Avian polyomavirus mutants with deletions in the VP4-encoding region show deficiencies in capsid assembly and virus release, and have reduced infectivity in chicken

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Avian polyomavirus (APV) is the causative agent of an acute fatal disease in psittacine and some non-psittacine birds. In contrast to mammalian polyomaviruses, the APV genome encodes the additional capsid protein VP4 and its variant VP4Δ, truncated by an internal deletion. Both proteins induce apoptosis. Mutation of their common initiation codon prevents virus replication. Here, the generation of replication competent deletion mutants expressing either VP4 or VP4Δ is reported. In contrast to infection with wild-type virus, chicken embryo cells showed no cytopathic changes after infection with the mutants, and induction of apoptosis as well as virus release from the infected cells were delayed. Electron microscopy revealed the presence of a high proportion of small particles and tubules in preparations of the VP4 deletion mutant, indicating a scaffolding function for VP4. Wild-type and mutant viruses elicited neutralizing antibodies against APV after intramuscular and intraperitoneal infection of chicken; however, VP4-specific antibodies were only detected after infection with wild-type virus. Using the oculonasal route of infection, seroconversion was only observed in chickens infected with the wild-type virus, indicating a strongly reduced infectivity of the mutants. Based on the biological properties of the deletion mutants, they could be considered as candidates for APV marker vaccines.

INTRODUCTION

Avian polyomavirus (APV) causes a multisystemic disease with high mortality rates in psittacine and some non-psittacine bird species (Lehn & Müller, 1986; Krautwald et al., 1989; Johne & Müller, 1998; Sandmeier et al., 1999; Literak et al., 2006). In contrast, the closely related mammalian polyomaviruses, e.g. Simian virus 40 (SV40) or Murine polyomavirus, do not cause disease in their natural immunocompetent hosts (Shah, 1996). Most of the mammalian polyomaviruses have been shown to induce tumours after inoculation into newborn laboratory rodents (Benjamin, 2001; Cole, 1996), whereas tumour induction has never been described for APV. Another significant difference between APV and SV40 has been shown in the apoptotic potential of these viruses: APV infection strongly induces apoptosis in chicken embryo (CE) cells, whereas SV40-infected Vero cells show no apoptotic phenotype (Johne et al., 2000). It has been speculated that the induction of apoptosis could play a role in APV pathogenicity, e.g. by allowing efficient virus release from the nucleus and rapid virus spread through the organism.

Polyomaviruses are non-enveloped, icosahedral particles containing a circular, double-stranded (ds) DNA genome which is transcribed bidirectionally for the expression of early and late genes (Cole, 1996). A comparison of the late mRNA coding capacity of mammalian polyomaviruses and APV revealed the presence of additional open reading frames (ORF) in the 5′-region of the polycistronic mRNAs of APV (Luo et al., 1995). The two predominant mRNA species encode two additional proteins with no amino acid homologies to any protein of mammalian polyomaviruses...
(Johne & Müller, 2001); mutation of their common initiation codon abolishes production of infectious progeny in CE cells (Johne et al., 2000).

Recently, the 176 aa gene product of the larger ORF has been identified as a structural protein in APV, designated VP4 (Johne & Müller, 2001). This protein, formerly called agnoprotein 1a (Luo et al., 1995; Johne et al., 2000), is regularly observed in polyomavirus particles in addition to the structural proteins VP1–3; however, it is not essential for the formation of virus particles (Johne & Müller, 2004). Interaction of VP4 with the major structural protein VP1 and dsDNA has been demonstrated (Johne & Müller, 2001). VP4A, a 112 aa protein previously designated agnoprotein 1b (Luo et al., 1995; Johne et al., 2000), is encoded by alternative splicing of the smaller ORF. The region deleted in VP4A (aa 69–132 in VP4) contains a leucine zipper-like motif, suggested to be involved in DNA binding of VP4 (Johne & Müller, 2001). After SDS-PAGE VP4 and VP4A show apparent molecular masses of 32 and 29 kDa, respectively; additional multiply phosphorylated subspecies have also been detected (Liu & Hobom, 2000; Liu et al., 2000). The expression of either protein induces apoptosis in insect cells as well as in CE cells (Johne et al., 2000). It has been speculated that, by the induction of apoptosis, VP4 and VP4A act as factors determining pathogenicity of APV infection.

