

Assembly of adeno-associated virus type 2 capsids *in vitro*

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Capsid proteins VP1, VP2 and VP3 of adeno-associated virus type 2 (AAV-2) were separately expressed by recombinant baculoviruses, purified under denaturing conditions and renatured in the presence of 0.5 M arginine, followed by dialysis against buffers of physiological ionic strength. At a protein concentration of 0.05 mg/ml, the three capsid proteins predominantly formed monomers and, to a lesser extent, oligomers, as determined by sedimentation analysis. Oligomerization increased at higher protein concentrations. The capsid protein oligomers consisted of globular, non-capsid-like structures, as detected by electron microscopy. Addition of a HeLa cell extract significantly stimulated oligomerization of the capsid proteins, probably due to interactions with HeLa cell proteins. Characterization of structures sedimenting around

60S by immunoprecipitation and electron microscopy showed that, in addition to other aggregates, empty capsid-like structures were formed *in vitro*. The identity of these structures as empty AAV capsids was verified by immunoelectron microscopy. Analysis of capsid formation in HeLa cells by transfection of VP expression constructs allowing separate expression of VP1, VP2 and VP3 showed that they were able to form capsids, although with a reduced efficiency as compared to VP proteins expressed from the wt cap gene. This finding suggests that the mutations introduced to allow separate capsid protein expression reduced the efficiency of capsid assembly *in vivo* and might also explain the reduced recovery of empty capsids employing the *in vitro* assembly procedure.

Introduction

Adeno-associated virus type 2 (AAV-2) contains a single-stranded DNA genome of 4.6 kb packaged into an icosahedral capsid 20–24 nm in diameter (Srivastava *et al.*, 1983; Hoggan, 1970; Arella *et al.*, 1990). The capsid is composed of three capsid proteins VP1, VP2 and VP3 with molecular masses of 90 kDa, 72 kDa and 60 kDa, respectively (Rose *et al.*, 1971; Buller & Rose, 1978; Johnson, 1984). The capsid proteins are expressed from one overlapping ORF which is transcribed from the AAV p40 promoter located at map unit 40. In order to generate the three proteins, a 2.6 kb transcript from the p40 promoter is spliced into two 2.3 kb mRNAs using the same splice donor site but different splice acceptor sites (Becerra *et*

al., 1988; Cassinotti *et al.*, 1988; Trempe & Carter, 1988). These sites are used with different frequencies, resulting in a minor and a major spliced p40 mRNA fraction. The initiation codon for VP1 is present only in the unspliced and the minor spliced mRNA, whereas the initiation codons for VP2 and VP3 are present on both spliced mRNAs and the unspliced mRNA. VP3 is efficiently translated from an AUG initiation site at position 2809, whereas VP2 is less efficiently initiated at an ACG codon at position 2614. This difference in translational initiation frequency, together with the relative abundance of the major and minor spliced mRNAs, leads to the synthesis of VP1, VP2 and VP3 in a relative ratio of about 1:1:10 (Muralidhar *et al.*, 1994). Since splicing of AAV mRNA is influenced by the helper virus, for example adenovirus, coinfection with a helper virus also has an influence on the stoichiometry of the capsid proteins (Trempe & Carter, 1988). At the protein sequence level, the three capsid proteins differ only in their N termini due to the alternative initiation sites. They terminate at a common site which is 27 amino acids further downstream and within a different ORF to that originally proposed in the AAV sequence (Srivastava *et al.*,

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1983; Ruffing *et al.*, 1994). These additional C-terminal amino acids are required for capsid formation (Ruffing *et al.*, 1994).

Studies on the assembly of AAV indicated a two-step process in which empty particles are first formed in a rapid reaction and subsequently become packaged with the single-stranded genome in a rather slow process (Myers & Carter, 1980). VP2 and VP3 are sufficient for accumulation and sequestration of single-stranded progeny DNA, suggesting that they are also able to form capsids (Smuda & Carter, 1991; Myers & Carter, 1981). VP1, however, is required for production of infectious particles (Hermonat *et al.*, 1984; Tratschin *et al.*, 1984; Smuda & Carter, 1991). Transfection studies with AAV genomes containing single mutated initiation codons are consistent with these findings (Muralidhar *et al.*, 1994).

In order to produce empty particles on a preparative scale sufficient for use in packaging AAV DNA *in vitro*, we expressed the AAV capsid proteins in insect cells using baculovirus expression vectors (Ruffing *et al.*, 1992), allowing separate expression of VP1, VP2 or VP3. Separate expression was achieved by mutating the initiation codons for VP2 and VP3. Coexpression of several combinations of capsid proteins led to the formation of empty capsid-like particles. However, none of these particles contained the capsid proteins in the desired stoichiometry. This demonstrates that the stoichiometry of the capsid proteins in the virions is not determined by the assembly process. We therefore started to work out conditions for *in vitro* assembly of capsids in which we could adjust the desired capsid protein ratios.

Methods

■ **Cell culture and virus infection.** *Spodoptera frugiperda* Sf9 cells were grown as suspension or monolayer cultures in TNMFH medium (Hink, 1970) supplemented with 10% foetal calf serum, 100 µg/ml gentamicin and 2 mM glutamine at 27 °C. For expression of capsid proteins, 2×10^8 Sf9 cells grown in suspension were pelleted and resuspended in recombinant baculovirus stocks with a m.o.i. of 2 to 20. Infection with recombinant baculovirus stocks for expression of VP1_{ex}, VP2_{ex} or VP3_{ex}, respectively, were performed in separate cultures (Ruffing *et al.*, 1992). After 1 h incubation at 27 °C, the suspension was transferred to a spinner flask, fresh medium was added to the initial volume of the suspension and culturing continued at 27 °C. Cells were harvested after 60 h by pelleting at 200 g for 10 min. The medium was removed and the cells were washed twice with PBS (140 mM NaCl, 6.5 mM Na₂HPO₄, 2.5 mM KCl, 1.5 mM KH₂PO₄ pH 7.4) and stored at -20 °C in aliquots of 10⁷ Sf9 cells per pellet.

AAV virus stocks were generated in HeLa cells as described by Wistuba *et al.* (1997). AAV was subsequently pelleted by centrifugation in a Sorvall SS 34 rotor for 3 h at 20000 r.p.m. The pellet was resuspended in 1% sodium deoxycholate containing 0.025% trypsin and then fractionated on a CsCl gradient according to published procedures (de la Maza & Carter, 1980). Fractions with a density of around 1.41 g/cm³ were collected and dialysed against 50 mM Tris pH 8.0 containing 0.3 M NaCl.

