Serological cross-reactions between four polyomaviruses of birds using virus-like particles expressed in yeast

Anja Zielonka,1,2 Alma Gedvilaite,3 Jochen Reetz,4 Uwe Rösler,2,5 Hermann Müller1 and Reimar Johne4

Correspondence
Anja Zielonka
zielonka@vetmed.uni-leipzig.de

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Polyomaviruses are aetiological agents of fatal acute diseases in various bird species. Genomic analysis revealed that avian polyomavirus (APyV), crow polyomavirus (CPyV), finch polyomavirus (FPyV) and goose hemorrhagic polyomavirus (GHPyV) are closely related to each other, but nevertheless form separate viral species; however, their serological relationship was previously unknown. As only APyV can be grown efficiently in tissue culture, virus-like particles (VLPs) were generated by expression of the genomic regions encoding the major structural protein VP1 of these viruses in yeast; these were used to elicit type-specific antibodies in rabbits and as antigens in serological reactions. For increased VLP assembly, a nuclear-localization signal was introduced into APyV-VP1. VLPs derived from the VP1 of the monkey polyomavirus simian virus 40 served as control. APyV-, GHPyV- and CPyV-VLPs showed haemagglutinating activity with chicken and human erythrocytes. CPyV- and GHPyV-specific sera showed slight cross-reactions in immunoblotting, haemagglutination-inhibition assay and indirect ELISA. The FPyV-specific serum inhibited the haemagglutination activity of APyV-VLPs slightly and showed a weak cross-neutralizing activity against APyV in cell-culture tests. Generally, these data indicate that the four polyomaviruses of birds are serologically distinct. However, in accordance with genetic data, a relationship between CPyV and GHPyV as well as between APyV and FPyV is evident, and grouping into two different serogroups may be suggested. The haemagglutinating activity of APyV, CPyV and GHPyV may indicate similar receptor-binding mechanisms for these viruses. Our data could be useful for the development of vaccines against the polyomavirus-induced diseases in birds and for interpretation of diagnostic test results.

INTRODUCTION

Polyomaviruses of birds are the causative agents of chronic and acute multisystemic diseases with high mortality rates in young birds (Johne & Müller, 2007). In contrast, mammalian polyomaviruses mainly cause innocuous subclinical infections in their natural immunocompetent hosts. Until now, the four bird polyomaviruses avian polyomavirus (APyV), goose hemorrhagic polyomavirus (GHPyV), crow polyomavirus (CPyV) and finch polyomavirus (FPyV) infecting various bird species have been characterized in more detail (Müller & Nitschke, 1986; Guérin et al., 2000; Johne et al., 2006). APyV is the causative agent of budgerigar fledgling disease, GHPyV causes haemorrhagic nephritis and enteritis of geese, and FPyV infection has been connected with skin lesions and increased mortality of bullfinches (Miksch et al., 2002; Johne & Müller, 2007; Wittig et al., 2007). Recently, an additional polyomavirus has been identified in diseased canary birds (Halami et al., 2010).

The genome of the avian polyomaviruses is a circular dsDNA encoding the capsid proteins VP1, VP2 and VP3, and two non-structural proteins designated the small and large tumour antigens. In addition, the APyV genome encodes the structural protein VP4, which interacts with genomic DNA and also has a function in the induction of
apoptosis (Johne et al., 2000). The other bird polyomaviruses have homologous proteins encoded by a genomic region designated ORF-X, which have not been further characterized (Johne & Müller, 2007). Phylogenetic and structural analyses indicate that bird polyomaviruses are also structurally distinct from mammalian polyomaviruses. Together with their biological properties, this led to a taxonomic proposal to group the bird polyomaviruses into a genus called Avipolyomavirus, separated from the two mammalian genera Orthopolyomavirus and Wukipolyomavirus within the family Polyomaviridae (Johne et al., 2011).

Among the bird polyomaviruses, APyV and FPyV as well as CPyV and GHPyV are genetically related to each other (Johne et al., 2006). Only APyV can be efficiently grown in tissue culture, and antigens of the other bird polyomaviruses cannot be produced by virus propagation. It has been shown for several polyomaviruses that recombinant expression of the major capsid protein VP1 in eukaryotic systems leads to the formation of virus-like particles (VLPs), which carry the major antigenic epitopes (Ulrich et al., 2009). VLPs are frequently generated in antigen production, particularly as they are known to be excellent antigens in diagnostic systems and to be safe and efficient vaccines for the prevention of various virus infections (Grbac & Anderson, 2006; Ludwig & Wagner, 2007; Roldao et al., 2010). In the case of bird polyomaviruses, the formation of VLPs has been achieved for GHPyV in insect and yeast cells and for APyV in yeast cells (Sasnauskas et al., 2002; Zielonka et al. 2006). The introduction of an additional nuclear-localization signal (NLS) into the N-terminal VP1-sequence of APyV has been shown to increase the efficiency of VLP assembly in chicken cells (Johne & Müller, 2004).