To elucidate the functions of VP4 and VP4A in the virus replication cycle, deletion mutants were created and their biological properties were tested in CE cells and chicken. As it had been previously observed that deletion of both VP4 and VP4A abolished APV infectivity (Johne et al., 2000), viruses were constructed with deletion of either VP4 or VP4A, which were subsequently characterized in tissue culture experiments. Infection experiments were performed with chicken to evaluate the ability of the mutants to establish infection in vivo. The ability to elicit neutralizing antibodies against APV as well as VP4-specific antibodies was analysed in order to examine the possible use of the mutants as APV marker vaccines.

METHODS

Cells and viruses. Primary cultures of CE cells maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum were infected with the viruses as previously described (Müller & Nitschke, 1986). APV strain BFDV-5 was generated by transfection of its genome, which had been excised from plasmid pAPV-wt (Johne & Müller, 1998), into CE cells. This strain, with more than 99.9% similarity to the reference strain BFDV-1 (at the nucleotide level), has two Bsu36I sites instead of three in BFDV-1, which was useful for the following cloning procedures.

Generation of recombinant viruses. To generate a full-length plasmid with deletion of intron 2a (Luo et al., 1995), the 328 bp fragment of Bsu36I-digested plasmid p1b-His (Johne & Müller, 2001), which contains the cDNA of VP4. The resulting plasmid pAPV-VP4A− is expected not to express VP4A (2 in Fig. 1a–d). To generate a full-length plasmid with deletion of intron 2b (Luo et al., 1995), the 328 bp fragment of Bsu36I-digested pAPV-wt was removed and replaced by the 72 bp fragment of Bsa36I-digested plasmid p1b-His (Johne & Müller, 2001), which contains the cDNA of VP4A. The resulting plasmid pAPV-VP4− should not be able to express VP4 (3 in Fig. 1a–d).

Wild-type and mutated viruses were generated by transfection of the excised and recircularized genomes into CE cells as previously described (Johne & Müller, 1998). After 5 days, cells were frozen and thawed three times and the supernatants were used to infect fresh CE cells. After an additional 5 days, the cells were observed for cytopathic changes. Thereafter, cells were harvested and analysed by PCR for the presence of the viral genome and subjected to immunoblotting for the demonstration of viral structural proteins.

Detection of the viral genomes and structural proteins. Total DNA was isolated from infected cells from proteinase K treatment and subsequent phenol/chloroform extraction (Sambrook et al., 1989) and a PCR was performed with the primers 5’-CAACCAAGATG-TCTACTCCAGCG-3’ and 5’-GTGCAGATCTATAGCAGGCG-3’ amplifying the VP4/VP4A-encoding region. The expected PCR products, with a length of 687, 623 and 431 bp in the case of pAPV-wt, pAPV-VP4− and pAPV-VP4A−, respectively, were analysed by electrophoresis on ethidium bromide-stained 2% agarose gels.

The structural proteins VP1, VP2, VP3 and VP4 were detected in infected cells by immunoblotting with antibodies directed against APV particles as described (Stoll et al., 1993). The antiserum z1a was used for detection of VP4 (Johne & Müller, 2001).