■ **Purification and renaturation of capsid proteins.** A pellet of approximately 10⁷ Sf9 cells, infected with recombinant baculoviruses

expressing VP1_{ex}, VP2_{ex} or VP3_{ex}, was thawed and resuspended in 3 ml NP40-buffer (1% NP40, 150 mM NaCl, 20 mM Tris pH 7.5, 15 mM β-mercaptoethanol, 5 mM Na EDTA). After sonication on ice with a Branson Sonifier (four times for 10 s at 1 min intervals, level 5) the suspension was centrifuged for 10 min at 3000 g, 4 °C. The supernatant contained very low amounts of soluble capsid proteins. The insoluble capsid proteins were resuspended in 2 ml NP40-buffer, sonicated and centrifuged under the same conditions. The pellet was sonicated in 2 ml DNase-buffer (50 mM Tris pH 7.5, 5 mM MgCl₂, 5 mM MnCl₂) twice for 10 s at 1 min intervals, level 5. One hundred µg DNase and 50 µg RNase A were then added and the mixture was incubated for 40 min at 37 °C. After centrifugation for 10 min at 3000 g, 4 °C, the insoluble pellet was resuspended in 2 ml NP40-buffer, sonicated (twice for 10 s at 1 min intervals, level 5) and again centrifuged. The pellet was finally dissolved in 200 µl urea-buffer (20 mM Tris pH 7.5, 5 mM EDTA, 5 mM DTT, 0.4 M NaCl, 8 M urea) and the tube was shaken for 30 min at room temperature. The solubilized pellet was added to 300 µl Q-Sepharose (washed twice before use with urea-buffer) and was again shaken for 30 min at room temperature. The capsid proteins could be recovered in the supernatant after centrifugation for 10 min at 17 600 g, 4 °C and were stored at -20 °C. Purity and capsid protein concentration was quantified by gel electrophoresis and staining with Coomassie blue in comparison to protein standards.

For renaturation, capsid proteins were thawed and 720 µg capsid protein in 100–250 µl of urea-buffer (VP1:VP2:VP3 = 1:1:10) was added to 10 ml arginine-buffer (0.5 M arginine, 2 mM EDTA, 0.1 M Tris pH 8) while shaking. After centrifugation for 10 min at 3000 g, 4 °C, the supernatant was dialysed against 10 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 2 mM MgCl₂, 5 mM mercaptoalcohol (TNEMM) and simultaneously concentrated by vacuum dialysis. The vacuum dialysis bag was washed with water, saturated for 30 min with 1% BSA in PBS and washed several times with water before use. After reduction of the volume to 3 ml, the dialysis buffer (350 ml) was changed and the sample was further concentrated to 2 ml with a capsid protein concentration of about 0.1–0.2 mg/ml. In order to achieve higher capsid protein concentrations, the sample was further concentrated by speed-vac centrifugation. For renaturation experiments with the chaperones GroEL and GroES, the proteins were purified as described by Buchner *et al.* (1991). Chaperones and capsid proteins were mixed in 1 M urea in molar ratios of 0.5:1 to 2:1. Capsid protein aggregation was monitored by measurement of light scattering in a Hitachi F 400 fluorimeter.

■ **Preparation of HeLa cell extracts.** A pellet of HeLa cells (5×10^9 cells), kept at -80 °C, was slowly thawed, carefully resuspended in 40 ml ice-cold PBS and centrifuged in four tubes for 3 min at 600 g, 4 °C. Each pellet was resuspended in 10 ml ice-cold, isotonic buffer (20 mM HEPES pH 7.8, 5 mM KCl, 1.5 mM MgCl₂, 1 mM DTT, 250 mM sucrose), centrifuged again for 3 min at 600 g, 4 °C, resuspended in 10 ml ice-cold, hypotonic buffer (20 mM HEPES pH 7.5, 5 mM KCl, 1.5 mM MgCl₂, 1 mM DTT) and incubated for 15 min at 4 °C. The cells were then homogenized by 10 strokes in a precooled type S Dounce homogenizer; most of the cells were broken as detected by microscopic examination. Large cellular debris was removed by centrifugation at 20000 g_{av} for 15 min at 4 °C. The supernatant was further cleared from subcellular particles by centrifugation at 100000 g_{av} for 1 h at 4 °C. The supernatant was shock-frozen in liquid nitrogen in aliquots, stored at -80 °C and used as 'cytoplasmic HeLa cell extract'.

The pellet of the 20000 g_{av} centrifugation step, containing nuclei and large cellular debris, was extracted with buffer B (50 mM HEPES pH 7.5, 10% sucrose, 0.4 mM EDTA, 0.3 mM PMSF, plus leupeptin and pepstatin added to final concentrations of 0.003 and 0.001 mg/ml,

respectively) containing 1 M NaCl for 1 h on ice and then centrifuged for 1 h at 100 000 g_{av} at 4 °C. The supernatant with the soluble nuclear fraction was dialysed against buffer C (20 mM HEPES pH 7.5, 1 mM DTT, 0.1 mM EDTA, 10% glycerin, 50 mM NaCl) and again centrifuged for 30 min at 27 000 g_{av} 4 °C. The soluble fraction was also shock-frozen, stored at -80 °C, and designated the 'nuclear HeLa cell extract' for the following experiments.