Serological cross-reactions between mammalian polyomaviruses have been studied in detail (Sasnauskas et al., 2002; Ulrich et al., 2009); however, no data on cross-reactions among bird polyomaviruses have been available so far. Here, suitable antigens for such studies were generated by recombinant expression of VP1 of four bird polyomaviruses in Saccharomyces cerevisiae, and the ability to form VLPs was investigated. The purified VLPs were used for the immunization of rabbits, and virus-specific sera were produced. Cross-reactions of these sera were assessed by immunoblotting, ELISA, haemagglutination-inhibition (HI) and neutralization tests. The obtained data should elucidate the serological relationship between the bird polyomaviruses and provide basic information for the generation of diagnostic tests and vaccines.

**RESULTS**

**Generation, characterization and purification of VLPs from recombinant yeast**

Expression plasmids were generated containing the VP1-encoding regions of APyV, CPyV or FPyV downstream of the galactose-inducible promoter and subsequently transformed into S. cerevisiae strain AH22-214. In the case of APyV, the amino acid sequence KRK (acting as an NLS) was introduced at the N-terminus as described previously (Johne & Müller, 2004) and the construct was designated APyV-VP1(KRK). Yeast transformed with pFX7 without the VP1 sequence and yeast transformed with plasmid pFX7-SV40-VP1, expressing VP1 of simian virus 40 (SV40) (Sasnauskas et al., 2002), were used as negative and positive controls, respectively. Yeast transformed with pFGG3-GHPyV-VP1 and expressing VP1 of GHPyV was available from an earlier study (Zielonka et al., 2006). After induction of protein expression for 24 h, yeast cells were harvested and analysed for the presence of the expected protein. By immunohistochemistry analysis of the recombinant yeast cells transformed with pFX7-APyV-VP1(KRK) using an APyV-VP1-specific mAb, cells stained red or red-orange, indicating VP1 expression (Fig. 1a).

Examination of ultrathin sections of such yeast preparations by electron microscopy revealed clusters of spherical structures in some of the cells (Fig. 1b, c).

Yeast lysates were subjected to CsCl density centrifugation. Analysis of the VP1-containing gradient fractions by SDS-PAGE and immunoblotting is shown in Fig. 2. In Coomassie brilliant blue-stained gels, a prominent band with an apparent molecular mass of approximately 40–50 kDa was found in all cases except the yeast negative control (Fig. 2a, lanes 1–5). In samples with CPyV- and GHPyV-VP1, additional faint bands, probably representing degradation products of VP1, were visible (Fig. 2a, lanes 4 and 5). These proteins could not be removed by further density-gradient centrifugations (not shown). Analysis of APyV particles, purified from infected tissue-culture cells, resulted in a prominent band of 45 kDa, corresponding to VP1 (Fig. 2a, lane 7). In the immunoblot, recombinant VP1 of APyV(KRK), SV40, FPyV, GHPyV and structural proteins of APyV were detected using the polyclonal serum #APV (Johne & Müller, 2004; Stoll et al., 1993) directed against APyV particles (Fig. 2b, lanes 1–3, 5, 7). There were no cross-reactions of this APyV-specific serum with recombinant CPyV-VP1 and the yeast proteins of the negative control (Fig. 2b, lanes 4, 6).

Mean yields in purified VP1 differed remarkably and, with 1.1 mg (g wet weight of yeast)⁻¹, were high in the case of SV40 but about tenfold lower in the cases of GHPyV- and CPyV-VP1 (0.1 mg g⁻¹). For APyV-VP1(KRK) and FPyV-VP1, yields of 0.3 and 0.4 mg g⁻¹ were achieved, respectively. Yield of VLPs with APyV-VP1(KRK) was approximately twofold higher than that reported for APyV-VP1 without an additional NLS (Sasnauskas et al., 2002).

