools, intermediate expression upon infection with one conventional and one MultiBac virus pool, and high expression upon infection with two MultiBac virus pools (Fig. 6). As expected, differences in L1 protein levels are reflected as correspondingly different VLP recovery rates in an amplified manner. Accordingly, coinfection of MultiBac virus with a conventionally derived AcNPV reduces recombinant protein expression in the host cell implicating that conventional viruses confer a dominant negative factor, which is absent in MultiBac-based particles. A major distinction between the employed baculovirus expression systems is the deletion of the genes encoding for chiA and V-CATH, which was shown to enhance protein yield due to depletion of viral cysteine protease activity (Hom and Volkman, 2000; Kaba et al., 2004; Slack et al., 1995; Suzuki et al., 1997). The detection of truncated L1 only in the cells infected with conventionally generated AcNPV is in accordance with these reports. Therefore, the dominant negative factor conveyed by conventional baculovirus may be V-CATH activity alleviating intracellular L1 protein and leading to markedly decreased VLP yield. However, in addition to the disproportionate correlation between L1 concentration and VLP production, a complementary effect might impact on the system efficacy. Truncated L1 proteins occurring in cells infected with conventionally generated virus may exert an inhibitory effect as abortive components in the process of capsid assembly. This hypothesis is in line with the observation that increased MOI, also increasing the hypothesized negative factor, does not enhance VLP yield significantly despite considerably elevated full-length L1 expression levels (Fig. 7).

In the case of coinfection with $conv57L1_{polh}$ and $multGFP_{polh}$ the achieved HPV 57 VLP yield was higher than that after infection with $conv57L1_{polh}$ alone. This rescuing effect would be consistent with the presence of a dominant negative factor conveyed by conventional AcNPVs as interaction of the truncated V-CATH or chiA conferred by the MultiBac-based virus may inhibit activity of the functional V-CATH or chiA introduced by the conventional virus. Note that chiA is required for the activity of the V-CATH protease (Hom and Volkman, 2000). In fact, according to our information, both enzymes have been inactivated by homologous recombination targeting the c-terminal part of either gene and leaving at least half of the protein-coding region intact (information was obtained from the international patent application WO2005085456). Such obstructing interaction, however, is likely to depend on stoichiometry as indicated by the decreased VLP yield in case of coinfection with $convGFP_{polh}$ and $mult57L1_{polh}$.

Infection with $mult57L1_{polh}$ at different MOIs revealed a virus dose optimal for HPV57 L1 VLP production between MOIs one and two (Fig. 7). At MOIs >2 the corresponding VLP yield was substantially decreased. Such decreased recovery rates at comparatively low MOIs may be due to formation of very high concentrations of nucleation centres consuming most L1 proteins before VLP assembly is completed. This phenomenon has been described as “kinetic trapping” for assembly of HBV capsids (Zlotnick and Stray, 2003).

It has been well described that VLP yield of different HPV 16 variants varies considerably using a baculovirus-mediated *in vitro* expression system (Touze et al., 1998). Hence, it is not unexpected that VLPs from different PVs are not equally straightforward to produce. In this report we provide evidence for more efficient VLP production of various PV types. In our study we show for the first time, that efficient production of VLPs from alpha-papillomaviruses can be achieved. For some of these PV types it had been problematic to produce large amounts of VLPs in our laboratory.

One issue not specifically addressed in our investigations is the genetic stability of MultiBac baculoviruses, which is an important aspect in the large-scale VLP production. In our study we compared multiple independently generated viruses containing single and double L1 insertions. These stocks led to consistent L1 expression after serial passage indicating at least mid-term genetic stability even when two copies of L1 are present. We anticipate that use of the MultiBac expression system will facilitate capsid production in general and particularly for challenging PV types and thereby enable the generation of vaccines preventing infections by many of the as yet untargeted HPVs.

