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Characterization of Aleutian Disease Virus as a Parvovirus

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We characterized a strain of Aleutian disease virus adapted to growth in Crandall feline kidney cells at 31.8°C. When purified from infected cells, Aleutian disease virus had a density in CsCl of 1.42 to 1.44 g/ml and was 24 to 26 nm in diameter. [^{3}H]thymidine could be incorporated into the viral genome, and the viral DNA was then studied. In alkaline sucrose gradients, Aleutian disease virus DNA was a single species that cosedimented at 15.5S with single-stranded DNA from adeno-associated virus. When the DNA was analyzed on neutral sucrose gradients, a single species was again observed, which sedimented at 21S and was clearly distinct from 16S duplex adeno-associated virus DNA. A similar result was obtained even after incubation under annealing conditions, implying that the bulk of Aleutian disease virus virions contained a single non-complementary strand with a molecular weight of about 1.4×10^6. In addition, two major virus-associated polypeptides with molecular weights of 89,100 and 77,600 were demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of virus purified from infected cultures labeled with [^{35}S]methionine. These data suggest that Aleutian disease virus is a nondefective parvovirus.

Aleutian disease of mink is an immune complex disease associated with persistent infection by the Aleutian disease virus (ADV) (3, 10, 17, 31). Hallmarks of the disease are glomerulonephritis, arteritis, plasmacytosis, and hypergammaglobulinemia with chronic viremia associated with extremely high levels of nonneutralizing antiviral antibody. The serum of infected minks contains infectious virus-antibody complexes (29), and immune deposits containing viral antigen and antiviral antibody have been demonstrated (32).

Studies performed on ADV purified from infected mink tissues and work with cell cultures suggest that ADV is probably a parvovirus (9-12, 30, 35). The parvoviruses are icosahedral viruses which are 18 to 26 nm in diameter and have reported particle densities in CsCl of from 1.38 to 1.42 g/ml (33, 39). These viruses replicate in nuclei and encapsidate single-stranded linear DNA genomes of 1.2×10^6 to 2.2×10^6 daltons (1, 33). They have either two or three structural polypeptides, which range in molecular weight from 92,000 to 55,000 (33, 39).

In the present study we characterized the nucleic acid and polypeptides of cell culture-adapted ADV. The virus genome was labeled with [^{3}H]thymidine, and the extracted viral DNA was a single species that sedimented in sucrose gradients and migrated in agarose gels like the single-stranded DNA of a nondefective parvovirus. In addition, sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) revealed two major viral polypeptides, which had molecular weights of approximately 89,100 and 77,600.

MATERIALS AND METHODS

Animals. Minks of either the Aleutian (sapphire) or the non-Aleutian (pastel) genotype were housed and maintained as previously described (3, 17). All animals were shown to be free of antibodies to ADV before use in any experiment. Infectivity titrations were performed as described previously, except that the results were expressed in terms of 50% mink infective doses based on development of antiviral antibodies, as calculated by the Spearman-Karber method (16). The criteria used for diagnosis of Aleutian disease were those reported previously (3, 17). All animals were kept under observation for at least 6 months.

CIE and determination of serum gamma globulin levels. Counterimmunoelectrophoresis (CIE) antigen was prepared and sera were assayed for anti-ADV antibodies as described previously (4). Serum gamma globulin levels were determined as described previously (4).

Virus strains. An isolate of Utah-I ADV adapted to growth at 31.8°C in Crandall feline kidney (CRFK) cells by John Gorham, Washington State University, Pullman, was obtained from him at passage levels 7 and 5 in CRFK cells. This cell culture isolate of Utah-I ADV is designated ADV-Gorham (ADV-G) here. The previously described CRFK cell-adapted isolate of Utah-I ADV (30) was obtained from David Porter, University of California School of Medicine, Los Angeles, and is designated ADV-Porter (ADV-P) here. Utah-I ADV was propagated by in vivo passage in minks, as previously described (9).

Cell cultures and passage of viruses in CRFK

cells. CRFK cells (15) were obtained from David Porter and were maintained in Eagle minimal essential medium containing 0.025 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 10% fetal calf serum, 0.05% lactalbumin hydrolysate, and 50 μg of gentamicin per ml (complete medium). Virus (ADV-G) was serially passaged in CRFK cells by seeding 150-cm^2 plastic tissue culture flasks (Corning Glass Works, Corning, N.Y.) with 4.5×10^6 CRFK cells at 37°C and infecting 1 day later with 5 ml of a 1:10 dilution of the previous CRFK cell passage of ADV-G. Virus was adsorbed for 2 h at 31.8°C, and flasks were then fed with 50 ml of complete medium and incubated at 31.8°C for 5 to 7 days. Virus was harvested by scraping the cells into medium, centrifuging the cell suspension at 3,000 rpm for 30 min, and resuspending the pellets in 1/10 the original volume of fresh medium. The pellets were subjected to four cycles of freeze-thawing, followed by sonication in an ultrasonicator equipped with a cup probe (four 15-s cycles at 105 W/cm^2). These treated pellets contained more than 90% of the infectivity and were stored at −70°C for use as virus stocks.

