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Structural Proteins of Adenovirus-Associated Viruses

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The structural proteins of adenovirus-associated virus (AAV) types 1, 2, and 3 were analyzed by acrylamide gel electrophoresis. In each case, one major protein (C) and two minor proteins (A and B) were identified. Component C had an estimated molecular weight of 62,000 daltons, and the molecular weights of components A and B were found to be 87,000 and 73,000 daltons, respectively. Coelectrophoresis of adenovirus and AAV proteins revealed an overlap only between the adenovirus fiber-penton component and the AAV C polypeptide. Among AAV serotypes, homologous components were electrophoretically identical, except that the C component of AAV-2 was of slightly lower molecular weight than the C components of AAV-1 and AAV-3. The relative incorporation of ^{14}C-arginine and ^{14}C-mixed amino acids into the three polypeptides of AAV-2 was similar, indicating an absence of an arginine-rich component. In addition, AAV-2 was found to have a substantially lower arginine content than helper adenoviruses.

The adenovirus-associated viruses (AAV) are small, deoxyribonucleic acid (DNA)-containing animal viruses whose size and physical properties resemble those of other members of the parvovirus group (1, 4, 7, 19, 22). The AAV differ, however, from other parvoviruses in two distinctive ways. (i) They are defective and multiply only in cell cultures which are also infected with an adenovirus (1, 7, 14, 25), and (ii), although AAV particles contain single-stranded DNA, each virion contains either a "plus" or a "minus" strand of complementary DNA (2, 13, 21). Thus far, four AAV serotypes have been clearly defined (7, 14, 20). Although genomes from all four serotypes appear to share a similar degree of nucleic acid sequence homology (20), antigenic relatedness can only be demonstrated between AAV-2 and AAV-3 (7). Neither antigenic relatedness nor nucleic acid sequence homology can be detected between AAV and their helper adenoviruses (1, 7, 20).

The single-stranded AAV genome has a molecular weight of 1.5×10^6 to 1.7×10^6 daltons (15, 19, 21) and is approximately $\frac{1}{12}$ as large as the double-stranded adenovirus genome. Although AAV are defective, AAV DNA is similar in size to DNA from other parvoviruses (4, 22). These genomes are among the smallest and least complex of all animal virus genomes and, based on molecular weight, would be expected to specify as few as 4 to 8 proteins. In recent studies with Kilham rat virus (KRV), Salzman and White have identified one major and two minor structural proteins (23). Toolan and Kongsvik also report that another parvovirus, H-1 virus, can similarly be fractionated into one major and two minor structural components (*personal communication*). This paper demonstrates that AAV, like KRV and H-1 virus, are also composed of three structural proteins. A comparison of three AAV serotypes reveals a close similarity in the pattern of electrophoretic mobility of the homologous polypeptides.

MATERIALS AND METHODS

Materials. Human embryonic kidney (HEK) cells were obtained from Flow Laboratories, Rockville, Md., and KB cells were from a line originally provided by M. Green. Reconstituted ^{3}H- and ^{14}C-labeled protein hydrolysates (^{3}H-RPH and ^{14}C-RPH) were purchased from the New England Nuclear Corp., Boston, Mass., and ^{14}C-arginine (312 mCi/mmole) was obtained from Schwarz BioResearch, Orangeburg, N.Y.

Production of radioactive virus. AAV-1 and AAV-3 were grown in HEK cells co-infected with the E46$^-$ strain of adenovirus type 7 (20), and AAV-2 was produced in KB cells in suspension culture with adenovirus type 2 as a helper (2). Conditions for the growth of adenovirus type 7 in KB cells have been described (18). Adenovirus type 7A was originally obtained from the National Institutes of Health Research Reference Branch. Viral protein was labeled by propagation of virus in Eagle's medium containing a 10-fold reduction of amino acids and 3 μCi or 0.5 μCi of ^{3}H- or ^{14}C-labeled amino acids per ml, respec-

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tively. AAV and adenovirus type 7A were purified as described previously (2, 19).

Polyacrylamide gel electrophoresis. The basic technique of gel electrophoresis has been described in detail (11, 12). Specific conditions are given with each figure.

Amino acid analysis. Amino acid composition was determined as before (8) by using a model 120-C Beckman amino acid analyzer.

RESULTS

Electrophoresis of viral proteins. Figure 1 shows a comparison of the proteins of AAV-1 and adenovirus type 7. There are one major and two minor AAV protein components. These have been designated A, B, and C in order of descending size. The minor components, A and B, with molecular weights of 87,000 and 73,000 daltons, respectively (Table 1), are smaller than adenovirus hexon protein, which has a molecular weight of 120,000 daltons (12). The major AAV component, C, has a molecular weight similar to that of adenovirus fiber protein, about 62,000 daltons (12). Only the C component is observed to overlap (12). Only the C component is observed to overlap an adenovirus polypeptide. This is of interest because of AAV dependence on adenoviruses. However, in view of the lack of antigenic and genetic relatedness between AAV and adenoviruses, the significance of this finding remains unknown.