■ **In vitro assembly reactions.** Renatured capsid proteins (VP1:VP2:VP3 = 1:1:10) with a concentration of 0.1–0.4 mg/ml were incubated with 5 vols HeLa cell extract. The HeLa cell extract consisted of cytoplasmic and nuclear extract in a proportion of 1:1. In addition, ATP was added to a final concentration of 2 mM, PMSF to a final concentration of 1 mM, and pepstatin and leupeptin to concentrations of 0.0025 mg/ml and 0.00375 mg/ml, respectively. This mixture was directly dialysed and concentrated by vacuum dialysis against TNEMM-buffer until an initial concentration of renatured capsid proteins of 0.1–0.4 mg/ml was achieved. For comparison with this assembly reaction, the renatured capsid proteins of the same concentration (0.1–0.4 mg/ml) were fivefold diluted in a protein mixture of aldolase (1.46 mg/ml), catalase (0.43 mg/ml) and thyroglobulin (0.33 mg/ml), incubated with ATP and protease inhibitors and reconcentrated to the initial capsid protein concentration as described above. For dialysis against 100 ml of different buffers, 0.025 ml aliquots of renatured capsid proteins with a protein concentration of 0.1 mg/ml were dialysed as drops on a membrane filter (Millipore; type VM). Four different buffers were tested: 10 mM Tris pH 7.5 and 1 mM EDTA (TE), TNEMM, TNEMM containing 10 mM EDTA and TNEMM containing 2 mM $CaCl_2$. After 2 h dialysis, the drops on the filters were removed and analysed by electron microscopy. Capsid proteins at a concentration of 0.4 mg/ml and a 1:1:10 stoichiometry were also dialysed in micro collodium bags (Sartorius) against 10 mM Tris pH 7.2 supplemented with (a) 1 mM EDTA, 150 mM NaCl, 15 mM β -mercaptoethanol, 5% glycerol or (b) 2 M ammonium sulfate, 5% glycerol or (c) 0.5 mM $CaCl_2$, 5% glycerol. Alternatively, 10 mM Tris pH 8.5 or 10 mM sodium acetate pH 5.0 were used with the same supplements. After 72 h dialysis at room temperature, samples were taken and prepared for electron microscopy.

■ **Sucrose gradients.** Samples of up to 0.6 ml of the *in vitro* assembly reactions were loaded onto a 10 ml sucrose gradient (5–30% sucrose in TNEMM) and centrifuged for 2 h at 160 000 g_{av} 4 °C in a swing-out rotor. Fractions of 0.5 or 1 ml volume were collected and used for immunoprecipitation or were precipitated with 20% (final concentration) trichloroacetic acid (TCA) and 10 μ g RNase A as precipitation aid for SDS-PAGE and Western blot analysis. Reference proteins were run on a parallel sucrose gradient: BSA (6.5S), thyroglobulin (19S), recombinant empty AAV VP2/3 capsids (60S) and purified AAV particles (110S).

■ **Western blot analysis and immunoprecipitation experiments.** TCA-precipitated proteins were analysed by gel electrophoresis on polyacrylamide gels in the presence of SDS (Thomas & Kornberg, 1975) as described previously (Wistuba *et al.*, 1995). Proteins were electrophoretically transferred to nitrocellulose membranes by standard procedures (Towbin *et al.*, 1979) and Western blots were developed using the MAb B1 (Wistuba *et al.*, 1995) according to published methods (Harlow & Lane, 1988).

Immunoprecipitations were performed as described elsewhere (Wistuba *et al.*, 1995) using monoclonal antibodies directed against AAV capsids (A20) or against capsid proteins (A69/A1) (Wistuba *et al.*, 1997).

■ **Transfection and immunofluorescence analysis.** For immunofluorescence, HeLa cells were transfected according to published

procedures (Chen & Okayama, 1987, 1988) and processed for immunofluorescence as described by Wistuba *et al.* (1997).

■ **Preparation of empty AAV capsids.** HeLa cells (5×10^7) were transfected with a plasmid containing the AAV cap gene under the control of the CMV promoter (pCMV-VP) and infected with adenovirus-2 as described above. Twenty hours post-infection the cells were harvested, washed with PBS, resuspended in 1 ml PBS with 0.004 mg/ml leupeptin, 0.002 mg/ml pepstatin, 0.4 mM PMSF and sonified on ice with a Branson sonifier three times for 10 s at 1 min intervals at level 5. The pellet obtained by a further centrifugation step (10 min, 16 500 g , 4 °C) was resuspended in 1 ml buffer D (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 5 mM $MgCl_2$) and digested for 3 h at 12 °C with RNase A and DNase at final concentrations of 0.025 mg/ml and 0.05 mg/ml, respectively. The digested extract was adjusted to 0.5 M NaCl, 10 mM DDT, 2 mM EDTA. The extract was then sonified again (as described above), shaken for 30 min at room temperature and centrifuged for 20 min at 3000 g , 4 °C. The supernatant (about 1 ml) was loaded onto a double sucrose cushion of 200 μ l 50% and 200 μ l 30% sucrose in TE and centrifuged for 2.5 h at 130 000 g_{av} 4 °C. The pellet was resuspended for 30 min at room temperature in 300 μ l TE containing 0.1 M NaCl and again pelleted for 1.5 h at 350 000 g_{av} 4 °C, to remove the sucrose completely. The pellet was resuspended in 200 μ l TE containing 0.3 M NaCl and agitated for 15 h at 4 °C. After centrifugation for 10 min at 16 500 g , 4 °C to get rid of aggregated material, the supernatant was used for negative staining.

■ **Electron microscopy.** A 4 ml aliquot of the fractions sedimenting at around 60S was further purified through a double sucrose cushion of 3 ml 50% sucrose and 3 ml 30% sucrose in TE (10 mM Tris pH 7.5, 1 mM EDTA) by centrifugation for 6.5 h at 130 000 g_{av} 4 °C in a swing-out rotor. The pellet was resuspended in 500 μ l TE containing 0.1 M NaCl, incubated for 1 h at room temperature and again pelleted by centrifugation for 1.5 h at 350 000 g_{av} 4 °C. The pellet was resuspended in 50 μ l TE containing 0.3 M NaCl. For electron microscopic analysis, 10 μ l of the purified sample was negatively stained with 2% aqueous uranyl acetate. Immunostaining of the particles was performed on glow-discharged 300 mesh copper grids by incubating 10 μ l of the purified 60S fraction on the grids for 2 min at room temperature. After washing three times with PBS for 5 min at room temperature, the grids were incubated face down on a 20 μ l drop containing purified mouse MAb (A20; Wistuba *et al.*, 1997) diluted 1:10 in PBS for 30 min at room temperature followed by a further wash with PBS. The specimens were then incubated face down on a 20 μ l drop containing the gold-coupled secondary antibody diluted 1:10 in PBS (Sigma) for 20 min at room temperature. After a further PBS wash step, the specimens were negatively stained with 2% aqueous uranyl acetate and examined by electron microscopy. Electron micrographs were taken with a Zeiss EM 10 electron microscope at 80 kV.

