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The Relationship between Capsid Protein (VP2) Sequence and Pathogenicity of Aleutian Mink Disease Parvovirus (ADV): a Possible Role for Raccoons in the Transmission of ADV Infections

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Aleutian mink disease parvovirus (ADV) DNA was identified by PCR in samples from mink and raccoons on commercial ranches during an outbreak of Aleutian disease (AD). Comparison of DNA sequences of the hypervariable portion of VP2, the major capsid protein of ADV, indicated that both mink and raccoons were infected by a new isolate of ADV, designated ADV-TR. Because the capsid proteins of other parvoviruses play a prominent role in the determination of viral pathogenicity and host range, we decided to examine the relationship between the capsid protein sequences and pathogenicity of ADV. Comparison of the ADV-TR hypervariable region sequence with sequences of other isolates of ADV revealed that ADV-TR was 94 to 100% related to the nonpathogenic type 1 ADV-G at both the DNA and amino acid levels but less than 90% related to other pathogenic ADVs like the type 2 ADV-Utah, the type 3 ADV-ZK8, or ADV-Pullman. This finding indicated that a virus with a type 1 hypervariable region could be pathogenic. To perform a more comprehensive analysis, the complete VP2 sequence of ADV-TR was obtained and compared with that of the 647-amino-acid VP2 of ADV-G and the corresponding VP2 sequences of the pathogenic ADV-Utah, ADV-Pullman, and ADV-ZK8. Although the hypervariable region amino acid sequence of ADV-TR was identical to that of ADV-G, there were 12 amino acid differences between ADV-G and ADV-TR. Each of these differences was at a position where other pathogenic isolates also differed from ADV-G. Thus, although ADV-TR had the hypervariable sequence of the nonpathogenic type 1 ADV-G, the remainder of the VP2 sequence resembled sequences of other pathogenic ADVs. Under experimental conditions, ADV-TR and ADV-Utah were highly pathogenic and induced typical AD in trios of both Aleutian and non-Aleutian mink, whereas ADV-Pullman was pathogenic only for Aleutian mink and ADV-G was noninfectious. Trios of raccoons experimentally inoculated with ADV-TR and ADV-Utah all became infected with ADV, but only a single ADV-Pullman-inoculated raccoon showed evidence of infection. Furthermore, none of the ADV isolates induced pathological findings of AD in raccoons. Finally, when a preparation of ADV-TR prepared from infected raccoon lymph nodes was inoculated into mink and raccoons, typical AD was induced in Aleutian and non-Aleutian mink, but raccoons failed to show serological or pathological evidence of infection. These results indicated that raccoons can become infected with ADV and may have a role in the transmission of virus to mink but that raccoon-to-raccoon transmission of ADV is unlikely.

The pathogenesis of a viral infection is a fluid mosaic governed by the complex interaction between a viral agent and its host. This disease mosaic is readily apparent in infections with the Aleutian mink disease parvovirus (ADV) (3, 15, 17, 20, 25). Classical Aleutian disease (AD) in adult mink is a protracted disorder associated with a persistent infection and a variety of abnormalities of the immune system (3, 5, 20, 23, 26, 30, 46, 47, 55, 56, 63, 64). These abnormalities include progressive immune system-mediated nephritis with both glomerular and interstitial components, marked polyclonal hypergammaglobulinemia, elevated levels of antibodies directed against structural and nonstructural viral proteins, and plasmacytosis. However, the specific virus strain as well as the age and genotype of the mink strongly influence whether this classical picture occurs after infection (23, 40, 42, 43).

A number of different isolates of ADV have been compared in adult mink (23, 38, 40–43). Highly pathogenic isolates, such as ADV-Utah, the Canadian ADV-Ontario, and the Danish ADV-K, reliably cause classical AD in adult mink of both Aleutian and non-Aleutian genotypes (3, 40, 42). ADV-Pullman, on the other hand, induces severe disease in Aleutian mink, but its pathogenicity is greatly reduced in non-Aleutian animals (23, 42, 43, 64). Furthermore, the cell culture-adapted ADV-G strain has a severely reduced capacity for in vivo replication and is nonpathogenic, although it replicates permissively in cell culture (19, 24). These findings point out that for ADV infections, both the viral strain and the genotype of the host are important in determining the disease picture.

