Avian polyomavirus agnoprotein 1a is incorporated into the virus particle as a fourth structural protein, VP4

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Introduction

Avian polyomavirus (APV) is the causative agent of budgerigar fledgling disease (BFD), an acute fatal disease affecting young budgerigars, with mortality rates of up to 100% (Lehn & Müller, 1986; Krautwald et al., 1989). The virus is also known to cause acute or chronic disease in other psittacine and non-psittacine birds (Johne & Müller, 1998; Ritchie et al., 1991; Marshall, 1989). Genome analysis of strains isolated from different species of birds has revealed a high degree of sequence similarity (Stoll et al., 1993; Johne & Müller, 1998; Phalen et al., 1999), leading to the suggestion that the name APV should be adopted for all known isolates, instead of the misleading designation BFD virus (BFDV) (Johne & Müller, 1998).

Morphologically, APV is characterized by a non-enveloped particle, 45–50 nm in diameter, containing a 4981 bp circular double-stranded (ds) DNA genome (Dykstra & Bozeman, 1982; Müller & Nitschke, 1986; Lehn & Müller, 1986; Rott et al., 1988). The genome is transcribed bidirectionally for the expression of early and late genes (Luo et al., 1995). Despite these overall similarities, remarkable differences from mammalian polyomaviruses have been found in the genome sequences, especially in the non-coding regulatory region and in the regions encoding the large tumour (T) antigen and the agnoproteins of APV (Luo et al., 1994). The differences in the nucleotide sequences and the ability of APV to cause an acute fatal disease, unusual in mammalian polyomaviruses, have led to the suggestion that this virus should be placed in a distinct subgenus, ‘Avipolyomavirus’, within the genus Polyomavirus (Stoll et al., 1993).

The agnoproteins of the mammalian polyomaviruses simian virus 40 (SV40), BK virus (BKV) and JC virus are small proteins encoded by an ORF located close to the 5′-end of the late mRNA, which also encodes the structural protein VP1 (Jackson & Chalkley, 1981; Rinaldo et al., 1998). The functions of these agnoproteins in the virus life-cycle are still unknown. In SV40 infection, the agnoprotein is expressed several hours after the major structural protein, VP1 (Jackson & Chalkley, 1981; Jay et al., 1981), it accumulates in a perinuclear location (Jay et al., 1981; Nomura et al., 1983) and is not incorporated into the virus capsid (Jackson & Chalkley, 1981). SV40 mutants that do not express the agnoprotein are still viable in tissue culture.
(Shenk et al., 1976). Recently, it has been shown that the agnoprotein of BKV interacts with cellular proteins, but not with viral proteins (Rinaldo et al., 1998).

In APV, late mRNAs show a high degree of heterogeneity due to two starting points of late mRNA transcription and partial as well as alternative splicing events. Based on the localization in the genome and the splicing pattern, seven ORFs have been proposed to encode agnoproteins in a subset of 18 mRNAs (Luo et al., 1995; Liu et al., 2000; Johne et al., 2000). Recently, Liu & Hobom (1999) have concluded that only two of these agnoproteins, 2a and 2b, represent the ‘real’ counterparts of SV40 agnoprotein, whereas agnoproteins 1a and 1b share few similarities, if any, with the agnoproteins of SV40.

The ORF of agnoprotein 1a encodes a protein of 176 aa. The ORF for agnoprotein 1b is created by alternative splicing. The deletion of the codons for amino acids 69–132 of agnoprotein 1a results in a final protein of 112 aa. A multiple phosphorylation pattern has been shown recently for agnoproteins 1a and 1b, resulting in a complex series of electrophoretically separable subtypes. Phosphatase treatment phosphorylation pattern has been shown recently for agnoproteins 1a and 1b, resulting in a complex series of electrophoretically separable subtypes. Phosphatase treatment yields the two primary proteins, with apparent molecular

Methods

Cells, viruses and bacteria. Avian polyomavirus strain BFDV-1 was used for infection of primary cultures of CE cells, as described previously (Müller & Nitschke, 1986). Vero cells, cultivated at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal calf serum, were used for the propagation of SV40. Viruses were concentrated and purified from infected cell cultures 5 days post-infection (p.i.) by repeated centrifugation on CsCl density gradients as described previously (Müller & Nitschke, 1986). Sf9 cells were cultivated at 27 °C in TNM-FH insect medium (Sigma) supplemented with 10% foetal calf serum and used for the propagation of recombinant baculoviruses. E. coli XL-1 Blue MRF’ cells (Stratagene) were used for DNA cloning and expression of recombinant proteins.