**Characterization of VLPs**

VP1-containing fractions were negatively stained with uranyl acetate and subjected to electron microscopy. In all cases, VLPs with a shape typical for polyomavirus particles were detected (Fig. 3a–e). As observed previously with other yeast-expressed polyomavirus VLPs (Sasnauskas et al., 2002) and also with APyV particles (Shen et al., 2011), the VLPs were heterogeneous in size, with predominant diameters
ranging from 42 to 46 nm. In addition, a smaller fraction of VLPs with diameters of 20–25 nm was observed. The highest number of VLPs was detected in fractions corresponding to the buoyant densities of 1.30–1.31 g ml\(^{-1}\), suggesting the formation of empty VLPs. In corresponding fractions of the yeast negative control, only isolated spherical structures of approximately 40 nm with a very thin wall and without the typical capsomer-structured surface were found (Fig. 3f). Although the same amount of total protein was analysed, a markedly higher number of VLPs were found in the SV40-VP1 and APyV-VP1(KRK) preparations than in the preparations of FPyV-, GHPyV- and CPyV-VP1. In the cases of GHPyV- and CPyV-VP1, a high number of additional structures with a diameter of approximately 6 nm, which may represent capsomeres resulting from disassembly of VLPs, were found (Fig. 3d, e).

The VLPs were tested for their ability to agglutinate chicken erythrocytes. As shown in Fig. 4, a purified APyV preparation with known haemagglutinating (HA) activity (Godde, 1994) had an HA activity down to a concentration of 800 ng ml\(^{-1}\). HA activities were also shown for APyV-VP1(KRK)-, GHPyV- and CPyV-VLPs down to concentrations of 800 ng ml\(^{-1}\), 3.1 µg ml\(^{-1}\) and 100 ng ml\(^{-1}\), respectively. As described previously (Zielonka et al., 2006), the preparation of SV40-derived VLPs did not show any HA activity with chicken erythrocytes. Also, the FPyV-VP1-specific VLPs, and the preparation derived from the pFX7-transformed yeast lysate serving as a control, did not show HA activity with this type of red blood cell. A repetition of these experiments using human erythrocytes yielded similar results (data not shown).

Serological cross-reactions in immunoblotting and indirect ELISA

For the assessment of serological cross-reactions between the different bird polyomaviruses, polyclonal sera were elicited in rabbits by inoculation of the purified VLP preparations. Additional rabbits were inoculated with purified APyV particles or yeast protein, serving as positive and negative controls, respectively. Cross-reactions between the sera and VLPs were investigated by immunoblotting (Fig. 5) and indirect ELISA (Fig. 6). In both test systems, each of the VP1 proteins showed a strong reaction with its homologous serum. Generally, only weak cross-reactions were recorded by testing sera with a heterologous VP1, indicating high
specificity of the sera and diversity of the antigens. The APyV-specific sera detected both of the APyV antigen preparations [APyV and APyV-VP1(KRK)-VLPs] with similar efficiency (Figs 5a, g and 6a, f). In both test systems, cross-reactions between CPyV and GHPyV were evident (Figs 5d and 6e). In addition, a weak cross-reactivity of the anti-GHPyV-VP1 serum with all of the other VP1 preparations was seen by immunoblotting (Fig. 5e), whereas FPyV-VP1 only was detected weakly by all sera in indirect ELISA (Fig. 6c); however, comparable reverse cross-reactions of all sera with GHPyV-VP1 by immunoblotting and of FPyV-VP1 serum with all VP1 preparations in ELISA were not observed (Figs 5a–d, f, g and 6a, b, d–f). No unspecific reactions between any of the VP1-specific sera and proteins of yeast lysate and between control serum raised against the yeast protein preparation, or the corresponding pre-immune serum and VP1-antigens were found.

Assessment of HI and neutralizing activity of antibodies elicited with VLPs

Cross-reactions were further characterized by HI test and serum neutralization test (SNT). In the HI test, the rabbit sera anti-APyV-VP1(KRK), anti-CPyV-VP1, anti-GHPyV-VP1 and anti-APyV showed HI titres of 8192, 4096, 512 and 2048 with their homologous antigens (Fig. 7a–d).

![Fig. 3. Negatively stained electron micrographs of recombinant APyV-VP1(KRK) (a), SV40-VP1 (b), FPyV-VP1 (c), CPyV-VP1 (d) and GHPyV-VP1 (e) expressed in S. cerevisiae. Lysates prepared from pFX7-transformed yeast (f) and purified APyV particles (g) served as negative and positive controls of particle formation. Bars, 100 nm. Narrow arrows show VLPs with a diameter of approximately 45 nm, transparent arrowheads indicate smaller particles with diameters ranging from 20 to 25 nm, and black arrowheads indicate smaller capsomere-like structures with a diameter of approximately 6 nm.]