Similar implications may be drawn from studies on AD in

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newborn mink (2, 3, 20). Kits from seronegative dams develop a fulminant interstitial pneumonia with respiratory distress following inoculation with antibody-free ADV (2, 7–9). Highly pathogenic ADVs produce 90 to 100% disease and mortality in newborns, whereas isolates of low virulence induce disease in only 50 to 70% of kits, with a reduced mortality (30 to 50%) (6–9). Taken together, results of these studies in both adult and newborn mink, carried out over many years at a number of institutions, show the complex interactions between virus and host in ADV infections.

A number of uncharacterized ADV isolates also exist (11, 39, 45, 49, 51), and animals other than mink may be reservoirs of ADV infection (10, 36, 44, 50). For example, seroprevalence in ferrets to ADV can be as high as 42% (49, 65, 66), but although ADV-ferret causes mild lesions in ferrets, it is not pathogenic for mink (66). A number of other mustelids, including weasels, fishers, marten, striped skunks, and otters, can also be infected with ADV, but studies are even more limited than in ferrets (44, 50, 73). In addition, serological evidence of ADV infection has been found in skunks, raccoons, wild mink, and foxes (10, 44, 50). In most instances, the viruses infecting these animals have not been characterized, and it is uncertain where they would fall in the spectrum of pathogenicity for mink and how closely related they may be to known virus isolates.

In other parvovirus systems, differences in pathogenicity, host range, and cellular tropism are influenced by the capsid protein sequences. For example, feline panleukopenia virus (FPV), canine parvovirus (CPV), and mink enteritis parvovirus (MEV) all cause acute diseases in their respective hosts (28, 32, 57, 60, 62, 67, 71). Although closely related to each other antigenically and by DNA sequence (>98%) (54, 60), these viruses nevertheless differ markedly in pathogenicity and host range for the alternate species (12, 54, 57, 62, 69). The relevant viral determinants map to capsid sequences (57, 59, 69). Furthermore, the determinants have been localized to specific sites on the viral particle (1, 29, 69, 70). In addition, the pathogenicity and host range of strains of minute virus of mice are also governed by capsid protein sequences (13, 14, 35).

Molecular virology studies have begun to elucidate genomic features that may influence pathogenicity and host range for isolates of ADV (16, 18, 19). The complete sequence and transcription map of the 4,748-nucleotide (nt) nonpathogenic ADV-G isolate of ADV are known (4, 16, 18). Furthermore, the capsid coding sequences have also been determined for the pathogenic ADV-Utah and Danish ADV-K (18, 37). Isolates of ADV can be distinguished by examination of a hypervariable DNA and amino acid sequence found in a ca. 600-bp *Eco*RV-*Bst*EII fragment within the capsid protein gene (map units 64 to 65) (20, 37, 38). Definition of the hypervariable region has led to a preliminary typing scheme based on this sequence (18, 19, 38). For example, the nonpathogenic cell culture-adapted strain, ADV-G, is defined as type 1, whereas the highly pathogenic ADV-Utah strain is designated type 2 (38), and the various sequence types appear to be quite stable (37). Interestingly, ADV-Utah and the pathogenic ADV-K were found to be mixtures of more than one sequence type on the basis of examination of the hypervariable region sequence (38). ADV-Utah contains both type 2 and type 1 viruses, whereas ADV-K contains type 2 as well as an additional type, designated type 3. However, it is not clear that the particular hypervariable region of a virus type determines host range, pathogenicity, or antigenicity.

Because the capsid genes play such a prominent part in parvovirus pathogenicity, we investigated the relationship of ADV VP2 sequence type to pathogenicity of several characterized isolates of ADV. A new isolate was derived from an ongoing outbreak of AD on mink ranches in Utah and included in the studies. In addition, we examined a possible role for raccoons in the transmission of ADV. We found that viruses with type 1 hypervariable regions can be pathogenic and that raccoons may play a role in the transmission of ADV.