Results

Expression, purification and renaturation of capsid proteins

Capsid proteins VP1, VP2 and VP3 of AAV-2 were expressed separately in insect cells using recombinant baculoviruses as described previously (Ruffing *et al.*, 1992). In order to allow separate expression of these viral proteins and hence the manipulation of their stoichiometry *in vitro*, the initiation codons for VP2 and VP3 were mutated within the expression

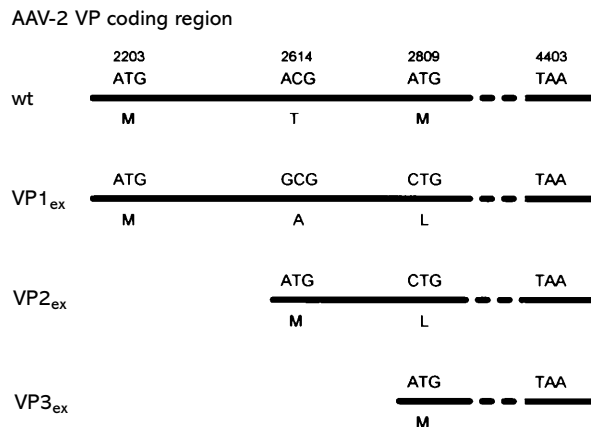


Fig. 1. Comparison of wt and mutant capsid protein coding regions used for protein expression. Translational initiation codons of the overlapping ORF for VP1, VP2 and VP3 code for M, T and M in the wt genome. For separate expression of VP1, VP2 or VP3 these initiation codons were mutated and cloned in different expression vectors (VP1_{ex}, VP2_{ex}, VP3_{ex}; Ruffing *et al.*, 1992). Translational termination occurs in all constructs at position 4403 of the corrected VP ORF nucleotide sequence (Ruffing *et al.*, 1994).

constructs for VP1 and VP2 to prevent coexpression of the overlapping lower molecular mass capsid proteins (Fig. 1). More than 90% of the expressed proteins accumulated in insoluble aggregates within the Sf9 cells. This property was used to selectively remove the cellular and baculovirus proteins from the capsid proteins with a buffer containing 1% NP40, which resulted in capsid protein preparations of about 70–90% purity. Since such preparations contained significant amounts of short nucleic acids, we included a DNaseI/RNase digestion step, followed by adsorption of the released nucleotides and oligonucleotides on Q-Sepharose in the presence of 8 M urea and 0.4 M NaCl. The capsid proteins were solubilized under these conditions and did not bind to the Q-Sepharose. Such capsid protein preparations showed a purity of more than 90% and contained less than 1% nucleic acids (Fig. 2*a, b*). Although the same mutations were introduced at the initiation codon of VP3 for separate expression of VP1 or VP2, in VP2 preparations we always observed a polypeptide migrating at the position of VP3, which was missing in VP1 preparations (Fig. 2*b*). We assume that this is due to proteolytic processing taking place on VP2 but not on VP1 based on the different N-termini of the two proteins.

Dilution of the capsid proteins solubilized in 8 M urea in buffers without detergents led to irreversible denaturation and precipitation. Also, stepwise dialysis against buffers with lower urea concentration under different reducing conditions (Rudolph, 1990) always resulted in precipitation of the capsid proteins when the urea was removed in the final dialysis step. Addition of purified chaperones GroEL and GroES to the renaturation buffer in the presence of 1 M urea also failed to renature the capsid proteins. Finally, we diluted the solubilized capsid proteins (in 8 M urea) directly into a buffer containing

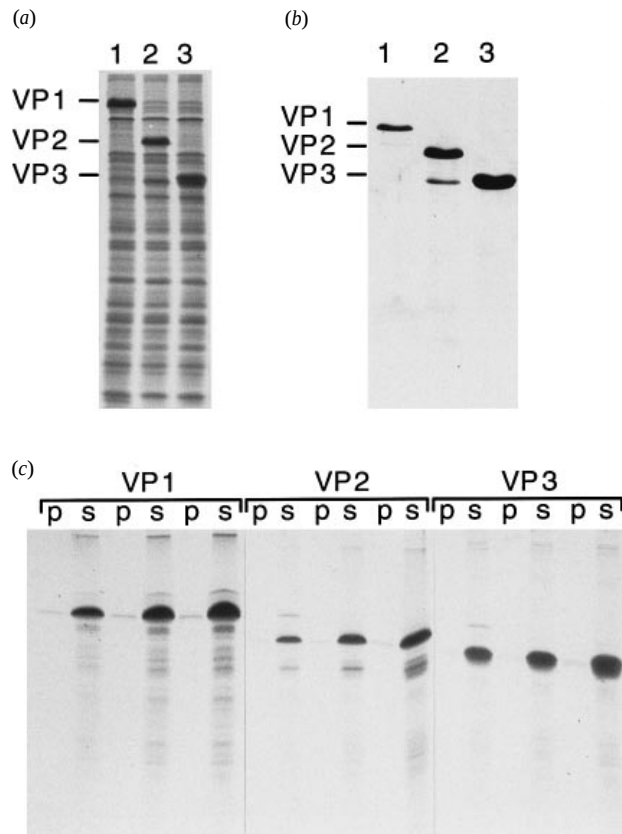


Fig. 2. Expression, purification and renaturation of capsid proteins. (a) Total cell lysates of insect cells infected with VP1_{ex} (lane 1), VP2_{ex} (lane 2) or VP3_{ex} (lane 3) recombinant baculovirus stocks were analysed by SDS-PAGE. (b) Purified capsid proteins as recovered in 8 M urea. (c) In the first renaturation step, the denatured capsid proteins were diluted into a buffer containing 0.5 M arginine. Soluble (s) and pelleted (p) material of increasing concentrations of VP1 (2.5–10 µg), VP2 (1–4 µg) and VP3 (2.5–10 µg) were analysed by SDS-PAGE. Marker proteins were myosin (210 kDa), β -galactosidase (115 kDa), bovine serum albumin (68 kDa), actin (43 kDa) and carbonic anhydrase (30 kDa). Proteins were stained with Coomassie blue.

0.5 M arginine (Buchner & Rudolph, 1991). After this treatment, the capsid proteins remained completely soluble up to a concentration of 0.1 mg/ml, as shown separately for VP1, VP2 and VP3 (Fig. 2*c*). At higher concentrations, we observed some precipitation of the capsid proteins. After dialysis against buffers of high or approximately physiological ionic strength, the capsid proteins remained soluble at low protein concentrations, whereas at low ionic strength the proteins partly precipitated (data not shown).