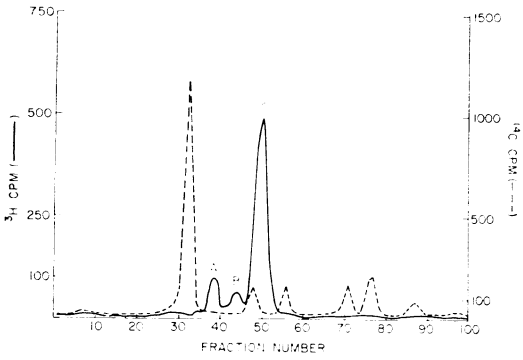

FIG. 1. *Comparison of the proteins of AAV-1 and adenovirus type 7A. Purified AAV-1 grown in the presence of ^{3}H-RPH (5,000 counts/min) and adenovirus type 7A grown in the presence of ^{14}C-RPH (5,800 counts/min) were mixed and dialyzed against 0.01 M sodium phosphate, pH 7.2. The mixture was then made 1.0% in sodium lauryl sulfate (SDS), 1.0% in 2-mercaptoethanol, and 10% in sucrose and heated at 100 C for 1 min. A 0.2-ml portion was applied to a 0.6 by 20 cm gel containing 5.0% acrylamide, 0.14% bis-N,N'-methyleneacrylamide, 0.1% SDS, 0.1 M sodium phosphate (pH 6.8), 0.05% tetramethylethylenediamine, and 0.1% ammonium persulfate. Electrophoresis was carried out at 50 v for 16 hr by using 0.1% SDS and 0.1 M sodium phosphate (pH 6.8) as electrode buffer. The gel was then fractionated and collected with a Savant Autogeldivider and Unifrac fraction collector and counted in a Beckman liquid scintillation spectrometer (10). In this and all subsequent figures, migration is from left to right.*

TABLE 1. *Adenovirus-associated virus polypeptides*

Gel component	Mol wt[a]	Weight fraction[b]	Relative no. of molecules[c]	Calculated no. of molecules per virion[d]
A	87,000	0.081	1.3	3.5
B	73,000	0.051	1.0	2.7
C	62,000	0.861	19.7	52.0

[a] Estimated by the method of Shapiro et al. (24) with adenovirus type 2 proteins (11) and β-galactosidase, bovine serum albumin, gamma globulin heavy and light chains, chymotrypsinogen, and cytochrome *c* as reference proteins.

[b] Fraction of total protein hydrolysate (RPH) radioactivity in each component.

[c] Weight fraction of each component divided by its molecular weight and normalized by dividing these quotients by that for the least plentiful component, B.

[d] Calculated by dividing the total daltons of protein of a given type in the virion by the molecular weight of that component. The total daltons of protein was obtained by subtracting the molecular weight of deoxyribonucleic acid (1.7×10^6 daltons; reference 21) from the molecular weight of the virion (5.4×10^6; reference 13). A similar virion molecular weight was also determined from a sedementation value of 125*S* as observed in 5 to 20% sucrose density gradients.

A comparison of the proteins of three AAV serotypes, AAV-1, AAV-2 and AAV-3, is shown in Fig. 2. In these studies AAV-1, labeled with ^{3}H-RPH, was mixed with either ^{14}C-RPH-labeled AAV-2 or AAV-3, and the viral proteins were electrophoretically separated as before. To be sure that electrophoretic differences did not arise on the basis of the differently labeled precursors, ^{3}H- and ^{14}C-RPH-labeled AAV-1 preparations were first compared. Panel I shows the electrophoretic separation of polypeptides in this mixture. The A, B, and C components are again seen, and there is no difference between the patterns of the ^{3}H-labeled and ^{14}C-labeled proteins. A comparison of AAV-1 and AAV-2 proteins is shown in panel II, and AAV-1 proteins are compared with those of AAV-3 in panel III. It can be seen that the C component of AAV-2 does not superimpose with that of AAV-1 (panel II). Since AAV-1C and AAV-3C are coincident (panel III), the C component of AAV-2 also differs from that of AAV-3C. No other electrophoretic differences are apparent among these serotypes. A summary of the electrophoretic data is shown in Table 1. Components A, B, and C are estimated to have molecular weights