Materials and methods

Cells and viruses

Spodopteria frugiperda 9 (Sf9; Invitrogen) cells were grown in suspension at 27 °C and maintained on Grace's insect medium (Gibco) supplemented with 10% foetal bovine serum (FCS; Sigma) and Pluronic F-68 (Sigma). *Trichoplusia ni* (TN) High Five cells (Invitrogen) were cultivated in Ex-Cell™ 405 serum-free medium (SAFC Biosciences) at 27 °C. The WT AcMNPV was obtained from BD Biosciences, the recombinant viruses were produced as described below.

Generation of baculovirus recombinants

All point mutations in the L1 genes were introduced using the QuikChange® Multi Site Directed Mutagenesis Kit (Stratagene). The generation of the chimeric HPV 16 $L1E7_{1–60}$ construct has been described previously (Muller et al., 1997). Full-length or mutated L1 genes were cloned into the transfer plasmids pVL1392 (Invitrogen) or

pFBDM (Berger et al., 2004) by PCR amplification with primers introducing restriction sites. All constructs were confirmed by DNA sequencing.

Recombinant AcNPVs referred to here as produced with a conventional system were generated as described in Muller et al. (1995). Briefly, 2 μg of the respective transfer plasmid and 0.2 μg of linearized DiamondBac™ baculovirus DNA (Sigma) were cotransfected by calcium phosphate precipitation into 5×10^6 Sf9 cells.

To generate the MultiBac recombinant AcNPVs, the strategy explicitly outlined previously (Fitzgerald et al., 2006) was applied. Briefly, 10 ng of recombinant plasmid was transformed into DH10MultiBac cells. Positive clones, as identified by blue/white selection, were amplified and MultiBac bacmid DNA was isolated. One microgram of bacmid DNA was transfected into 5×10^6 Sf9 cells by calcium phosphate precipitation.

All recombinant Ac viruses were amplified at least three times before their employment for a productive infection of TN High Five cells. The titer of all AcNPVs was determined by a plaque assay as described previously (Matsuura et al., 1987).

Virus-like particle production and purification

PV virus-like particles (VLPs) were produced as described in Muller et al. (1997). Briefly, 2×10^8 TN High Five cells were infected with WT or recombinant baculovirus at an MOI of 2 unless indicated otherwise. Three days post-infection, cells were harvested and lysed by sonication. Subsequently, the lysate was cleared by centrifugation, layered onto a two-step gradient with 14 ml of 40% sucrose on top of 8 ml of a 57.5% CsCl solution, and centrifuged for 3 h at 96,500 ×*g* at 10 °C in a SW32 rotor (Beckman). The interphase was collected and transferred into a Quick-seal tube (Beckman). A CsCl gradient was produced by a 16 hour-centrifugation at 184,000 ×*g* at 20 °C in a Sorval TFT 65.13 rotor and fractionated into 1 ml specimen. Purity and L1 content of the collected fractions were assessed by SDS-PAGE and Coomassie-staining.

The peak fractions were pooled, dialyzed against 50 mM Hepes (pH 7.4, 0.3 M NaCl), and cleared from residual debris by centrifugation at 20,000 ×*g* for 10 min at 4 °C. The samples were further purified by affinity chromatography using 1 ml HiTrap™ Heparin HP columns (GE Healthcare). Elution of VLPs was carried out with 50 mM Hepes (pH 7.4) containing 1 M NaCl. The eluates were analysed by SDS-PAGE and Coomassie-staining and western-blot analysis. The capsid quality was verified by electron microscopy.

Detection and quantification of L1 proteins

PV L1 proteins were analysed by SDS-PAGE and stained with colloidal Coomassie dye (GelCode Blue stain reagent, Pierce) or immunoblotted and probed with the anti-L1 monoclonal antibody (MAb) MD2H11. L1 protein concentrations were determined using image densitometry software ImageJ (http://rsb.info.nih.gov/ij/) and bovine serum albumin or HPV 57 L1 as standards for the Coomassie-stained SDS-PAGE gels or the immunoblots respectively.

Sedimentation analysis

Samples were loaded onto a linear gradient of 5%–50% sucrose in 50 mM Hepes (pH 7.4) containing 0.5 M NaCl and centrifuged at 222,000 ×*g* for 3 h at 4 °C using a SW41 Ti rotor. Fractions (600 μl) were collected from the bottom of the gradient and analysed by SDS-PAGE and immunoblotting.