MATERIALS AND METHODS

Viruses and plasmids. The following preparations of ADV were used in this study. (i) The nonpathogenic type 1 ADV-G strain was derived from a molecularly cloned stock of virus (XXI-Q-3-15) (19). (ii) The type 2 ADV-Utah 1 strain, prepared from acutely infected adult mink, was derived from the same preparation used in previous studies at Rocky Mountain Laboratories (6, 7, 23, 31, 42); for convenience, it is referred to herein simply as ADV-Utah. (iii) The ADV-Pullman strain was from the same preparation described in former work (23, 42). (iv) ADV-TR was a 10% (wt/vol) suspension in phosphate-buffered saline (PBS) prepared from mesenteric lymph nodes of naturally infected mink from a single ranch in Utah (see below). (v) A suspension of ADV-Ontario was obtained from W. J. Hadlow (42, 48).

Plasmids pXXI-Q-3-15 (containing the entire ADV-G sequence) (16, 19) and pXX-B-1 (a full-length plasmid substituting the ADV-Utah sequence for the segment from map units 15 to 88) (19) were used to provide templates of known sequence for PCR. Additional molecular clones of ADV-Utah, designated ADV-Utah kit, were derived from duplex replicative-form DNA (21) from the lungs of ADV-Utah-infected mink kits (6, 16, 19, 21, 27). The sequences for ADV-Utah and the Danish type 3 virus isolated from ADV-K (designated ADV-ZK8) are published (16, 18, 19, 37). The ADV-Utah sequence extends only to the *Hin*dIII site at 88 map units, and the remaining capsid protein sequence was assumed to be identical with that of ADV-Utah kit.

Animals. ADV-negative sapphire (Aleutian genotype) and pastel (non-Aleutian genotype) mink (*Mustela vison*) were caged individually and maintained in modified small primate cages essentially as described previously (19, 22). Weanling raccoons (*Procyon lator*) were obtained from Ruby Fur Farms (New Sharon, Iowa), caged individually in modified primate cages, and fed standard dog chow. Mink and raccoons were segregated. All experimental work involving animals was performed under the supervision of the Rocky Mountain Laboratory Animal Care and Use Committee.

Animals were inoculated intraperitoneally with 0.5 ml of viral suspensions. The specific inocula contained approximately 10^7 50% mink infectious doses of ADV-Utah, 10^6 50% mink infectious doses of ADV-Pullman, or 10^7 fluorescence-forming units of ADV-G. The infectivity titer in mink of the ADV-TR preparations was not determined.

Mink and raccoon blood samples were obtained under ketamine-acepromazine anesthesia by venipuncture of the jugular and cephalic veins, respectively, in order to avoid potential cross-contamination that might result from toenail clipping. Whole-cell DNA was isolated from mesenteric lymph node at the time of necropsy essentially as noted in previous publications (5, 22, 26), omitting the RNase treatment. Tissue blocks from liver, spleen, mesenteric lymph node, and kidney were prepared according to standard histopathological procedures. The diagnosis of progressive AD was confirmed by the presence of typical gross and microscopic lesions (20, 23, 33, 55, 64).

Additional serum and mesenteric lymph node samples from naturally infected mink and vicinal raccoons were obtained by Gary Durrant on three mink ranches in Utah (see Results), using cardiac puncture and standard necropsy techniques.

Serum samples from 22 rabies-negative, free-ranging skunks from South Dakota were provided by Elisabeth S. Williams, University of Wyoming. This population of skunks was reported to have a seroprevalence to ADV of about 50% when tested by counterimmunoelectrophoresis (CEP) by a commercial laboratory.

PCR methods. PCRs (37, 53, 68) were performed in 50-μl reaction mixtures containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.02 mM each deoxynucleoside triphosphate, 0.45 μM each primer, and 1.25 U of *Taq* polymerase (Perkin-Elmer, Norwalk, Conn.). Following a hot start employing AmpliWax PCR Gems (Perkin-Elmer), amplification was done in 40 cycles consisting of 1 min at 95°C, 1 min at 50°C, and 1.5 min at 72°C, with a final 7-min elongation step at 72°C, using a model 480 Thermal Cycler (Perkin-Elmer). Reactions were performed in 0.5-ml thin-walled Gene Amp tubes (Perkin-Elmer). PCR products (10 μl) were subjected to electrophoresis in 2.5% agarose gels in Tris-borate-EDTA buffer and visualized under UV light following ethidium bromide staining.

