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Assembly of Viruslike Particles by Recombinant Structural Proteins of Adeno-Associated Virus Type 2 in Insect Cells

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The three capsid proteins VP1, VP2, and VP3 of the adeno-associated virus type 2 (AAV-2) are encoded by overlapping sequences of the same open reading frame. Separate expression of these proteins by recombinant baculoviruses in insect cells was achieved by mutation of the internal translation initiation codons. Coexpression of VP1 and VP2, VP2 and VP3, and all three capsid proteins and the expression of VP2 alone in Sf9 cells resulted in the production of viruslike particles resembling empty capsids generated during infection of HeLa cells with AAV-2 and adenovirus. These results suggest a requirement for VP2 in the formation of empty capsids. Individual expression of the AAV capsid proteins in HeLa cells showed that VP1 and VP2 accumulate in the cell nucleus and VP3 is distributed between nucleus and cytoplasm. Coexpression of VP3 with the other structural proteins also led to nuclear localization of VP3, indicating that the formation of a complex with VP1 or VP2 is required for accumulation of VP3 in the nucleus.

The adeno-associated virus (AAV), a human parvovirus, has a single-stranded DNA genome packaged in an icosahedral capsid with a diameter of 20 to 24 nm (for a review, see reference 3). The capsid consists of three structural proteins, VP1, VP2, and VP3, with molecular weights of 90, 72, and 60 kDa, respectively. They are expressed from overlapping sequences of the same open reading frame by using alternative initiation codons (1, 2, 28). A 2.6-kb precursor RNA is encoded by the right half of the AAV type 2 (AAV-2) genome (18, 19) and is spliced into two 2.3-kb mRNAs by using the same donor site but different acceptor sites. In the presence of adenovirus, most of the 2.6-kb RNA is spliced to the acceptor site at nucleotide 2228 (major splice), whereas the minority of the transcript is spliced to a more-proximal acceptor site at nucleotide 2201 (minor splice; 1, 6, 45). VP1 is expressed from an AUG initiation codon at position 2203 which is present only in the minor-splice RNA, while VP2 and VP3 can be expressed from both differentially spliced mRNAs. VP3 is efficiently translated from an AUG initiation codon at nucleotide 2809, and VP2 is expressed about 10-fold less efficiently from an unusual initiation codon, ACG, at position 2614. The relative accumulation of the alternatively spliced mRNAs together with the reduced translation initiation frequency at the ACG initiation codon leads to the synthesis of VP1, VP2, and VP3 in relative ratios of about 1:1:10, the mass ratio which is found in mature AAV virus particles (5).

Kinetic experiments allow us to distinguish two steps in the assembly process of infectious AAV particles: (i) rapid assembly of empty capsids and (ii) packaging of single-stranded DNA into preformed capsids (30). While capsid assembly and association with the viral DNA are rapid processes, maturation to infectious particles requires several hours. It is not known which proteins compose the empty capsids and whether additional proteins are required for DNA packaging. VP2 and VP3 are sufficient for accumulation or sequestration of single-stranded progeny DNA. VP1, however, is required for production of infectious particles (15, 37, 44).

Packaging signals have been postulated to be present in the inverted terminal repeats, since defective interfering particles contain at least one terminal repeat (9). This assumption has been corroborated by the successful packaging of DNA which contained only the terminal repeats of the AAV genome into AAV particles (35).

In order to study the requirements for the synthesis of empty capsids, we expressed the three capsid proteins VP1, VP2, and VP3 separately and in combination in *Spodoptera frugiperda* Sf9 cells by using baculovirus expression vectors. As has been observed with autonomous parvoviruses (4, 20, 34), this resulted in formation of empty viruslike particles (VLPs). Unexpectedly, however, there seemed to be a strict requirement for the minor capsid component, VP2, for detectable formation of VLPs in Sf9 cells. We failed to observe capsid formation with the proteins VP1 and VP3 alone. Separate expression of the capsid proteins in HeLa cells showed that VP1 and VP2 were able to accumulate in the cell nucleus, whereas VP3 was evenly distributed between nucleus and cytoplasm. Coexpression of VP3 with VP1 and/or VP2, however, resulted in accumulation of VP3 in the nucleus, too, suggesting that AAV capsid proteins need to form complexes for efficient nuclear accumulation and particle assembly.

MATERIALS AND METHODS

Mutagenesis and construction of recombinant plasmids. For oligonucleotide-directed mutagenesis, a 2.9-kb fragment comprising the right half of the AAV-2 genome starting from nucleotide 1882 was isolated from the *Hin*dIII- and *Sst*I-digested pTAV-2 plasmid (14) and cloned into the M13mp18 vector. This DNA fragment contains the complete capsid protein-coding sequences. For separate expression of the structural proteins, the internal translation initiation codons were mutated as indicated in Fig. 1 essentially according to the methods described in reference 41 by using an in vitro mutagenesis kit (Amersham, Braunschweig, Germany). Mutants were identified by DNA sequencing according to the

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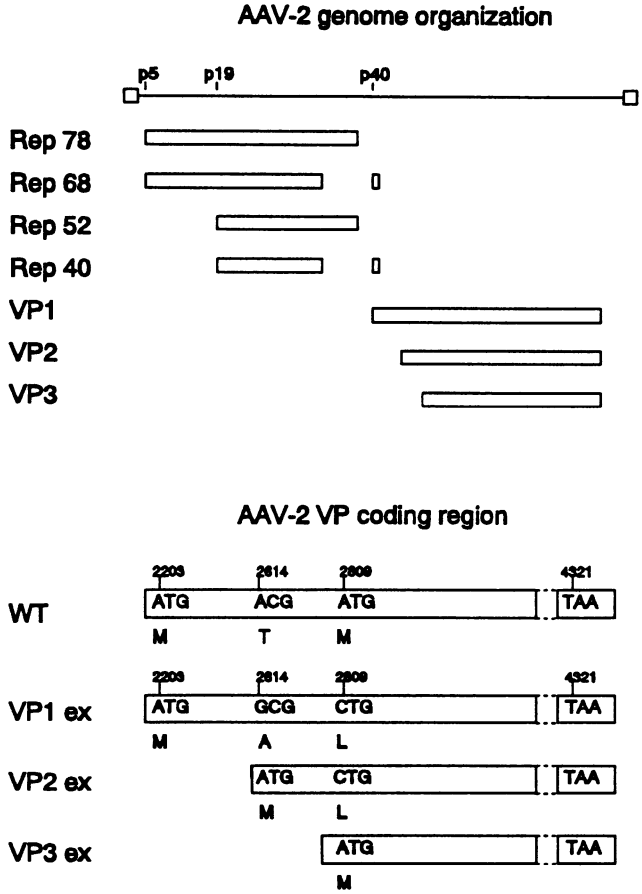

