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DNA Encapsidation by Viruslike Particles Assembled in Insect Cells from the Major Capsid Protein VP1 of B-Lymphotropic Papovavirus

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Capsids of polyomaviruses—small, nonenveloped DNA viruses—consist of the major structural protein VP1 and the minor structural proteins VP2 and VP3. The contributions of the individual capsid proteins to functions of the viral particle, such as DNA encapsidation, cell receptor attachment, entry, and uncoating, are still not clear. Here we show that viruslike particles assembled in nuclei of insect cells from VP1 of the monkey B-lymphotropic papovavirus (LPV) are sufficient to unspecifically encapsidate DNA. LPV VP1 expressed in large amounts in insect cells by a baculovirus vector assembled spontaneously in the nuclei to form viruslike particles. After metrizamide equilibrium density gradient purification and nuclease digestion, a fraction of these particles was shown to contain VP1-associated linear, double-stranded DNA with a predominant size of 4.5 kb. The fraction of DNA-containing VP1 particles increased with time and dose of baculovirus vector infection. The DNA-containing particles, further purified by sucrose gradient centrifugation, appeared as "full" particles in negative-staining electron microscopy. As shown by DNA hybridization, the encapsidated DNA consisted of insect cell and baculoviral sequences with no apparent strong homology to LPV sequences. Three non-LPV VP1-derived host proteins with apparent molecular masses of approximately 14, 15, and 16 kDa copurified with the DNA-containing particles and may represent insect cell histones encapsidated together with the DNA. A similar species of host DNA was also found in purified LPV wild-type virions. These data suggest that LPV VP1 alone can be sufficient to encapsidate linear DNA in a sequence-independent manner.

Polyomaviruses are small, nonenveloped animal viruses with a circular covalently closed double-stranded DNA genome of about 5,000 bp. The viral capsid consists of the major structural protein VP1 (360 molecules per particle) and the two minor structural proteins VP2 and VP3 (together up to 72 molecules per particle). The icosahedral capsid, with a diameter of 45 to 50 nm, is built of 72 pentamers of VP1 (27). VP2 and VP3 have been tentatively localized in the center of the VP1 pentamer (1, 11, 17). The viral DNA encapsidated in the virions is associated with host histones H2A, H2B, H3, and H4 (10). Functional studies indicate a sequence-independent DNA-binding activity for the VP1 proteins of the two polyomaviruses simian virus 40 (SV40) (32) and mouse polyomavirus (4, 22). Contradictory data are available for the minor structural proteins VP2 and VP3. DNA-binding activity of SV40 VP2 and VP3 has been reported (6), but this activity has not been found for the corresponding mouse polyomavirus proteins (4).

VP1 of mouse polyomavirus, when expressed alone from a baculovirus vector in insect cells, assembles spontaneously into viruslike particles in the cell nucleus (8, 21). Such particles not only provide a model system for the study of polyomavirus capsid assembly but also have been used as viral vectors after experimental attachment of exogenous DNA in vitro (7).

The African green monkey B-lymphotropic papovavirus (LPV) is another, yet less-well-analyzed, polyomavirus (25, 38). In tissue culture of human cells, LPV displays a highly restricted host range. It can productively infect only a subset of human B-lymphoma-derived cell lines (e.g., BJA-B [3]). LPV binds with very high specificity and affinity to cell surface receptors on cultured human B-lymphoma cells (13, 14). The presence of LPV receptors has been identified as a major determinant restricting the viral host range (13). The tropism and narrow host range of LPV could provide a basis for the development of a tissue-specific viral vector. Here we show that in insect cells, LPV VP1 also assembles into viruslike particles and that a substantial fraction of the particles has encapsidated linear DNAs of host cell and baculoviral origins with a predominant size of approximately 4.5 kb.

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MATERIALS AND METHODS

Cells and viruses. The human B-lymphoma cell line BJA-B (19), subclone K88 (15), was propagated as a suspension culture in glass Erlenmeyer flasks at 37°C in RPMI 1640 medium with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml in a humidified 5% CO_2 atmosphere. *Spodoptera frugiperda* (Sf9) cells were cultivated in suspension in spinner culture flasks (Beckman, Palo Alto, Calif.) at 28°C in TNM-FH insect medium (Sigma, Munich, Germany) with the same supplements as described above.

To generate stocks of LPV (LPV strain P12 [3]), 2×10^7 BJA-B cells were electrotransfected with 20 μg of *Bam*HI-digested plasmid DNA of pLPV-P12 (complete LPV P12 genome cloned with *Bam*HI into pUC19). After cultivation for 7 days, when approximately 50% of the transfected cells were LPV VP immunofluorescence positive, the cells were washed and extracted as described previously (13).

Construction of recombinant LPV VP1 baculoviruses. To express the complete, 1,104-bp VP1 reading frame of LPV in insect cells, an *Nco*I restriction site was generated at the VP1 start codon (AG→CC, nucleotides 1650 and 1651 [25]) in the cloned wild-type LPV (LPV_{wt}) genome pLPVK38 by site-directed mutagenesis. The 1,714-bp *Nco*I-*Pst*I restriction fragment (nucleotides 1651 to 3365) encompassing the complete VP1 reading frame and part of the 3′ end of the early region was inserted into the multiple cloning site of the baculovirus expression vector pVL 1393 (Invitrogen, Leek, The Netherlands) between the *Sma*I and *Pst*I

sites to yield plasmid $pBac_{VP1}$. Recombinant baculoviruses expressing LPV VP1 were generated by cotransfection of $pBac_{VP1}$ with wild-type *Autographa californica* nuclear polyhedrosis virus DNA into Sf9 cells by methods described by Summers and Smith (33). Sf9 cells expressing LPV VP1 were identified by indirect immunofluorescence with a polyclonal rabbit anti-LPV particle serum or monoclonal anti-VP1 antibodies specific for conformational epitopes as described previously (13). High-titer stocks of recombinant baculoviruses (8×10^8 infectious and LPV VP1-expressing units per ml) were obtained from the supernatant of infected Sf9 cells in suspension cultures. The stocks produced initially still contained wild-type baculovirus, which was subsequently removed by limiting-dilution purification in 96-well plate cultures.

Purification of recombinant LPV VP1 ($VP1_{bac}$) viruslike particles and LPV_{wt} particles. For standard purification of $VP1_{bac}$ particles, an LPV VP1 baculovirus stock was added to a 500-ml Sf9 culture (7×10^5 cells per ml; multiplicity of infection [MOI] of approximately 100), and the cells were incubated for 3 days at 28°C in a spinner flask. The dependence of particle assembly and DNA encapsidation on the initial infectious dose was analyzed with MOIs of 1, 10, and 100 3 days postinfection (p.i.); time dependence was analyzed with an MOI of 10 at days 2, 4, and 5 p.i.

For LPV_{wt} purification, BJA-B K88 cells pretreated for 48 h with 150 ng of tunicamycin (Boehringer Mannheim, Mannheim, Germany) per ml to induce a maximal number of LPV receptors (15) were infected with LPV stock virus (MOI of approximately 1) and cultivated for 3 days. Baculovirus- or LPV-infected cells were sedimented ($400 \times g$, 20 min, 25°C), washed once in phosphate-buffered saline, and stored as pellets at −20°C until extraction.