Construction of plasmids. For the expression of agnoproteins 1a and 1b in E. coli and subsequent purification by affinity chromatography, total RNA of APV-infected CE cells was isolated 4 days p.i. by proteinase K treatment and phenol–chloroform extraction (Sambrook et al., 1989). RT–PCR was performed by using the Titan RT–PCR system (Boehringer Mannheim) with the isolated RNA as template and the primers 5’-CAACCAACATGCTTACTCCAGGG-3’ and 5’-GTGCAGATCTATA-CCGAGCGG-3’. The PCR products, of 545 bp (agnoprotein 1a) and 350 bp (ag noprotein 1b), were digested with AflIII and BglIII and the resulting fragments were ligated to NcoI/BglII-restricted vector pQE-60 (Qiagen). The resulting plasmids, p1aHis and p1bHis, contain the coding sequences for agnoproteins 1a and 1b with additional codons for a C-terminal tail with the sequence YRSHHHHH (Fig. 1).

A plasmid for the expression of a polypeptide consisting of amino acids 69–132 of agnoprotein 1a, which are absent in agnoprotein 1b, was constructed by amplification of the corresponding coding region with the Expand High Fidelity PCR system (Boehringer Mannheim), the APV full-length plasmid pAPVinf (Johne et al., 2000) as template and the primers 5’-GGGAGATCTCTGTGAAGGAGGGACA-3’ and 5’-CCITCCATTGGGACATCGCGCCGCCTC-3’. The PCR product, with a length of 211 bp, was digested with NcoI and BglII and ligated to NcoI/BglII-restricted vector pQE-60. The resulting plasmid, p1a69–132His, encodes the polypeptide with an additional N-terminal methionine and a C-terminal tail with the sequence RSHHHHHHH (Fig. 1).

The plasmid pAPVnc, containing nucleotides 1–202 of the non-coding regulatory region of the APV genome (numbering according to Rott et al., 1988), was constructed for the generation of a DNA probe to be used in mobility-shift DNA-binding assays. Plasmid pAPVinf was cleaved with NcoI and HindIII and a 202 bp fragment was isolated. After a fill-in reaction with Klenow enzyme (AGS), the fragment was cloned into the HindIII site of the vector pBluescript SK(+) (Stratagene).

Expression and purification of His-tailed proteins. Saturated cultures of E. coli transformed with the appropriate expression plasmid were diluted 1:100 in 1 litre fresh medium (LB broth supplemented with 100 µg ampicillin per ml). Cells were grown until the OD_{600} reached 0.8 and expression was induced with 1 mM IPTG. After 5 h, bacteria were harvested by centrifugation, resuspended in sonication buffer (10 mM Tris–HCl pH 7.8, 50 mM KH_{2}PO_{4}, 300 mM NaCl, 10 mM β-mercaptoethanol) and subsequently lysed by sonication on ice for six 30-s bursts. The lysate was centrifuged at 10000 g for 20 min and the supernatant was used for purification of the His-tailed proteins with Ni^{2+}–NTA affinity resin (Qiagen). All purification steps were performed at room temperature. The resin (4 ml) was added to a 20 ml column (Qiagen) and equilibrated with 20 ml sonication buffer. The cell lysates were loaded on the column, which was then washed with 20 ml washing buffer (40 mM Tris–HCl, pH 7.5, 20%

Fig. 1. Schematic representation of plasmids p1aHis, p1bHis and p1a69-132His. Regions encoding different parts of agnoprotein 1a are shown as shaded boxes (amino acid positions are indicated). The amino acid sequences of the tails are given in the one-letter code. In p1a69-132His, the N-terminal methionine (M) was introduced by cloning.