![Fig. 4. HA activity of VLPs. A twofold dilution series of purified VLPs expressed in S. cerevisiae consisting of APyV-VP1(KRK), SV40-VP1, FPyV-VP1, CPyV-VP1 and GHPyV-VP1 were tested in an HA assay with 1% chicken erythrocytes. Purified APyV particles and pFX7-transformed yeast lysate were used as positive and negative controls, respectively. The bar indicates the highest antigen dilution at which haemagglutination of chicken erythrocytes is observed (HA titre).](http://vir.sgmjournals.org)
Cross-reactions observed in immunoblot were confirmed by cross-HI activities between CPyV and anti-GHPyV-VP1 and between GHPyV and anti-CPyV-VP1, with HI titres of 1024 and 64 (Fig. 7b, c). A weaker cross-reactivity was observed between FPyV and APyV. The anti-FPyV-VP1 serum showed cross-HI activities with APyV-VP1(KRK)-VLPs and APyV, with HI titres of 64 (Fig. 7a, d).

The positive-control anti-APyV serum and the serum prepared with APyV-VP1(KRK)-VLPs and APyV, with HI titres of 64 (Fig. 7a, d).

The positive-control anti-APyV serum and the serum prepared with APyV-VP1(KRK)-VLPs showed high neutralization titres of $10^{2.96}$ and $10^{2.06}$, respectively, in SNT with APyV and chicken embryo cells (Fig. 8a). The serum prepared with FPyV-VLPs showed a cross-neutralizing activity with APyV with a ND50 titre of $10^{-3.67}$. With the other heterologous VP1-specific sera, the negative-control anti-pFX7 serum and the pre-immune sera, only low ND50 titres between $10^{-2.17}$ and $10^{-2.06}$, were recorded, which were regarded as unspecific reactions. In SNT with SV40 and Vero cells, only the positive-control anti-SV40 serum and the serum prepared with SV40-VP1-VLPs showed virus-neutralizing activities, with ND50 titres of $10^{-2.96}$ and $10^{-4.16}$, respectively, whereas the other sera showed only unspecific reactions, with low titres of $10^{-0.85}$ (Fig. 8b).

**DISCUSSION**

In contrast to the mammalian polyomaviruses, bird polyomaviruses have been investigated only scarcely and their serological relationships have so far remained unknown. However, as these viruses are the aetiological agents of acute and chronic diseases, studies on their serological characteristics are needed in order to develop reliable diagnostic tests and effective vaccines. Here we present a comprehensive study of serological reactions among some bird polyomaviruses, enabling an assessment of the serological relationships of the viruses that may provide a basis for the development of serological diagnostic tests and vaccines for these viruses.

Until now, the efficient propagation of bird polyomaviruses in tissue culture was only possible in the case of APyV (Johne & Müller, 2007). In order to provide antigens for serological investigations for the other identified bird polyomaviruses, recombinant expression of VP1-VLPs in yeast has been applied in our studies. The yeast expression system offers several advantages that might be relevant for the efficient formation of VLPs. One important aspect is that *S. cerevisiae* has been attributed to the GRAS (generally recognized as safe) micro-organisms and is considered to
be free of toxins hazardous to the health of humans or animals. Additionally, cultivation of yeast and recombinant protein expression are relatively cheap and have been widely used for VLP production, e.g. in the cases of hepatitis B virus and human papillomavirus (McAleer et al., 1984; Hofmann et al., 1995), as well as a large variety of other viruses. It has been demonstrated that the VP1 expression of polyomaviruses from humans, monkeys and rodents resulted in high VLP yields (Sasnauskas et al., 1999, 2002; Zielonka et al., 2011). In recent investigations, we were able to show that VP1 of APyV and GHPyV also assembled as VLPs; yields, however, were about tenfold lower than those for mammalian polyomaviruses (Sasnauskas et al., 2002; Zielonka et al., 2006).

In the present study, we were able to show that the yeast system applied was also suitable for the efficient production of VLPs in the case of other bird polyomaviruses. By electron microscopy, different particle sizes have been observed in CsCl gradient-purified VLP preparations of all virus types, in accordance with published data (Sasnauskas et al., 2002; Shen et al., 2011). Most interestingly, we were able to generate VLPs of GHPyV-VP1 with diameters of approximately 45 nm. This is in contrast to results of a former study, in which only smaller GHPyV-VLPs could be generated using different expression systems (Zielonka et al., 2006). Differences in the specific conditions applied in protein expression (e.g. medium, aerobic/anaerobic growth conditions) and VLP purification (use of CsCl or sucrose gradients, temperature, storage) may be responsible for that finding.