Identical extraction and purification procedures were used for LPV_{wt} and $VP1_{bac}$ particles. Frozen cell pellets were thawed in 40 ml of extraction buffer (8) (10 mM Tris-HCl [pH 7.5], 50 mM NaCl, 0.01 mM $CaCl_2$, 0.01% Triton X-100, 10 μg of aprotinin [Boehringer Mannheim] per ml), and particles were extracted on ice by sonicating three times for 20 s at level 5 with a Branson Sonifier 250. Cell debris was pelleted by centrifugation (10 min, $6{,}000 \times g$, 4°C) and reextracted in 20 ml of extraction buffer. After centrifugation of the pooled extracts (20 min, $23{,}000 \times g$, 4°C), the supernatant was incubated at 37°C for 30 min with RNase and DNase I (both from bovine pancreas, 10 μg per ml each; Boehringer Mannheim). Particles from 10 ml of supernatant were subsequently sedimented (2.5 h, $180{,}000 \times g$, 20°C, Beckmann SW41Ti rotor) through 1.5 ml of 50% (wt/vol) sucrose in extraction buffer onto a 0.5 ml cushion of 80% (wt/vol) metrizamide (Gibco BRL, Eggenstein, Germany) (28) in extraction buffer. The metrizamide cushion and the sucrose-metrizamide interphase were collected (approximately 0.7 ml from each tube), pooled, adjusted to 43% (wt/vol) metrizamide, and subjected to equilibrium density gradient centrifugation (20 h, $150{,}000 \times g$, 10°C, Beckmann VTi65 rotor). Twenty-four fractions (approximately 250 μl each) were collected from each gradient.

Further purification was achieved by centrifugation in a continuous preformed 20 to 50% sucrose gradient in extraction buffer. Metrizamide gradient fractions were diluted 1:3 to reduce the density before loading on the gradient. After centrifugation in an SW60Ti rotor for 60 min at $188{,}000 \times g$ and 4°C, the gradient was fractionated in 140-μl aliquots. Analyses of purification products by silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), LPV VP1-specific Western blotting (immunoblotting), and enzyme-linked immunosorbent assay (ELISA) were performed as described in detail previously (13, 15). The amount of VP1 antigen present in the purified particle preparations was quantified by LPV VP1-specific ELISA (13, 14), which recognizes only native, nondenatured VP1, and total protein was quantified with the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany).

Electron microscopy. For electron microscopy of baculovirus-infected Sf9 cells, the washed cell pellet was fixed with 2.5% glutaraldehyde in 50 mM sodium cacodylate (pH 7.6) for 30 min at 4°C. Cells were collected by low-speed centrifugation ($200 \times g$, 10 min, 4°C), fixed with 2% osmium tetroxide, stained with 0.5% uranyl acetate, and processed for ultrathin sectioning. For negative staining of particles, carbon-coated copper grids were glow discharged, and 10-μl aliquots of metrizamide gradient fractions, diluted in water, were allowed to adsorb to the coat for 30 to 120 s. After one wash with 20 μl of water, excess liquid was removed and the specimens were stained for 30 s with 2% aqueous uranyl acetate and were finally air dried. Specimens were examined in a Zeiss EM10A electron microscope at 80 kV. The magnification indicator was routinely controlled by using a grating replica.

Extraction and analysis of particle DNA. To isolate nucleic acids from purified particle preparations, metrizamide gradient fractions were digested at 37°C for 30 min with RNase and DNase I (both from bovine pancreas, 10 μg per ml each; Boehringer Mannheim); this was followed by denaturation with 1% SDS and extraction with phenol-chloroform-isoamyl alcohol (25:24:1). In some experiments, LPV $VP1_{bac}$ particles before nucleic acid extraction were further separated from copurified contaminants by immunoprecipitation. Two milliliters of hybridoma supernatant containing about 10 μg of monoclonal mouse antibody 520-1 (13) directed against native LPV VP1 and 100 μl of a 50% (vol/vol) suspension of swollen protein A-Sepharose (Pharmacia, Freiburg, Germany) were incubated for 30 min at room temperature. After washing in 125 mM NaCl–10 mM Tris-HCl (pH 7.5)–0.05% (vol/vol) Nonidet P-40–4 mM EDTA–8 mM $CaCl_2$–10 μg of aprotinin per ml, the antibody-Sepharose complex was incubated with 10 μg of $VP1_{bac}$ particles in 500 μl of the same buffer for 60 min at room temperature. The supernatant and extensively washed pellet were digested with nucleases and extracted as described above.

To purify baculovirus DNA, nonoccluded virus present in the supernatant of infected Sf9 cells were pelleted and extracted as described by Summers and Smith (33). Cellular DNAs were isolated as described previously (23). Extracted nucleic acids were ethanol precipitated, enzyme digested, and analyzed by agarose gel electrophoresis and ethidium bromide staining by standard methods. DNA fragments were isolated from agarose gels through GenElute agarose spin columns (Sigma-Aldrich-Supelco, Munich, Germany). For Southern blotting, agarose gels were blotted onto GeneScreen Plus (DuPont, Boston, Mass.) nylon membranes by alkaline transfer and hybridized under stringent conditions (T_m − 18°C) or, for the detection of cross-hybridizing sequences, under low-stringency conditions (T_m − 50°C) with probes labeled with ^{32}P as described previously (23).

RESULTS

Assembly of LPV VP1 into viruslike particles in nuclei of insect cells. Recombinant baculoviruses expressing LPV VP1 were generated by cotransfection of a baculovirus vector plasmid containing the complete LPV VP1-coding sequence under control of the baculovirus polyhedrin promoter with wild-type *A. californica* nuclear polyhedrosis virus DNA into *S. frugiperda* (Sf9) cells. Sf9 cells expressing LPV VP1 ($VP1_{bac}$) were identified by indirect immunofluorescence. $VP1_{bac}$ in immunofluorescence was reactive with six monoclonal anti-VP1 antibodies that recognize conformational epitopes (13) indicating the presence of protein in the native conformation (data not shown). $VP1_{bac}$ was seen in the cytoplasm as well as in the nucleus.

Viruslike particles approximately 45 nm in diameter and comparable in shape to LPV_{wt} virions were found in abundance in the nuclei by electron microscopy of ultrathin sections (Fig. 1a). Such particles were present only in the nuclei of Sf9 cells infected with LPV VP1 recombinant baculovirus and not in the cytoplasm or in cells infected with wild-type baculovirus.

Purification of viruslike $VP1_{bac}$ particles. To isolate the viruslike particles, whole infected Sf9 cells were extracted twice by sonication in low-salt and low-detergent buffer (8). After digestion of the pooled extract with DNase and RNase, particles were purified by centrifugation through 50% sucrose onto a 80% metrizamide cushion. This was followed by equilibrium density ultracentrifugation in metrizamide as described previously for LPV_{wt} particles (13).

Viruslike particles in metrizamide gradient fractions were identified by negative-staining electron microscopy. In the material isolated at a density of around 1.29 g/cm^3, particles approximately 45 nm in diameter were found in abundance (Fig. 1b). Sixty-five percent of these particles were stain-excluded particles consistent with the appearance of LPV virions (hereafter termed full particles), and the other 35% appeared as empty capsids. Smaller particles with a similar morphology and a diameter of approximately 30 nm were also present; however, these were 15 times less abundant than the 45-nm-diameter particles. In addition, smaller structures presumably representing aberrantly assembled capsomere complexes were found. Also, individual rods 60 to 70 nm in diameter and with various lengths were seen at a frequency of about one rod per 20 of the 45-nm-diameter particles; we assume that these represent aberrant baculovirus capsids. In identically purified LPV_{wt} preparations at this density, only the 45-nm-diameter particles, and mostly those of the full type, were seen (data not shown). In the $VP1_{bac}$ fractions of lower densities down to a density of 1.25 g/cm^3, 45- and 30-nm-diameter particles also were found; however, both were present at a much lower extent (Fig. 1c).