Titration of viral infectivity. As no cytopathic changes were visible in cells infected with the deletion mutants, a limiting dilution method combined with immunofluorescence was applied for the determination of infectivity. Briefly, virus suspensions were diluted and inoculated on CE cells grown on 96-well plates. After 5 days, the presence of viral antigen was determined by indirect immunofluorescence using the monoclonal antibody 3G11G2 (Fattaey et al., 1992) directed against APV VP1 (kindly provided by R. A. Consigli, Kansas State University, Manhattan, KS, USA). Virus titres were calculated according to Kärber (1931) and the data of three independent experiments were averaged.

DNA fragmentation analysis and TUNEL assay. Fragmented DNA indicating apoptosis was demonstrated by electrophoresis of isolated DNA or in situ using a terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay (Gavrieli et al., 1992) as previously described (Johne et al., 2000). The TUNEL assay was performed using an in situ Cell Death Detection kit (Roche). The percentage of apoptotic cells was determined by counting 200 cells in each experiment and averaging the results of three tests.

Electron microscopy. CE cells grown on 15 (or 150 in the case of APV-VP4−) tissue culture dishes with a diameter of 140 mm (Greiner Bio One) were harvested at 5 days after infection, lysed by ultrasonication and cellular debris was removed by centrifugation at 4000 r.p.m. for 10 min (Megafuge 1.0R; Heraeus). The supernatant was subjected to density-gradient centrifugation as described recently (Johne & Müller, 2004). Briefly, the supernatant was underlaid with caesium chloride (CsCl) solutions with densities of 1.3 and 1.4 g cm−3, respectively, and subsequently centrifuged at 30 000 r.p.m. for 2 h in the rotor SW41 of the LE-80 Ultracentrifuge (Beckman). Opalescent virus bands were extracted and concentrated by centrifugation at 40 000 r.p.m. for 2 h. The pellet was resuspended in 100 μl PBS and adsorbed to a carbon-coated copper grid. After negative staining using 2% uranyl acetate, the specimens were subjected to electron microscopy.

Infection of chicken. Three groups, with a total of 49 1-day-old specific-pathogen-free (SPF) chickens each (breed White Leghorn,
Intervet International), were placed in different isolators and used for infection with the wild-type virus, the VP4-deletion mutant or the VP4\(_D\)-deletion mutant. Each of the groups was divided into four subgroups containing 13 birds, which were inoculated intramuscularly with 1 \( \times 10^4 \) TCID\(_{50} \); 13 birds, which were inoculated intraperitoneally with 1 \( \times 10^4 \) TCID\(_{50} \); 13 birds, which were inoculated via the oculonasal route with 5 \( \times 10^3 \) TCID\(_{50} \) (the lower dose is due to the low titre of APV-VP4\(_2\) and the maximal volume which could be administered by this route) and 10 birds which were not infected, serving as contact controls. A further group of 10 chickens was placed in a separate isolator and served as a non-infected control group. At days 0, 8, 15 and 22 after infection, two birds of each inoculated subgroup were euthanized and blood was taken for serological analysis. At 30 days after infection, serum samples were taken from all birds and at 56 days after infection, all of the remaining birds were sacrificed and blood was taken for serological analysis. The sacrificed birds were subjected to pathological examination and samples taken from the heart, kidney and liver were examined histologically, focusing on subendocardial haemorrhages and mononuclear infiltration.

Serological analysis. Serum samples derived from chickens were tested for the presence of APV-specific antibodies using indirect ELISA essentially as described (Khan et al., 2000). Briefly, APV particles purified by caesium chloride density centrifugation (Müller & Nitschke, 1986) or VP4–His, expressed in *Escherichia coli* and purified by Ni\(^{2+}\)–NTA affinity chromatography [designated agno 1a-His in the paper by Johne & Müller, (2001)], were used as antigens in ELISA. The sera were placed in duplicate wells at a fixed dilution of 1:500 in PBS along with a diluent control. Sera collected from a chicken at 0 and 28 days after subcutaneous inoculation of purified APV particles (Khan et al., 2000) were used as negative and positive controls, respectively. Detection of specific antibodies was carried out using biotinylated anti-chicken IgG (Dianova) and streptavidin horseradish peroxidase (Sigma). After addition of substrate, the absorbance was measured at 490 nm. The data were used for the determination of staining intensity of the samples relative to the positive control serum by the formula: relative staining intensity = 100 \( \times (\text{test serum–diluent})/(\text{positive control–diluent}) \).