Electron microscopy

VLPs (100 ng) were applied onto carbon coated grids and stained with 2% uranyl acetate. Grids were analysed using a transmission electron microscope CM200 FEG (FEI) operating at 200 kV. Pictures were taken at a 27,000 fold magnification using a 2 k×2 k CCD camera.

Generation and purification of the monoclonal antibody TS57-1

For the generation of MAb TS57-1 BALB/c mice were immunized s.c. three times in 4 week intervals with 10 μg HPV 57 VLPs emulsified in complete Freund's adjuvant for the first injection or incomplete Freund's adjuvant for the subsequent injections. An additional boost was performed i.p. three days before the mice were sacrificed and the spleens were isolated. Fusion of spleen cells with myeloma cell line SP2/0-Ag14 (ratio 5:1) was carried out using polyethylenglycol (PEG; Sigma-Aldrich) (Kohler and Milstein, 1975). Hybridoma supernatants were screened by enzyme-linked immunosorbent assay (ELISA) using GST-L1 fusion protein as described in Sehr et al. (2001).

The hybridoma supernatant was purified by affinity chromatography using GammaBind™Plus Sepharose™ (GE Healthcare). Bound antibodies were eluted with 100 mM glycine (pH 3.0), collected in 1 ml fractions, and 50 μl of 1 M Tris (pH 8.0) was added to each fraction. Highly reactive fractions as identified by GST-capture ELISA were pooled and dialyzed overnight against PBS. Protein content was determined by Bradford assay and purity was verified by SDS-PAGE followed by Coomassie-staining.

Immunofluorescence

On 18 mm round cover slips, 5×10^5 Sf9 cells were seeded and infected with recombinant or WT baculovirus. TN High Five cells were infected in suspension and, after a three-day incubation, 1×10^4 cells were centrifuged onto microscope slides by means of cytospin (Shandon). Cells were fixed with 4% paraformaldehyde (PFA) and washed three times with phosphate buffered saline (PBS). Immunostaining was performed with the MAb TS57-1 as primary antibody (1:500) and Cy™3-linked goat anti-mouse IgG (1:200) (GE Healthcare) or Fluor 488-conjugated goat anti-mouse IgG (1:300) (Invitrogen) as secondary antibodies. Cells were washed five times with PBS after incubation with each antibody. DAPI (Sigma) was used to stain the nuclei. As mounting reagent ProLong® Gold (Invitrogen) was applied. Digital images were captured using a Leitz DM-RBE microscope (Leica) equipped with an F-View camera (Olympus) controlled by cell^F software (Olympus).

Transcript analysis

RNA from 3×10^6 infected TN High Five cells was isolated using the RNeasy kit (Qiagen). Poly(A)$^+$-RNA was selected using DynaBeads oligo(dT)25 (Invitrogen). For Northern blot analysis, 2 μg of the poly (A)$^+$-RNA was size-fractionated on a denaturing agarose gel and transferred to a Hybond membrane (GE Healthcare). A probe was generated by radiolabelling of PCR-amplified full-length HPV 57 L1 DNA with an α[^{32}P]ddNTPs/dNTP blend using the random priming method (GE Healthcare). For sequence analysis, poly(A)$^+$-RNA samples were reverse transcribed and amplified applying the Phusion™ RT-PCR kit (Finnzymes) or the LongRange 2Step RT-PCR kit (Qiagen) for transcript specimen <2500 nucleotides or >2000 nucleotides respectively. In both cases, the forward primer CATGACATTCTGGGATGTGG and an oligo(dT) reverse primer were applied for the amplification reaction. The same primer set was used to verify the sequence identities of the obtained cDNAs.

Acknowledgments

We thank Imre Berger for providing the MultiBac plasmids and Birgit Aengeneyndt and Corinna Klein for their expert assistance. We

are grateful to Elisabeth Schwarz and Bo Xu for their help with the northern blot. The work was in part supported by the Wilhelm Sander Stiftung (awarded to MM) and by DKFZ-Canceropole Grand-Est (granted to LG).