FIG. 1. AAV-2 genome organization and mutant VP coding regions used for protein expression. (Top) AAV-2 genome, with inverted terminal repeats indicated by open boxes at the ends of the genome and the three promotors (p) at map unit positions 5, 19, and 40. The coding regions for regulatory proteins Rep78, Rep68, Rep52, and Rep40 and structural proteins VP1, VP2, and VP3 are shown by open rectangular boxes. (Bottom) Overlapping open reading frames of the three capsid proteins with the translation initiation codons which code for the amino acids M, T, and M in the wild-type (WT) genome. For separate expression of VP1, VP2, and VP3 in different expression vectors, these initiation codons were mutated as indicated in boxes VP1ex, VP2ex, and VP3ex. Translation was terminated in all constructs at the same stop codon at position 4321.

method of Sanger et al. (36). In plasmid M13mp18-VP1ex, the ACG translation initiation codon (nucleotides 2614 to 2616, according to reference 6) was changed to GCG, and ATG (nucleotides 2809 to 2811) was changed to CTG. In M13mp18-VP2ex, ACG (nucleotides 2614 to 2616) was changed to ATG, and an *Nde*I restriction site was simultaneously introduced by changing nucleotide 2611 from A to C and nucleotide 2613 from G to T. ATG at nucleotide positions 2809 to 2811 was mutated to CTG. In M13mp18-VP3ex at the ATG translation initiation codon (nucleotides 2809 to 2811), an *Nde*I restriction site was introduced by mutating nucleotides 2806 to 2808 from ACG to CAT. The plasmid pVL-Vp1 was constructed by insertion of a *Dra*I-*Xba*I fragment (*Dra*I site blunt ended) from M13mp18-VP1ex into the *Sma*I-*Xba*I-digested baculovirus vector pVL1393, a derivative of the plasmid pVL941 (27). Generation of the plasmids pVL-Vp2 and pVL-Vp3 was performed by cloning *Nde*I-*Bal*I fragments (nucleotides 2614 to 4552 and nucleotides 2809 to 4552, respectively; *Nde*I site blunt ended) into the *Sma*I site of pVL1393. The junction sequences were confirmed by dideoxy sequence analysis.

For expression of the AAV capsid proteins in HeLa cells, the corresponding mutated viral capsid protein (VP)-coding regions were inserted into the expression vector pKEX-2-XR (32). The plasmid pKEX-VP1 was generated by ligation of a *Bam*HI-*Xba*I (*Bam*HI site blunt ended) fragment of pVL-VP1 into pKEX-2-XR digested with *Xho*I and *Xba*I (*Xho*I site also blunt ended). The constructs pKEX-VP2 and pKEX-VP3 resulted from cloning the respective *Nde*I-*Xba*I fragments from M13mp18-VP2ex and M13mp18-VP3ex into the plasmid pUC131 (kindly provided by H. Schaller, Zentrum für Molekulare Biologie, Heidelberg, Germany). The plasmids pUC-VP2 and pUC-VP3 were digested with *Cla*I and *Xba*I, respectively. The resulting 2.1- and 1.8-kb DNA fragments were inserted into pKEX-2-XR digested with the same enzymes.

Cell culture and virus stocks. *S. frugiperda* Sf9 cells were grown as suspension or monolayer cultures in TNMFH medium (16) with 10% fetal calf serum, 100 μg of gentamicin per ml, and 2 mM glutamine at 27°C and 95% humidity. For generation of recombinant baculovirus stocks, 1×10^7 to 5×10^7 cells in suspension were infected for 1 h at 27°C at a multiplicity of infection (MOI) of 2 to 5. After infection, cells were transferred into Spinner flasks and stirred for 5 to 6 days at 27°C. Cells were removed by centrifugation, and the supernatant with the recombinant baculoviruses was stored at 4°C. For expression of the capsid proteins, 10^6 Sf9 cells from a monolayer culture were infected by baculovirus recombinants for 1 h at 27°C at an MOI of 5 to 10. After infection, cells were incubated for 48 h at 27°C. Medium was removed, cells were washed with phosphate-buffered saline (PBS; 140 mM NaCl, 6.5 mM Na_2HPO_4, 2.5 mM KCl, 1.5 mM KH_2PO_4, pH 7.4), scraped off, and resuspended in 150 μl of sample buffer (24) for protein analysis by electrophoresis.

HeLa cells were propagated in Dulbecco's modified Eagle's medium with 5% fetal calf serum, 100 μg each of penicillin and streptomycin per ml, and 2 mM glutamine at 37°C and 5% CO_2. For generation of AAV-2 (strain H; American Type Culture Collection) stocks, cells were grown to 40 to 60% confluency (5×10^4 cells per cm^2). After the medium was removed, cells were first infected for 1 h at 37°C with AAV-2 (MOI = 10) and then with adenovirus (MOI = 2) under the same conditions. When more than 90% of the HeLa cells showed a cytopathic effect, cells were removed by centrifugation. Supernatant was incubated for 1 h at 56°C for inactivation of adenovirus and then stored at −20°C.

Generation of recombinant baculoviruses. Recombinant pVL plasmids were used to generate recombinant baculoviruses according to the protocol of Summers and Smith (40). For transfection of Sf9 cells with recombinant pVL plasmids and wild-type *Autographa californica* nuclear polyhedrosis virus DNA, a calcium phosphate precipitation technique was used. Recombinant baculoviruses were purified by a plaque assay procedure.