To analyze the protein composition of fractions during the purification and from the metrizamide gradient, proteins were separated by SDS-PAGE and silver stained (Fig. 2A). In total proteins of recombinant baculovirus-infected cells, a promi-

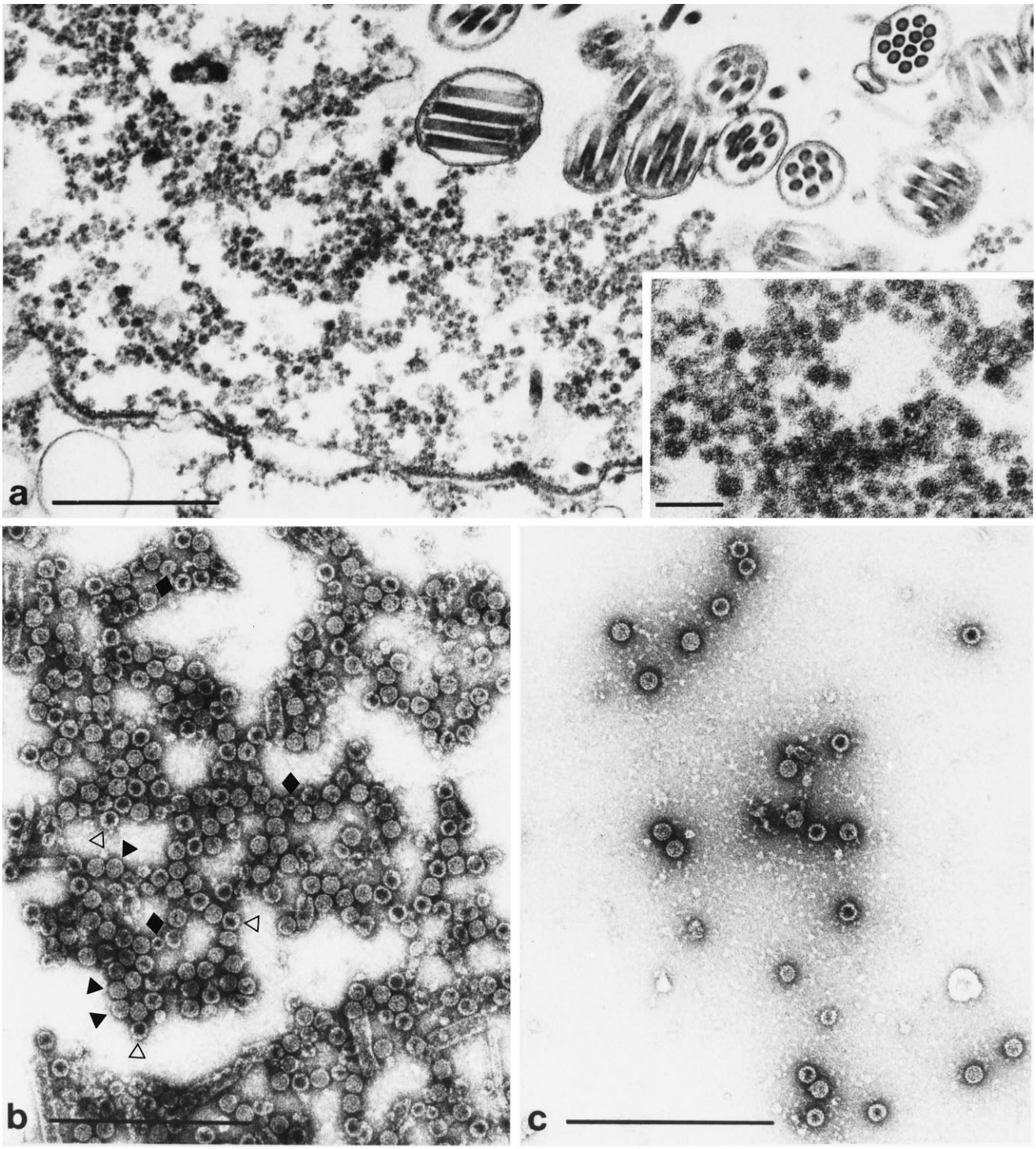

FIG. 1. Electron micrographs of LPV VP1 baculovirus-infected Sf9 cells and of metrizamide gradient-purified $VP1_{bac}$ viruslike particles. (a) Ultrathin section of a cell nucleus and some neighboring cytoplasm (at bottom) 72 h p.i., showing particles consistent with the appearance of virions of the polyomavirus group (middle and left, enlarged in inset) and baculovirus particles, i.e., 7 to 12 rodlike capsids surrounded by a common envelope, cut at different angles (top and right corners). Bar, 500 nm; inset bar, 100 nm. (b and c) Negative staining of material corresponding to fractions 13 (b) and 16 (c) of the metrizamide gradient (see Fig. 2), with equal volumes applied to the grids. Individual stain-excluded 45-nm-diameter particles consistent with the appearance of LPV virions (▲), empty 45-nm-diameter capsids (△), and 30-nm-diameter particles (◆) are indicated. Bar, 500 nm.

nent protein with an apparent molecular mass of approximately 40 kDa was present (Fig. 2A, lane C); this protein was absent in wild-type-baculovirus-infected cells (not shown). The size of the protein corresponds to the calculated VP1 size of 39,976 Da (25), and its reactivity with a polyclonal anti-LPV VP1 serum on Western blots allowed us to identify it as recombinant LPV VP1. In addition to the expected predominant 40-kDa band, minor smaller polypeptides of 31 to 35 kDa

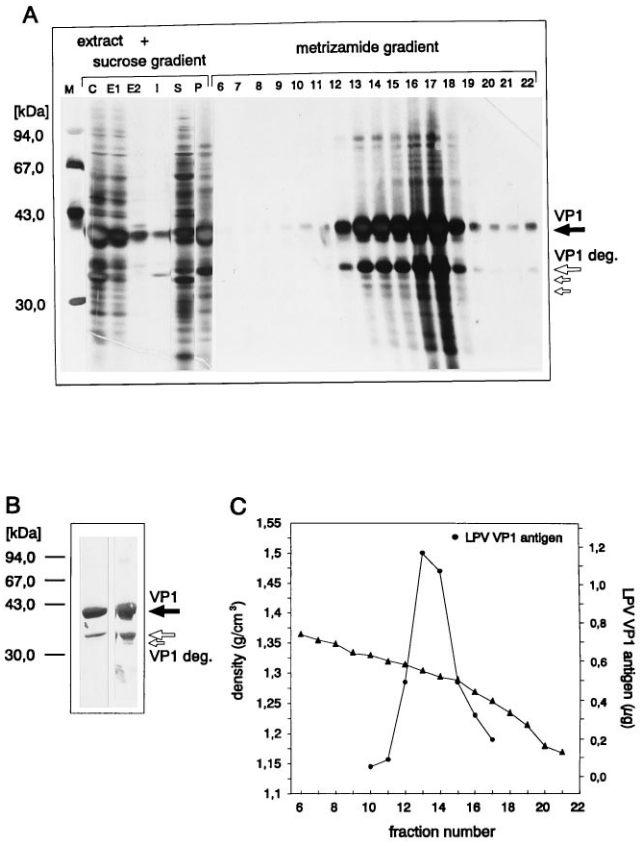

FIG. 2. Purification of $VP1_{bac}$ viruslike particles from recombinant baculovirus-infected Sf9 cells by sucrose step and metrizamide equilibrium density gradient centrifugation. (A) Equal amounts (1%) from various fractions of the particle purification procedure were separated by SDS-PAGE, and proteins were silver stained. The lanes contain size markers (M), whole-cell lysate (C), extracts 1 and 2 (E1 and E2), the insoluble fraction (I), the supernatant (S), and the pellet (P) from the sucrose step gradient and metrizamide equilibrium density gradient fractions 6 to 22. Arrows on the right indicate the positions of immunoreactive $VP1_{bac}$ and smaller $VP1_{bac}$ degradation products (VP1 deg.). (B) Western blot of extract E1 (left lane) and metrizamide gradient fraction 13 (right lane) (see panel A for comparison) stained with rabbit anti-LPV particle serum. (C) The density (▲) and content of native LPV VP1 antigen (●) (micrograms per fraction) as determined by conformation-dependent ELISA are shown for individual metrizamide gradient fractions.

which copurified with particles (see below) and which in Western blot analysis also reacted with polyclonal rabbit anti-LPV VP1 serum (Fig. 2B) were also found. The relative amount of these shorter VP1 species increased with the dose and the time of infection as well as with the length of time needed for the extraction and initial purification steps. These minor bands are therefore assumed to represent $VP1_{bac}$ degradation products. It cannot be excluded that the 35-kDa VP1 species is the product of an internally initiated form, which, in analogy to the domain organizations of the SV40 and polyomavirus VP1 proteins, would then be missing the DNA-binding domain located at the VP1 amino terminus.