References

Airenne, K.J., Peltomaa, E., Hytonen, V.P., Laitinen, O.H., Yla-Herttuala, S., 2003. Improved generation of recombinant baculovirus genomes in *Escherichia coli*. Nucleic Acids Res. 31 (17), e101.

Baker, T.S., Newcomb, W.W., Olson, N.H., Cowsert, L.M., Olson, C., Brown, J.C., 1991. Structures of bovine and human papillomaviruses. Analysis by cryoelectron microscopy and three-dimensional image reconstruction. Biophys. J. 60 (6), 1445–1456.

Berger, I., Fitzgerald, D.J., Richmond, T.J., 2004. Baculovirus expression system for heterologous multiprotein complexes. Nat. Biotechnol. 22 (12), 1583–1587.

Bieniossek, C., Richmond, T. J., and Berger, I. (2008). MultiBac: multigene baculovirus-based eukaryotic protein complex production. Curr Protoc Protein Sci Chapter 5, Unit 5 20.

Bosch, F.X., Lorincz, A., Munoz, N., Meijer, C.J., Shah, K.V., 2002. The causal relation between human papillomavirus and cervical cancer. J. Clin. Pathol. 55 (4), 244–265.

Casini, G.L., Graham, D., Heine, D., Garcea, R.L., Wu, D.T., 2004. *In vitro* papillomavirus capsid assembly analyzed by light scattering. Virology 325 (2), 320–327.

Christensen, N.D., Reed, C.A., Cladel, N.M., Hall, K., Leiserowitz, G.S., 1996. Monoclonal antibodies to HPV-6 L1 virus-like particles identify conformational and linear neutralizing epitopes on HPV-11 in addition to type-specific epitopes on HPV-6. Virology 224 (2), 477–486.

Clifford, G.M., Smith, J.S., Aguado, T., Franceschi, S., 2003. Comparison of HPV type distribution in high-grade cervical lesions and cervical cancer: a meta-analysis. Br. J. Cancer 89 (1), 101–105.

Cory, J.S., Hails, R.S., 1997. The ecology and biosafety of baculoviruses. Curr. Opin. Biotechnol. 8 (3), 323–327.

Cubie, H.A., Plumstead, M., Zhang, W., de Jesus, O., Duncan, L.A., Stanley, M.A., 1998. Presence of antibodies to human papillomavirus virus-like particles (VLPs) in 11–13-year-old schoolgirls. J. Med. Virol. 56 (3), 210–216.

DiFalco, M.R., Bakopanos, E., Patricelli, M., Chan, G., Congote, L.F., 1997. The influence of various insect cell lines, p10 and polyhedrin promoters in the production of secreted insulin-like growth factor-interleukin-3 chimeras in the baculovirus expression system. J. Biotechnol. 56 (1), 49–56.

Fitzgerald, D.J., Berger, P., Schaffitzel, C., Yamada, K., Richmond, T.J., Berger, I., 2006. Protein complex expression by using multigene baculoviral vectors. Nat. Methods 3 (12), 1021–1032.

Fitzgerald, D.J., Schaffitzel, C., Berger, P., Wellinger, R., Bieniossek, C., Richmond, T.J., Berger, I., 2007. Multiprotein expression strategy for structural biology of eukaryotic complexes. Structure 15 (3), 275–279.

Harper, D.M., Franco, E.L., Wheeler, C., Ferris, D.G., Jenkins, D., Schuind, A., Zahaf, T., Innis, B., Naud, P., De Carvalho, N.S., Roteli-Martins, C.M., Teixeira, J., Blatter, M.M., Korn, A.P., Quint, W., Dubin, G., 2004. Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial. Lancet 364 (9447), 1757–1765.

Harris, R., Polayes, D.A., 1997. Focus® 19, 6–8.

Hom, L.G., Volkman, L.E., 2000. *Autographa californica* M nucleopolyhedrovirus chiA is required for processing of V-CATH. Virology 277 (1), 178–183.

