

Structural Proteins of Adenovirus-Associated Virus Type 3

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Three major structural proteins were found in adenovirus-associated virus (AAV) type 3H virions which were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The molecular weights of the polypeptides were determined to be approximately 66,000 (VP1), 80,000 (VP2), and 92,000 (VP3). The component having a molecular weight of 66,000 comprised about 80% of the total virion protein in the major AAV-3H particle, and the other two components comprised about 10% each. Proteins of the same molecular weight were found in the minor dense AAV-3H virion, but the 80,000- and 92,000-molecular-weight components were present at about one-half the concentration. The AAV-3H virion contains about 72 molecules of VP1 and 8 and 7 molecules of VP2 and VP3, respectively.

Adenovirus-associated viruses (AAV; also referred to in the literature as adeno-satellite viruses or adeno-associated satellite viruses) are small (20 to 25 nm) defective parvoviruses containing single-stranded deoxyribonucleic acid (DNA) and were first noted as contaminants in adenovirus stocks. These agents require co-infection with serologically unrelated adenovirus for production of infectious progeny (1, 6, 16). There are four recognized serological types of AAV differentiated by complement fixation, immunodiffusion, neutralization, and fluorescent-antibody techniques (4).

It has been found in our laboratory that parvovirus preparations frequently show three distinct virus bands when centrifuged to equilibrium in isopycnic CsCl gradients (5). In the case of AAV preparations, the major AAV infectious band is found in the density range of 1.388 to 1.445 g/ml depending on the serological type. This band is referred to as the major AAV band. A lighter band which contains little AAV infectivity is found in the density range of 1.33 to 1.36 g/ml. Examination by electron microscopy reveals that this band consists of a mixture of empty and partially empty AAV capsids and adenovirions. A minor infectious AAV band is frequently found at a density greater than 1.45 g/ml. Although the particles found in this band are immunologically indistinguishable from those found in the major band (5), they appear to be smaller, having a diameter which is 12% smaller, as observed in examination by electron microscopy (4). This band is referred to as the denser, or minor, band.

The AAV particle has a molecular weight of about 7.5×10^6 daltons and contains DNA of a molecular weight of approximately 1.5×10^6 daltons (11), sufficient to code for only a small number of proteins. As part of a long range study to delineate the complete function of the virus genome, the proteins of purified AAV type 3 strain H were studied by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis after dissociation in SDS and dithiothreitol (DTT). Three major virion proteins were found.

MATERIALS AND METHODS

Virus production and purification. AAV-3H was grown in KB cell spinner cultures with adenovirus type 2 helper. The cells (originally provided by James A. Rose, Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases) were grown in Eagle's Spinner no. 2 medium containing 5% heat-inactivated horse serum and 0.03% glutamine. One-liter cultures containing 200,000 to 280,000 cells per ml were infected with adenovirus 2 at an input multiplicity of 2 to 3 median tissue culture infective doses (TCID₅₀) per cell. After 2 hr, the cells were superinfected with AAV-3H at a multiplicity of 2 to 6 TCID₅₀ per cell and incubated for 40 hr at 37 C. The infected cells were harvested by centrifugation at $150 \times g$ for 10 min, suspended in 16 ml of 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.2, and frozen and thawed twice. These cells were further disrupted by sonication at 4 C for 5 sec in a Branson W-140-D sonifier at maximum intensity with a microtip probe. Four milliliters of 10% sodium deoxycholate and 0.125% trypsin contained in 0.01 M Tris buffer (pH 8.2) was added and incubated at 37 C for 30 min. Deoxyribonuclease I (500 units; Worthing-

ton Biochemical Corp., Freehold, N.J.) was added, and the suspension was then incubated for an additional 30 min at 37 C. The AAV in this suspension was purified by banding three times in isopycnic CsCl gradients (average density, 1.40 g/ml), and fractions were collected as previously described (6).