Since higher protein concentrations were desirable for *in vitro* assembly studies, we tried to reconcentrate the capsid proteins using a number of concentration techniques. The method of choice finally turned out to be vacuum dialysis, in which we simultaneously dialysed the arginine out and concentrated the sample. Despite major losses of capsid proteins into the dialysis bag, we routinely achieved concentrations of 0.1–0.2 mg/ml at a final arginine concentration of

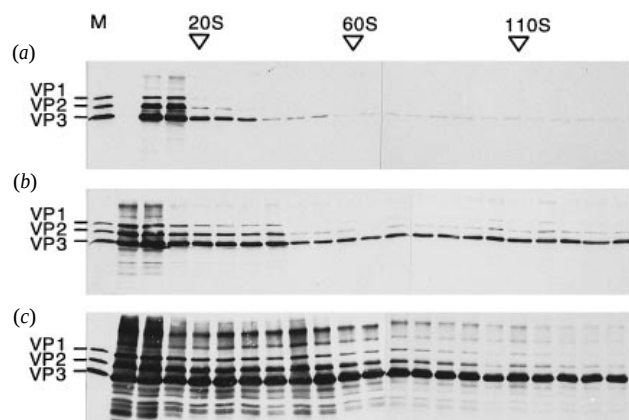


Fig. 3. Sedimentation analysis of capsid proteins renatured at a stoichiometry of 1:1:10 for VP1:VP2:VP3. Three different concentrations, (a) 0.06 mg/ml, (b) 0.124 mg/ml and (c) 0.46 mg/ml, of renatured capsid proteins were analysed by sucrose density gradient centrifugation (2 h at 160000 g_{av} , 4 °C in a swinging-bucket rotor). The gradients were fractionated, capsid proteins were precipitated with TCA, separated by SDS-PAGE and detected by Western blotting using the MAb B1 (Wistuba *et al.*, 1995). Western blots were developed by a secondary phosphatase-coupled antibody. Sucrose gradients were calibrated by sedimentation of thyroglobulin (19S), empty AAV VP2/3 capsids (60S) and purified AAV virus (110S) in parallel gradients.

0.03 mM. Further concentration by vacuum dialysis or by microseparation devices (Centriprep) was always associated with high losses of capsid proteins. For experiments with higher capsid protein concentrations, further concentration was achieved by speed-vac centrifugation. In this fashion, concentrations of up to 1 mg/ml could be obtained without any protein precipitation. When, however, such concentrated capsid protein solutions were dialysed against buffers of lower salt concentration, protein was invariably lost by precipitation.

Assembly studies with purified capsid proteins

Sucrose gradient centrifugation was employed to characterize the oligomerization status of the renatured capsid proteins either separately or after mixing in a stoichiometry of 1:1:10. At a protein concentration of about 0.05 mg/ml, the separate capsid proteins, as well as the mixture of VP1, VP2 and VP3, sedimented predominantly at the monomer position around 6.5S. Small amounts of oligomers of up to 110S and more, but without a defined peak, were also detectable by Western blotting. Sedimentation analysis of the capsid proteins at increased protein concentrations of 0.124 mg/ml and 0.46 mg/ml showed larger amounts of capsid proteins in the oligomerized state. However, the overall sedimentation pattern did not change and no defined size class of capsid protein oligomers could be detected (Fig. 3). This result suggests that the capsid proteins are able to oligomerize, but that they do not form defined assembly products such as capsomers or empty capsids in quantities which could be detected by this sedimentation analysis. This interpretation was confirmed by electron microscopy (data not shown). The capsid proteins

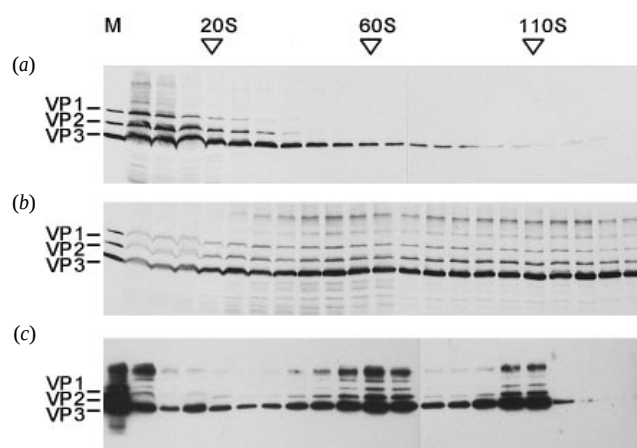


Fig. 4. Influence of a HeLa cell extract on the sedimentation profile of renatured capsid proteins. Renatured capsid proteins with a concentration of 0.4 mg/ml were incubated under assembly conditions (see Methods) with a mixture of aldolase, catalase and thyroglobulin (a) or with HeLa cell extract (b), and analysed by sucrose density gradient centrifugation as outlined in Fig. 3. The sedimentation positions of empty and full AAV virions (60S and 110S) were determined on a parallel sucrose gradient of an AAV preparation (c).

showed a strong tendency to precipitate at protein concentrations above 0.2 mg/ml. Even under a variety of ionic and pH conditions no indication of capsid formation was observed at concentrations of 0.1–0.2 mg/ml.

Assembly studies with purified capsid proteins in the presence of a HeLa cell extract

Since capsid protein oligomerization is a prerequisite for capsid formation, we studied the influence of a HeLa cell extract on the oligomerization of the three capsid proteins as separate proteins or in combination. As shown in Fig. 4, the sedimentation profile of the capsid proteins was significantly changed when they were incubated with a HeLa cell extract (Fig. 4b) as compared to capsid proteins incubated with three randomly selected proteins (aldolase, catalase and thyroglobulin) (Fig. 4a). Either the capsid protein oligomerization was stimulated or the capsid proteins associated with cellular proteins or both, thereby forming complexes or oligomers sedimenting in the range 20S to more than 110S. The increase in the S-values of the capsid proteins incubated with the HeLa cell extract was independent of the presence of ATP or of Ca^{2+} ions. When the influence of the HeLa cell extract on the single capsid proteins was analysed, the sedimentation pattern of all three capsid proteins was found to have shifted towards increased oligomer formation (data not shown). Variation in the ratio of HeLa cell extract to capsid proteins from 1:1 to 5:1 or increase of the capsid protein concentration from 0.1 to 0.4 mg/ml had no detectable effect on capsid protein oligomerization.