$VP1_{bac}$ could be efficiently extracted (Fig. 2A, lanes E1 and E2), with only little protein remaining in an insoluble fraction (Fig. 2A, lane I). Most of the $VP1_{bac}$ protein could be sedimented through 50% sucrose onto a metrizamide cushion (Fig. 2A, lane P). In the metrizamide equilibrium density gradient, $VP1_{bac}$ was found between fraction 12 (density, 1.31 g/cm^3) (Fig. 2A, lane 12) and fraction 18 (density, 1.22 g/cm^3) (Fig. 2A, lane 18). The distribution of $VP1_{bac}$ appeared to be biphasic. A broad peak was present at approximately 1.25 g/cm^3, with a prominent shoulder in the fractions around 1.29 g/cm^3, which in some experiments also appeared as a clear peak (not shown) and which by electron microscopy had shown the viruslike particles. LPV_{wt} as well as mouse polyomavirus particles purified by the same protocol banded between 1.27 and 1.30 g/cm^3 but did not show VP1 around 1.25 g/cm^3 (data not shown).

In the LPV VP1 ELISA, which employs monoclonal antibody 456-1 with specificity for a conformational VP1 epitope as the catching antibody (13), $VP1_{bac}$ of the dense fractions reacted much more strongly than $VP1_{bac}$ in the less dense fractions (Fig. 2C). This suggested that $VP1_{bac}$ present in the dense fractions is in the native conformation and that the material in the less dense fractions is largely in a nonnative conformation. The native conformation coincides with the presence of particles, which indicates that a native conformation is necessary to form the viruslike structures.

From the staining intensities of the VP1 band in silver-stained protein gels compared with that of a quantitated LPV_{wt} VP1 standard (13), we estimated that 1 liter of cell culture infected for 3 days with recombinant LPV VP1 baculovirus at an MOI of 100 contains about 5 mg of $VP1_{bac}$ protein, of which about 1 mg is found in the dense, particle-containing metrizamide gradient fractions (Fig. 2A, lanes 12 to 14). On the basis of silver stain intensities, more than 90% of the protein in these dense fractions appears to be of $VP1_{bac}$ origin.

Nucleic acids of defined size are encapsidated in a substantial portion of recombinant LPV VP1 particles. The presence in the dense purified $VP1_{bac}$ preparations of many full viruslike particles with a high degree of similarity to LPV_{wt} particles led to the hypothesis that nucleic acids might be encapsidated into these particles. Metrizamide gradient fractions were incubated with DNase and RNase to digest copurified but unprotected nucleic acids, and then proteins were removed by phenol extraction and putative nucleic acids were precipitated, separated by agarose gel electrophoresis, and stained with ethidium bromide (Fig. 3A). Fractions 12 to 15, with a peak in fraction 13, contained nucleic acids which migrated predominantly like 4.5-kb linear, double-stranded DNA fragments (Fig. 3A, left panel). In addition, a second, small species of nucleic acids of less than 0.6 kb length was present, which, however, extended also into neighboring, less dense fractions containing aggregated, nonnative VP1 proteins and very few viruslike particles. The amount of DNA of this species varied strongly in different preparations. The nucleic acids purified from fraction 13 were further characterized (Fig. 3A, right panel). Both species were resistant to RNase digestion and sensitive to DNase digestion. The 4.5-kb species could be digested to small fragments by the frequently cutting restriction enzymes *Alu*I and *Ava*II, indicating that it consists entirely of double-stranded DNA. In agarose gels of different concentrations, the band migrated like a 4.5-kb, linear double-stranded DNA fragment (not shown). Electron microscopy of purified DNA after cytochrome *c* spreading and metal shadowing showed linear double-stranded DNA fragments of approximately 4.5 kb as well as short fragments. Both DNA species were not detectable in unextracted $VP1_{bac}$ particle preparations (not shown).

The distribution of the 4.5-kb DNA species in the metrizamide gradient (Fig. 3A) coincided with the concentration of native VP1 as measured by LPV VP1 ELISA (Fig. 2C) and also with the presence of viruslike particles. Immunoprecipitation was used to demonstrate a physical association of the DNA with $VP1_{bac}$ particles (Fig. 3C). Monoclonal antibody 520-1, which recognizes native LPV VP1, coprecipitated both