The serum neutralization test (SNT) was performed using APV strain BFDV-5 and CE cells as described (Khan et al., 2000). Briefly, heat-inactivated sera, diluted in 10-fold steps, were mixed with 100 TCID\(_{50}\) BFDV-5 and incubated at 38°C for 30 min. Thereafter, the mixture was inoculated onto cells. SNT titres were determined as the highest serum dilution which totally inhibited the development of a cytopathic effect after incubation of cells at 38°C for 5 days.

**RESULTS**

**Generation of viruses with deletion of VP4 or VP4Δ**

A full-length plasmid with a deleted intron 2a sequence resulting in deletion of VP4Δ was generated and designated pAPV-VP4Δ\(_2\). A second full-length plasmid with a deleted...
intron 2b sequence, which should not be able to express VP4, was designated pAPV-VP4\(^{-}\) (Fig. 1a–d). The viral genome sequences of these plasmids or of plasmid pAPV-wt were excised, recircularized and used for transfection of CE cells. The viruses generated (designated APV-VP4\(\Delta\), APV-VP4\(^{-}\) and APV-wt) were passaged after 5 days. Cytopathic changes, characterized by rounding and enlargement of the CE cells, were only observed in the case of APV-wt (not shown). Infected cells were analysed by PCR for the presence and identification of the viral genomes. In all cases, PCR products with the expected lengths were demonstrated by gel electrophoresis (Fig. 1b). Immunoblotting of the infected cells using an antisera directed against APV particles detected bands corresponding to VP1, VP2 and VP3 in all cases (Fig. 1c). An additional band, in a position corresponding to VP4, however, was only detected in the case of APV-wt and APV-VP4\(\Delta\). Using specific antibodies, these bands were identified as VP4 (Fig. 1d). VP4\(\Delta\), however, was observed in none of the transfected or infected CE cell cultures, even in the case of APV-wt. This is in accordance with previously published observations (Johne et al., 2000; Johne & Müller, 2001).

**Time-course analysis of virus replication in CE cells**

Infectivity in the supernatants of the CE cells infected with the recombinant viruses was titrated and diluted to infect the cells at an m.o.i. of 0.1. At 24 h intervals from day 1 to day 5 after infection, the culture supernatants were harvested for infectivity titrations and replaced by equal volumes of fresh medium. Thereafter, the cells were subjected to three cycles of freezing and thawing and the supernatants were used for titrations of cell-associated infectivity. It is evident from the titres reached at individual time points (Fig. 2a) that virus release into the supernatant is delayed in the case of APV-VP4\(\Delta\) and particularly in the case of APV-VP4\(^{-}\). Compared to APV-wt, the final titres are approximately 10-fold lower in the case of APV-VP4\(\Delta\)- and 100-fold lower in the case of APV-VP4\(^{-}\). No remarkable differences between APV-VP4\(\Delta\) and APV-wt were obvious when the cell-associated virus released by repeated freezing and thawing was titrated; however, the titres reached by APV-VP4\(^{-}\) were approximately 100-fold lower than those reached by APV-wt.

To analyse the course of viral protein expression, equal volumes of the cell remnants obtained after freezing and thawing were analysed by immunoblotting using antibodies directed against APV particles (Fig. 2b). In all cases, viral structural proteins were first detected at 4 days after infection and the protein amounts increased until day 5 after infection. Again, no protein band corresponding to VP4 was observed in cells infected with APV-VP4\(^{-}\); remarkably, however, the amounts of the other structural proteins did not correspond to the low infectivity titres.