All DNA, serum samples, and reagents destined for PCR studies were processed and manipulated in a remote laboratory suite where no ADV molecular biology had been performed. Aliquots of serum samples to be tested were held at 70°C for 45 s in the thermocycler. Five microliters of serum was used as the template in 50-μl reactions (34). Whole-cell DNA samples contained 0.5 to 2.0 μg of DNA in 1.0 μl. Reduction of the total volume to 25 μl with the use of 2.5 μl of serum did not affect sensitivity or reproducibility of the results. Negative controls, included in each set of amplifications, consisted of normal serum and reactions from which template was omitted.

Oligonucleotides were prepared on an Applied Biosystems model 380B DNA synthesizer. A specific primer pair was devised to detect and amplify ADV DNA sequences from unknown samples after inspection of published ADV sequences (16, 18, 37). The designations of these two primers, based on their positions (map units) on the ADV-G genomic map, and their sequences were as follows: 54.47(+), 5′-CTTGTCACGCTACTAGAATGGT-3′; and 68.49(−), 5′-AGCT TAAGGTTAGTTTACATGGTTTACT-3′. Other oligonucleotides used for amplification and sequencing were based on the ADV-G sequence (16, 18).

DNA sequencing and sequence analysis. Direct PCR sequencing of the amplification products was performed by using the GIBCO-BRL dsDNA Cycle Sequencing System (Life Technologies, Gaithersburg, Md.) according to the supplier's recommendations (52). About 100 ng of template was used, and the sequencing primers were end labeled with [γ-^{33}P]ATP. The thermal cycling program consisted of 20 cycles of 95°C for 30 s, 55°C for 30 s, and 70°C for 1 min, followed by 10 cycles of 95°C for 30 s and 70°C for 1 min. Samples were electrophoresed on a 0.4-mm 6% acrylamide gel in Tris-borate-EDTA buffer (16, 18). Removing unlabeled primers from the amplification products before direct sequencing with the Wizard PCR Preps DNA Purification System (Promega, Madison, Wis.) proved crucial to obtaining consistent results. Alternatively, some PCR products, following purification on Centricon 100 columns (Amicon, Inc., Beverly, Mass.), were directly sequenced by using the *Taq* DyeDeoxy Terminator Cycle Sequencing protocol in association with the requisite kit supplied by the manufacturer. The templates were subjected to 25 cycles of amplification, each consisting of 95°C for 20 s, 50°C for 20 s, and 60°C for 3 min. Subsequent electrophoresis and analysis of the samples were performed on an ABI 373A DNA Sequencer and associated software (Applied Biosystems, Foster City, Calif.). Sequencing of recombinant plasmids by standard dideoxy sequencing was accomplished as previously noted (16, 18). Analysis of compiled sequences was done with GeneRunner (Hastings Software, Inc.) and ALIGN Plus (Scientific and Educational Software, State Line, Pa.).

Antiviral antibody determinations. Antibodies to ADV were determined by CEP (23). A horizontal agarose gel electrophoresis chamber, trays, and modified combs were used. This modification resulted in precipitin lines more easily evaluated than with the previously used punched round wells.

Use of PCR to identify ADV viremia. As a surrogate for viremia (5, 26), viral DNA was identified in serum from infected mink by direct PCR. Although previous studies indicate that the viral DNA present in serum largely represents DNase-resistant encapsidated virion DNA (26), the number of samples to be tested and the risk of contamination made DNase treatment of serum impractical. Thus, the term positive or negative serum PCR was used.

Aliquots of serum were heat treated (34) and amplified as detailed above. To validate the method, we prepared serial dilutions of a serum in which we had previously determined the number of ADV genomes by DNA hybridization (5, 26). Aliquots were used as the template and subjected to PCR using primers 54.47(+) and 68.49(−). With this technique, it was possible reliably to detect <1 fg (<10 genomes) of ADV DNA in a 2.5-μl sample of serum. This value corresponded to <4,000 ADV genomes per ml of serum, a level approximately 100 times more sensitive than that detected by Southern blot hybridization (5).