Immunofluorescence assay. For the direct immunofluorescence assay of virus, the method of Porter et al. (30) was modified slightly. Circular cover slips (12 or 15 mm) were placed in the wells of 24-well tissue culture trays (Linbro Div., Flow Laboratories, Hamden, Conn.) and were seeded with 1.5×10^5 CRFK cells in 1 ml of complete medium. The next day, subconfluent cultures were infected with 0.2-ml portions of virus dilutions for 2 h at 31.8°C and fed with 1 ml of medium; they were then incubated at 31.8°C for 3 days. The cover slips were washed with Dulbecco phosphate-buffered saline, fixed in cold acetone for 60 min, and stained with fluorescein conjugates of immunoglobulin prepared from the sera of Aleutian disease-affected or normal minks (14). Cover slips were examined with a fluorescent microscope, and the number of stained cells per unit area was related to the number of fluorescence-forming units (FFU) per milliliter of ADV, as described previously (30).

Passage of ADV-G in vivo in mink spleens. Two normal sapphire minks were inoculated intraperitoneally with 10^7 FFU of ADV-G from CRFK cell passage 10. After 21 days they were killed, and their spleens were collected under sterile conditions. A pooled spleen suspension (20%, wt/vol) in complete medium was made as described previously (9), and 1 ml of this suspension was then inoculated intraperitoneally into two additional sapphire minks. Three such serial passes were made.

Purification and CsCl density gradient analysis of virus. Flasks (150 cm^2) were seeded with CRFK cells and infected as described above with passage 10 ADV-G at a multiplicity of infection of 10 FFU/cell. Virus was purified 5 days after infection by a modification of the method of Muller et al. (27). Infected cells from 150-cm^2 flasks were scraped into medium and centrifuged at 3,000 rpm for 30 min at 4°C, and the cell pellets were suspended in 1 ml of 0.01 M Tris (pH 7.4), freeze-thawed, and then sonicated as before. The pellets were digested for 60 min at 37°C with 100 μg of DNase and 20 μg of RNase in 0.006 M $MgCl_2$. After addition of EDTA to 0.01 M and Sarkosyl to 1% and incubation at 37°C for another 60 min, the mixture was centrifuged for 30 min at 4,000 rpm and 25°C. The supernatant containing the partially purified virus was brought to 5 ml with 0.05 M Tris–0.002 M EDTA–0.1% Sarkosyl (pH 8.0) (TEB-S), and enough solid CsCl was added to effect a final density of 1.40 g/ml, as determined on a calibrated Abbe refractometer. A gradient was formed by ultracentrifugation in a Beckman SW50.1 rotor at 28,000 rpm for 40 h at 15°C. Fractions (0.3 ml), which were collected through a hole in the bottom of the tube, were analyzed for density and after dialysis against phosphate-buffered saline were assayed for infectivity in CRFK cells and for viral antigen by CIE. Electron microscopy was performed as reported previously (9), except that 1% uranyl acetate was used as the stain.

Thymidine incorporation and DNA extraction. For radioactive labeling of viral nucleic acid, infected cultures were incubated with 50 μCi of [^{3}H]thymidine (50 Ci/mmol) per ml or 0.4 μCi of [^{14}C]thymidine (40.8 mCi/mmol) per ml (both from New England Nuclear Corp.) from 48 h and then harvested as described above at 5 days. The partially purified virus was sedimented through a 2-ml layer of 30% sucrose in TEB-S into a 7-ml self-generating gradient of CsCl in TEB-S (average density, 1.38 g/ml) in an SW41 rotor at 15°C and 28,000 rpm for 24 h (27). Fractions (0.45 ml) of the CsCl gradient were assayed for density and for radioactivity by liquid scintillation counting. Fractions containing the peak of radioactivity were dialyzed into TEB-S, denatured in 0.3 M NaOH for 10 min at room temperature, and sedimented at 20°C in 5 to 20% alkaline sucrose gradients either in an SW50.1 rotor for 3.5 h at 42,000 rpm or in an SW41 rotor for 6 h at 40,000 rpm (5, 23). Fractions (0.25 ml) were collected, and samples were assayed by liquid scintillation counting. Peak DNA-containing fractions were dialyzed into 0.01 M Tris–0.001 EDTA (pH 7.5) for analysis on 5 to 20% neutral sucrose gradients (2.5 h in an SW50.1 rotor at 48,000 rpm and 20°C) (5). Some DNA preparations were allowed to anneal in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate) for 1 h at 60°C before sedimentation in neutral sucrose gradients (2, 17). DNA markers were either [^{14}C]thymidine-labeled virion DNA (form I and form II) from simian virus 40 (a gift from Norman P. Salzman) or [^{14}C]-thymidine-labeled virion DNA from adenovirus type 2 and adeno-associated virus type 2 (AAV) prepared as described previously (4, 23). *Eco*RI digestion of adenovirus type 2 DNA (28) was performed as described previously (5) by using *Eco*RI restriction endonuclease purchased from Miles Laboratories.