**Induction of apoptosis by the viruses**

The apoptotic potential of the recombinant viruses was analysed by TUNEL assay and agarose gel electrophoresis of cellular DNA at different time points after infection. In the TUNEL assay, a high number (27.3 ± 6.8 %) of apoptotic cells were found 3 days after infection of CE cells with APV-wt, whereas infection with APV-VP4\(\Delta\) or APV-VP4\(^{-}\) resulted in 17.6 ± 4.0 or 9.3 ± 2.5 % apoptotic cells, respectively (Fig. 3a); in the mock-infected culture, 7.6 ± 2.1 % apoptotic cells were detected. Slight DNA fragmentation (Fig. 3b), indicative of apoptosis, was observed 2 days after APV-wt infection by agarose gel electrophoresis. In APV-VP4\(\Delta\)- or APV-VP4\(^{-}\)-infected cells, DNA fragmentation was observed on day 3 and DNA laddering was less intense, particularly on day 4 after infection.

**Morphology of viral particles**

CE cells infected with the viruses were subjected to CsCl density centrifugation. A virus band could be extracted in the case of APV-wt and APV-VP4\(\Delta\). As no virus band was visible in the case of APV-VP4\(^{-}\), a fraction corresponding to that of the other viruses was extracted and subsequently analysed. Using electron microscopy and negative staining,
icosahedral particles with a diameter of approximately 45 nm were demonstrated in all cases (Fig. 4a–c). However, a very low concentration was achieved in the case of APV-VP4Δ2. To enable further comparison of virion morphology, a second purification of APV-VP4Δ2 particles was performed using a 10-fold amount of infected CE cells. Electron microscopy of this preparation indicated a considerably high percentage (20–30 %) of tubular structures and smaller virus-like particles with diameters of 20–35 nm (Fig. 4d) compared to the preparations of pAPV-wt or pAPV-VP4Δ−, where only up to 10 % of these assemblies were detected.

Infection of chicken

To test the infectivity and immunogenicity of the viruses generated in birds, 1-day-old chickens were infected via the i.p., i.m. or o.n. route. During a period of 56 days, no clinical
signs were recorded either in non-infected chickens or in chickens infected with any one of the three viruses. In addition, no gross lesions were observed in the pathological inspection of euthanized chickens. Mild to moderate multifocal mononuclear infiltrations were observed in the liver and in the heart of several animals by histopathological examination; however, no correlation between these findings and virus infection could be drawn from the data (not shown).

Sera derived from infected chickens or control animals at different time points after infection were tested for the presence of APV-specific antibodies in an indirect ELISA (Fig. 5a–d). Using APV particles as antigen, seroconversion was observed in all cases after infection via the i.p. or i.m. route. In the case of i.p. infection, no significant differences in antibody development were obvious by comparison of the relative staining intensity at different time points after infection with the three different viruses (Fig. 5a). The development of APV-specific antibodies was slightly delayed after i.m. infection with APV-VP4\textsuperscript{D} compared to i.m. infection with the other viruses (Fig. 5b). Using the o.n. route, only the chickens inoculated with APV-wt developed APV-specific antibodies, some of these late after infection (Fig. 5c). All of the other chickens including all contact controls remained negative over the whole period of 56 days (Fig. 5d). Selected sera of all groups were also tested by SNT using the APV-strain BFDV-5 and CE cells. A high degree of correlation was found between the relative staining intensity in the APV-specific ELISA and the SNT titres of these sera (Table 1).

Using purified VP4–His expressed in \textit{E. coli} as antigen in an indirect ELISA, seroconversion was observed only in chickens that had been infected with APV-wt, but not in chickens infected with the other viruses or the contact controls (Fig. 6a–d). The antibody titres detected in the sera of APV-wt-infected animals varied remarkably between the individuals as indicated by large differences of the relative staining intensity at day 56 post-infection, ranging from 4.6 to 83.6 % within this group (see SD in Fig. 6a, b). Generally, VP4-specific antibodies were observed late after infection, beginning at about 30 days post-infection, and remained at a low concentration even 56 days after infection (Fig. 6a–c).