To ensure that sera testing negative by PCR did not contain inhibitory components, all negative sera were spiked with a diluted sample of the positive control serum so that 10 fg (100 genomes) was present in the reaction mixture. Following this addition, ADV DNA could be amplified from all samples.

Nucleotide sequence accession numbers. The complete VP2 sequences for ADV-TR, ADV-Pullman, and ADV-Utah kit were entered into GenBank with accession numbers U39013, U39014, and U39015.

RESULTS

Analysis of samples from an outbreak of ADV infection in Utah. Since the early 1990s, a severe outbreak of AD has been occurring on commercial mink ranches in Utah. Several went from ADV-free status to greater than 90% ADV infected in less than 6 months. The reasons for the extremely rapid spread were unclear. Differences in husbandry techniques may have been involved (32a); however, several ranches were experiencing problems with vagrant raccoons and skunks entering the mink sheds. Furthermore, ranchers reported that the disease was more severe than that previously encountered.

We questioned whether this outbreak was due to the "old" ADV-Utah (22, 31, 42, 64), to a known isolate of ADV, or to a new one. We also wondered if other animals, such as raccoons, were playing a role. Consequently, we examined mesenteric lymph node and serum samples from six individual seropositive mink from each of three mink ranches. Two ranches (TR and DF) were near Morgan, Utah, and the third (RE) was near Summit, Utah. These communities are about 14 miles (ca. 22.5 km) apart. Lymph node and serum samples from several raccoons were also analyzed. One raccoon (RAC1) had been taken in the Morgan, Utah, area, and another (RAC2) had been taken in the Summit area. Sera from both had anti-ADV antibodies.

Initial efforts focused on analyzing the hypervariable segment of the capsid protein gene (18, 37). PCR amplification was performed on samples of serum and mesenteric lymph node DNA. A single band corresponding in size to the expected ADV fragment was readily detected in the amplification products from the two raccoons and from all but one mink. Negative controls included samples from uninfected sapphire mink and from uninfected raccoons obtained commercially. The primers also failed to react with a sample of MEV (61). The findings confirmed that the outbreak was indeed due to ADV infection, that raccoons as well as mink harbored viral sequences, and that the primers we had selected were suitable for this kind of analysis.

The PCR products from two mink from each ranch (TR3 and -6, DF1 and -3, and RE1 and -6) as well as from the two raccoons were subjected to direct DNA sequencing (52) with primers situated to capture the hypervariable region. A core 161-bp sequence was obtained for all samples (Fig. 1A), and it was apparent that the viruses from the Utah outbreak were highly (98 to 100%) related to each other.

No skunk samples were available from the Utah outbreak, but 22 sera from free-ranging South Dakota skunks were tested. Four (SKU12, SKU14, SKU15, and SKU21) were positive by CEP for anti-ADV antibody. Three sera (SKU2, SKU8, and SKU15) were positive by PCR, and amplification products from two (SKU8 and SKU15) were also directly sequenced (Fig. 1A). These sequences were unlike those obtained from mink and raccoons in the Utah outbreak.

Sequence analysis of ADV hypervariable regions. The next goal was to compare the hypervariable region sequences of the viruses from the ongoing outbreak with those from other isolates of ADV. To perform a more comprehensive analysis, we also amplified and sequenced analogous segments of DNA from ADV-Pullman (23, 42) and ADV-Ontario (42, 48) (Fig. 1A) and compared them with the sequences for ADV-G (16, 18), ADV-Utah (18), ADV-Utah kit, the type 3 sequence from the Danish ADV-K (ADV-ZK8) (37), and the two skunk specimens.

Analysis of this set of 15 ADV DNA sequences yielded a number of interesting observations (Fig. 1A). First, in this short region of the viral genome, the samples from the current outbreak in Utah were almost as closely related to ADV-G (98 to 100%) as to each other, but all were less than 90% related to any of the other samples examined. ADV-Utah and the new ADV-Utah kit sequence were identical for this region, so only the ADV-Utah kit sequence is shown. None of the other sequences were extremely closely related to ADV-Utah, although ADV-Ontario had a 96% homology. Finally, the only virus closely related to the Danish ADV-ZK8 at the DNA level was ADV-Pullman (98%).