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glycerol, 100 mM KCl, 1 mM β-mercaptoethanol) supplemented with 20 mM imidazole. The proteins were eluted in fractions of 4 ml containing 50, 80, 120, 200, 300 and 500 mM imidazole in washing buffer. Aliquots of each fraction were analysed by SDS–PAGE and immunoblotting. Fractions with large amounts of recombinant protein were pooled and the concentration of total protein was measured by the Pierce BCA protein reagent assay. The purity of the recombinant proteins was determined by densitometric analysis of Coomassie brilliant blue-stained gels after SDS–PAGE by using Gel-Pro Analyzer software (Media Cybernetics).

Expression of proteins in insect cells. Generation of recombinant baculovirus expressing agnoprotein 1a has been described recently (Johne et al., 2000). The APV structural proteins VP1 and VP2 were expressed by using the baculovirus transfer vector pL546 (kindly provided by M. Pawlita, Heidelberg), with the C-terminal undecapeptide KPPTPPPEPET of the SV40 large T antigen as a tag.

The VP1-encoding region was amplified by PCR with pAPVinf as template and the primers 5′-GGCTACATGTCCAAAAGAAAA-GGAACG-3′ and 5′-TATGCTACATGTCGGCCAGGAGGGCAT-3′. The PCR product was digested with AflI and BglII and ligated to NcoI/BamHI-restricted vector pL546. The VP2-encoding region was amplified by PCR with the primers 5′-CTAAGCCATGGGAGCTAACATGTCCCAAAAAGGAAAA-GGAACG-3′ and 5′-GTTGTTGGATCCTCTGGACCCTATGAGATCTGCGGGGAGCTTTGGGGGG-3′. The PCR product was digested with NcoI and BamHI and ligated as above. The resulting plasmids were used to generate recombinant baculoviruses for the production of the tagged structural proteins VP1tag and VP2tag. Generation and amplification of recombinant baculoviruses were carried out by using the BaculoGOLD kit (Pharmingen) and SF9 cells as described previously (Pawlita et al., 1996).

Preparation of antisera. His-tailed proteins agno 1a–His, agno 1b–His and agno 1a–His, purified by affinity chromatography on Ni2+-NTA resin, were mixed with Freund’s incomplete adjuvant (Sigma) and used for the intracutaneous immunization of rabbits. Two subcutaneous booster immunizations were performed at 2-week intervals. Sera were collected 2 weeks after the last immunization.

Analysis of proteins. Proteins were analysed by SDS–PAGE and immunoblotting as described previously (Stoll et al., 1993). The monoclonal antibody KT3 (kindly provided by M. Pawlita, Heidelberg), with the C-terminal undecapeptide KPPTPPPEPET of the SV40 large T antigen (MacArthur & Walter, 1984). The low molecular mass markers calibration kit (Pharmacia) and biotinylated low molecular mass markers (Bio-Rad) were used as standards.

Immunofluorescence. For the subcellular localization of proteins by immunofluorescence, CE cells grown on glass coverslips were fixed with 3% paraformaldehyde in PBS containing 0.1% Triton X-100, 2 mM EDTA, 50 mM β-mercaptoethanol, 20 mM sodium pyrophosphate, 2 mM Pefabloc protease inhibitor (Boehringer Mannheim). Cellular debris was removed by centrifugation and the supernatant was incubated with the specific antibodies for 1 h at 4 °C. After the addition of 20 µl of protein A–agarose (Sigma), the mixture was shaken gently at 4 °C for 1 h. Thereafter, the agarose beads were pelleted by centrifugation, washed five times with lysis buffer and subsequently analysed by immunoblotting.

Mobility-shift DNA-binding assay. The DNA probes were prepared by restriction endonuclease digestion of plasmid DNA and subsequently labelled with [α-32P]dCTP (Hartmann Analytic) by using the Klenow enzyme (AGS) as described previously (Buratowski & Chodos, 1996).