Agarose gel electrophoresis of DNA. Purified DNAs from AAV and ADV were analyzed in 1.4% agarose slab gels as described by Sharp et al. (36) in a horizontal electrophoresis apparatus (Bio-Rad Laboratories). DNA samples (0.5 μg) were precipitated with 3 volumes of 95% ethanol at −20°C in the presence of 0.3 M sodium acetate (pH 6.0) and 50 μg of yeast tRNA (42) and dissolved in 15 μl of 0.01 Tris–0.001 M EDTA (pH 7.5). Alternate samples were electrophoresed either as purified or after denaturation in 0.3 M NaOH at room temperature for 10 min. After electrophoresis for 4 h at 50 mA (constant current) and 100 V at room temperature, the gel was stained with ethidium bromide for 30 min and photographed through a Kodak 23A Wratten filter with Polaroid P/

N55 film while being transilluminated with a UV illuminator.

Incorporation of [^{35}S]methionine. For labeling with [^{35}S]methionine, cultures were seeded with CRFK cells and infected as described above. At 48 h after infection, cultures were washed twice with phosphate-buffered saline (prewarmed to 31.8°C) and then incubated for 30 min at 31.8°C with 5 ml of phosphate-buffered saline containing 40 μCi of [^{35}S]methionine (specific activity, >400 Ci/mmol; New England Nuclear Corp.) per ml; after this incubation 10 ml of medium reduced 10-fold with respect to methionine was added. After 2 h, 30 ml of complete medium was added, and the culture was incubated and harvested 5 days after infection. The CsCl gradient fractions containing the [^{35}S]methionine-labeled peak were prepared for analysis as described below.

PAGE. SDS-PAGE was performed by using the SDS-Tris-glycine system of Laemmli in a Bio-Rad model 221 slab gel apparatus (24). Resolving gels (12.5% polyacrylamide, 12.5% acrylamide, 0.3% bisacrylamide) and stacking gels (5% polyacrylamide, 5% acrylamide, 0.13% bisacrylamide) were cast at room temperature and were electrophoresed for 4 h at 40 mA (constant current) while being cooled with tap water. No SDS was included in either the resolving gel or the stacking gel (38). After electrophoresis, gels were fixed overnight in 25% isopropanol–7% glacial acetic acid, prepared for fluorography by the method of Laskey and Mills (26), and exposed to Kodak X-Omat XR-5 film at −70°C. Autoradiographs were developed in Kodak D-19 developer. Samples of CsCl gradients to be analyzed were prepared for SDS-PAGE as described previously (41) by precipitation with 10 volumes of 90% methanol in the presence of 2 μg of gelatin as a coprecipitant at −20°C overnight. Labeled material was recovered by centrifugation at 10,000 rpm for 30 min in the HB-4 head of a Sorvall RC-2B centrifuge, and the pellets were suspended by boiling in sample buffer (2% SDS, 10% glycerol, 0.012% bromophenol blue, 4% β-mercaptoethanol, 0.0625 M Tris, pH 6.8) for 2 min before analysis. Molecular weights were estimated by plotting mobilities of labeled polypeptides relative to a ^{14}C-labeled mixture of protein standards (Amersham). Acrylamide was purchased from Eastman Kodak, SDS was from BDH Chemicals, and other SDS-PAGE reagents were from Bio-Rad Laboratories.