Analysis of protein expression. For analysis of protein expression by gel electrophoresis, 2×10^6 pelleted cells were lysed by heat at 100°C for 5 min in 150 μl of sample buffer (24). After sonication (10 s at level 4; Branson Sonifier), total cellular polypeptides were analyzed by electrophoresis on a 15% polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS) as described in reference 42. Proteins were visualized by staining with 0.2% Coomassie blue. For Western blot (immunoblot) analysis, proteins were electrophoret-

ically transferred to nitrocellulose membranes (43). AAV capsid proteins were detected by incubations with an anti-VP3 antiserum and a phosphatase-coupled second antibody (13).

Preparation of anti-VP3 serum. The DNA fragment coding for the AAV-2 VP3 capsid protein was isolated from the M13mp18-VP3ex plasmid by digestion with *Nde*I and *Bal*I and cloned into the *Escherichia coli* expression vector pET3a (39). *E. coli* BL21 (DE3) cells (39) transformed with the recombinant plasmid pET-VP3 expressed the VP3 capsid protein. Cells were lysed by sonication in lysis buffer (50 mM Tris [pH 8], 5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl, 2.5 mM dithiothreitol, 1 mg of lysozyme per ml), and the unsoluble proteins were spun down at 4,000 × *g* for 5 min. After the pellet had been heated for 5 min at 100°C in sample buffer, polypeptides were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and visualized by staining with 1% Coomassie blue in water. The region corresponding to the VP3 protein was excised, and the protein was eluted in 0.02% SDS overnight at room temperature. Gel pieces were spun down, and proteins in the supernatant were precipitated by acetone. VP3 (100 to 200 μg) was suspended in PBS for each immunization, mixed with an equal volume of Freund's adjuvant, and injected subcutaneously into a rabbit (chinchilla bastard). The rabbit was immunized three times in 3 months. One week after the third immunization, serum was tested by an immunoblot analysis.

Preparation of VLPs and electron microscopy. Sf9 cells (2×10^8 to 5×10^8) were (co-)infected with recombinant baculoviruses at an MOI of 10 to 20. At 70 to 72 h postinfection, cells were harvested by centrifugation and suspended in 1 ml of a buffer containing 1% deoxycholate (DOC), 0.1% SDS, 10 mM Tris (pH 8), 0.1 mM EDTA and 2 mM phenylmethylsulfonyl fluoride. After sonication, cell debris were removed by centrifugation (12,000 × *g*, 5 min), and the supernatant was layered onto a double sucrose cushion of 50 and 30% sucrose (wt/vol) in PBS. After centrifugation at 100,000 × *g* for 2 h, the particle-containing sediment was resuspended in PBS and centrifuged again (200,000 × g, 1 h). VLPs were resuspended in PBS, stained with 2% uranyl aqueous acetate, and examined in a Zeiss EM 10 electron microscope. The magnification was routinely controlled by using a grating replica. In parallel, the same fractions were analyzed by SDS-PAGE and Western blotting.

Purification of AAV-2 virus particles. AAV-2 virus stock was prepared as described above. A 12-ml portion of this stock was spun down for 1 h at 100,000 × *g*. The sedimented viral particles were suspended in 200 μl of 10 mM Tris (pH 8.0)–1 mM EDTA (TE buffer) with 1% DOC for 30 min at 37°C and diluted 1:10 with TE buffer. CsCl was added to a final density of 1.4 g/cm^3, and equilibrium centrifugation was performed in a TST60-4 rotor (65 h, 35,000 rpm, 20°C; Kontron T-1065 ultracentrifuge). Fractions with a density of 1.409 to 1.459 g/cm^3 were collected, dialyzed against TE buffer, and analyzed by electron microscopy.

Transfection of HeLa cells and immunofluorescence. Transfection of HeLa cells was performed by the protocol of Chen and Okayama (7) in a slightly modified form. Briefly, the day before transfection, 4×10^5 cells were seeded in a petri dish (diameter, 9.4 cm) with 8 ml of medium and incubated at 37°C and 5% CO_2. DNA (15 μg) was mixed with 2× BBS buffer (7) (250 μl) to which $CaCl_2$ had been added to a final concentration of 175 mM and incubated for 20 min at room temperature. The transfection solution was added to the cells, and cells were incubated for 18 to 22 h at 35°C and 3% CO_2. After the supernatant had been removed, cells were washed twice with serum-free medium and then incubated in medium with fetal calf serum for 24 h at 37°C and 5% CO_2. For immunofluorescence, cells were grown on coverslips in tissue culture dishes and transfected as described above. After the coverslips had been washed in PBS, cells were first fixed for 10 min at −20°C in methanol and then for 5 min at −20°C in acetone. Cells were air dried, rehydrated in PBS for 5 min at room temperature, and then incubated with the anti-VP3 serum (diluted 1:50 in PBS with 1% bovine serum albumin [BSA]) for 15 min at room temperature. Cells were washed three times in PBS and then incubated with a fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin serum (Nordic, Talburg, Germany) diluted 1:50 in PBS with 1% BSA. After being washed in PBS, coverslips were embedded in Elvanol (1 g of polyvinyl alcohol mixed with 8 ml of PBS and 4 ml of 86% glycerol, incubated overnight at 80°C, and stored at −20°C) and visualized under a Leitz UV microscope.