In the mobility-shift DNA-binding assay, the DNA probes (about 20000 c.p.m.) and purified proteins were incubated for 5 min at room temperature in binding buffer (40 mM Tris–HCl, pH 7.4, 10 mM HEPES–NaOH, 50 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, 0.8 mM DTT, 12% glycerol). Protein–DNA complexes were resolved on 6% polyacrylamide gels under non-denaturing conditions (40:1 acrylamide: bisacrylamide, 0.25 M Tris base, 1–9 M glycerol, 10 mM EDTA) by electrophoresis at 20 mA until bromophenol blue, added in a separate lane, approached the bottom of the gel. The gel was then dried and the DNA bands were localized by autoradiography.

In the competition mobility-shift DNA-binding assay, unlabelled competitor DNA was mixed with the DNA probe in binding buffer before the purified protein was added. For specific competition, unlabelled DNA probe was used. Unspecific competitors were poly(dA–dT) (Boehringer Mannheim), poly(dA) (Boehringer Mannheim) and 16S and 23S rRNA from E. coli (Boehringer Mannheim). The concentration of DNA was determined by measuring the A260 with a DU-64 spectrophotometer (Beckman).

Software. Sequence data were compared by using the Lasergene System software (DNASTAR). Calculation of the apparent molecular mass of a protein and determination of its relative abundance in a protein mixture were performed by densitometric analysis of Coomassie brilliant blue-stained gels using the Gel-Pro Analyzer software (Media Cybernetics).

Results

Expression and purification of agnoproteins in E. coli

The His-tailed proteins agno 1a–His, agno 1b–His and agno 1a–His were expressed in E. coli and purified under native conditions with Ni2+-NTA resin. SDS–PAGE revealed that all of the recombinant proteins were purified to near homogeneity by this procedure (Fig. 2). Agno 1a–His appeared as a double band with apparent molecular masses of 34 and 35 kDa. The yield of purified protein was 0.12 mg/ml with about 92% purity. Agno 1b–His appeared as a broad band with an apparent molecular mass of 32–34 kDa. The yield was 0.41 mg/ml and the purity about 95%. The apparent molecular mass of agno 1a–His could not be determined as this polypeptide migrated faster than the 14 kDa molecular mass marker. The yield of protein was 0.08 mg/ml, with a purity of about 90%.

Preparation of antisera

The purified recombinant proteins were used to elicit specific antibodies in rabbits. The specificities of the antisera α1a, α1b and α1a–His, directed against agno 1a–His, agno 1b–His and agno 1a–His, were tested by immunoblotting.
with the recombinant proteins as antigens. A rabbit antiserum directed against APV particles (Stoll et al., 1993) was designated αAPV and included as a control. All of these antisera detected agno 1a–His (Fig. 2b). Agno 1b–His showed the strongest reaction with antiserum α1b, but was also recognized by antisera αAPV and α1a. As expected, no reaction was observed between agno 1b–His and α1a. The polypeptide agno 1a69–132–His was recognized by all antisera with the exception of α1b (Fig. 2d). Based on these results, the highly specific antibodies required for further investigations were available.

**Detection and localization of agnoprotein 1a in APV-infected CE cells**

The antisera described above were used for the detection of the agnoproteins in APV-infected CE cells by immunoblotting. A double band with an apparent molecular mass of 32/33 kDa,
corresponding to agnoprotein 1α, was detected by all of the antisera; however, none of the antisera detected a protein band corresponding to agnoprotein 1β. As expected, the control antisera αAPV recognized additional bands representing the structural proteins VP1, VP2 and VP3 of APV (Fig. 3a; 3 days p.i.). No proteins were detected in mock-infected cells (not shown). The presence of the agnoproteins was also determined in a time-course experiment by immunoblot analysis of constant volumes taken from individual infected CE cell cultures at various times after infection. Increasing amounts of agnoprotein 1α were detected between 24 and 72 h p.i. with the antisera α1a (Fig. 3b). A similar pattern was observed when the antisera α1b and α1a69–132 were used (not shown). Again, agnoprotein 1β should never be detected.

The antibodies described above were also used to investigate the subcellular localization of the agnoproteins in APV-infected CE cells by immunofluorescence 3 days p.i. (Fig. 4). Using antisera α1a or α1a69–132, strong intranuclear fluorescence was observed. Intranuclear and moderate intracytoplasmic fluorescence was observed with the antisera α1b. A similar picture was obtained with zAPV. No fluorescence was observed in mock-infected cells.