The purity of the AAV preparations was determined by examination by electron microscopy after the preparations had been found free of adenovirus and tissue culture antigens in the complement fixation test. They were negative for infectious adenovirus when inoculated into human embryonic kidney (HEK) cells. The absence of adenovirus antigens was further verified by the fact that these preparations did not produce adenovirus antibodies when injected into the foot pad of guinea pigs with complete Freund's adjuvant. This latter technique has been found in our own laboratory as well as in the laboratory of H. Pereira (*personal communication*) to be the most sensitive test for detecting small amounts of the various adenovirus antigens.

Protein determination. Protein concentrations were determined by the method of Lowry et al. (7), with bovine serum albumin as the standard protein. These determinations were done on disrupted virus, as whole virus gave no color reaction in the Lowry test. Further, because these determinations cannot be made in the presence of large concentrations of the reducing agent DTT, virus to be tested was disrupted in SDS in the absence of reducing agent.

Polyacrylamide gel electrophoresis. The polypeptide subunits were analyzed in 7.5% SDS-polyacrylamide gels by using the procedure of Maizel (8). The virus suspensions were dialyzed in 0.01 M sodium phosphate buffer, pH 7.2, and disrupted in either 1% SDS and 0.01 M DTT at 100 C for 1 min, or 10 M urea and 0.01 M DTT at 100 C for 30 min. The virus sample, adjusted to contain between 5 and 20 μ g of protein, 10% sucrose, and 1 μ g of Pyronin Y tracking dye, was layered over either 5-cm or 10-cm gels. Overloading the gels with greater concentrations of protein did not reveal the existence of other minor polypeptides. Electrophoresis was carried out in 0.1 M sodium phosphate buffer (pH 7.2) containing 0.1% SDS at 4 ma per gel until the tracking dye front reached the bottom of the gel (about 3 hr for 5-cm gels and 6 to 6.5 hr for 10-cm gels). The gels were fixed in 20% trichloroacetic acid and stained with 0.05% Coomassie Brilliant Blue in 10% trichloroacetic acid for 4 hr and destained in 7% acetic acid. For molecular weight determination the procedure of Shapiro et al. (15) was used, and 16- μ g amounts each of bovine serum albumin (Armor Pharmaceutical Co., Kankakee, Ill.), ovalbumin and ovalbumin dimer, pepsin, and trypsin (Nutritional Biochemicals Corp., Cleveland, Ohio) were used on parallel gels. The relative distance of migration (millimeters of band migration/millimeters of tracer dye migration) was plotted versus the \log_{10} molecular weight of the standard proteins. The unknown molecular weights were determined from the standard plot. Standards were included in each electrophoretic run.

RESULTS

Polyacrylamide gel electrophoresis of viral proteins. The results of the experiment in which the

dissociated viruses were subjected to electrophoresis on 10-cm 7.5% polyacrylamide gels are shown in Fig. 1. The major AAV-3H band with a density of 1.395 to 1.408 g/ml in CsCl is shown on gel A. The virus on this gel was dissociated by the SDS technique. Virus from the dense minor AAV-3H band (1.467 to 1.468 g/ml) dissociated in SDS is shown on gel B. Three major proteins were found in virions of both densities. The fastest migrating protein, which was the protein of highest concentration, was designated virus protein 1 (VP1). The other proteins were designated VP2 and VP3. Infrequently a minor high-molecular-weight band (117,000) was observed which, per microgram of protein, was more prominent in the denser AAV-3H particle. In some determinations this band appeared from both light and dense virions, but it appeared more