RESULTS

Comparison of isolates of ADV for infectivity in cell cultures and in minks. The Utah-I strain of ADV passaged in vivo in mink spleens was compared with ADV-G from CRFK cell passages 5, 7, and 10, as well as with ADV-P from CRFK cell passage 9, for infectivity in CRFK cells (Table 1). ADV-G had high titers of infectivity for CRFK cells (>10^7 FFU/ml) at all passage levels; Utah-I ADV had a titer of $10^{6.8}$ FFU/ml, but the titer of ADV-P was about 1,000-fold lower ($10^{4.1}$ FFU/ml). These preparations were then tested in minks for infectivity (as measured by induction of antibody) and also for ability to cause Aleutian disease (Table 1). Both ADV-P and Utah-I ADV had levels of infectivity in minks about 10-fold higher than the levels in CRFK cells. Furthermore, all infected animals developed typical Aleutian disease. When ADV-G was tested in 64 minks (40 sapphires and 24 pastels), the infectivity for minks was significantly (at least 1,000-fold) less than the infectivity observed in cell cultures (Table 1). Infectivities, as determined by appearance of anti-ADV antibody, were equal for minks of the two genotypes (data not shown). Of 11 infected sapphires, 8 developed progressive disease; however, none of the pastels that had antibody developed disease.

Because this in vivo attenuation was so pronounced, the relationship of ADV-G to Utah-I ADV was examined in several ways. First, serological comparisons by using CIE and immunofluorescence showed a close relationship between ADV-G and Utah-I ADV (data not shown). In addition, ADV-G from CRFK cell passage 10 was serially passaged three times in sapphire mink spleens and then tested as described above (Table 1). The titer of this mink-passaged ADV-G in cell cultures fell about 1,000-fold (from $10^{7.4}$ to $10^{4.9}$ FFU/ml), but the infectivity for minks rose dramatically, increasing from $10^{0.5}$ to $10^{5.3}$ 50% mink infective doses per ml. Thus, the in vivo and in vitro levels of infectivity were similar, as was the case for Utah-I ADV and ADV-P. Nevertheless, because ADV-G could be serially passaged in CRFK cells at such high titers, ADV-G from CRFK cell passage 10 was chosen for further study.

TABLE 1. *Infectivity of ADV strains for cell cultures and for minks*

Virus tested	Virus titer in CRFK cells (FFU/ml)[a]	Virus titer in minks (MID_{50}/ml)[b]
Utah-I ADV[c]	$10^{6.8}$	$10^{7.8}$
ADV-P (CRFK cell passage 9)[d]	$10^{4.1}$	$10^{5.3}$
ADV-G (CRFK cell passage 5)[e]	$10^{8.0}$	$10^{4.5}$
ADV-G (CRFK cell passage 7)	$10^{7.2}$	$10^{2.8}$
ADV-G (CRFK cell passage 10)	$10^{7.4}$	$10^{0.5}$
Mink-passaged ADV-G[f]	$10^{4.9}$	$10^{5.3}$

[a] Samples (0.2 ml) of virus dilutions were assayed on cover slip cultures of CRFK cells as described in the text.

[b] Samples (0.5 ml) of virus dilutions were inoculated intraperitoneally into pairs of minks whose sera were then examined at monthly intervals by CIE for development of antiviral antibodies. The results are expressed as 50% mink infective doses (MID_{50}) per milliliter, as determined by the Spearman-Karber method.

[c] Utah-I strain of ADV as passaged in mink spleens.

[d] Utah-I strain of ADV adapted to growth in CRFK cells at 31.8°C by David Porter.

[e] Utah-I strain of ADV adapted to growth in CRFK cells at 31.8°C by John Gorham.

[f] CRFK cell passage 10 ADV-G that was serially passaged in sapphire minks three times at 21-day intervals, as described in the text.

Purification of ADV-G from infected cells. A preliminary study showed that ADV-G was highly cell associated, and further characterization required a technique for purifying virus from infected cells. A satisfactory method for doing this was developed, as described above, and ADV was subsequently analyzed on CsCl density gradients for infectivity in CRFK cells and for the presence of viral antigen. A representative gradient (Fig. 1) had a major peak of infectivity at 1.44 g/ml. Similar results were obtained from 10 gradients in which the density of the peak fraction was always in the range of 1.42 to 1.44 g/ml. As much as 14% of the initial infectivity could be recovered from peak gradient fractions. Electron microscopy of the fractions in the 1.42- to 1.44-g/ml range revealed viral particles 24 to 26 nm in diameter (Fig. 2A); viral antigen was detected in these fractions by CIE. A smaller peak of infectivity was observed at densities between 1.33 and 1.35 g/ml. Viral antigen was more readily detected in this region, and numerous 24- to 26-nm particles resembling empty viral capsids were observed by electron microscopy (Fig. 2B).