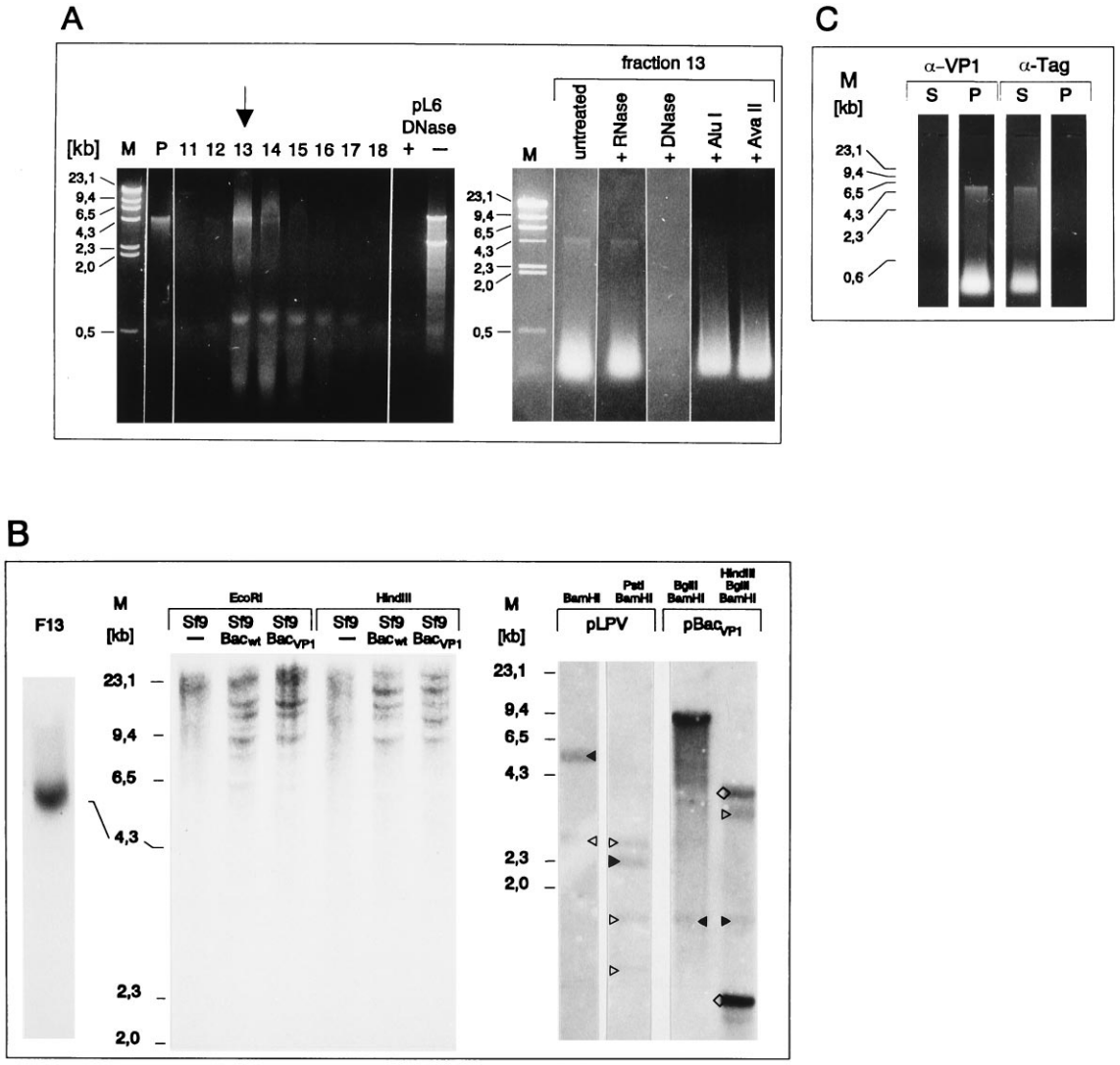

FIG. 3. Presence and characteristics of DNA in VP1$_{bac}$ viruslike particles. (A) Ethidium bromide-stained agarose gel of nucleic acids extracted from fractions of the VP1$_{bac}$ particle purification shown in Fig. 2. Lanes M, size markers; lane P, pellet obtained after centrifugation through 50% sucrose; lanes 11 to 18, metrizamide equilibrium density gradient fractions 11 to 18, respectively. To demonstrate complete DNase digestion of unprotected DNA, purified DNA of plasmid pL6 was incubated in 50% metrizamide with (+) and without (−) DNase under the same conditions as applied for the fractions before nucleic acid extraction. The resistance or susceptibility of the extracted nucleic acids from the peak fraction of the metrizamide gradients (taken from another purification experiment, corresponding to fraction 13 in Fig. 2) to digestion by RNase, DNase, and the frequently cutting restriction enzymes *Alu*I and *Ava*II are shown in the right panel. (B) In Southern blot analysis the isolated and ^{32}P-labeled DNA of the 4.5-kb band from metrizamide gradient fraction 13 was hybridized to itself (lane F13); to *Eco*RI- or *Hin*dIII-digested DNA (10 μg per lane) from insect cell line Sf9 without baculovirus infection (lanes Sf9 −), with wild-type baculovirus infection (lanes Sf9 Bac$_{wt}$), or with recombinant LPV VP1 baculovirus infection (lanes Sf9 Bac$_{VP1}$); and to purified, restriction enzyme-digested plasmid DNAs (1 μg per lane) of pLPV (complete LPV genome cloned with *Bam*HI into vector pUC19) and pBac$_{VP1}$ (LPV VP1-coding sequence in baculovirus vector pVL1393). Plasmid DNA restriction fragments containing LPV VP1 sequences (◁), bacterial vector sequences and LPV sequences outside the VP1-coding region (◀), and baculovirus sequences flanking the LPV VP1 sequences in pBac$_{VP1}$ (◇) are indicated. All hybridizations were performed under conditions of high stringency (T_m − 18°C) except for the hybridization to pLPV, which was performed under conditions of low stringency (T_m − 50°C) to detect cross-hybridizing sequences. (C) Coimmunoprecipitation of VP1$_{bac}$ and 4.5-kb DNA. An excess of either LPV VP1-specific monoclonal antibody 520-1 (α-VP1) or an equal amount of SV40 T-antigen-specific control antibody (α-Tag) of the same heavy-chain class complexed with protein A-Sepharose was incubated with 10 μg of VP1$_{bac}$ from fraction 13 and subsequently precipitated by centrifugation. Nucleic acids were extracted from the supernatant (lanes S) and precipitate (lanes P) and visualized after agarose gel separation by ethidium bromide.

DNA species, whereas with a control antibody the DNA remained in the supernatant.

The 4.5-kb DNA was purified from the gel and used as a ^{32}P-labeled hybridization probe. It reacted with multiple species of Sf9 cell DNA, and in total DNA extracted from baculovirus-infected cells it reacted more intensely with many fragments of baculovirus origin (Fig. 3B, left panel). However, this does not indicate that baculoviral DNA was preferentially present in the probe, because as a result of the high replication level, baculovirus DNA fragments were highly overrepresented in the blot relative to the cellular sequences, as was seen in ethidium bromide-stained gels (not shown).

Hybridization to defined restriction fragments from purified plasmid DNAs was used to analyze whether the LPV sequences present in the recombinant baculovirus vector or cellular or baculoviral sequences cross-hybridizing with LPV sequences were selectively encapsidated (Fig. 3B, right panel). The probe recognized LPV VP1 sequences slightly better than

sequences of the prokaryotic vector pUC, which indicates that some VP1 sequences were present in the encapsidated DNA. No increased hybridization to other regions of the LPV genome was observed, even under conditions of low stringency. In contrast, slightly increased hybridization to baculovirus sequences present immediately downstream of the inserted LPV VP1-coding sequence was observed. However, hybridization to Southern blots of purified baculovirus DNA digested with various restriction enzymes did not reveal predominantly hybridizing regions within the baculoviral genome (data not shown).

We estimated from ethidium bromide-stained gels that in the preparation derived 3 days p.i. from an infection with an MOI of 100, 1 μg of $VP1_{bac}$ particles from the peak fraction of the metrizamide gradient (fraction 13 in Fig. 2A and 3A) contained about 20 ng of the 4.5-kb DNA species. If one assumes that at maximum all VP1 is assembled in particles, with 360 VP1 molecules forming one particle, then at least 1 of 10 particles contains one molecule of this DNA species.

Taken together, these data indicate that at least 10% of the metrizamide gradient-purified LPV VP1 particles which spontaneously assembled in the insect cell nucleus contain linear double-stranded DNA with a predominant size of approximately 4.5 kb. The encapsidated DNA consists of nonselectively encapsidated host cell and baculovirus sequences with no apparent strong homology to LPV sequences.

Increase of DNA encapsidation by LPV $VP1_{bac}$ particles with time and dose of recombinant baculovirus infection. VP1 particles were harvested and purified from cells infected with LPV VP1 baculoviruses at an MOI of 10 for 2, 4, and 5 days. At day 2 p.i. VP1 was already expressed but at a rather low level, yielding only about 5 μg of VP1 particles from 1 liter of culture. The particle preparation showed no VP1 degradation but because of the low level of expression was still considerably contaminated with cellular proteins (Fig. 4A). This early particle preparation contained a smear of DNA fragments smaller than 800 bp, but no distinct 4.5 kb species could be detected, even after long-term exposure of the Southern blot (Fig. 4B). At days 4 and 5 high-level VP1 expression was achieved, resulting in purer preparations (390 and 620 μg of VP1 particles, respectively, per liter of culture), and also VP1 degradation was clearly detectable (Fig. 4A). At day 4 p.i. the 4.5-kb DNA species was present together with some of the small fragments that were predominant at day 2 p.i. Finally, VP1 particles harvested at day 5 p.i. contained the largest amounts of DNA, which consisted almost exclusively of the large, 4.5-kb species and smaller fragments down to 3.5 kb in size.