These findings were essentially confirmed when the translated amino acid sequences (53 residues) were aligned (Fig. 1B). The hypervariable regions from the two raccoons and the mink from DF and TR were identical. Furthermore, although the sequences from the RE mink differed slightly, all samples from the current outbreak were more closely related to ADV-G (94 to 100%) than to ADV-Utah kit (85%) or ADV-ZK8 (85%). ADV-type 3, ADV-Ontario, and ADV-Pullman were 94, 92, and 92% related to ADV-Utah kit. ADV-Ontario and ADV-Pullman were both 96% related to the ADV-ZK8. Finally, the two samples from the skunks had the lowest homology to any of the other samples (ca. 80%).

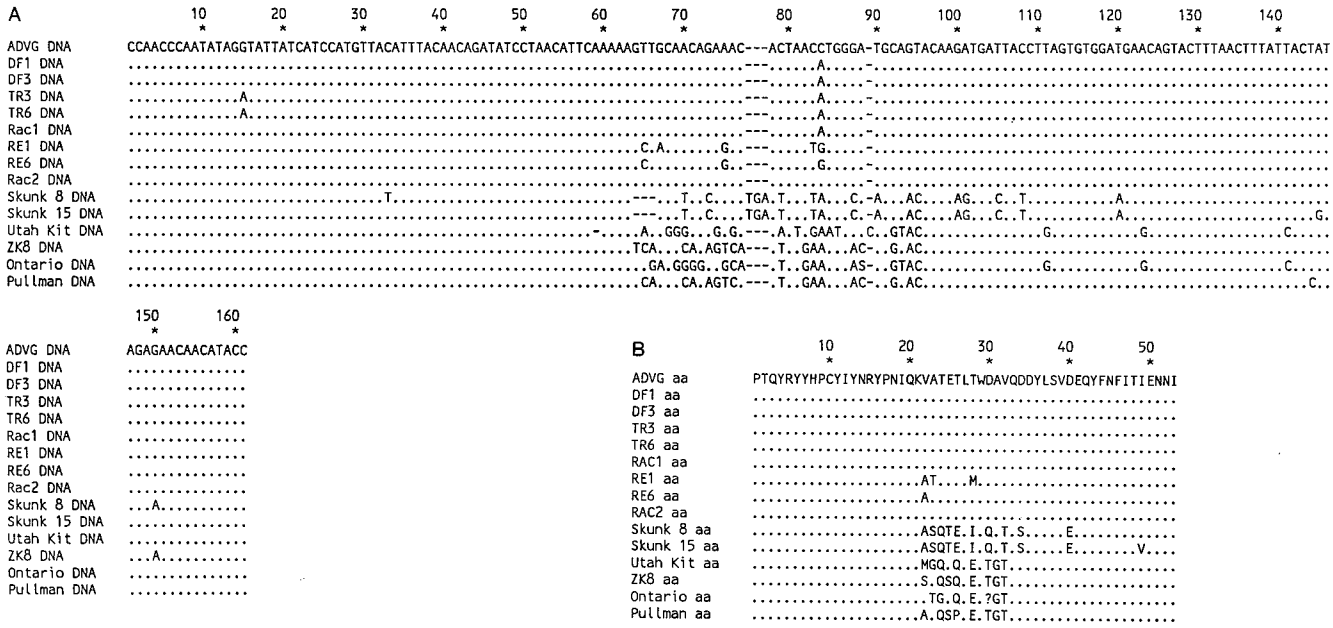

FIG. 1. Sequence analysis and comparisons of the hypervariable regions of ADV. (A) A 161-nt segment capturing the hypervariable region from 15 different ADV DNA sequences was aligned on the ADV-G sequence (ADV-G nt 3036 to 3196), using the sequence analysis program ALIGN. (B) The predicted 53-amino-acid sequences from the same sequences were aligned on the ADV-G sequence (ADV-G VP2 amino acid residues 211 to 263). ADVG, Utah Kit, ZK8, Ontario, and Pullman represent the ADV isolates described in the text. DF1 and -3, TR3 and -6, and RE1 and -6 refer to specimens from individual mink from the three ranches mentioned in the text. Rac1, Rac2, Skunk 8, and Skunk 15 refer to the specimens mentioned in the text.