Incorporation of [^{3}H]thymidine into ADV-G and analysis of viral DNA. When cultures infected with ADV-G were incubated with [^{3}H]thymidine, we could demonstrate incorporation of the isotope into the 1.42- to 1.44-g/ml peak and, to a lesser extent, into the 1.33-

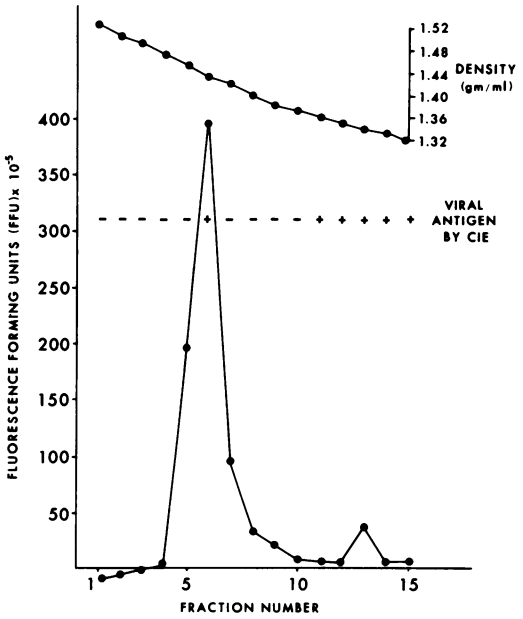

FIG. 1. *Infectivity of ADV-G in a CsCl equilibrium density gradient. Five 150-cm^2 flasks of CRFK cells were infected with passage 10 ADV-G at multiplicity of infection of 10 and incubated at 31.8°C for 5 days. Virus was purified and analyzed in a 5-ml CsCl gradient as described in the text. Fractions (0.3 ml) were assayed for density and, after dialysis into phosphate-buffered saline, for infectivity in CRFK cells, as well as for viral antigen by CIE.*

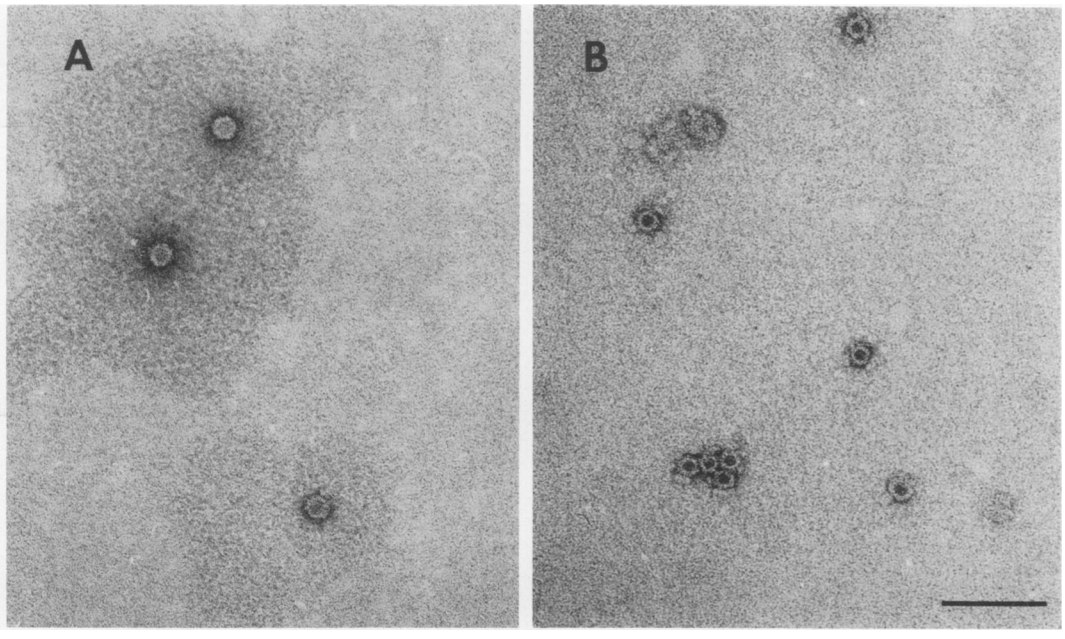

FIG. 2. *Electron microscopy of ADV-G purified from infected cells on CsCl density gradients. (A) Virus particles observed in the 1.42- to 1.44-g/ml region of the gradient. (B) Virus particles resembling empty capsids, which were observed in the 1.33- to 1.35-g/ml region of the gradient. The bar represents 100 nm.*