Encapsidated DNA is associated with full particles and distinct species of small host proteins, probably histones. We expected that the full and empty particles isolated in the metrizamide gradient fraction (Fig. 1B) should be similar in size and shape but should differ in mass according to their DNA contents. Therefore, further separation of the particles was attempted by centrifugation in a continuous sucrose gradient. In several gradients the distribution of VP1 antigen, DNA, and total proteins showed a consistent pattern. VP1 antigen was found spread over seven fractions (Fig. 5A). On the left, "heavy" side, the VP1 antigen concentration profile rose sharply to reach a peak in fraction 13. LPV_{wt} particles subjected to the same purification procedure in parallel gradients sharply banded at a position corresponding to fraction 12. Towards the right, "lighter" side, the VP1 concentration fell more slowly, forming a shoulder.

The particle-associated large DNA fragments appeared mostly in fractions 12 and 13 (Fig. 5B). Whereas in fraction 12 the fragments predominantly were 4.0 to 4.5 kb, in the neighboring, lighter fraction 13 somewhat smaller DNA fragments

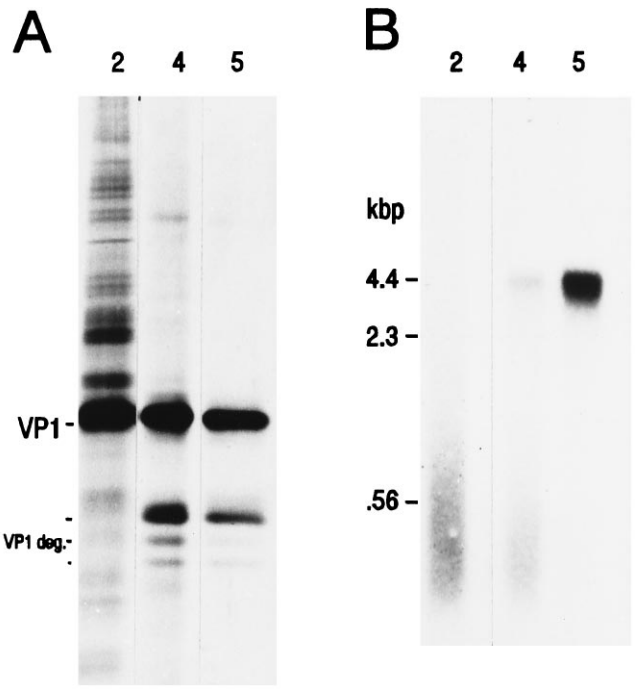

FIG. 4. Increase of VP1 degradation and DNA encapsidation in VP1 particles with time of baculovirus infection. VP1 particles were purified by metrizamide gradient centrifugation from cells infected for 2, 4, or 5 days (lanes 2, 4, and 5 respectively) with recombinant LPV VP1 baculovirus (MOI of 10). (A) From each particle preparation the equivalent of 200 ng of LPV VP1 ELISA antigen was separated by SDS-PAGE and silver stained. The positions of full-length VP1 protein (VP1) and the three VP1 degradation products (VP1 deg.) are indicated on the left. (B) DNA extracted from the particle preparations (equivalent of 200 ng VP1 antigen) was separated by electrophoresis, blotted, and hybridized with a labeled probe representing the encapsidated 4.5-kb DNA. DNA size markers are indicated on the left.

of about 3 to 4 kb appeared. In the following two fractions, 14 and 15, the DNA decreased in amount and fragment size. The distribution of total proteins suggested that towards the lighter fractions the content of VP1 protein not reactive in the ELISA as well as of the 31- to 35-kDa VP1 degradation products increased (Fig. 5C). However, even in the highly purified DNA-containing particle preparations a substantial amount of VP1 degradation products remained.

The particles apparently were separated by the sucrose gradient centrifugation according to their mass; heavier particles contained the DNA and migrated largely in fractions 11 to 14, and lighter particles without DNA overlapped with the heavier particle population in fraction 14 and extended up to fraction 17. Negative-staining electron microscopy supported this view. Fraction 12, which was regarded as representative of the DNA-containing particle population, showed nearly exclusively full particles, whereas in fraction 16 mostly empty particles had accumulated (Fig. 6).

In these experiments gels with higher resolution in the lower molecular mass range (17% polyacrylamide) were used in order to detect smaller, additional non-VP1 proteins that might be associated with the encapsidated DNA. Indeed, in fractions 12 to 14, in which the most of the large DNA fragments were found, three proteins of approximately 14, 15, and 16 kDa were detected by silver staining (Fig. 5C). This protein pattern is suggestive of the histone pattern described for baculovirus-infected insect cells (36). We assume that these three proteins represent insect cell histones, encapsidated together with the