The reasons for different efficiencies in VLP formation among the various polyomaviruses under investigation are not known. Misfolding of the expressed proteins, lack of assembly into VLPs or disassembly of the VLPs during purification may be involved. As shown previously, APyV-VLPs were not assembled after protein expression in Sf9 insect cells, but were formed efficiently in yeast (Sasnauskas et al., 2002). It has been shown for APyV that an intranuclear localization of VP1 is crucial for the efficient assembly into virus particles in chicken cells (Johne & Müller, 2004). In our studies, we showed that the insertion of an NLS in the APyV-VP1 increased VLP assembly compared with a study using native APyV-VP1 (Sasnauskas et al., 2002). It may be speculated that optimization of the NLS could also improve VLP assembly in the cases of GHPyV and CPyV, which showed only low VLP yields in our study. For these viruses, electron micrographs showed the presence of a large number of capsomere-like structures,

Fig. 7. HI titres of the rabbit immune sera assessed with APyV-VP1(KRK)-VLPs (a), CPyV-VP1-VLPs (b), GHPyV-VP1-VLPs (c) and APyV particles (d). HI titres of all VLP-specific sera anti-APyV-VP1(KRK), anti-SV40-VP1, anti-FPyV-VP1, anti-CPyV-VP1, anti-GHPyV-VP1, or anti-pFX7 and anti-APyV as negative- and positive-control sera, were investigated with 8 U haemagglutinating antigen. The HI titre was determined from the highest serum dilution in which inhibition of HA activity of antigens with chicken erythrocytes was observed.
which may be a result of inefficient assembly or disassembly of VLPs.

The buoyant densities of all VLP preparations in CsCl gradients were similar to those of VLPs obtained without encapsidated DNA in a former study (Sasnauskas et al., 2002). Encapsidation of cellular DNA has been shown for polyomavirus VLPs after expression in insect cells (Pawlita et al., 1996). However, other studies revealed that recombinant protein expression in yeast cells did not incorporate host DNA into VLPs because of the differences in chromatin organization between yeast and animal cells (Puig et al., 1999). Preliminary results of attempts to isolate DNA from our SV40-, APyV- and FPyV-VLPs indicate the presence of empty VLPs (data not shown); however, more sensitive methods and the inclusion of other VLPs are necessary to generally exclude packaging of DNA. The availability of empty VLPs would be advantageous for their use as safe vaccines and prospective vectors for gene therapy.

Until now, the HA activity of bird polyomaviruses was only known for GHPyV and APyV (Godde, 1994; Zielonka et al., 2006). Here, we showed that CPyV, but not FPyV, is also capable of haemagglutinating chicken red blood cells. Some of the mammalian polyomaviruses, e.g. MPyV and JCpyV, also show HA activity, which is conferred by binding to sialic acid residues (Imperiale & Major, 2007); others, e.g. SV40, have no HA activity and binding of the virus particles is conferred by other molecules, e.g. MHC-I complexes. The binding of SV40 to sialic acid residues present on ganglioside GM1 has also been described, but the steric arrangement of the binding amino acids differs from that of haemagglutinating viruses such as MPyV (Neu et al., 2008; Ewers et al., 2010; Magaldi et al., 2012). Interactions with sialic acid-containing structures, such as gangliosides, may therefore also be a feature of the haemagglutinating bird polyomaviruses. However, as FPyV showed no HA activity with chicken and human erythrocytes, it may be concluded either that the virus interacts with sialylated glycans that are not present on these cells, or that another binding mechanism to a putative receptor exists for FPyV. This finding is somewhat surprising, because in both phylogenetic sequence analysis (Johne et al., 2006) and the results of the serological analysis presented here, FPyV is related closely to APyV, which readily agglutinates erythrocytes. Further studies analysing the binding partners and the capsid structure of FPyV will be necessary to clarify the reasons behind these differences.

Serological cross-reactions between the avian polyomaviruses were analysed using specific rabbit sera against the respective VLPs. Rabbit hyperimmune sera were selected for this investigation for reasons of practicability and comparability with previous studies, including early studies on primate polyomaviruses (Takemoto & Mullarkey, 1973; Shah et al., 1977), as well as recent studies with yeast-expressed VLPs of mammalian polyomaviruses (Sasnauskas et al., 2002). In general, most of the rabbit sera reacted highly specifically, and only slight cross-reactions with heterologous antigens were evident. These cross-reactions are limited to GHPyV and CPyV on one side, as evident from immunoblotting, ELISA and HI assay, and to APyV and FPyV in HI and neutralization assays on the other side. Our serological data confirm the phylogenetic relationship of these viruses, showing that CPyV is related most closely to GHPyV, whereas FPyV is related most closely to APyV; remarkably, all of these viruses represent clearly distinct species (Johne et al., 2006, 2011).