RESULTS

Separate expression of individual AAV-2 capsid proteins. In order to analyze the contributions of the three viral capsid proteins of AAV-2 to the assembly of VLPs, we expressed VP1, VP2, and VP3 separately. For separate expression of VP1, we started with a subfragment of plasmid pTAV-2 (14) which contained the complete open reading frame coding for all three capsid proteins and changed the internal translation start sites of VP2 and VP3 at nucleotide positions 2614 and 2809 to GCG and CTG, respectively, by site-directed mutagenesis (Fig. 1, VP1ex; numbering according to reference 6). These mutations introduced a change of amino acid 138 of VP1 from T to A and of amino acid 202 from M to L. For separate expression of VP2, we mutated its unusual ACG translation initiation codon to ATG, thereby also changing the N-terminal amino acid T to M (Fig. 1, VP2ex). The internal start site of VP3 at 2809 was also mutated to CTG to prevent simultaneous expression of VP3. In addition, we introduced a unique *Nde*I restriction site at the new translation initiation codon of VP2, which allowed the separate subcloning of the VP2 open reading frame into different expression vectors. Similarly, the translation codon of VP3 was converted into a unique *Nde*I restriction site, which allowed expression of VP3 without VP1 and VP2 after VP3 was subcloned into a suitable expression vector (Fig. 1, VP3ex). The three open reading frames were expressed in Sf9 cells under the control of the baculovirus polyhedrin promoter. Analysis of proteins of total cell extracts by SDS-PAGE shows that VP1, VP2, and VP3 were strongly expressed (Fig. 2a, lanes 2, 3, and 4), constituting up to 5% of total cellular protein compared with extracts of cells expressing only wild-type baculovirus proteins (Fig. 2a, lane 1). The relative molecular weights of the recombinant capsid proteins correspond to those of the viral capsid as shown by Western blot analysis (Fig. 2b). The kinetics of AAV-2 capsid protein expression in Sf9 cells showed that levels of VP1 and VP3 reached a maximum about 72 h after infection, whereas the VP2 content did not increase significantly after 48 h (data not shown). In extracts of cells expressing VP1 or VP2, variable amounts of polypeptides in the molecular weight range of VP3 which reacted with our anti-VP3 serum (Fig. 2b, lane 2, 3) could be observed. These and products with even lower molecular weights were more evident in VLP preparations from cells expressing VP1 and VP2 (Fig. 3a) or VP2 alone (data not shown). In addition, in cells

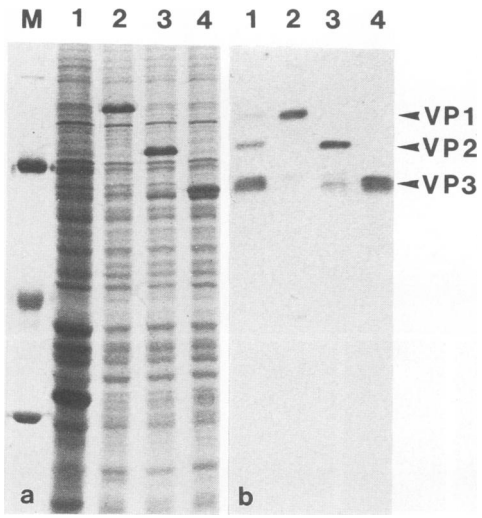

FIG. 2. Expression of AAV-2 structural proteins VP1, VP2, and VP3 in insect cells by recombinant baculoviruses. (a) Total proteins of *S. frugiperda* Sf9 cells infected with *A. californica* nuclear polyhedrosis virus (lane 1) or VP1 (lane 2), VP2 (lane 3), and VP3 (lane 4) recombinant baculovirus were separated by SDS-PAGE and stained with Coomassie blue. Cells were harvested 48 h postinfection. Molecular weight markers (lane M): β-galactosidase (115 kDa), BSA (68 kDa), egg albumin (45 kDa), and carbonic anhydrase (29 kDa). (b) shows the corresponding immunoblot analysis of extracts of insect cells infected with recombinant baculovirus expressing VP1 (lane 2), VP2 (lane 3), or VP3 (lane 4) with a rabbit antiserum raised against VP3. For comparison, an extract of HeLa cells infected with AAV-2 and adenovirus-2 is analyzed in lane 1.

expressing solely VP3, the resultant overexpressed VP3 protein often appeared as a double polypeptide band on SDS-PAGE (Fig. 2a, lane 4). Although the variable appearances of these polypeptides suggest that they are degradation products of VP1, VP2, or VP3, we cannot exclude the possibility that they arise from usage of alternative initiation codons (for example, at nucleotide positions 2833 to 2835) near the authentic translation initiation codon AUG for VP3.

Assembly of VLPs by recombinant capsid proteins. Individually expressed capsid proteins accumulated as insoluble aggregates in Sf9 cells. They were recovered in a low-speed-centrifugation sediment of cell extracts prepared by sonication of the cells in buffers with physiological salt concentrations and neutral pH. Addition of low- and high-ionic-strength buffers or low concentrations of different detergents or organic solvents did not solubilize the capsid