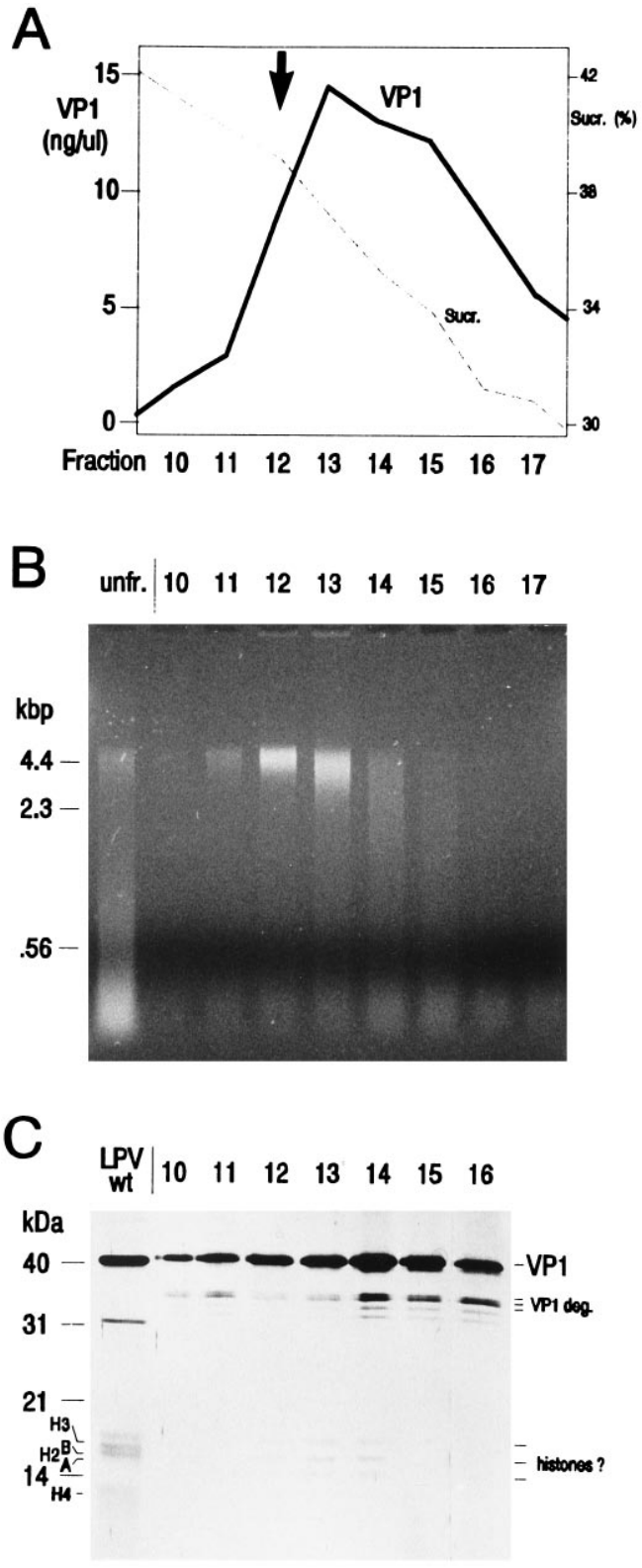

FIG. 5. Purification of LPV VP1 particles by continuous sucrose gradient centrifugation, showing distribution of VP1 ELISA antigen (A), ethidium bromide-stained DNA (B), and silver-stained protein (C) after sucrose gradient fractionation of VP1 particles that had been isolated by metrizamide gradient centrifugation (3 days after baculovirus infection, MOI of 10, corresponding to fraction 13 in Fig. 2). (A) The arrow indicates the banding position of purified LPV_{wt} particles analyzed in parallel gradients. (B) DNA extracted from equal volumes of each fraction was applied in each lane. Fraction numbers are given at the top; size markers are indicated on the left. The leftmost lane (unfr.) contains the unfractionated material loaded onto the sucrose gradient. (C) Loading of equal amounts of VP1 ELISA antigen per lane was attempted. The amount of silver-stained VP1 appears to increase from left to right, indicating that the amount of in the VP1 ELISA nonreactive VP1 increases in the lighter fractions. The positions of the three small, 14- to 16-kDa proteins detectable in the DNA-containing VP1 particle fractions are indicated on the right and tentatively labeled (histones?). In lane 14 more protein accidentally was loaded. The leftmost lane (LPV wt) contains purified LPV_{wt} particles separated as a reference; the mammalian histone proteins H3, H2B, H2A, and H3 that are encapsidated together with the LPV genome in these particles are identified on the left.

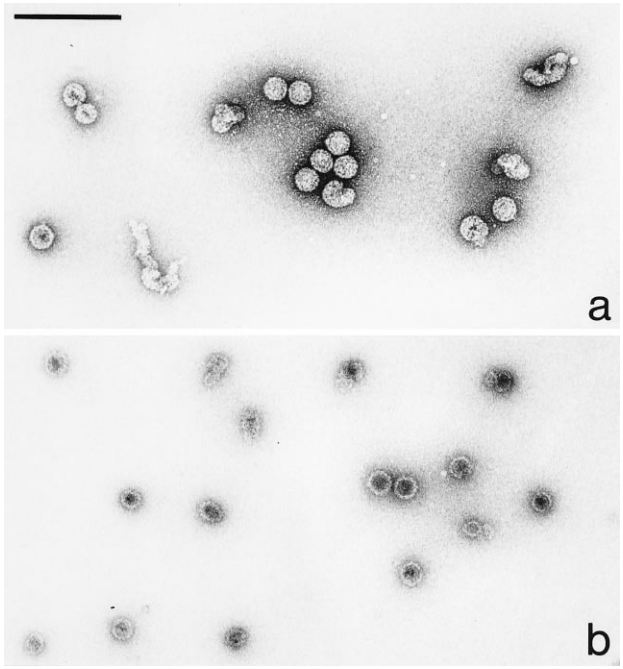

FIG. 6. Electron micrographs of negatively stained full and empty LPV VP1 particles separated by sucrose gradient centrifugation. Particles of fraction 12 (a) and fraction 16 (b) from the gradient of Fig. 5 are shown. Bar, 200 nm.

DNA into the VP1 particles. The 15-kDa protein could also represent the basic DNA-binding protein of baculoviruses known to associate with baculoviral DNA in baculovirus particles (35).

Host cell DNA is also encapsidated in LPV_{wt} particles. The apparently nonselective encapsidation of host cell and baculovirus DNAs into $VP1_{bac}$ viruslike particles raised the possibility that some LPV_{wt} particles also might have encapsidated DNA of their respective host cell instead of the viral genome. To test this hypothesis, DNA was extracted from LPV_{wt} virions that had been purified and nuclease digested prior to DNA extraction exactly as described above for $VP1_{bac}$ particles. The particle DNA was digested with *Bam*HI to linearize the viral genome, separated by agarose gel electrophoresis, blotted, and hybridized with labeled DNA of the LPV host cell, the human B-lymphoma cell line BJA-B. This probe did not hybridize to the linear 5.3-kb LPV genome but reacted with DNA fragments of around 4.5 kb and a decreasing smear of smaller fragments (Fig. 7). The pattern and size of the hybridization signals were similar to those obtained by cross-hybridization to the insect cell DNA encapsidated in $VP1_{bac}$ particles. In the ethidium bromide-stained agarose gel of the LPV_{wt} particle DNA, a faint smear of DNA fragments of 4.5 kb and smaller

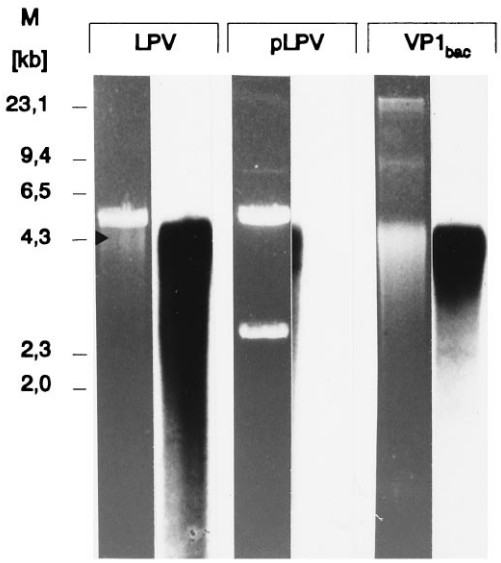

FIG. 7. Presence of host cell DNA in LPV_{wt} particles. Nucleic acids (1 μg) from purified and DNase-digested LPV_{wt} particles from BJA-B cells (LPV), from plasmid pLPV (pLPV), and from LPV $VP1_{bac}$ particles from Sf9 cells ($VP1_{bac}$) were separated by agarose gel electrophoresis and stained with ethidium bromide (dark lanes on the left sides of the panels) or blotted and hybridized with ^{32}P-labeled BJA-B (bright lanes on the right sides of the panels). pLPV DNA cut with *Bam*HI to separate the 5.3-kb complete LPV genome sequences from the 2.7 kb vector was used to show the absence of cross-hybridization of the BJA-B DNA probe with LPV sequences.

can be detected (Fig. 4); this probably represents the encapsidated cellular DNA sequences detected by hybridization.

DISCUSSION

The main structural protein VP1 of polyomaviruses is sufficient for the assembly of viruslike capsid structures, at least when expressed by baculovirus vectors in the *S. frugiperda* cell line Sf9. This had been demonstrated previously for mouse polyomavirus VP1 (8, 21) and is shown here for VP1 of the African green monkey B-lymphotropic papovavirus LPV.

In the purified LPV $VP1_{bac}$ particle preparations, in addition to the expected predominant 45-nm-diameter particles we observed a small fraction of approximately 30-nm-diameter particles. Similar small particles have been observed previously in wild-type polyomavirus and SV40 infections (16, 18) but were not present in LPV_{wt} particles purified by the same protocol as used for the LPV $VP1_{bac}$ particles. The in vitro assembly of viruslike particles from purified recombinant mouse polyomavirus VP1 has been described (29). Depending on the ionic strength, pH, and concentration of divalent cations during in vitro self-assembly, in addition to the normal 45- to 50-nm-diameter particles two types of smaller particles are also found, i.e., 32-nm-diameter particles with octahedral symmetry consisting of 24 close-packed pentamers and 26-nm-diameter particles with icosahedral symmetry consisting of 12 capsomeres (30). The approximately 30-nm-diameter LPV $VP1_{bac}$ particles might represent one or both of these smaller particle types.