### Table 1. Comparison of ELISA and SNT results of selected sera using BFDV-5 particles as antigen

Sera of chicken inoculated with APV-wt, APV-VP4\textsuperscript{D} and APV-VP4\textsuperscript{2} by different routes were analysed at 30 or 56 days after inoculation. i.p., intraperitoneal; i.m., intramuscular; o.n., oculonasal.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Route of inoculation</th>
<th>Days after inoculation</th>
<th>Relative staining intensity (%) in ELISA</th>
<th>SNT titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>APV-wt</td>
<td>i.p.</td>
<td>30</td>
<td>73.1</td>
<td>1:32</td>
</tr>
<tr>
<td>APV-wt</td>
<td>i.m.</td>
<td>30</td>
<td>89.9</td>
<td>1:64</td>
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<tr>
<td>APV-wt</td>
<td>o.n.</td>
<td>56</td>
<td>72.3</td>
<td>1:16</td>
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<tr>
<td>APV-VP4\textsuperscript{D}</td>
<td>i.p.</td>
<td>30</td>
<td>80.8</td>
<td>1:64</td>
</tr>
<tr>
<td>APV-VP4\textsuperscript{D}</td>
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<td>76.9</td>
<td>1:32</td>
</tr>
<tr>
<td>APV-VP4\textsuperscript{D}</td>
<td>o.n.</td>
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<td>8.7</td>
<td>&lt;1:2</td>
</tr>
<tr>
<td>APV-VP4\textsuperscript{2}</td>
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<td>74.5</td>
<td>1:32</td>
</tr>
<tr>
<td>APV-VP4\textsuperscript{2}</td>
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<td>30</td>
<td>60.2</td>
<td>1:8</td>
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<td>APV-VP4\textsuperscript{2}</td>
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<td>5.4</td>
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<td>None</td>
<td>–</td>
<td>56</td>
<td>7.3</td>
<td>&lt;1:2</td>
</tr>
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**Fig. 5.** APV-specific antibody response following (a) intraperitoneal (i.p.), (b) intramuscular (i.m.), or (c) oculonasal (o.n.) infection of chicken with APV-wt, APV-VP4\textsuperscript{D} or APV-VP4\textsuperscript{2}. Contact controls are presented in (d). The relative staining intensity of the sera was determined by an indirect ELISA with purified APV particles as antigen. Days p.i., days post-infection.
Fig. 6. VP4-specific antibody response following (a) intraperitoneal (i.p.), (b) intramuscular (i.m.), or (c) ocular nasal (o.n.) of chicken with APV-wt, APV-VP4Δ" or APV-VP4-". Contact controls are presented in (d). The relative staining intensity of the sera were determined by an indirect ELISA with purified VP4–His, expressed in E. coli, as antigen. Days p.i., days post-infection.

DISCUSSION

In contrast to mammalian polyomaviruses, APV and the recently discovered other three polyomaviruses of birds (Guérin et al., 2000; Johne et al., 2006) have been recognized as pathogenic agents leading to acute multisystemic disease in several bird species. The VP4-encoding region, only present in APV and the other polyomaviruses of birds (Johne et al., 2006), has been suggested to be responsible for this pathogenicity as it had been shown that its gene product VP4 and VP4Δ are apoptotic inducers (Johne et al., 2000). To verify this hypothesis, APV mutants with deletion of either VP4 or VP4Δ were constructed to study the significance of these deletions in CE cell culture as well as in chicken. It had been shown previously that deletion of both VP4 and VP4Δ abolishes APV infectivity (Johne et al., 2000); as shown here, however, deletion mutants with one of these proteins retained are replication competent.