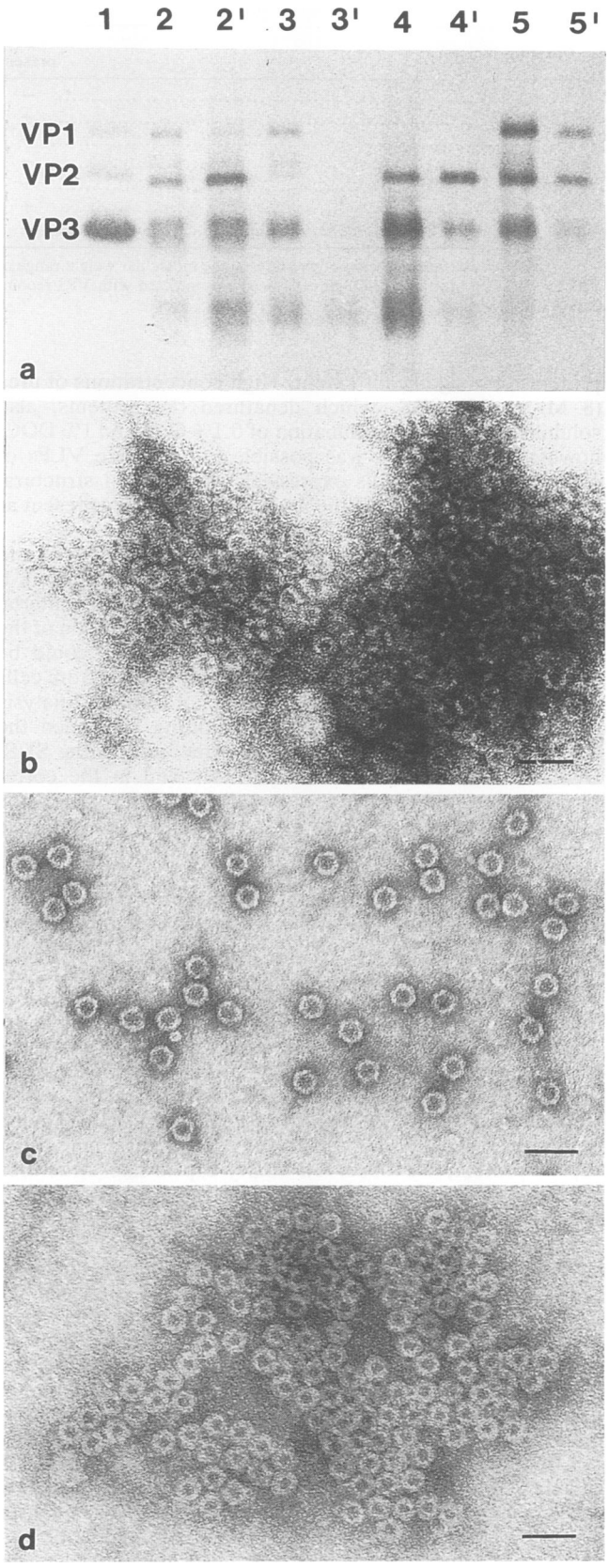

FIG. 3. Analysis of capsid formation by coexpression of different structural proteins in insect cells. The insoluble fractions of sonicated Sf9 cells containing recombinant AAV-2 capsid proteins were treated with 0.1% SDS and 1% DOC. Soluble (a, lanes 2', 3', 4', and 5') and insoluble (a, lanes 2, 3, 4, and 5) materials were analyzed by immunoblotting with an anti-VP3 serum. Lane 1, authentic AAV-2 capsid proteins produced in HeLa cells infected with AAV-2 and adenovirus-2; lanes 2 and 2', cells coexpressing VP1 and VP2; lanes 3 and 3', cells coexpressing VP1 and VP3; lanes 4 and 4', cells coexpressing VP2 and VP3; and lanes 5 and 5', cells coexpressing VP1, VP2 and VP3. Electron micrographs are of negatively stained empty-capsid-like structures recovered from the solubilized fractions of insect cells coexpressing VP1 and VP2 (b) (cf. panel a, lane 2'), VP2 and VP3 (c) (cf. panel a, lane 4'), and VP1, VP2, and VP3 (d) (cf. panel a, lane 5'). Bars = 50 nm.

TABLE 1. Detection of VLPs in insect cells expressing AAV-2 capsid proteins

Capsid protein(s)	VLPs present
VP1 (+ VP3)[a]	−
VP2 (+ VP3)[a]	+
VP3	−
VP1 + VP3	−
VP1 + VP2 (+ VP3)[a]	+
VP2 + VP3	+
VP1 + VP2 + VP3	+

[a] In these cases, we observed polypeptides in the molecular weight range of VP3 by Western blotting, although cells were not infected with VP3 recombinant baculoviruses.

proteins to a significant extent. High concentrations of urea (8 M) or 1% SDS, which denatured the proteins, also solubilized them. A combination of 0.1% SDS and 1% DOC, however, by which it was possible to solubilize VLPs of rotavirus from Sf9 cells expressing recombinant structural proteins, also solubilized the AAV capsid proteins present as capsidlike structures.

VLPs could be prepared from cells coexpressing VP1 and VP2, VP2 and VP3, or all three capsid proteins (Fig. 3, b through d) by sedimentation of the solubilized material through a double sucrose cushion and resedimentation of the resuspended pellet. The same type of particles could be solubilized from cells expressing VP2 only but not from cells expressing VP1 only or VP3 only (data not shown). Analysis of protein expression by Western blotting confirmed the presence of polypeptides with the correct size in the SDS-DOC-solubilized and -purified particles and in the corresponding insoluble fraction, although with different relative proportions (Fig. 3a, lanes 2, 2′, 4, 4′, 5, and 5′). The detection of immunologically cross-reactive polypeptides comigrating with VP3 in extracts of cells which were not infected with VP3 recombinant baculoviruses (for example, Fig. 3a, lanes 2 and 2′) complicates the interpretation when VP1 and VP2 or VP2 alone was expressed. Although the origin and nature of these polypeptides are unclear, as stated above, we cannot exclude the possibility of a contribution of these VP3-like molecules to empty-capsid formation. Coexpression of VP1 and VP3, however, did not promote the formation of empty-capsid-like structures which were soluble in 0.1% SDS–1% DOC (Fig. 3a, lanes 3 and 3′), which suggests that VP3 is not supporting capsid formation. On the contrary, whenever VP2 is coexpressed with any other capsid protein, we observed empty-capsid-like structures (Table 1). A detailed comparison of the particles derived from recombinant proteins (Fig. 4c, d, and e) with different images obtained from AAV-2 virus preparations (Fig. 4a and b) shows that in addition to the full particles in the virus preparation, there are also empty capsids (arrowheads in Fig. 4a and some examples in Fig. 4b) with morphologies very similar to those of capsids obtained in Sf9 cells.