In summary, agnoprotein 1α appears as a 32/33 kDa intranuclear protein in APV-infected CE cells.

Detection of agnoprotein 1α in purified virus particles

APV particles as well as SV40 particles purified by repeated CsCl gradient centrifugation were analysed by SDS–PAGE and Coomassie brilliant blue staining. The structural proteins VP1, VP2 and VP3 and bands with smaller apparent molecular masses, representing cellular histones, were detected in both virus preparations (Fig. 5a). Remarkably, however, an additional double band with an apparent molecular mass of 32/33 kDa was present in the preparation of APV particles. These bands represented about 13% of the total protein (Fig. 5b).

In immunoblots, this band was recognized in APV particles by all of the antisera directed against the agnoproteins (Fig. 5c). The antisera αAPV detected three additional bands, representing the APV structural proteins VP1, VP2 and VP3 (Fig. 5c, lane 1). As expected, no proteins were detected by the antisera α1a, α1b or α1a69–132 (Fig. 5d) in immunoblots of SV40 particles. However, the structural proteins VP1, VP2 and VP3 were readily detected by the antisera αAPV (Fig. 5d, lane 1), due to the cross-reaction described previously (Stoll et al., 1993).

Interactions between the viral structural proteins and agnoprotein 1α

Sf9 cells were infected with recombinant baculoviruses expressing agnoprotein 1α and the tagged proteins VP1tag and VP2tag and were analysed by immunoblotting 4 days p.i. (data not shown). Agnoprotein 1α, VP1tag and VP2tag were detected by the antisera αAPV. A minor band was present in VP1tag-expressing cells, presumably representing a degradation product of VP1tag. An additional band with an apparent molecular mass of 31 kDa, representing VP3, was found in cells infected with the VP2tag-expressing baculovirus. The monoclonal antibody KT3, which detects the tag peptide, detected VP1tag, VP2tag and the 31 kDa protein, but not agnoprotein 1α.

Sf9 cells were then co-infected with baculovirus expressing agnoprotein 1α and baculoviruses expressing either VP1tag or VP2tag, lysed after 4 days and subjected to immunoprecipitation followed by immunoblotting. Cells infected with baculoviruses expressing VP1tag, VP2tag or agnoprotein 1α only served as controls.

In a first series of experiments, the monoclonal antibody KT3 was used for immunoprecipitation and the antisera αAPV was used for immunoblotting. As shown in Fig. 6a, the tagged proteins were precipitated and the expected proteins, VP1tag and VP2tag, became visible by immunoblotting. In the
VP1tag-expressing cells, the putative degradation product mentioned above was again observed (lanes 1 and 4). As before, a protein band corresponding to VP3 was present in the VP2tag-expressing cells (lanes 2 and 5). An additional, faint band in the position of agnoprotein 1a was visible in cells that had been co-infected with baculoviruses expressing VP1tag and agnoprotein 1a (lane 1). In a second series of experiments, the antiserum α1a was used for immunoprecipitation and the monoclonal antibody KT3 for immunoblotting. It is evident from Fig. 6(b) that a band corresponding to VP1tag was only visible in cells that had been co-infected with baculoviruses expressing VP1tag and agnoprotein 1a (lane 1).

The results of these experiments suggest a specific interaction between agnoprotein 1a and VP1. Due to the type of experiments and the low intensities of the bands detected, a detailed stoichiometric analysis of the relative amounts of the two proteins in the presumed complex has not been performed.