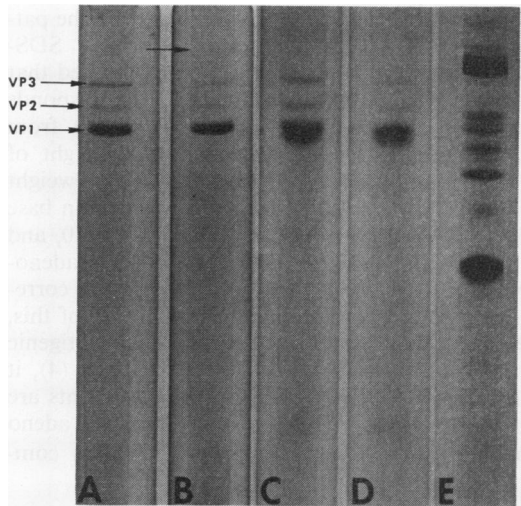


FIG. 1. Electrophoresis of dissociated AAV-3H and adenovirus type 2 on 7.5% polyacrylamide gels. Migration is from the top toward the anode on the bottom of the gels. The polypeptides were stained with Coomassie Brilliant Blue. (A) Gel showing the polypeptide bands of SDS-dissociated AAV-3H from the major CsCl band (density, 1.395 to 1.408 g/ml); (B) the polypeptides of AAV-3H dissociated in SDS from the minor or denser CsCl band (density, 1.467 to 1.468 g/ml); (C) AAV-3H from the major CsCl band dissociated in 10 M urea. (D) AAV-3H from the minor CsCl band dissociated in 10 M urea; (E) the polypeptides of purified adenovirus 2 dissociated in SDS. Three major polypeptides (VP1, VP2, and VP3) were found in AAV virions of both densities. The VP1 band corresponds to an area on the adeno-containing gel which probably represents the penton polypeptides. A minor high-molecular-weight fourth band infrequently appeared in gels of SDS-dissociated AAV and is probably a polymer of one or more of the other polypeptides, as it did not appear in gels of virus dissociated in urea.

frequently and in larger quantity in the denser particle. Neither the lighter virions (gel C) nor the denser virions (gel D) dissociated in 10 M urea yielded the 117,000-molecular-weight minor band. Urea-dissociated virus yielded only three polypeptide bands of the same apparent molecular weights as the three major peptides shown for SDS-treated virus. The 117,000-molecular-weight minor band, therefore, probably represents a polymer of at least one of the major polypeptides. Alkylation of the proteins with iodoacetic acid after SDS dissociation and reduction with 0.1 M 2-mercaptoethanol did not alter the described protein profile. The same three polypeptides remained after dissociation in 2% SDS, 0.01 M DTT and boiling for 1 hr. The fact that these various methods of dissociation and electrophoresis yielded the same three major polypeptides indicated that these polypeptides were dissociated to the monomeric level.

The last gel in Fig. 1 (gel E) illustrates the pattern obtained upon electrophoresis of SDS-degraded adenovirus type 2. It can be noted that only the major AAV constituent VP1 corresponds to polypeptides of any molecular weight from adenovirus 2 virions. The molecular weight of VP1 corresponds to the same molecular weight range reported for the adenovirus 2 penton base (70,000) and fiber (62,000; references 9, 10, and 12). The other polypeptide constituents of adenovirus 2 are either too large or too small to correspond to the AAV polypeptides. In view of this, and because of the total absence of antigenic cross-reactivity between these two viruses (4), it is highly unlikely that the AAV constituents are related to or derived from the prominent adeno-penton antigens or any other adenovirus component.

The molecular weights of the three proteins found in AAV-3H virions of both densities are given in Table 1. The figures given for the major particles were obtained from 25 molecular weight determinations; those given for the minor denser particle were obtained from 11 determinations. It

can be seen that proteins of similar weights are found in both types of particles.

Proportions of viral proteins in the virion. To estimate the relative quantities of each protein in the virions, the stained gels were scanned at 550 nm in a Gilford 2400 spectrophotometer. The fraction of the total protein represented by each peak was calculated and is given in Table 1. In AAV-3H from the major CsCl band, about 80% of the protein was VP1 and the other two constituents were found in about equal concentration at 10% each. The denser particle had a similar concentration of VP1 but had about half as much VP2 and VP3.