to 1.35-g/ml peak (data not shown). Mock-infected cultures showed no such labeling. DNA was then extracted from the virions in the 1.42- to 1.44-g/ml peak, denatured, and sedimented into alkaline sucrose gradients. A single homogeneous species of labeled ADV DNA was observed, which sedimented slightly slower than the 16S component of form II DNA from simian virus 40 (34) (data not shown). When the ADV DNA was sedimented in neutral sucrose gradients, a single peak was again observed, which sedimented just ahead of 21S form I DNA from simian virus 40 (34) (data not shown). For further study, purified ^{3}H-labeled ADV DNA was analyzed on another alkaline sucrose gradient, using ^{14}C-labeled AAV DNA as an internal marker. The position of ADV DNA was indistinguishable from that of 15.5S single-stranded AAV DNA in the same alkaline sucrose gradient (18) (Fig. 3A). The peak containing single-stranded AAV DNA and ADV DNA was dialyzed into 0.01 M Tris–0.001 M EDTA (pH 7.5) and centrifuged in a neutral sucrose gradient. The ADV DNA again sedimented as a single peak at 21S, whereas AAV DNA sedimented as a mixture of 16S duplex molecules and larger partial polymers under these conditions (18). A similar result was obtained if the AAV and ADV DNAs were allowed to anneal at 60°C in 1× SSC before centrifugation (C_0t for AAV DNA, 0.13 mol-s/liter; C_0t for ADV DNA, 0.02 mol-s/liter) (2, 6, 7) (Fig. 3B). The reported $C_0t_{1/2}$ for intact AAV DNA is 7×10^{-4} mol-s/liter (7), and, as expected, reannealing of AAV DNA was readily evident (Fig. 3B). The failure to observe any slower-sedimenting species of ADV DNA implied the absence of a significant percentage of complementary strands, even though annealing conditions were not identical for both AAV DNA and ADV DNA. Finally, equal amounts (0.5 μg) of AAV DNA and ADV DNA were electrophoresed in a neutral agarose gel before and after alkaline denaturation (Fig. 4); *Eco*RI restriction fragments of adenovirus type 2 DNA (28) were

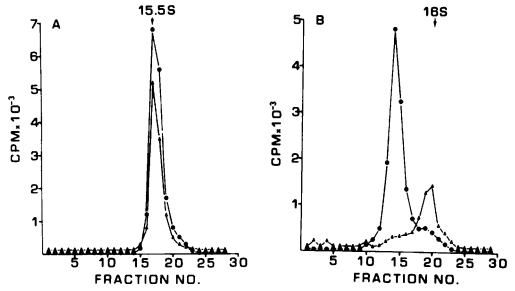

FIG. 3. *Sedimentation of ADV DNA in alkaline (A) and neutral (B) sucrose gradients. (A) ^{3}H-labeled ADV DNA purified from virus as described in the text was dialyzed into 0.01 M Tris–0.001 M EDTA (pH 7.5) and mixed with ^{14}C-labeled AAV. The mixture was denatured in 0.3 N NaOH for 10 min and sedimented in a 5 to 20% (wt/wt) alkaline sucrose gradient in a Beckman SW50.1 rotor, as described in the text. Fractions (0.25 ml) were collected, and samples of each fraction were assayed for ^{3}H (●) or ^{14}C (△). (B) The 16S peak from (A) was dialyzed into 0.01 M Tris–0.001 M EDTA (pH 7.5) and, after incubation in 1× SSC for 1 h at 60°C, was sedimented in a 5 to 20% (wt/wt) neutral sucrose gradient in an SW50.1 rotor, as described in the text. Fractions were assayed as described above in (A). The direction of sedimentation was from right to left.*