**Agno 1a–His and the polypeptide agno 1a$^{69–132}$–His, but not agno 1b–His, bind dsDNA**

Binding of agnoproteins expressed in *E. coli* to DNA was tested in mobility-shift DNA-binding assays. As specific interactions with the regulatory region of the APV genome had been expected, the *XhoI/HindIII*-digested DNA fragment of plasmid pAPVnc containing 202 bp of the non-coding region was used as a probe in the first experiments. Aliquots of 5 µl containing 0·6 µg purified agno 1a–His, 2·0 µg purified agno 1b–His, 0·4 µg purified agno 1a$^{69–132}$–His or 0·02 µg of a negative control, purified from *E. coli* transformed with the vector pQE-60, were used in binding reactions with $^{32}$P-labelled DNA. The autoradiograph presented in Fig. 7(a) shows slowly migrating bands in lane 1 with agno 1a–His and in lane 3 with agno 1a$^{69–132}$–His, indicative of protein–DNA complexes. Only the free DNA probe was visible in the case of agno 1b–His (lane 2) or the negative control (lane 4).

In order to determine the amount of agno 1a–His necessary to produce a band shift, dilutions containing 25–300 ng of the purified protein were tested. Fig. 7(b) shows that bands migrating slower than the free probe appeared after the addition of more than 25 ng agno 1a–His (lane 2). In the range between 50 and 200 ng agno 1a–His, multiple bands were detected, as well as a smear of slowly migrating DNA. When 300 ng agno 1a–His was added, the probe remained close to the starting position (lane 8).

**Specificity of DNA-binding activity of agno 1a–His**

The specificity of DNA binding was analysed by using specific and unspecific competitors (Fig. 7c). The incubation of the labelled probe with 300 ng agno 1a–His in the presence of a 100-fold excess of the unlabelled DNA probe as a specific competitor resulted in an increase in free labelled probe (lanes 3 and 4), whereas a 1000-fold excess prevented the band shift totally (lane 5). Competition with a 1000-fold excess of poly(dA–dT) as an unspecific dsDNA competitor also resulted in an increase in free labelled probe (lane 6). Incubation with a 1000-fold excess of poly(dA) as a single-stranded DNA competitor did not alter the banding pattern (lane 7), whereas incubation with *E. coli* 16S and 23S rRNA as single-stranded RNA competitors increased the amount of free labelled probe slightly (lane 8). When 300 ng agno 1a–His was incubated with each of the $^{32}$P-labelled dsDNA probes, prepared from different plasmids as described in the legend of Fig. 7, a mobility shift was observed with all of these probes, indicating sequence-unspecific binding of this protein (Fig. 7d).
Specific antisera have been prepared that are directed against agnoproteins 1a and 1b and a polypeptide consisting of 64 aa not present in agnoprotein 1b. As expected, all of these antisera reacted with agnoprotein 1a in immunoblots with APV-infected CE cells as antigen (Fig. 3a). Remarkably, however, although the antibodies directed against agnoprotein 1b reacted strongly with the recombinant protein used for its induction (Fig. 2c), no reactivity was observed with a protein of the corresponding size in immunoblots of APV-infected CE cells (Fig. 3a). This may be because of inefficient translation of the abundant mRNA present in infected cells or rapid proteolytic degradation of agnoprotein 1b.

The intranuclear localization of agnoprotein 1a was determined by immunofluorescence studies with APV-infected CE cells. Strong intranuclear fluorescence was observed with the antisera α1a and α1a,1b. Antiserum α1b, however, showed intranuclear as well as moderate cytoplasmic
fluorescence. It may be speculated that α1b reflects the intranuclear location of agnoprotein 1a and the intracytoplasmic location of agnoprotein 1b. It must be taken into account, however, that the presence of agnoprotein 1b in APV-infected CE cells has not been confirmed by immunoblotting. A potential nuclear localization signal, not present in agnoprotein 1a, has been recognized at aa 70–77 of agnoprotein 1a (HRRRPYDR; Fig. 8). Another mechanism of nuclear transport could be the co-transport of agnoprotein 1a together with other viral proteins, in a manner similar to that described for APV VP1, which is co-transported into the nucleus together with VP2 (An et al., 1999). The intranuclear localization of APV agnoprotein 1a is in marked contrast to the perinuclear localization of the agnoproteins of SV40 and BKV (Nomura et al., 1983; Rinaldo et al., 1998).