We further analysed the renatured capsid proteins either mixed with aldolase, catalase and thyroglobulin or with HeLa

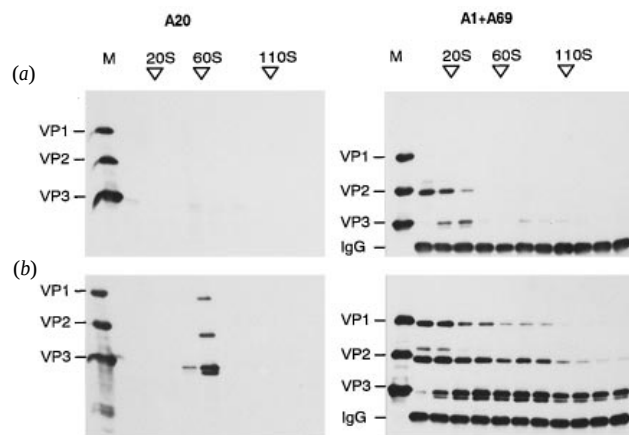


Fig. 5. Immunoprecipitation of assembly products. Renatured capsid proteins with a concentration of 0.124 mg/ml were incubated under assembly conditions (see Methods) with a mixture of aldolase, catalase and thyroglobulin (a) or with HeLa cell extract (b), fractionated on sucrose gradients and analysed by immunoprecipitation and SDS-PAGE. For specific precipitation of capsids, mouse MAb A20 was used, which recognizes empty or DNA-filled AAV capsids but not free capsid proteins (Wistuba *et al.*, 1997). MABs A1 and A69 recognize VP1 and VP1 plus VP2, respectively, preferentially in the non-assembled state (Wistuba *et al.*, 1997). The precipitated proteins were visualized by Western blot analysis using MAb B1, which reacts with all three capsid proteins. The blots were developed with a peroxidase-coupled secondary antibody and enhanced chemoluminescence.

cell extract by immunoprecipitation of every second fraction from the sucrose gradients (Fig. 5). For precipitation, we used a MAb (A20), which according to several criteria reacts with AAV capsids but not with free capsid proteins (Wistuba *et al.*, 1997). For comparison, we also precipitated the capsid proteins with a mixture of two monoclonal antibodies (A1 and A69), which specifically recognize VP1 and VP2 and preferentially precipitate non-assembled capsid proteins (Wistuba *et al.*, 1997). The precipitated proteins were detected by Western blotting with another MAb (B1) recognizing all three capsid proteins. Immunoprecipitation of the capsid proteins from sucrose gradient fractions without incubation with the HeLa cell extract showed no precipitate with the A20 antibody indicating that capsids had not been formed under these conditions (Fig. 5a). Precipitation with the A1/A69 monoclonal antibodies showed that, in the monomer range, no VP3 was precipitated together with VP2, whereas in the sedimentation range of VP oligomers, some VP3 was also coprecipitated. This suggests that not only were homooligomers of VP proteins present under these conditions, but also heterocomplexes of VP1 and/or VP2 with VP3. VP1 was only weakly recovered in this particular experiment for unknown reasons. Precipitation of capsid proteins incubated with the HeLa cell extract with the A20 antibody showed only precipitates sedimenting around 60S containing all the capsid proteins, although capsid proteins were distributed throughout the gradient as shown by immunoprecipitation with the A1/A69 monoclonal antibodies (Fig. 5b). This result suggests

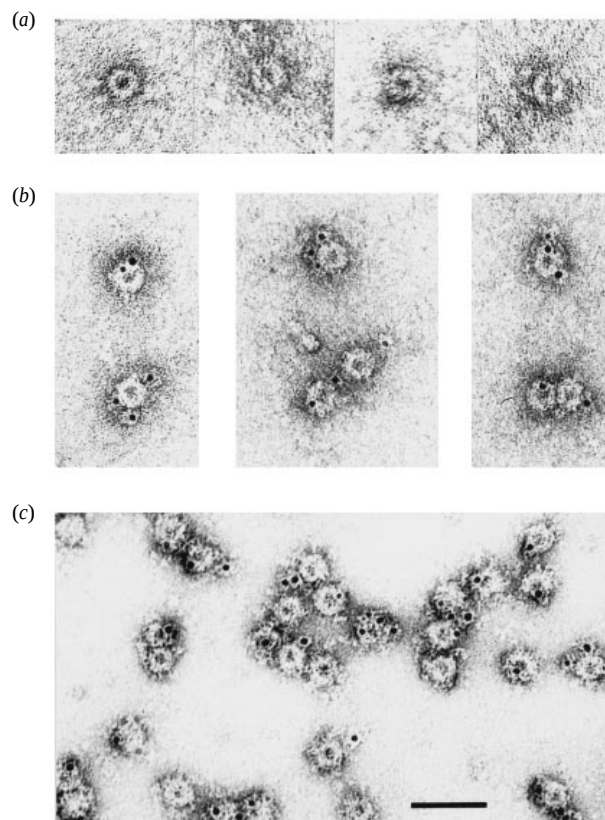


Fig. 6. Electron microscopic analysis of assembled empty capsids. Renatured capsid proteins with a concentration of 0.2 mg/ml were further concentrated to 0.4 mg/ml by speed-vac centrifugation and then incubated with HeLa cell extract under assembly conditions (see Methods). After fractionation by sucrose gradient centrifugation, 60S fractions were pooled and further purified by pelleting through a double sucrose cushion. The pelleted material was resuspended and examined in the electron microscope after negative staining (a). For identification of AAV capsid proteins, the purified material was first reacted with the MAb A20, followed by a gold-coupled secondary antibody before negative staining (b). For controls, empty AAV capsids were prepared from HeLa cells transfected with an AAV cap gene under the control of the CMV promoter and labelled by immunogold staining after incubation with the MAb A20 (c). Bar, 60 nm.

that among the VP oligomers sedimenting throughout the sucrose gradient there are some capsid-like structures in the range of 60S. Coprecipitation of relatively large amounts of VP3 together with VP1 and VP2 with the A1/A69 antibodies again indicates heteromeric complex formation with an increasing amount of VP3 at higher sedimentation values.