Thus, it seemed that a single ADV, designated ADV-TR, was responsible for the current outbreak and that this ADV had a type 1 hypervariable region sequence. The sequence differed dramatically in that region from the old type 2 ADV-Utah or any other of the recognized pathogenic ADV isolates. Furthermore, the results suggested that the vicinal raccoons were carrying the same virus.

Comparisons of the complete VP2 capsid protein sequences of ADV isolates. Because a type 1 ADV has not previously been identified as pathogenic and because of the potential importance of the capsid genes in pathogenicity, we wanted to compare the entire VP2 sequence of ADV-TR with those of the type 1 ADV-G, the type 2 ADV-Utah, and ADV-Utah kit, the type 3 Danish ADV-ZK8 virus, and the previously unsequenced ADV-Pullman.

Information for ADV-TR and ADV-Pullman was obtained by direct PCR sequencing, using additional primer pairs based on the ADV-G sequence (16, 18). The extremely G+C-rich polyglycine (GGG) region (18) proved refractory to direct sequencing. Consequently, amplified portions of ADV-Pullman and ADV-TR were cloned and sequenced by standard dideoxy methods (16, 18). ADV-TR and ADV-Pullman were 27 and 39 nt shorter in this region than ADV-G or ADV-Utah, a difference that was also noted on agarose gels used for analyzing the PCR products. Although these deletions may be PCR artifacts, amplification of the analogous fragment from ADV-G did not produce a truncated product (data not shown). Variability in the length of the GGG region among clones from a single preparation of viruses has been previously noted (18), and consequently, it was not possible to evaluate the significance of this finding.

When the DNA sequences were aligned to the 1,944-nt sequence for ADV-G VP2 (Fig. 2A), the homology varied from 98% for ADV-TR to 92% for ADV-Pullman. The most concentrated area of variation was between nt 690 and 730 of the VP2 sequence, corresponding to the hypervariable region. However, there were a number of dispersed nucleotide residues at which all or the majority of the pathogenic isolates differed from ADV-G. Furthermore, most nucleotides at which ADV-TR differed from ADV-G were also found where other isolates differed from ADV-G.

When the predicted VP2 molecules were aligned with the 647-amino-acid ADV-G VP2, a similar set of observations emerged (Fig. 2B). There were 12 differences between ADV-G and ADV-TR, as well as the nine-residue abbreviation in the ADV-TR GGG region. Each of the differences was at a position where one or more of the pathogenic isolates differed from ADV-G. In fact, 8 of these 12 changes were found at residues where all pathogenic isolates differed from ADV-G (A for T at amino acid position 4, P for A at position 6, F for Y at position 115, V for I at position 352, Q for H at position 395, N for D at position 433/434, D or E for N at position 491, and D for H at position 534). Interestingly, ADV-Pullman had a 13-residue and ADV-ZK8 had a single-residue abbreviation in the GGG region. In other words, with the exception of the hypervariable region, the majority of amino acid differences in VP2 between ADV-G and the pathogenic isolates, including ADV-TR, were confined to a limited number of residues.

In vivo response of mink and raccoons to selected isolates of ADV. Because we wanted to examine the pathogenicity of ADV-TR and the susceptibility of raccoons to ADV infection under controlled conditions, we next inoculated groups of three sapphire (Aleutian) mink, three pastel (non-Aleutian) mink, and three raccoons with ADV-Utah, ADV-G, ADV-Pullman, and the new ADV-TR. Several parameters of infection and disease induction were examined, including the development of anti-ADV antibody by CEP (23), ADV DNA in the serum as assayed by serum PCR (a surrogate measure for viremia) (5, 26), the presence of characteristic lesions of AD by gross and microscopic examination at necropsy (60 days for