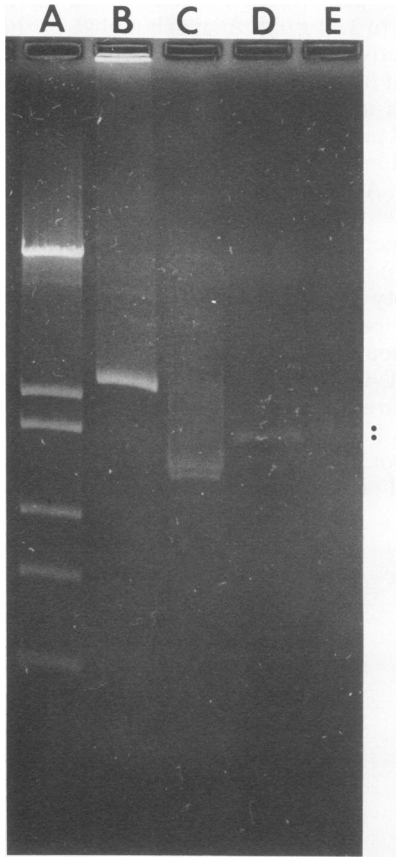

FIG. 4. *Electrophoresis of ADV DNA in an agarose gel. Equal amounts (0.5 μg) of AAV DNA (tracks B and C) and ADV DNA (tracks D and E) were electrophoresed in a 1.4% neutral agarose gel either before (tracks B and D) or after (tracks C and E) alkaline denaturation. After electrophoresis, the gel was stained with ethidium bromide and photographed as described in the text. The dots on the right represent the mobilities of native (lower dot) and denatured (upper dot) ADV DNAs. Track A contained EcoRI restriction endonuclease fragments of adenovirus type 2 DNA prepared as described in the text and electrophoresed without denaturation. The molecular weights of the fragments were as follows (from the top): 13.6×10^{6}, 2.7×10^{6}, 2.3×10^{6}, 1.7×10^{6}, 1.4×10^{6}, and 1.1×10^{6} (28).*

used as relative markers (Fig. 4, track A). Duplex AAV DNA (Fig. 4, track B) migrated slightly slower than the 2.7×10^6-dalton fragment of adenovirus type DNA (28) at approximately 3×10^6 daltons, which is in agreement with reported size estimates (1, 7, 18, 33); however, upon denaturation to single strands, the mobility was greatly increased (Fig. 4, track C). Resolution of the complementary strands of AAV DNA was observed, as reported previously (21). On the other hand, the mobility of ADV DNA (Fig. 4, tracks D and G) was essentially unaffected by alkaline denaturation, as expected for a single-stranded molecule (42). The mobility of ADV DNA was somewhat less than that of denatured AAV DNA, but since ADV DNA comigrated with the plus strand of AAV DNA in an alkaline gel (data not shown), this observation may reflect complementary regions within the ADV genome, such as terminal hairpins (5), that would rapidly reanneal while running in a neutral gel. Taken together, these data suggested that ADV contained a single non-complementary strand of DNA with a molecular weight very similar to the molecular weight of AAV (approximately 1.4×10^6) (18, 33, 37).

Incorporation of [^{35}S]methionine into ADV-G and analysis of viral proteins. Incorporation of [^{35}S]methionine into cultures of CRFK cells infected with ADV-G could be demonstrated, and the labeled virus had a density in CsCl gradients which was coincident with peaks of infectivity and [^{3}H]thymidine incorporation (data not shown). No such labeling was observed in preparations of uninfected cells. When this ^{35}S-labeled ADV-G was precipitated from the CsCl and analyzed on SDS-PAGE, two major polypeptides were observed (Fig. 5, track B). The molecular weights were estimated to be 89,100 and 77,600. The 77,600-dalton species appeared to be the most abundant. Upon prolonged exposure, three additional polypeptides with molecular weights of 61,600, 50,100, and 30,000 were also observed; these may represent either minor viral polypeptides, degradation products, or cellular contaminants. A more detailed analysis will be reported later.

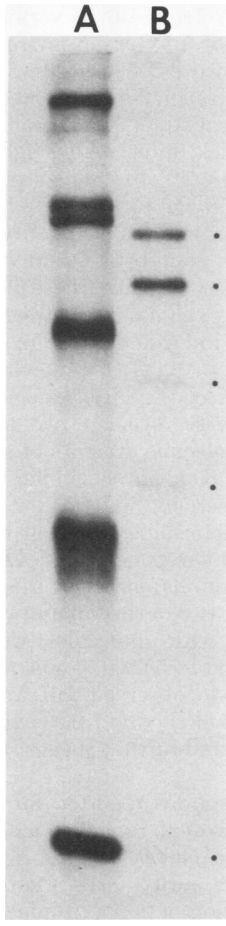

FIG. 5. *Electrophoresis of ADV polypeptides in an SDS-polyacrylamide gel (track A). The ^{14}C-labeled protein markers (Amersham) were (from the top) myosin (molecular weight, 200,000), phosphorylase B (100,000 and 92,500), bovine serum albumin (69,000), ovalbumin (46,000), and carbonic anhydrase (30,000). Track B contained [^{35}S]methionine-labeled ADV which was purified, and this labeled virus was precipitated from CsCl with methanol as described in the text. The samples were electrophoresed in an SDS–12.5% polyacrylamide gel, and the gel was fluorographed as described in the text. A dot has been placed just to the right of each observed band.*

DISCUSSION

We characterized a cell culture-adapted isolate of ADV (ADV-G), and our studies show that it is a parvovirus. The purified virus was 24 to 26 nm in diameter, had a density in CsCl of 1.42 to 1.44 g/ml, contained a DNA genome, and had two major viral polypeptides. Sedimentation of the DNA in alkaline sucrose gradients and electrophoresis in agarose gels suggested that the virions contain a single 15.5S species of DNA, which has an apparent molecular weight of about 1.4×10^6. Since the genome also sedimented as a single 21S species in neutral sucrose gradients, the bulk of the ADV virions probably contain only one of the two possible complementary DNA strands. Thus, ADV resembles nondefective parvoviruses, like minute virus of mice (5), which encapsidate only the minus strand of DNA, as opposed to the defective AAVs, which contain plus and minus strands in roughly equal proportions (1, 33).