Ikonomou, L., Schneider, Y.J., Agathos, S.N., 2003. Insect cell culture for industrial production of recombinant proteins. Appl. Microbiol. Biotechnol. 62 (1), 1–20.

Kaba, S.A., Salcedo, A.M., Wafula, P.O., Vlak, J.M., van Oers, M.M., 2004. Development of a chitinase and v-cathepsin negative bacmid for improved integrity of secreted recombinant proteins. J. Virol. Methods 122 (1), 113–118.

Kaufmann, A.M., Nieland, J.D., Jochmus, I., Baur, S., Friese, K., Gabelsberger, J., Gieseking, F., Gissmann, L., Glasschroder, B., Grubert, T., Hillemanns, P., Hopfl, R., Ikenberg, H., Schwarz, J., Karrasch, M., Knoll, A., Kuppers, V., Lechmann, M., Lelle, R.J., Meissner, H., Muller, R.T., Pawlita, M., Petry, K.U., Pilch, H., Walek, E., Schneider, A., 2007. Vaccination trial with HPV16 L1E7 chimeric virus-like particles in women suffering from high grade cervical intraepithelial neoplasia (CIN 2/3). Int. J. Cancer 121 (12), 2794–2800.

Kirnbauer, R., Taub, J., Greenstone, H., Roden, R., Durst, M., Gissmann, L., Lowy, D.R., Schiller, J.T., 1993. Efficient self-assembly of human papillomavirus type 16 L1 and L1–L2 into virus-like particles. J. Virol. 67 (12), 6929–6936.

Kohler, G., Milstein, C., 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256 (5517), 495–497.

Koutsky, L.A., Ault, K.A., Wheeler, C.M., Brown, D.R., Barr, E., Alvarez, F.B., Chiacchierini, L.M., Jansen, K.U., 2002. A controlled trial of a human papillomavirus type 16 vaccine. N. Engl. J. Med. 347 (21), 1645–1651.

Li, M., Beard, P., Estes, P.A., Lyon, M.K., Garcea, R.L., 1998. Intercapsomeric disulfide bonds in papillomavirus assembly and disassembly. J. Virol. 72 (3), 2160–2167.

Lowy, D.R., Schiller, J.T., 2006. Prophylactic human papillomavirus vaccines. J. Clin. Invest. 116 (5), 1167–1173.

Luckow, V.A., Lee, S.C., Barry, G.F., Olins, P.O., 1993. Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. J. Virol. 67 (8), 4566–4579.

Mao, C., Koutsky, L.A., Ault, K.A., Wheeler, C.M., Brown, D.R., Wiley, D.J., Alvarez, F.B., Bautista, O.M., Jansen, K.U., Barr, E., 2006. Efficacy of human papillomavirus-16 vaccine to prevent cervical intraepithelial neoplasia: a randomized controlled trial. Obstet. Gynecol. 107 (1), 18–27.

Matsuura, Y., Possee, R.D., Overton, H.A., Bishop, D.H., 1987. Baculovirus expression vectors: the requirements for high level expression of proteins, including glycoproteins. J. Gen. Virol. 68 (Pt 5), 1233–1250.

Muller, M., Gissmann, L., Cristiano, R.J., Sun, X.Y., Frazer, I.H., Jenson, A.B., Alonso, A., Zentgraf, H., Zhou, J., 1995. Papillomavirus capsid binding and uptake by cells from different tissues and species. J. Virol. 69 (2), 948–954.

Muller, M., Zhou, J., Reed, T.D., Rittmuller, C., Burger, A., Gabelsberger, J., Braspenning, J., Gissmann, L., 1997. Chimeric papillomavirus-like particles. Virology 234 (1), 93–111.

Munoz, N., Bosch, F.X., de Sanjose, S., Herrero, R., Castellsague, X., Shah, K.V., Snijders, P.J., Meijer, C.J., 2003. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N. Engl. J. Med. 348 (6), 518–527.

Neeper, M.P., Hofmann, K.J., Jansen, K.U., 1996. Expression of the major capsid protein of human papillomavirus type 11 in *Saccharomyces cerevisiae*. Gene 180 (1–2), 1–6.