Subcellular localization of AAV capsid proteins. Since it was difficult to determine the subcellular distribution of individually expressed capsid proteins in Sf9 cells, we expressed them under the control of the human cytomegalovirus (HCMV) immediate-early promoter-enhancer in HeLa cells (Fig. 5, lanes 2, 3, and 4) and localized the proteins by indirect immunofluorescence. Besides the expressed capsid proteins of expected sizes (Fig. 5, lanes 2 through 4, arrowheads), we observed in all cases lower-molecular-weight products (Fig. 5, unlabeled bands), which are especially

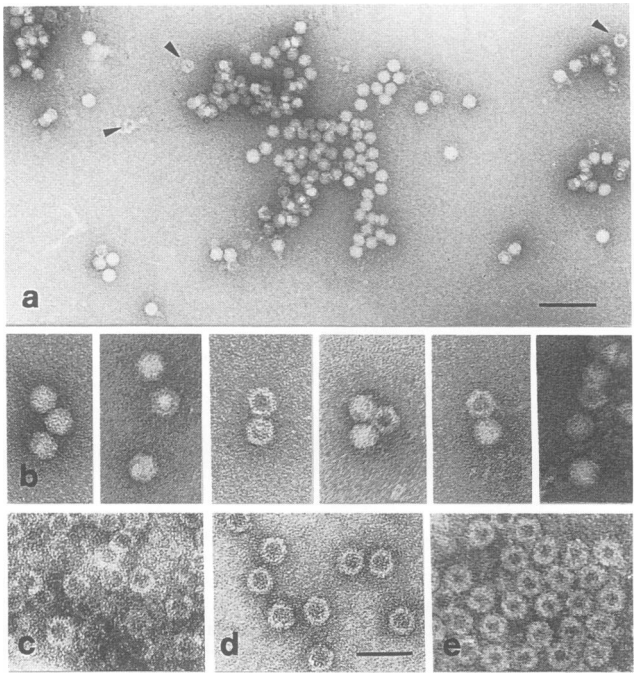

FIG. 4. Comparison of reconstituted and wild-type capsid structures of AAV-2. Different images of negatively stained AAV-2 capsid particles present in an AAV-2 virus preparation were compared with empty capsids formed in Sf9 cells after coinfection with recombinant baculoviruses expressing VP1 and VP2 (c), VP2 and VP3 (d), and VP1, VP2, and VP3 (e). Arrows in panel a indicate ringlike structures which probably represent empty capsids, some of which are shown in details in panel b. Specimens were negatively stained with uranyl acetate. Bars correspond to 100 nm in panel a and 40 nm in panels b through e.

pronounced in the case of VP1 expression (Fig. 5, lane 2). Since these expression products did not interfere with our conclusions drawn from the subcellular localization data, we did not investigate their identities any further. While VP1 and VP2 efficiently accumulated in the cell nucleus (Fig. 6a and b), VP3 was distributed throughout the nucleus and cytoplasm and seemed in some cases even partially excluded from the nucleus (Fig. 6c). In about 25% of VP3-positive cells, we observed a granular staining in the cytoplasm (Fig. 6c, inset). The nuclear staining of VP1 and VP2 is rather homogeneous and not in the clusters observed in HeLa cells infected with AAV-2 and adenovirus (17). In the presence of coexpressed VP1 or VP2 or both, VP3 disappears from the cytoplasm, suggesting that it is accumulated in the nucleus (Fig. 7). This assumption is supported by Western blot analysis, which shows that VP3 is not degraded (Fig. 5, lanes 6, 7, and 8). Especially when VP1 and VP3 are coexpressed, VP3 is more abundant in the cells than VP1 (Fig. 5, lane 6); thus, it should be possible to detect cytoplasmic staining of VP3 if the protein is not transported into the nucleus (Fig. 7b). This result suggests that complexing of VP1 and/or VP2 with the major capsid protein VP3 has to occur in order for VP3 to accumulate in the nucleus, the site of viral DNA packaging.

DISCUSSION

Assembly of AAV-2 VLPs in insect cells. The three capsid proteins of AAV-2 are encoded by overlapping reading

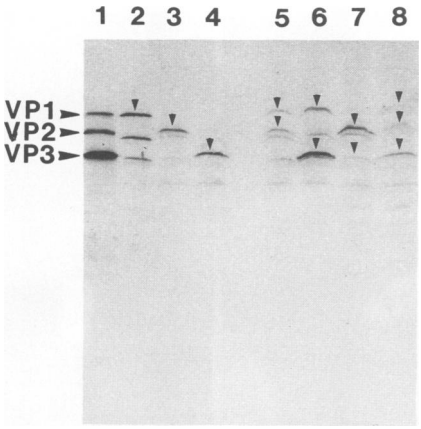

FIG. 5. Western blot analysis of AAV-2 capsid protein expression in HeLa cells under the control of the HCMV immediate-early promoter-enhancer. Lane 1 shows electrophoretic positions of VP1, VP2, and VP3 polypeptides present in an extract of HeLa cells coinfected with AAV-2 and adenovirus-2 after separation by SDS-PAGE and detection with an anti-VP3 serum. VP1, VP2, and VP3 individually expressed in HeLa cells under the control of the HCMV immediate-early promoter are shown in lanes 2, 3, and 4, respectively. Expressed capsid proteins of correct sizes are indicated by arrowheads, whereas lower-molecular-weight products which are also recognized by the antiserum are not marked. Coexpression of VP1 and VP2 (lane 5), VP1 and VP3 (lane 6), VP2 and VP3 (lane 7), and VP1, VP2, and VP3 (lane 8) are also shown.

frames, resulting in obligatory coexpression of the three proteins in the wild-type situation. In order to achieve separate expression of single capsid proteins, we mutated the internal translation initiation sites of VP2 and VP3. This resulted in a change of two amino acids in VP1 (T 138 to A, and M 202 to L) and two amino acids in VP2 (T 1 to M, and M 64 to L). The amino acid sequence of VP3 was not altered. By choosing conservative substitutions, we tried to minimize the influence on the capsid protein structure and capsid assembly. Using these mutant open reading frames, we expressed the three capsid proteins in all possible combinations by recombinant baculoviruses. Unexpectedly, whenever VP2 was expressed, we observed formation of VLPs, whereas in cells expressing VP1 or VP3 or both, we did not detect any viruslike structures. There may be several reasons for these occurrences. It is possible that particles are formed by VP1 and/or VP3 but are not stable in the presence of the detergents used during the particle preparation procedure. Alternatively, the particles might not be solubilized by 0.1% SDS and 1% DOC. Our Western blot control does not distinguish between these two possibilities. A number of alternative procedures for solubilizing the expressed capsid proteins, including VP2, in a native state have failed so far (data not shown). Since both VP1 and VP3 assembled into VLPs in the presence of VP2, we tend to favor the interpretation that VP2 somehow promotes capsid assembly, perhaps by preventing VP1 and VP3 from forming insoluble aggregates. However, we cannot completely rule out the possibility that VP2 only facilitates the accessibility of the assembled viruslike structures under the conditions used in this study.