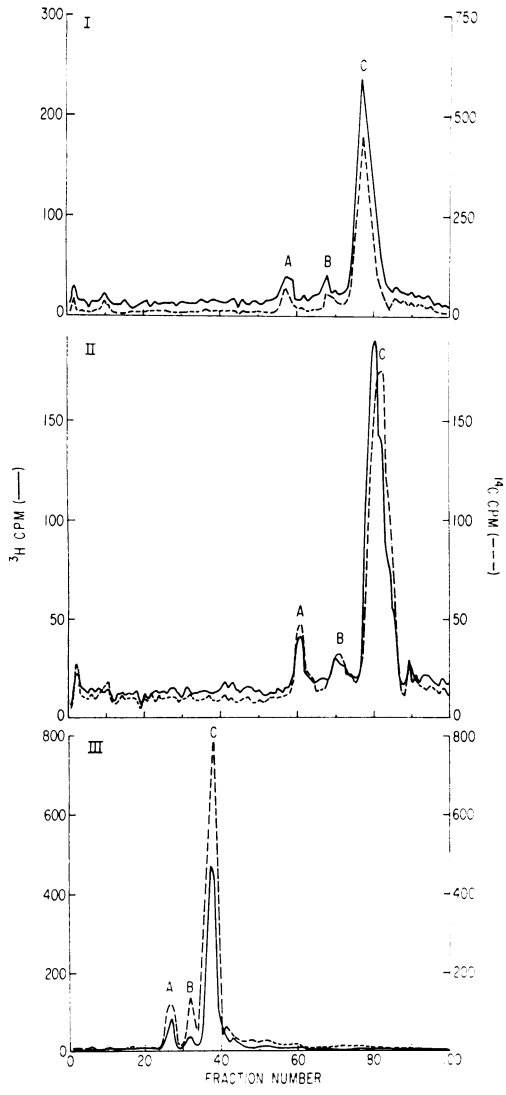

FIG. 2. *Comparison of the polypeptides of AAV types 1, 2, and 3. (I) Reproducibility of purification and analysis of AAV-1. AAV-1 labeled with ^{14}C-RPH (4,000 counts/min) was mixed with AAV-1 labeled with ^{3}H-RPH (2,300 counts/min) and dissociated and electrophoretically analyzed as in Fig. 1 except that the conditions were 90 v for 16 hr. (II) Comparison of AAV-1 and AAV-2. AAV-2 labeled with ^{14}C-RPH (7,350 counts/min) was mixed with AAV-1 ^{3}H-RPH (2,300 counts/min) and analyzed as in part I. (III). Comparison of AAV-1 and AAV-3. AAV-3 labeled with ^{14}C-RPH (7,893 counts/min) was mixed with ^{3}H-labeled AAV-1 (5,661 counts/min) and analyzed exactly as in Fig. 1.*

of 87,000, 73,000, and 62,000 daltons, respectively. Component C accounts for 86% of total structural protein. There are approximately 20 molecules of component C for each molecule of components A or B. Based on a virion molecular weight of 5.4×10^6 daltons (13), the number of C molecules per virion is calculated to be 52. If the AAV capsid is icosahedral, the minimum number of polypeptide units required for its construction would be 60 (3), i.e., 5 molecules for each of 12 vertices. Component C molecules would thus appear to be repeating units of capsid structure. The small proportion of A and B molecules suggests either that they are internal components, or, if they are in the capsid, they are not distributed according to fully occupied icosahedral symmetry.

Relative arginine content of AAV proteins. In the case of helper adenoviruses, internal components have been shown to have a high content of the basic amino acid, arginine (11, 16, 17). This possibility was also investigated with respect to AAV proteins. Figure 3 shows the electrophoretic patterns of AAV-2 protein labeled with ^{14}C-arginine (panel I) and ^{14}C-RPH (panel II). No relative difference in the incorporation of radioactive precursors is seen.

Other minor components. At least four components are detected in very small amounts in both the AAV-2 preparations, by using the sodium lauryl sulfate-containing disc electrophoresis buffer system and autoradiography (Fig. 3). Their mobilities indicate that they range in size from 15,000 to 40,000 daltons. It cannot be deduced at this time whether they represent integral and important virion constituents or whether they are merely degradation products or minor cell contaminants.

Amino acid analysis of AAV protein. An amino acid analysis of AAV-2 protein is shown in Table 2. The 4.8 moles per cent arginine content of this protein is considerably lower than an average value of 8 moles per cent found by Green et al. for several human adenovirus (16). The other major difference between AAV and adenovirus proteins is the relatively low alanine content of AAV protein, about half that found in adenovirus protein.