The distinct antibody response may vary between different animal species; therefore, further analyses should focus on the investigation of sera from naturally infected birds. However, as the natural hosts of CPyV and FPyV are crows and finches, respectively, prior development of serological tests capable of investigating these bird species is necessary. This may also include the development of competitive inhibition assays, which have been shown to be most suitable in determination of serological cross-reactivities between mammalian polyomaviruses (Viscidi & Clayman, 2006).
In summary, the results of our study presented here and of previous investigations may indicate that the four polyomaviruses of birds could be classified into two different serogroups. Interpretation of results obtained with serological tests for polyomavirus infection of birds, which are in use for APyV and GHPyV (Khan et al., 2000; Zielonka et al., 2006; Deb et al., 2010), should take these cross-reactions into consideration. The demonstration of cross-HI activities, as well as cross-neutralization activities of the rabbit sera directed against individual virus types, may indicate protective effects after immunization with VLPs. However, because the observed cross-reactions were only low, it must be concluded that homologous antigens should be used for the development of effective vaccines. Therefore, the use of an available APV vaccine (Ritchie et al., 1996) for the efficient immunization of other bird species in order to protect against infection with GHPyV or FPyV cannot be suggested. Further investigations should focus on increasing the yield and stability of the specific VLPs and their applicability in diagnostic tests and vaccines.

**METHODS**

**Cloning and generation of recombinant yeast.** Plasmids containing the genomes of APyV, CPyV and FPyV (GenBank accession numbers AF241168, DQ192570 and DQ192571, respectively) were used as templates in PCRs with primers containing NheI cleavage sites upstream of the initiation codon and downstream of the stop codon of the VP1-encoding region. A synthetic NLS was introduced into the APyV-VP1 sequence as described previously (Johne & Müller, 2004) in order to increase the efficiency of VLP formation. The amplification products were cloned into the XbaI site of the yeast expression vector pFX7 (Sasnauskas et al., 1999), placing it downstream of a galactose-inducible promoter. Sequences of the inserts were confirmed by DNA sequencing. The generation of the plasmid pFX7-SV40-VP1, used as positive control for VP1 expression and VLP formation, has been described previously (Sasnauskas et al., 2002; Zielonka et al., 2011). The expression plasmid pFGG3-GHPyV-VP1 (GenBank accession no. AY140894) was available from a former study (Zielonka et al., 2006). The plasmids were transformed into S. cerevisiae AH22 derivative 214 (a, leu2-3,112 his4-519). Yeast transformation, cultivation of yeast and the induction of recombinant protein expression were performed as described previously (Sasnauskas et al., 2002; Zielonka et al., 2006). Yeast transformed with vector pFX7 without any insert was used as a negative control.

**Immunohistochemical staining of VP1-expressing yeast cells.** Yeast cells were suspended in aqueous 30 % alcohol (1:1), agitated for 1 min, centrifuged at 1000 r.p.m. (rotor F-45-18-11; Eppendorf) for 4 min and the supernatant was removed. This procedure was repeated using 50, 70, 80, 96 and 100 % alcohol, as well as xylol. The resulting cell pellet was dried with filter paper and placed in a metallic chamber for fluid paraffin embedding according to standard procedures. Histological sections were prepared and deparaffinized, resulting cell pellet was dried with filter paper and placed in a metallic chamber for fluid paraffin embedding according to standard procedures. Histological sections were prepared and deparaffinized, and finally incubated for 5 min with a 3 % aqueous solution of H2O2 (Merck). Thereafter, the APV-specific mAb 3G10G5 (Fattaey et al., 1992), diluted 1:50 in PBS (Oxoid) with 1 % BSA, was added for 1 h. The secondary antibody goat anti-mouse IgG was used at a 1:17 dilution in PBS and incubated for 30 min. Then, ExtrAvidin-peroxidase (Sigma), diluted 1:17 in PBS, was added for 30 min. AEC (3-amino-9-ethylcarbazole; Sigma) was used as substrate chromogen. Slides were counterstained with haematoxylin (Merck), covered with Kaiser’s glycerol gelatine (Merck) and examined by light microscopy (BX50; Olympus).