We also cannot definitively state that VP2 alone or VP1 and VP2 alone formed the capsidlike structures isolated from insect cells expressing these proteins. In all particle preparations from cells which should not express VP3, we detected polypeptides in the molecular weight range of VP3 that cross-reacted with our antiserum. The possibility of a contribution of these molecules to particle formation cannot be excluded, because the presence of VP3 subspecies has also been observed in purified virions and empty capsids

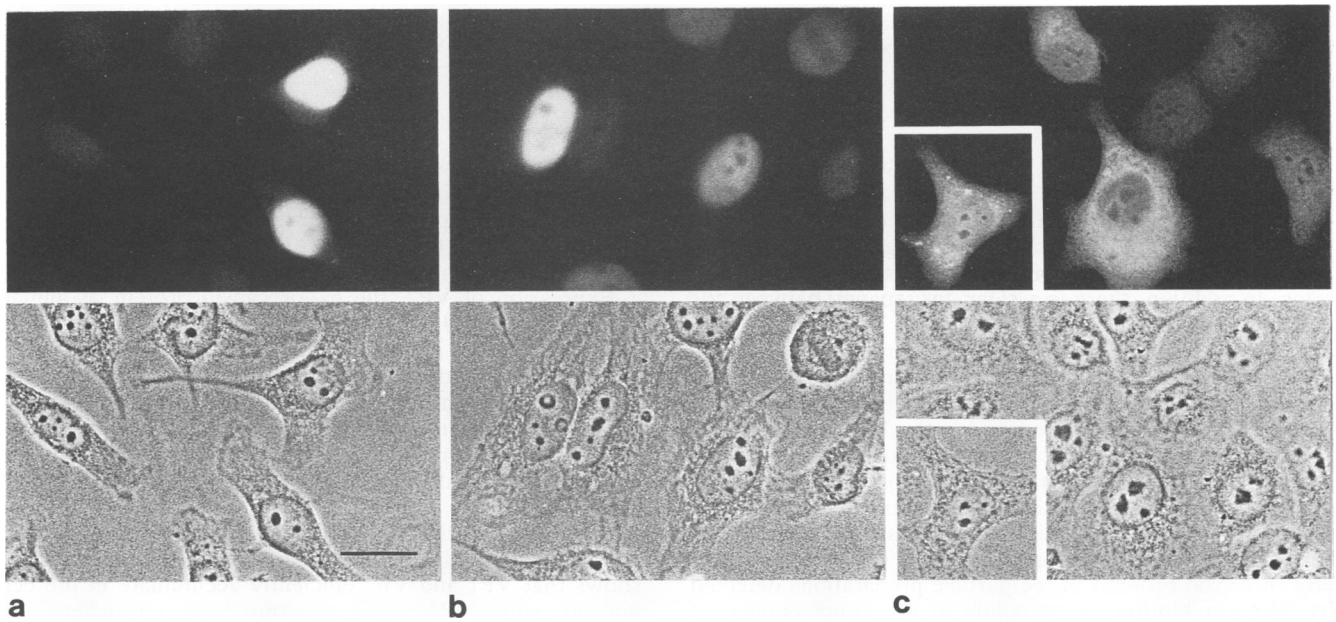

FIG. 6. Subcellular localization of individually expressed AAV-2 capsid proteins in HeLa cells. Plasmids expressing VP1, VP2, or VP3 (Fig. 1) under the control of the HCMV immediate-early promoter-enhancer were transfected into HeLa cells. The subcellular distributions of VP1 (a), VP2 (b), and VP3 (c) were analyzed by indirect immunofluorescence with an anti-VP3 serum. The corresponding phase-contrast pictures are shown in each panel. Bar in panel a corresponds to 20 μm.