We therefore prepared putative capsid-like structures by further purification of the appropriate sucrose gradient fractions by repeated sedimentation through a sucrose cushion and analysed the pelleted material by negative staining and electron microscopy. We observed capsid-like structures, although with a low frequency so that only single particles could routinely be detected in one electron microscopic image (Fig. 6a). Immunogold staining with the A20 antibodies confirmed that the particles which we observed were composed

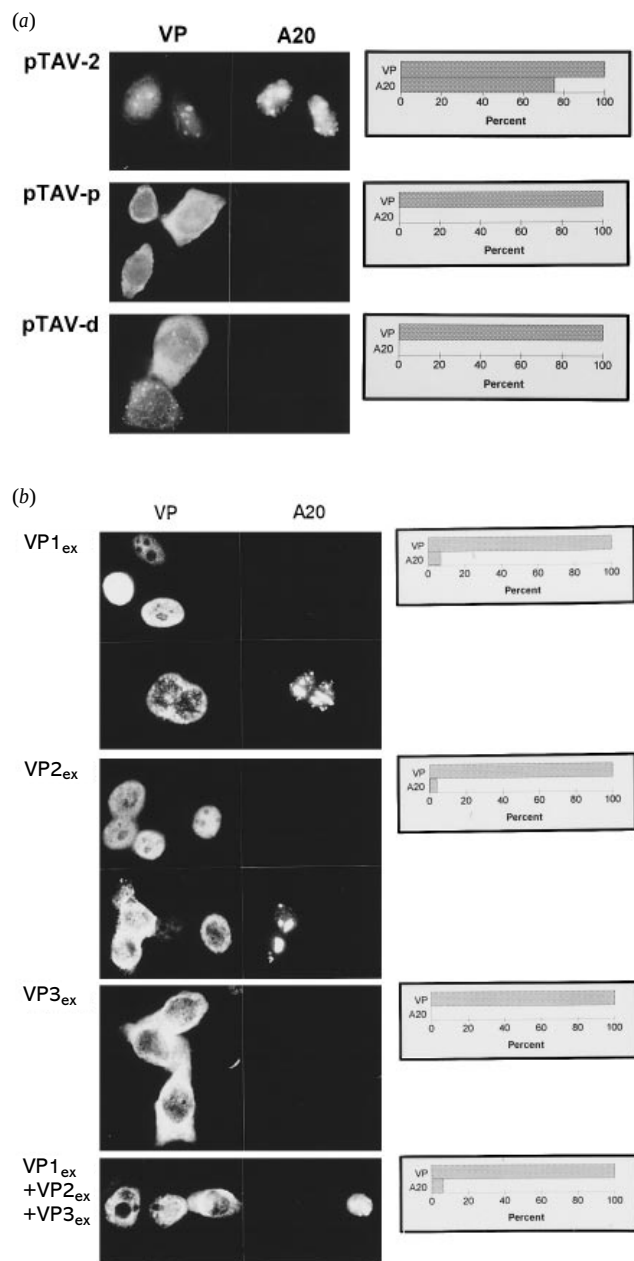


Fig. 7. Analysis of *in vivo* capsid formation with VP_{ex} expression constructs. HeLa cells were transfected with different VP expression constructs, infected with adenovirus-2 and examined for capsid formation by double immunofluorescence with a VP antiserum (detecting capsid protein expression) and MAb A20 (detecting assembled capsids but not free capsid proteins). (a) Cells transfected with an infectious clone of AAV-2 (pTAV-2) showed capsid formation in more than 70% of the cells which expressed capsid proteins (panel pTAV-2). Cells transfected with AAV-2 genomes containing mutations in the capsid ORF which prevent capsid assembly (Ruffing *et al.*, 1994; pTAV-p or pTAV-d) showed no capsid formation although capsid proteins were highly expressed. (b) Cells transfected with the VP1_{ex}, VP2_{ex} or a 1:1:5 mixture of VP1_{ex}, VP2_{ex} and VP3_{ex} expression plasmids showed capsid formation only in a few percent of the cells expressing the capsid proteins. Transfection of VP3_{ex} alone led to a predominantly cytoplasmic accumulation of capsid proteins, which did not form capsids in detectable amounts.

of AAV capsid proteins (Fig. 6b) and that they appeared indistinguishable from empty AAV capsids prepared by transfection of HeLa cells with an AAV capsid gene under the control of a CMV promoter (Fig. 6c).

Assembly studies in HeLa cells

Although these data show that we were able to form capsids *in vitro*, we were puzzled by the rather low efficiency of capsid formation. We therefore attempted to analyse the assembly capacity of the capsid proteins expressed from VP1_{ex}, VP2_{ex} or VP3_{ex} constructs *in vivo*. As already mentioned, the initiation codons for VP2 and VP3 had been mutated to achieve separate expression of VP1 or VP2 (Fig. 1) (Ruffing *et al.*, 1992). Of particular interest was the unusual initiation codon of VP2 (ACG) which encodes a T in wt AAV and which was mutated into A in VP1_{ex} and into M in VP2_{ex}. The mutated ORFs of the VP proteins were expressed under the control of the human CMV promoter after transfection into HeLa cells. Capsid protein expression and capsid formation were monitored by double immunofluorescence staining with a polyclonal VP antiserum recognizing both free and assembled capsid proteins and a MAb (A20) which specifically recognizes an epitope present in capsids but not in free capsid proteins (Wistuba *et al.*, 1997). In a control experiment, we first transfected a plasmid containing the AAV wt genome as well as two plasmids containing mutations in the capsid ORF which prevent capsid assembly (Ruffing *et al.*, 1994). We observed capsid formation in more than 70% of the cells successfully transfected with the wt AAV plasmid (Fig. 7a). In contrast, we observed no capsid formation in cells expressing the assembly-defective capsid proteins (Fig. 7a, pTAV-p and pTAV-d). In cells transfected with the single capsid protein expression constructs VP_{ex} we observed only a few cells which were positive for capsid formation after expression of VP1_{ex} or VP2_{ex}, although most of the cells showed strong capsid protein expression (Fig. 7b). VP3_{ex} alone almost never showed capsid formation and accumulated predominantly in the cytoplasm. Coexpression of the three capsid proteins did not improve capsid formation over the level we observed in cells expressing VP1_{ex} or VP2_{ex} alone. These results strongly suggest that the recombinant capsid proteins, mutated for separate capsid protein expression, have a reduced assembly capacity.

Discussion

In this report we have shown that the three capsid proteins of AAV-2, expressed as separate proteins by recombinant baculoviruses, could be purified, renatured and used for *in vitro* reconstitution of empty capsids. Capsid formation required HeLa cell proteins, showing that this *in vitro* reconstitution is not a self-assembly process. The process is still rather inefficient which is, at least partially, due to mutations in the VP1 and

VP2 ORFs which had been introduced to achieve separate expression of VP1, VP2 and VP3.