**Purification of VLPs and APyV particles.** After mechanical disruption of yeast cells using glass beads and buffer DB 450 (450 mM NaCl, 1 mM CaCl2, 0.001 % Triton X-100, 0.25 M L-arginine in 10 mM Tris/HCl buffer, pH 7.2), the supernatant was collected and loaded onto a stepwise CsCl gradient with densities of 1.31 and 1.42 g ml⁻¹. Ultracentrifugation at 37 000 r.p.m. (rotor SW41; Beckman) was performed for 3 h. Thereafter, collected fractions were subjected to SDS-PAGE. Fractions showing a band with an apparent molecular mass between 40 and 50 kDa (corresponding to polyomavirus VPI) were pooled, diluted in buffer DB 150 (150 mM NaCl, 1 mM CaCl2, 0.001 % Triton X-100, 0.25 M L-arginine in 10 mM Tris/HCl buffer, pH 7.2) and centrifuged again overnight on a CsCl gradient as described above. For a better VLP purification in the cases of GHPyV- and CPyV-VP1, the centrifugation protocol was modified as follows: the first centrifugation of supernatant was at 25 000 r.p.m. (rotor SW28; Beckman) for 3 h at 4 °C; the second centrifugation was performed overnight at 25 000 r.p.m. (rotor SW28; Beckman) using a multiple-step CsCl gradient (1.24, 1.29, 1.31, 1.34, 1.38 and 1.42 g ml⁻¹); the further centrifugation steps were done as described above. The buoyant densities of the collected fractions were determined by using a refractometer. The preparation of purified APyV particles from infected chicken embryo cells, which were used as a comparative control, has been described previously (Johne & Müller, 2007).

**Electron microscopy.** For the investigation of ultrathin sections by transmission electron microscopy, the yeast cell suspension was centrifuged (8000 r.p.m., 5 min) and the supernatant was removed. The resulting cell pellet was suspended in an aqueous 2.5 % solution of glutaric dialdehyde (TAAB Laboratories) for 24 h and centrifuged at 8000 r.p.m. (rotor F-45-18-11; Eppendorf) for 5 min. The pellet was mixed with an aqueous 3 % solution of low-melting-point agarose (MP Biomedicals), centrifuged at 14 000 r.p.m. for 5 s and refrigerated immediately for 10 min. This agarose/yeast-cell pellet was cut into little cubes that were soaked in 1 % aqueous osmium tetroxide (Electron Microscopy Sciences) solution before embedding in Epon resin (Agar Scientific) according to standard procedures. Thereafter, ultrathin sections were cut, post-contrasted with aqueous 2 % aqueous uranyl acetate solution for 20 min and 2.7 % alkaline lead citrate (Serva) solution for 15 min. CsCl-gradient fractions containing recombinant VP1 were dialysed overnight against PBS and negatively stained using 2 % aqueous uranyl acetate. The negatively stained specimens and the ultrathin sections were examined with a JEM-1010 electron microscope at 80 kV acceleration voltages.

**Generation of specific antisera in rabbits.** The animal study was approved by the local ethics committee of the state Saxony (Regierungspräsidium Leipzig, Germany). After collection of pre-immune sera, the rabbits were immunized intradermally with 100 µg antigen dissolved in 500 µl PBS and emulsified with 500 µl Freund’s incomplete adjuvant. One rabbit was immunized with a preparation of pFX7-transformed yeast lysate and another rabbit was immunized with APyV serving as negative and positive control, respectively. The first and second booster immunizations were performed by intramuscular injections at 3 week intervals, each with 100 µg antigen in PBS without adjuvant. Sera were collected 3 weeks after the second booster immunizations.

**SDS-PAGE and immunoblotting.** Proteins were analysed by electrophoresis on SDS–12.5 % polyacrylamide gels followed by Coomassie brilliant blue staining or immunoblotting. The polyclonal serum 2APV elicited in rabbits with purified APyV particles and cross-reaction with VP1 of other polyomaviruses (Stoll et al., 1993; Sasnauskas et al., 2002) was used for VP1 detection. Immunoblotting
was performed as described by Stoll et al. (1993). The apparent molecular mass of VP1 was calculated by comparison of the band position with SDS-PAGE standards (Bio-Rad). The concentration of purified VP1 was assessed by SDS-PAGE by comparing the intensities of individual stained protein bands with those of serially diluted BSA bands with known protein concentration.