It has been speculated that the induction of apoptosis could result in an efficient release of virus from the nuclei of infected cells (Johne et al., 2000). According to this hypothesis, the considerably lower percentage of apoptotic cells observed in CE cells infected with APV-VP4Δ" or APV-VP4Δ" should correlate with the amount of virus retained within the infected cells. Indeed, particularly in the case of APV-VP4Δ", a relatively high percentage of virus was present in the cells which could be released by cell disruption. Therefore, in the case of this mutant, the reduced release of progeny from infected cells seems to be directly related to the lower apoptotic potential.

In the case of the mutant APV-VP4Δ", however, only a low percentage of infectious virus was released by repeated freezing and thawing. Since the structural proteins VP1, VP2 and VP3 were present in abundance in the infected cells as demonstrated by immunoblotting, the absence of VP4 should be directly responsible for the reduction of the virus titre. In contrast to VP4Δ, never observed in the virion, VP4 is incorporated into the viral capsid, interacts with VP1 and is able to bind to dsDNA (Johne & Müller, 2001). Therefore, it has to be taken into consideration that VP4 exerts a distinct function within the APV particle, e.g., during virus assembly, stabilization of the virus particle or packaging of viral DNA. The absence of this protein could, therefore, result in deficiencies during the assembly of virions. Indeed, as shown by electron microscopy, a high proportion of small virus-like particles and tubular structures was observed in the case of APV-VP4Δ". Both particles with a 20–35 nm diameter and tubular structures are regularly observed during replication of APV (Literak et al., 2006), as well as in other polyomaviruses (Nilsson et al., 2005; Pawlita et al., 1996), in addition to standard particles with a 45 nm diameter. However, the relative amount of these structures was remarkably increased in the case of APV-VP4Δ", which might indicate a scaffolding function for VP4 during virion assembly.

The phenotypes of the deletion mutants APV-VP4Δ" and APV-VP4Δ" observed in vitro do not necessarily reflect the biological properties of the mutants in infected birds. As infection experiments with SPF parrots are difficult to perform and ethical considerations limit the use of a large number of parrots in infection experiments, 1-day-old chickens were used in the experiments described to assess infectivity and immunogenicity of the mutants. This species was also chosen because of the availability of secondary antibodies for immunological analyses. As no clinical signs or histopathological changes were observed even after infection with pAPV-wt, it has to be concluded that APV is non-pathogenic for SPF chickens. These observations are in accordance with studies indicating that clinical and histopathological changes are only observed in chicken which had been chemically immunosuppressed by treatment with cyclophosphamide prior to APV infection (Fitzgerald et al., 1996, 1999). As immunosuppressed chickens could not be used to characterize the immune response to the mutants, further experiments determining the virulence and protective potential of the mutant viruses have to be carried out using psittacine birds.

Virus replication of the mutants as well as the wild-type virus in chicken was demonstrated by showing seroconversion to high APV-specific titres after i.m. or i.p. infection. Using the o.n. route of inoculation, however, only chickens infected with APV-wt developed APV-specific antibodies. Although the distinct reasons for this difference have not been investigated in detail until now, a reduced infectivity of...
the mutants or a reduced stability of their virion are supposed to be responsible.

As both mutants were able to elicit neutralizing antibodies against APV, which are esteemed to be the major factor for protection against APV infection (Ritchie et al., 1994), these viruses should be considered as vaccines against the disease caused by APV, on condition that they are non-pathogenic for parrots. The finding that antibodies against VP4 were only detected in the case of infection with APV-wt, but not in the case of infection with the mutants, may be useful for the development of a marker vaccine. However, as the titres of VP4-specific antibodies determined were generally low, increasing even late after infection and varying between individuals of a group, serological tests with higher sensitivity have to be developed for the detection of VP4-specific antibodies. Using these tests, VP4-specific antibodies may be used as a marker for differentiation between naturally infected birds and those vaccinated with the mutant virus strains.

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