DISCUSSION

Three AAV serotypes (AAV-1, AAV-2, and AAV-3) each have three structural proteins, components A, B, and C. Among these serotypes, homologous components are electrophoretically similar, except for a small difference between AAV-2C and the C components of AAV-1 and AAV-3. The molecular weights of the A, B, and C polypeptides were found to be 87,000, 73,000 and 62,000 daltons, respectively. It should be noted that the estimated molecular weights of the AAV polypeptides are about 15% greater than those reported for the respective A, B, and C polypeptides of KRV (23). The combined molecular weights of these proteins total 222,000 daltons, a

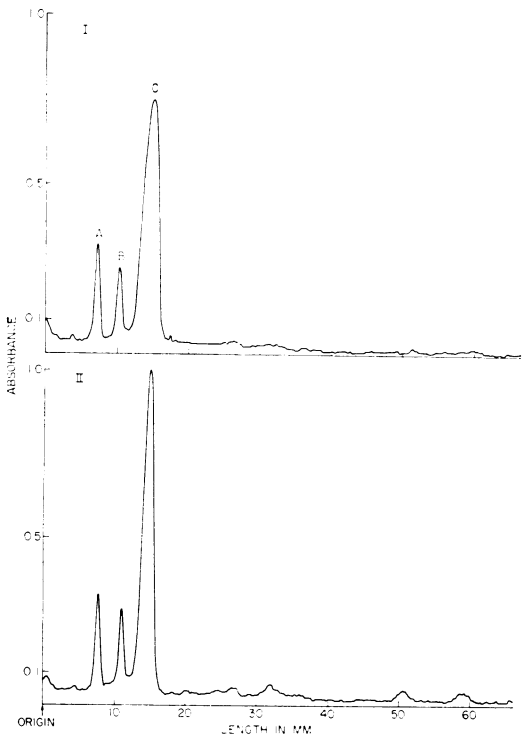

FIG. 3. *Distribution of arginine and mixed amino acid label among the polypeptides of AAV-2. Preparations of AAV-2 labeled separately with ^{14}C-RPH (88,750 counts/min) or ^{14}C-arginine (48,750 counts/min) were mixed and dissociated in 1.0% sodium lauryl sulfate (SDS), 0.06 M tris(hydroxymethyl)-aminomethane-hydrochloride (pH 6.8), 0.1% 2-mercaptoethanol, and 10% glycerol. The mixtures were electrophoretically analyzed in the "Disc" gel system (5) modified by the addition of 0.1% SDS to all buffers (9; J. V. Maizel, Jr. and U. K. Laemmli, in preparation). Resolving gels (10-cm long) containing 13% acrylamide and 0.34% bis-methylene acrylamide were used. Electrophoresis was for 4 hr at 90 v. The gels were then fixed and stained in 0.2% Coomassie Brilliant Blue R 250 in methanol-water-acetic acid [50:50:7 (v/v)], destained electrophoretically (26), sliced, dry-fixed to filter paper, and radioautographed by a modification (J. V. Maizel, Jr., in press) of the procedure of Fairbanks et al. (6). Suitably exposed (73 hr) and developed radioautograms were densitometrically analyzed in a Gilford spectrophotometer equipped with a linear transport mechanism.*

value in excess of the estimated 170,000-dalton coding capacity of the AAV genome. There are several possible explanations for this discrepancy. (i) Small errors in measurements of both protein and DNA molecular weight could have accumulated to give this difference; (ii) host cell protein(s) may be incorporated into the virion; (iii) virion structural proteins may arise by post-translational cleavage of a high-molecular-weight primary protein, i.e., have overlapping amino acid sequences. The C polypeptide of AAV protein appears to be the main unit of capsid structure, whereas the A and B polypeptides may be internal components. In contrast, the polypeptide corresponding to the B component is the major structural unit of KRV(23). Based on the relative incorporation of ^{14}C-RPH and ^{14}C-arginine into A, B, and C polypeptides, there is no AAV structural component relatively rich in arginine as is the case for helper adenoviruses. Furthermore, the arginine content of AAV protein is considerably less than that of adenovirus protein.

TABLE 2. *Amino acid composition of adenovirus-associated virus type 2*[a]

Amino acid	Mole (%)
Lysine	3.8
Histidine	2.1
Arginine	4.8
Aspartic acid	14.7
Threonine[b]	8.6
Serine[b]	8.3
Glutamic acid	10.6
Proline	6.6
Half-cystine	0.3
Glycine	9.9
Alanine	4.5
Valine	5.3
Methionine[b]	1.7
Isoleucine	3.3
Leucine	6.2
Tyrosine[b]	4.6
Phenylalanine	4.8

[a] Analysis of a 24-hr hydrolysate.

[b] Small corrections were made for losses due to hydrolysis and were based on data from other proteins (8).

Our findings demonstrate distinct differences between AAV and adenovirus proteins. The results suggest that adenovirus complementation of AAV replication does not depend on a contribution of an adenovirus polypeptide(s) to the AAV virion.

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