In the purified viruslike LPV $VP1_{bac}$ particle preparations, there are full particles which appear to have encapsidated linear double-stranded DNA of approximately 4.5 kb and also shorter fragments down to less than 1 kb. The interpretation that this DNA is indeed encapsidated in $VP1_{bac}$ particles is based on (i) its copurification with full $VP1_{bac}$ particles in metrizamide equilibrium density gradients as well as in the heavy fractions of sucrose gradients, (ii) its resistance to DNase digestion prior to protein extraction, and (iii) its coimmunoprecipitation with native LPV $VP1_{bac}$. Additionally, in the sucrose gradient the VP1 particles containing the largest DNA banded at a position very similar to that of LPV_{wt} particles. This finding is compatible with the view that the mass of these VP1 particles, which lack the minor structural proteins VP2 and VP3, is only slightly lower than that of LPV_{wt}. The sucrose gradient fractionation also indicated that the DNA-containing particles can be separated by the size of their encapsidated DNA.

The two groups which have analyzed mouse polyomavirus $VP1_{bac}$ particles described only empty particles (8, 21); however, Forstova et al. (8) did mention that some of their "capsid-like particles appeared to contain DNA." We do not favor the hypothesis that the DNA encapsidation described here is a specific feature of LPV VP1. There are experimental differences which could explain the different results. First, DNA encapsidation in substantial quantity occured only late in baculovirus infection and at higher infection doses. Mouse polyomavirus $VP1_{bac}$ particles apparently have not been analyzed under such extreme conditions. Second, the metrizamide and sucrose gradient purification protocols used here allow particle purification without the use of CsCl gradients and therefore without conditions of high ionic strength, which might negatively affect the stability of the full $VP1_{bac}$ particles.

We found that three small non-VP1 proteins with apparent molecular masses of approximately 14, 15, and 16 kDa copurified with the DNA-containing particles. At present we have no direct evidence to definitely identify these proteins; however, we assume that they represent insect cell histone proteins. The observed protein pattern is very similar to that described for histones present in insect cells infected for 24 h by baculovirus (36). In baculovirus-infected cells, synthesis of new histone proteins is suppressed, and instead a basic DNA-binding protein of *A. californica* nuclear polyhedrosis virus with an apparent molecular mass of 15 kDa in SDS-PAGE is synthesized (35) and packaged into the baculovirus nucleocapsid (34). We cannot exclude the presence of this protein in the DNA-containing $VP1_{bac}$ particles; however, it cannot be a major species. In LPV_{wt} (shown here) as well as in other polyomaviruses (10), host histones H3, H2A, H2B, and H4 but not H1 are coencapsidated together with the viral genome.

For SV40 wild-type capsids, Oppenheim et al. (24) have described an SV40 encapsidation signal (*ses*) which comprises the viral origin of replication, six GC boxes, and 26 bp of the enhancer element. The two latter elements of *ses* were found to be necessary in close proximity to the viral origin of replication for efficient encapsidation of replicated episomal DNA into wild-type SV40 capsids in COS cells. It is not known whether the LPV sequence in this region, which is highly homologous to SV40 (25), also harbors an encapsidation signal. The linear 4.5-kb double-stranded host cell and baculovirus DNA fragments encapsidated in LPV $VP1_{bac}$ particles did not cross-hybridize to a specific LPV sequence including the sequence homologous to *ses* even under conditions of low stringency (T_m − 50°C). This is taken as evidence that DNA encapsidation was not mediated via homology to an LPV encapsidation signal.

Wild-type baculovirus infection in *S. frugiperda* cell lines is lytic; cell lysis with degradation of host cell DNA normally occurs after 72 h or longer. In the early phase of the infection, up to at least 36 h, the cellular DNA is not fragmented (5). The clear increase of the amount of the dominant 4.5-kb linear

DNA encapsidated in $VP1_{bac}$ particles with the time of the ongoing baculovirus infection may indicate that host DNA fragmentation occuring late in baculovirus infection is favorable for host DNA encapsidation, although the contribution of other factors cannot be excluded. If indeed the concentration of host DNA fragments in the nucleus is the main host variable determining DNA encapsidation, this together with the absence of LPV-related sequences in the encapsidated DNA would argue in favor of an "availability" model for encapsidation and against a specific encapsidation signal sequence.

The encapsidated DNA has characteristics very similar to those of host cell DNAs observed previously in purified wild-type mouse polyomavirus and SV40 particle preparations (12, 20, 26, 37) and also found here in purified LPV_{wt} particles. In LPV_{wt} particles, far less than 1% of the encapsidated DNA appears to be of host origin, whereas in the LPV $VP1_{bac}$ particles, 10% or more of the particles can contain this DNA species. This might be due to the fact that in the insect cells no episomal viral DNA of the proper, packable size (and perhaps carrying the proper packaging signal) with which host cell DNA fragments would have to compete for encapsidation is present.

For encapsidation two models appear possible, depending on whether self-assembly of the viral proteins precedes or follows the interactions between the viral proteins and the viral chromatin. The former model predicts the existence of preformed empty capsids as intermediates in encapsidation, which after insertion of the chromatin would be sealed to yield virions. This model has not been proven; however, the finding that the addition of purified DNA to empty polyomavirus capsids in vitro can result in encapsidation of some of the added DNA in the pseudovirions (2, 31) could be taken as evidence in favor of it. The latter model suggests that encapsidation occurs by gradual addition and organization of capsid proteins around the chromatin, and some in vivo evidence supporting it has been found for SV40 (9). We cannot distinguish by which of the two models the host DNA was encapsidated in the LPV $VP1_{bac}$ particles. Irrespective of the sequence of the assembly steps, the predominant length of encapsidated 4.5-kb linear double-stranded DNA fragments may point to the upper encapsidation limit of LPV particles for linear DNA. In addition, it may suggest, in regard to the mechanism of encapsidation, that initially larger DNA fragments are part of the assembly process and that these subsequently, either in the cell or during the extraction and purification procedure, are trimmed by nucleases before assembly is completed. Apparently, small DNA fragments less than 1 kb in size can also be encapsidated. They were present in the LPV $VP1_{bac}$ particle preparations, especially early in the baculovirus infection cycle, and were also coimmunoprecipitated with $VP1_{bac}$ protein.

VP1 proteins of mouse polyomavirus and SV40 are able to bind purified double-stranded DNA in a sequence-unspecific manner. This binding activity has been located to the first seven to nine amino-terminal residues, which contain a stretch of three basic amino acids. (4, 6, 22). The LPV VP1 sequence in this region is highly conserved (25), suggesting a similar binding activity for LPV VP1. Despite this DNA-binding potential of polyomavirus VP1 proteins, it remains remarkable that DNA encapsidation can occur without the minor structural proteins VP2 and VP3. In SV40, these proteins carry rather strong sequence-independent DNA-binding activity at their common carboxy terminus (6); however, no DNA-binding activity has been found for murine polyomavirus VP2 and VP3 (4). The stretches of basic amino acids in the carboxy-terminal region are highly conserved between LPV and SV40 (25) and therefore can be assumed to have similar functions, but obviously such functions are not necessary for DNA encapsidation in LPV $VP1_{bac}$ particles. At present it remains unclear both for particle assembly and for DNA encapsidation whether functions present in baculovirus-infected insect cells can replace VP2 and VP3 functions or whether indeed VP1 alone is sufficient for encapsidation of histone-associated DNA. Except for the putative cellular histones, no additional host-derived non-VP1 proteins were found in the highly purified particle preparations. However, we cannot exclude the possibility that the 31- to 35-kDa VP1 degradation products that remain even in the sucrose gradient-purified preparation exert unknown functions and contribute to DNA encapsidation.

The capacity of $VP1_{bac}$ particles to rather efficiently but apparently unspecifically encapsidate host cell or other viral DNA could be used in the development of viral vectors based on these particles, provided that a suitable heterologous DNA replication system could be established in the host cells. However, in experiments employing these particles as vaccines or potential viral genetic vectors after attaching or encapsidating DNA in vitro (7), unspecific DNA encapsidation should also be taken into account as a potential safety hazard.

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