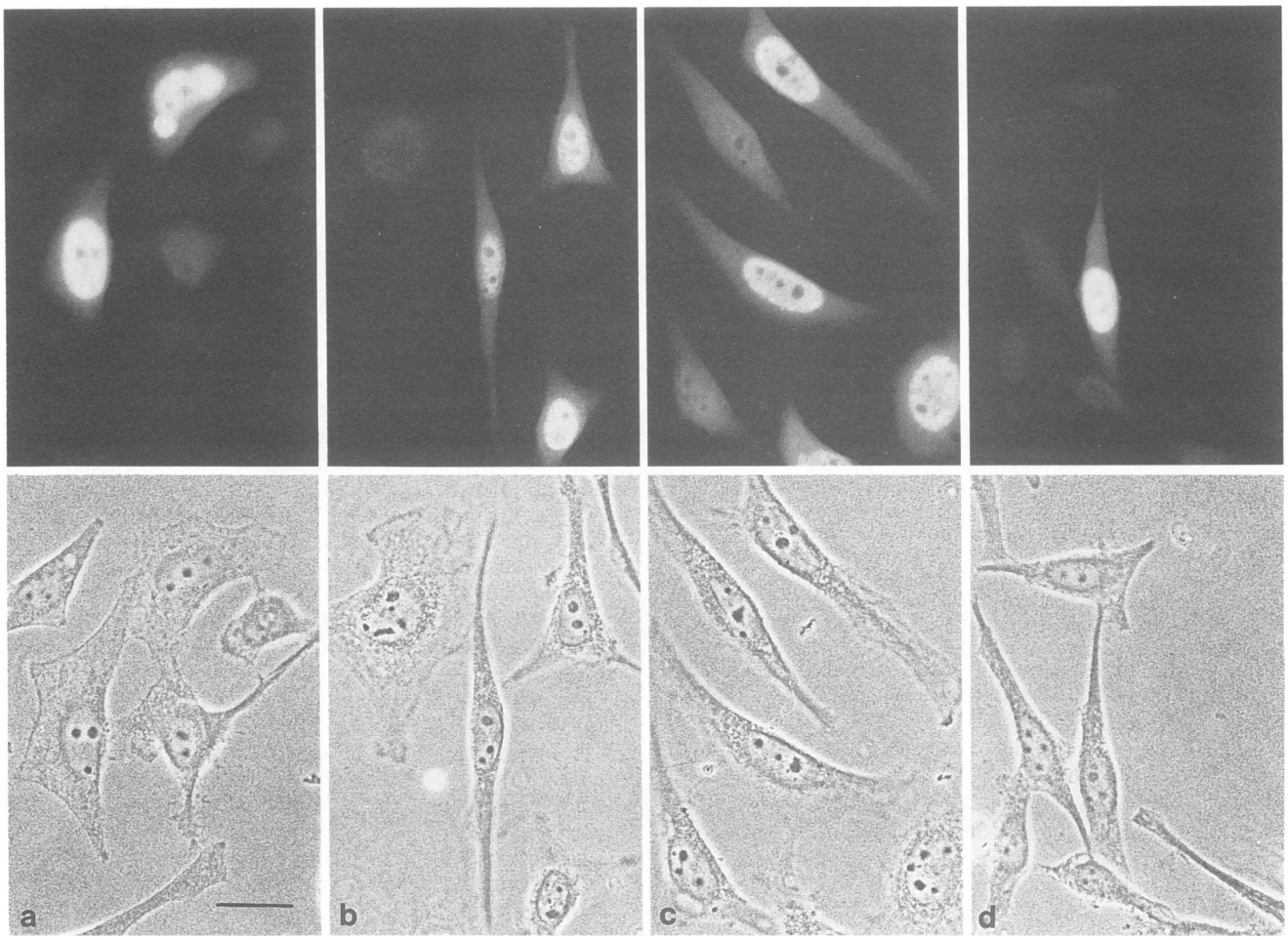

FIG. 7. Coexpression of VP1 or VP2 with VP3 leads to nuclear accumulation of VP3. Various combinations of plasmids for individual expression of VP1, VP2, or VP3 under the control of the HCMV immediate-early promoter-enhancer were cotransfected into HeLa cells. Nuclear accumulation of capsid proteins was analyzed by indirect immunofluorescence with an anti-VP3 serum. (a) Coexpression of VP1 and VP2; (b) coexpression of VP1 and VP3; (c) coexpression of VP2 and VP3; (d) coexpression of VP1, VP2, and VP3. Bar in panel a corresponds to 20 μm.

(28). The nature of these N- and/or C-terminal-truncated VP1 or VP2 molecules has not yet been clarified.

Preparations of VLPs from insect cells infected with different combinations of recombinant baculoviruses showed different relative proportions of the expressed proteins. In addition, there is a clear discrepancy between the stoichiometry of the capsid proteins in VLPs from insect cells and in purified wild-type AAV-2 virions. Whereas the ratio of the capsid proteins VP1, VP2, and VP3 is approximately 1:1:10 in infectious AAV particles (5), the relative amount of VP3 is always reduced in the VLP preparations. In principle, this difference may be explained by the lack of DNA in the VLP preparations. It is more plausible, however, that the capsid protein stoichiometry is controlled by the supply of capsid proteins for the assembly reaction. It cannot be deduced from our experiments that the stoichiometry of capsid proteins in the particle preparations detected by Western blotting corresponds to the stoichiometry of capsid proteins incorporated into empty capsids. It is difficult to correlate a quantitatively representative protein analysis of a fraction by SDS-PAGE with a rather selective visualization of structures in this fraction by electron microscopy. Nevertheless, in spite of the possible differences at the molecular level, the structures of VLPs and empty capsids found in AAV preparations are strikingly similar. This result also confirms that the mutations introduced for single capsid protein expression do not prevent capsid assembly.

Expression of VP2, the major capsid protein of human parvovirus B19 (4, 20) and canine parvovirus (34), led to particle formation in insect cells. This contrasts with the fact that we could not detect AAV VLPs in cells expressing VP3, the major capsid protein of AAV-2. On the other hand, it is also the expression of VP2, the middle-sized capsid protein, which allows formation of empty-capsid-like structures of AAV-2, although it represents a minor structural component in this virus.

Subcellular localization of capsid proteins. Immunolocalization of individually expressed capsid proteins clearly shows that VP1 and VP2 efficiently accumulate in the cell nucleus, while VP3 equilibrates only between nucleus and cytoplasm. This result is remarkable, because VP3 is too large to passively diffuse through nuclear pores (10). On the other hand, VP3 has no basic sequences which could allow selective entry into the nucleus. In about 25% of the cells

transfected by VP3, we observed a granular appearance of VP3 in the cytoplasm that indicates aggregation with itself or with cytoplasmic structures.

Coexpression of VP1 or VP2 with VP3 led to the disappearance of VP3 from the cytoplasm and also of the granular structures. Since VP3 is not degraded in coexpression experiments, one has to assume that it is accumulated in the nucleus either by cotransport or by trapping within the nucleus due to complex formation with VP1 or VP2. Cotransport of polypeptides lacking a nuclear localization signal by complex formation with polypeptides which actively accumulated in the nucleus was implicated in several reports (29, 46, 48).

The AAV-2 capsid proteins VP1 and VP2 contain several basic amino acid stretches with some similarity to the nuclear localization signal of the simian virus 40 large-T antigen (PKKKRKV; 12, 21, 22, 25, 26). However, they are shorter and separated from each other by sequences of different lengths. VP1, for example, contains the sequence AKKRV (positions 121 to 125), and the overlapping open reading frame of VP1 and VP2 contains the motif GKKRP (positions 141 to 145) and the motif ARKRL (positions 167 to 171). For several viral and cellular nuclear proteins, bipartite nuclear localization signals with such short basic stretches of amino acids have been described (23, 31, 33, 47).

In a recent publication, Hunter and Samulski (17) showed that the AAV structural proteins concentrate in nuclear subcompartments when cells are coinfected with AAV-2 and adenovirus 2. This additional compartmentalization obviously requires further protein-protein and/or protein-DNA interactions which are not provided in uninfected cells.

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