Possee, R.D., 1986. Cell-surface expression of influenza virus haemagglutinin in insect cells using a baculovirus vector. Virus. Res. 5 (1), 43–59.

Roden, R.B., Hubbert, N.L., Kirnbauer, R., Christensen, N.D., Lowy, D.R., Schiller, J.T., 1996. Assessment of the serological relatedness of genital human papillomaviruses by hemagglutination inhibition. J. Virol. 70 (5), 3298–3301.

Roelvink, P.W., van Meer, M.M., de Kort, C.A., Possee, R.D., Hammock, B.D., Vlak, J.M., 1992. Dissimilar expression of *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus polyhedrin and p10 genes. J. Gen. Virol. 73 (Pt 6), 1481–1489.

Sapp, M., Fligge, C., Petzak, I., Harris, J.R., Streeck, R.E., 1998. Papillomavirus assembly requires trimerization of the major capsid protein by disulfides between two highly conserved cysteines. J. Virol. 72 (7), 6186–6189.

Sehr, P., Zumbach, K., Pawlita, M., 2001. A generic capture ELISA for recombinant proteins fused to glutathione S-transferase: validation for HPV serology. J. Immunol. Methods 253 (1–2), 153–162.

Slack, J.M., Kuzio, J., Faulkner, P., 1995. Characterization of v-cath, a cathepsin L-like proteinase expressed by the baculovirus *Autographa californica* multiple nuclear polyhedrosis virus. J. Gen. Virol. 76 (Pt 5), 1091–1098.

Suzich, J.A., Ghim, S.J., Palmer-Hill, F.J., White, W.I., Tamura, J.K., Bell, J.A., Newsome, J.A., Jenson, A.B., Schlegel, R., 1995. Systemic immunization with papillomavirus L1 protein completely prevents the development of viral mucosal papillomas. Proc. Natl. Acad. Sci. U.S.A. 92 (25), 11553–11557.

Suzuki, T., Kanaya, T., Okazaki, H., Ogawa, K., Usami, A., Watanabe, H., Kadono-Okuda, K., Yamakawa, M., Sato, H., Mori, H., Takahashi, S., Oda, K., 1997. Efficient protein production using a *Bombyx mori* nuclear polyhedrosis virus lacking the cysteine proteinase gene. J. Gen. Virol. 78 (Pt 12), 3073–3080.

Touze, A., El Mehdaoui, S., Sizaret, P.Y., Mougin, C., Munoz, N., Coursaget, P., 1998. The L1 major capsid protein of human papillomavirus type 16 variants affects yield of virus-like particles produced in an insect cell expression system. J. Clin. Microbiol. 36 (7), 2046–2051.

Villa, L.L., Costa, R.L., Petta, C.A., Andrade, R.P., Ault, K.A., Giuliano, A.R., Wheeler, C.M., Koutsky, L.A., Malm, C., Lehtinen, M., Skjeldestad, F.E., Olsson, S.E., Steinwall, M., Brown, D.R., Kurman, R.J., Ronnett, B.M., Stoler, M.H., Ferenczy, A., Harper, D.M., Tamms, G.M., Yu, J., Lupinacci, L., Railkar, R., Taddeo, F.J., Jansen, K.U., Esser, M.T., Sings, H.L., Saah, A.J., Barr, E., 2005. Prophylactic quadrivalent human papillomavirus (types 6, 11, 16, and 18) L1 virus-like particle vaccine in young women: a randomised double-blind placebo-controlled multicentre phase II efficacy trial. Lancet. Oncol. 6 (5), 271–278.

Volpers, C., Schirmacher, P., Streeck, R.E., Sapp, M., 1994. Assembly of the major and the minor capsid protein of human papillomavirus type 33 into virus-like particles and tubular structures in insect cells. Virology 200 (2), 504–512.

Zlotnick, A., Stray, S.J., 2003. How does your virus grow? Understanding and interfering with virus assembly. Trends. Biotechnol. 21 (12), 536–542.

zur Hausen, H., 1999. Papillomaviruses in human cancers. Proc. Assoc. Am. Physicians 111 (6), 581–587.