The genome size of ADV-G (molecular weight,

approximately 1.4×10^6) is very similar to the genome sizes of AAV, minute virus of mice, and other parvoviruses (1, 5, 23). The DNA of Guelph ADV extracted from infected mink tissues by Shahrabadi et al. (35) consisted of three discrete species (molecular weights, 1.2×10^6, 0.5×10^6, and 0.2×10^6). The size of the largest molecule is similar to the size of the molecule which we observed, but the presence of two smaller pieces within the virion would be novel for parvoviruses. Possibly, these pieces represent contaminating cellular DNA fragments or fragments of the viral genome produced during DNA extraction.

SDS-PAGE of the virus-associated polypeptides of ADV-G revealed two major species, which had molecular weights of approximately 89,100 and 77,600. Three smaller minor species were also observed, and further study will be required to determine which of these proteins are structural components of ADV. Studies on a number of unrelated parvoviruses (33, 36, 41) showed either two or three major viral structural polypeptides with molecular weights in the range of 92,000 to 55,000. Thus, the major proteins which we observed fall well within the range of sizes of reported parvovirus structural polypeptides and further suggest that ADV is a parvovirus.

The polypeptides reported for Guelph ADV by Shahrabadi et al. ranged in molecular weight from 30,000 to 14,000 (35); however, the conditions used to purify virus (namely, repeated Freon extraction of 20 kg of infected livers and spleens) may have permitted extensive proteolysis. Alternatively, the structural composition of the virus in vivo may differ markedly from that in vitro. We are currently examining these possibilities.

ADV-G was originally derived from Utah-I spleen ADV (9, 19, 31) but became progressively attenuated for mink during passage in cell culture. This attenuation was not an invariable consequence of in vitro passage since ADV-P, which was also derived from Utah-I ADV, retained the virulence of the parent strain (Table 1) (9, 19, 31). The decreased infectivity of ADV-G for mink may reflect a severely diminished potential for in vivo replication, but since infectivity for mink could be restored by serial in vivo passage of ADV-G, it seemed likely that a virus population infectious for mink was still present. Perhaps preparations of ADV-G have large amounts of defective interfering virus particles that hamper initiation of in vivo infection (22). Our findings of apparently empty viral capsids associated with a small peak of [^{3}H]thymidine incorporation at a density of 1.33 to 1.35 g/ml might suggest that such defective interfering particles are present. Several other parvoviruses have been shown to produce populations of defective interfering viruses that interfere with the growth of the standard virus (8, 27).

The progressive attenuation of ADV-G by serial passage in CRFK cells may in fact provide a model for studying why some strains of ADV produce nonpersistent infections (3, 17, 25). Nonpersistent infections might relate to an intrinsic inability of some viruses to maintain productive infections in minks, and this could indicate a role for defective interfering particles in vivo. Alternatively, there may be a cellular restriction on viral reproduction that explains the limited expression of ADV observed in mink cells (20, 30), and this restriction may also be operative in vivo under some circumstances (40). The ability to radiolabel the viral DNA of ADV-G may enable us to examine these possibilities.

Earlier studies characterizing ADV have given conflicting results about the density of ADV and are at variance with our present findings. First, the density in CsCl of the ADV-G purified from CRFK cells (1.42 to 1.44 g/ml) was similar to that described by Porter et al. for ADV-P (30), but it was distinctly different from the density which we reported previously for Utah-I ADV purified from mink tissues (1.37 to 1.38 g/ml) (9) and also from the density initially found for the Guelph strain of ADV by Cho and co-workers (10–12, 35). No effort has been made to resolve these differences, but we have found that the density of spleen Utah-I ADV can be changed from 1.38 to 1.42 g/ml simply by varying the buffer in which the CsCl gradient is prepared (data not shown). The reason for this density shift is as yet unclear. Heavy and light populations of infectious minute virus of mice have been described (13, 39), and experiments are currently under way to determine whether alterations in the structural composition of ADV occur.

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