Formation of polyomavirus capsids and encapsidation of the viral DNA take place within the nucleus of the infected cell. The intranuclear location of agnoprotein 1a raised the question of whether this protein is also present in the virus particle, particularly as unidentified protein bands in addition to the structural proteins VP1, VP2 and VP3 have been observed regularly (Müller & Nitschke, 1986; Krautwald et al., 1989) in Coomassie brilliant blue-stained gels after SDS–PAGE of highly purified APV particles (Fig. 5a, b). By immunoblotting with monospecific sera, these bands were identified as agnoprotein 1a (Fig. 5b). The large amount of agnoprotein 1a found in the virion argues against this observation being due to non-specific contamination of the preparation. This protein was detected regularly as a double band, which may represent different phosphorylated subspecies of agnoprotein 1a (Liu & Hobom, 2000). The incorporation of agnoprotein 1a into APV particles seems to be completely different from the situation in mammalian polyomaviruses; for example, it has been demonstrated that the agnoprotein of SV40 is not present in virus particles (Jackson & Chalkley, 1981).

The presence of an almost constant amount [13% of total structural proteins (Fig. 5b); 15% in Müller & Nitschke (1986)] of agnoprotein 1a in the APV particle suggests an interaction(s) of this protein with other components of the virion. By precipitating either VP1 or agnoprotein 1a followed by immunoblotting, co-immunoprecipitation of agnoprotein 1a and VP1 was observed, indicating a specific interaction between these viral proteins. It must taken into account, however, that the experiments were performed with tagged proteins, which does not represent natural conditions. The interaction of agnoprotein 1a with the viral DNA was investigated by using proteins expressed in E. coli. In band-shift assays, agno 1a–His and the peptide agno 1a*–His were shown to bind DNA, whereas no DNA-binding activity of agno 1b–His was observed. The presence of multiple bands (Fig. 7b) suggests binding of increasing numbers of agno 1a–His molecules to the DNA probe. It became evident from further experiments that binding was not dependent on the nucleotide sequence of the DNA probe, notwithstanding the...
fact that a low-affinity sequence specificity was observed, as
competition with a specific probe was more efficient than
competition with the unspecific DNA probes.

Agnoprotein 1a consists of 176 aa, of which 25 are proline residues (Fig. 8a). The molecular mass deduced from the nucleotide sequence is 18.6 kDa, whereas the molecular mass apparent from SDS–PAGE was 32/33 kDa. A detailed sequence analysis of amino acids 69–132 of agnoprotein 1a, deleted in agnoprotein 1b, reveals a potential leucine zipper motif (Landschulz et al., 1988) that might be responsible for DNA-binding activity. A region of basic amino acids at positions 70–77, necessary for the interaction with DNA, is followed by leucine residues at positions 84, 98, 105 and 112, which could enhance DNA binding through dimerization of the protein.

The suggested functions of the agnoproteins of mammalian polyomaviruses remain controversial. In the case of SV40, agnoprotein-mediated effects have been suggested on virus assembly (Carswell & Alwine, 1986; Margolkskee & Nathans, 1983), maturation (Hou-Jong et al., 1987) and regulation of transcription (Alwine, 1982; Hay & Aloni, 1985). For the agnoproteins of SV40 and BKV, a function in the release of mature virus is favoured (Resnick & Shenk, 1986; Rinaldo et al., 1986). The function of agnoprotein 1a of APV seems to be completely different. Recently, induction of apoptosis has been described as one function of agnoproteins 1a and 1b of APV (Johne et al., 2000). The presence of agnoprotein 1a in the virus capsid suggests a second function, as a fourth structural protein, in addition to VP1, VP2 and VP3 observed regularly in polyomaviruses.

Because of the presence of two initiation codons, the region encoding the agnoproteins of APV reveals two sets of agnoproteins. As mentioned above, no structural or functional homologies can be observed between agnoprotein 1a or 1b of APV and the agnoproteins of mammalian polyomaviruses. Agnoproteins 2a and 2b (Fig. 8b), however, show distinct similarities with regard to structure (basic and hydrophobic proteins), function (non-essential genes) and subcellular localization (Liu & Hobom, 1999). These observations and the presence of agnoprotein 1a within the APV particle justify the renaming of this protein as VP4, indicating its function as a fourth structural protein of APV, instead of the misleading designation agnoprotein 1a.

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