**ELISA.** Wells of a Nunc MaxiSorb ELISA plate were coated overnight at 4°C with 50 μl coating buffer (0.1 M sodium carbonate, pH 9.6) containing purified antigen preparations (0.3 μg ml⁻¹); other wells were coated with coating buffer without antigen. Thereafter, the wells were washed with PBS-Tween (0.05 %), and 200 μl blocking solution (10 % skim milk powder in PBS-Tween) was added and incubated for 1 h at 37°C. Thereafter, the wells were washed three times with PBS-Tween and 200 μl of individual serum dilutions were added to individual wells in duplicate. In order to eliminate yeast protein-specific antibodies in the rabbit immune sera prepared with proteins expressed in yeast, they were incubated with pFX7-transformed yeast lysate for 1 h at 37°C and thereafter centrifuged at 14 000 r.p.m. (rotor F-45-30-11; Eppendorf) for 10 min before being investigated.

After incubation for 1 h at 37°C, wells were again washed three times and 100 μl biotinylated anti-rabbit IgG, diluted 1:1000 in PBS-Tween, was added to each well. After 1 h at 37°C and three washes, each well was incubated for 1 h at 37°C with 100 μl streptavidin–HRP conjugate, diluted 1:2000 in PBS-Tween. A final washing was performed using PBS-Tween twice and PBS once. A 100 μl substrate solution containing o-phenylenediamine hydrochloride was added and colour development was stopped using 50 μl M H₂SO₄. A₄₉₀ was measured with a reference wavelength of 650 nm. The A₄₉₀ values from the wells without antigen were subtracted from each value with antigen. The values determined in three independent tests were averaged and standard deviations were calculated. For assessment of cross-reactivities, end-point titrations of each serum were performed using its homologous antigen, and a 64-fold higher concentration than the end-point dilution was used thereafter. The mean A₄₉₀ value detected by sera with its homologous antigen was set as 100 % and the percentages of relative staining intensities with heterologous sera were calculated accordingly.

**HI assay.** To test for HA activity, 25 μl volumes of the VLPs (25 μg ml⁻¹) were diluted in twofold steps with PBS in a U-shaped microtitre plate. Thereafter, 25 μl of a 1 % (v/v) chicken erythrocyte suspension were added to each well. After 1 h at 4°C, haemagglutination was recorded and the titre was defined as the reciprocal of the highest antigen dilution clearly showing haemagglutination. In addition, the haemagglutination of all VLPs was tested with the same procedure, but using human red blood cells (blood group O).

For the HI assay, sera were pre-treated by heating (56°C, 30 min). In order to remove heat-resistant inhibitors, 25 μl heat-inactivated serum was incubated with 150 μl kaolin-borate-NaCl buffer (acid-washed kaolin in 0.15 M NaCl and 0.05 M boric acid, pH 9) for 15 min at room temperature. Thereafter, the suspension was mixed with chicken erythrocyte suspension (50 %) in PBS for 5 min. The haemagglutinins and kaolin were removed by two centrifugation steps at 3000 r.p.m. (rotor F-45-30-11; Eppendorf) for 5 min. Volumes (25 μl) of the different sera were diluted in twofold steps with PBS in a microtitre plate (starting at 1:8), and 25 μl antigen with 8 HA units was added to each well. After 1 h at 37°C, 25 μl of 1 % erythrocytes in PBS was added and the plate was incubated again for 1 h at 4°C. The HI titre was defined as the reciprocal of the highest serum dilution that inhibited HA activity completely.

**SNT.** Volumes (25 μl) of heat-inactivated serum (56°C, 30 min, diluted 1:10) were diluted in twofold steps in a microtitre plate with serum-free Dulbecco medium (Biochrom) in duplicate. Thereafter, 25 μl Dulbecco medium with 100 TCID₅₀ APyV or SV40 were added to each serum dilution, mixed and incubated at 38°C for 1 h. As a positive control for virus neutralization, anti-APyV or anti-SV40 sera were used. For the determination of toxic reactions of sera, cells were incubated with serum (25 μl each, diluted 1:10) but without virus in control reactions. Thereafter, 50 μl primary chicken embryo fibroblasts or Vero cells (approx. 2 x 10⁶ cells ml⁻¹) in Dulbecco medium with 5 % FCS were added to each well. After 5–6 days incubation at 38°C and 5 % CO₂, cells were analysed for cytopathic effects. Viral antigens in infected cells were stained by indirect immune fluorescence with APyV-specific mAb 3G10G5 (Fattaey et al., 1992) or mAb anti-SV40-T-AG (MacArthur & Walter, 1984) and FITC-labelled anti-mouse IgG as secondary antibody. The neutralization titre (ND₅₀) was calculated from values of at least three independent tests according to the formula of Behrens and Kärber (Mayr et al., 1977).

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