It is important to note that it was necessary to assume similar amino acid composition for the three polypeptides (3). This assumption is supported by other data, including radiolabeling with mixed amino acids which show proportions of the three polypeptides similar to those reported when Coomassie Blue staining was used. In any case, the degree of difference noted in Coomassie Blue staining between VP1 and VP2-VP3 cannot be explained on the basis of different amino acid compositions.

The number of polypeptide units per virion was calculated, based on the assumption that the molecular weight of the nucleic acid is 1.5×10^6 daltons (2) and that 20% of the AAV-3 particle weight is nucleic acid (11). It follows that the AAV-3 virion contains about 6.0×10^6 daltons of protein. By using the known approximate amount of each component and its molecular weight, the number of peptide units for the major virus particle was calculated as shown in Table 1. Since comparable size data are not available for the virus particles in the denser band, it was not possible to make the calculation of polypeptide units per virion for the particles in this band.

DISCUSSION

The results of this study show that the protein moiety of AAV-3H is composed of at least three major polypeptides of different molecular sizes

TABLE 1. *Polypeptides of adenovirus-associated virus type 3H analyzed by polyacrylamide gel electrophoresis*

CsCl band	Virus protein	Mol wt	Fraction of virion protein (%)	Calculated no. of polypeptides per virion
Major, (density, 1.395 to 1.408 g/ml)	1	65,900 \pm 2,600	78.5 \pm 5.2	72
	2	79,300 \pm 3,300	10.5 \pm 2.8	8
	3	91,600 \pm 2,600	11.1 \pm 2.7	7
Minor (density, 1.467 to 1.468 g/ml)	1	68,600 \pm 1,500	85.3 \pm 5.4	
	2	80,300 \pm 2,300	6.6 \pm 3.9	
	3	92,500 \pm 3,100	6.5 \pm 3.1	

Preliminary data indicate that AAV Type 1 grown in KB cells with adenovirus 2 helper also has three major polypeptides in infectious virions of both densities. The polypeptides had molecular weights similar to those described for AAV-3H. We have also noted that AAV-3H produced in HEK cells with adenovirus 7 helper and in Vero cells with SV-15 helper have 3 polypeptides with molecular weights similar to those from virus produced in KB cells with adenovirus 2 helper. Three major structural proteins have also been found in another parvovirus, Kilham rat virus. One of these structural proteins, having a molecular weight of 62,000, comprised about 75.5% of the virion protein (14).

The three polypeptides of the virus were present in markedly different concentrations with 79% attributable to a single polypeptide. Using Coomassie Blue staining to determine the relative concentrations of the three polypeptides, we observed, as has been reported (3), that at high protein concentrations densitometric analyses become nonlinear. Consequently, a variety of concentrations of the main band and lower band preparations were done, and the relative concentrations presented were determined under conditions of linearity. Under these conditions, there was reproducibly less of the two minor components in the virions of the more dense band. Although possible solvation differences in CsCl cannot be ruled out, the greater relative specific density of the particles found in the minor infectious band strongly suggests that these particles have a lower protein to nucleic acid ratio than those particles found in the major infectious band. The finding of less of the two minor components in the virions of the denser band, together with their reported smaller diameter (4), favors the interpretation that there is a lack of some peptide units in the denser virions as opposed to the possibility that they contain extra DNA. Other functions and the structural relationship of the three peptides in the infectious virions are not yet known.

The AAV-3 genome contains about 1.5×10^6 daltons of single-stranded DNA (2, 13), sufficient to code for about 118,000 daltons of protein. The combined molecular weight of the AAV-3H peptides is about 237,000, twice the amount for which the genome can code. The possibility exists that the adenovirus helper contributes peptides which become part of the AAV virion. However, our molecular weight data and other results on immunologic studies (4; unpublished data) tend to argue against this possibility. Alternatively, the three distinct polypeptides could arise by cleavage of a precursor protein of a molecular weight compatible with the genome size (17), combination of of small-molecular weight polypeptide subunits,

or, less likely, utilization of cell-specified protein. These and other studies on the synthetic origin of the peptides and their structural relationship are in progress.

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