From other work it has been suggested that AAV-2 assembly occurs in two steps involving first the formation of empty capsids followed by introduction of the newly replicated ssDNA into these preformed capsids (Myers & Carter, 1980). In order to reconstruct these steps in an *in vitro* system it is necessary to provide empty capsids with the VP proteins in the correct stoichiometry. A major difficulty in producing such capsids in *E. coli* or with recombinant baculoviruses is the compact genomic organization of the cap gene in three overlapping reading frames. This organization does not allow expression of the capsid proteins in the desired stoichiometry of 1:1:10 in these organisms, since this stoichiometry is achieved by the abundance of alternatively spliced mRNAs and the initiation frequency at the unusual translational initiation codon for VP2 (Muralidhar *et al.*, 1994; Becerra *et al.*, 1985; Trempe & Carter, 1988; Cassinotti *et al.*, 1988). The splicing activity of the host cells is influenced by the helper virus (Trempe & Carter, 1988) and the initiation frequency at an ACG codon is probably different in *E. coli* as compared to eukaryotic cells. We therefore introduced mutations at the initiation codons for VP2 and VP3 in order to achieve separate expression of each capsid protein by recombinant baculoviruses (Ruffing *et al.*, 1992). Although coexpression of these capsid proteins in insect cells led to the formation of empty capsids under a variety of combinations (Ruffing *et al.*, 1992), in none of these combinations was the stoichiometry of capsid proteins in the empty particles 1:1:10 as it is observed in AAV wt virions. We therefore tried to use the baculovirus expressed capsid proteins for *in vitro* assembly in which we could directly determine the desired protein stoichiometry.

The fraction of soluble capsid proteins expressed in insect cells was very low, even when they were expressed for shorter times (20 h or 40 h) to prevent strong overexpression (data not shown). The same observation was made when all three VP proteins were expressed in HeLa cells in their natural genomic configuration from overlapping reading frames with a CMV-driven cap expression construct in the presence or absence of adenovirus (data not shown). This suggests that the capsid proteins have an intrinsic propensity to associate with cellular structures and that this behaviour is not due to the mutations introduced for separate capsid protein expression. The strong tendency of the capsid proteins to associate with insoluble structures might also explain the difficulties which we had in renaturing them after overexpression and solubilization in 8 M urea in the absence of any stabilizing structure. A number of classical renaturation procedures failed, and the addition of molecular chaperones (Buchner *et al.*, 1991; Ellis & van der Vies, 1991; Frydman *et al.*, 1992; data not shown) also did not prevent irreversible precipitation of the capsid proteins when urea was completely removed. The surprising effect of arginine on the solubility of the capsid proteins cannot be explained in molecular terms (Buchner & Rudolph, 1991). One speculation

is that arginine might partially mimic the structure and polar nature of urea and, on the other hand, might less efficiently disturb the water structure and therefore allow the minimum of hydrophobic interactions needed for folding of the capsid proteins. Upon further dialysis, the capsid proteins were more soluble in buffers of higher ionic strength. Also, increasing the ionic strength during speed-vac centrifugation allowed greater concentration of the proteins, which again supports the interpretation that hydrophobic interactions play a critical role in their solubility.

The assembly of biological macromolecular structures is usually strongly dependent on the concentration of the respective monomers, independent of whether it is a nucleated or an equilibrium-dependent process (Oosawa & Asakura, 1975; Pollard & Cooper, 1986; Zlotnick, 1994). Attempts to study AAV capsid protein oligomerization showed that oligomers were already observed at low protein concentrations (0.05 mg/ml) when the majority of the proteins remained in a monomeric state. Although the amount of oligomers increased when the protein concentration was raised up to 0.5 mg/ml, most of the capsid proteins remained monomeric. Analysis of the sedimentation pattern and electron microscopic examination of samples with different protein concentrations showed that this increase in protein concentration was not sufficient for a switch from pure oligomerization to capsid assembly. Also, adjustment to different pH values, to different ionic strengths, addition of Ca^{2+} or Mg^{2+} ions or removal of bivalent ions, as described in other successful *in vitro* assembly procedures, did not lead to detectable capsid formation (Salunke *et al.*, 1989; Ready *et al.*, 1988; Zhao *et al.*, 1995; Colomar *et al.*, 1993; Rodgers *et al.*, 1994; Salunke *et al.*, 1986; Schneemann *et al.*, 1994). Addition of a HeLa cell extract, however, changed the pattern of sedimentation in sucrose gradients especially in the range of capsid protein monomers, but also in the higher molecular mass range of the oligomers. Under these conditions we were able to detect a significant number of capsids by immunoprecipitation and electron microscopy. Nevertheless, the sedimentation pattern mostly did not show a peak around 60S as would have been expected if empty capsid formation had occurred with a majority of the capsid proteins.

Cellular fractionation and immunofluorescence studies showed that AAV capsid assembly occurs in the cell nucleus (Wistuba *et al.*, 1995, 1997). These observations suggest the need for nuclear accessory factors for the assembly process. For our *in vitro* reconstitution system a cytoplasmic HeLa cell extract was prepared as it is used for *in vitro* replication studies, which contains only a fraction of highly soluble nuclear proteins. Therefore it is possible that nuclear factors required for the assembly process were present in limiting amounts. Experiments designed to precisely localize the site of capsid formation within the AAV/adenovirus-infected cells suggest that capsid formation begins in the nucleolus and then expands to other sites of the nucleus (Wistuba *et al.*, 1997). If nucleolar

components or structures are involved in the assembly process, it might be necessary to provide them for efficient capsid formation *in vitro*. An alternative interpretation is that the cytoplasm and also the cytoplasmic extract contains a capsid dissociating activity, which would result in a futile cycle of assembly and disassembly of the capsids if the dissociation activities and the assembly activities are not compartmentalized. Experiments aimed at identifying cellular proteins which interact with the capsid proteins are in progress.

Finally, we explored another possibility which might explain the low assembly efficiency of our *in vitro* reconstitution system. Analysis of capsid formation of the single capsid proteins *in vivo* showed that the mutated capsid proteins also have a reduced assembly capacity. This is most likely due to the mutations in the VP2 and VP3 initiation sites. The fact that an unusual and inefficient translational initiation codon ACG is conserved at the VP2 initiation site has always been interpreted as a prerequisite for achieving the 1:1:10 stoichiometry of capsid proteins under the given genome organization. The threonine encoded by the ACG initiation codon of VP2, however, might also be a critical amino acid for correct folding and efficient capsid assembly. A future task will be to identify the structural requirements and cellular factors involved in AAV capsid assembly in order to increase the efficiency of *in vitro* reconstitution of AAV-2 capsids.

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