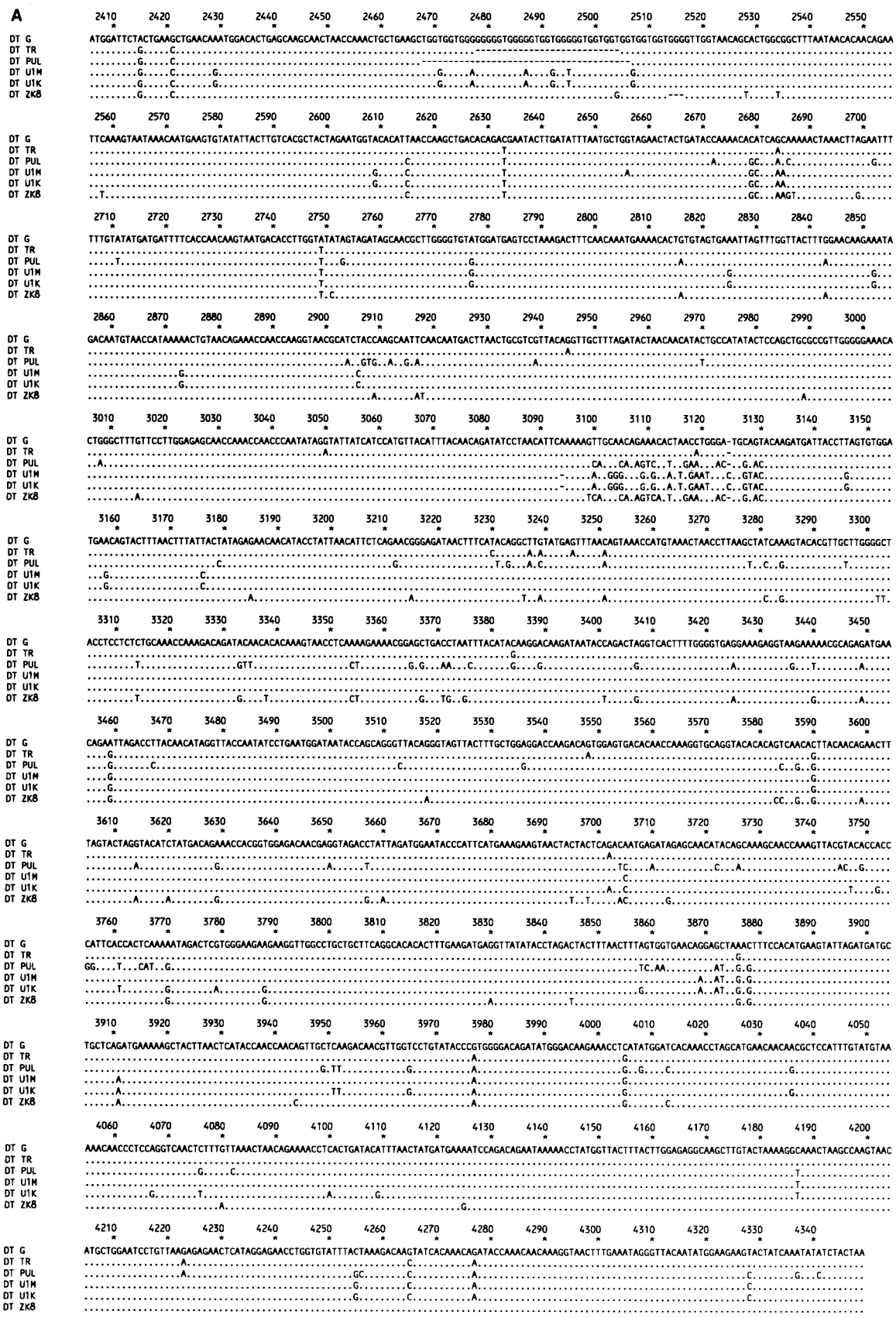

FIG. 2. Sequence analysis and comparisons for the entire VP2 coding regions of six isolates of ADV. (A) The DNA segments corresponding to the entire VP2 coding regions for ADV-G (DT G), ADV-TR (DT TR), ADV-Pullman (DT PUL), ADV-Utah (DT U1M), ADV-Utah-kit (DT U1K), and ADV-ZK8 (DT ZK8) were aligned with the ADV-G sequence (ADV-G nt 2406 to 4349), using the sequence analysis program ALIGN. (B) The predicted VP2 amino acid sequences from ADV-TR (PVP2 TR), ADV-Pullman (pvp2 pul), ADV-Utah (pvp2 ut1mod), ADV-Utah-kit (pvp2 u1k), and ADV-ZK8 (pvp2 zk8) were aligned with the 647-amino-acid residue ADV-G VP2 sequence (pvp2 g).

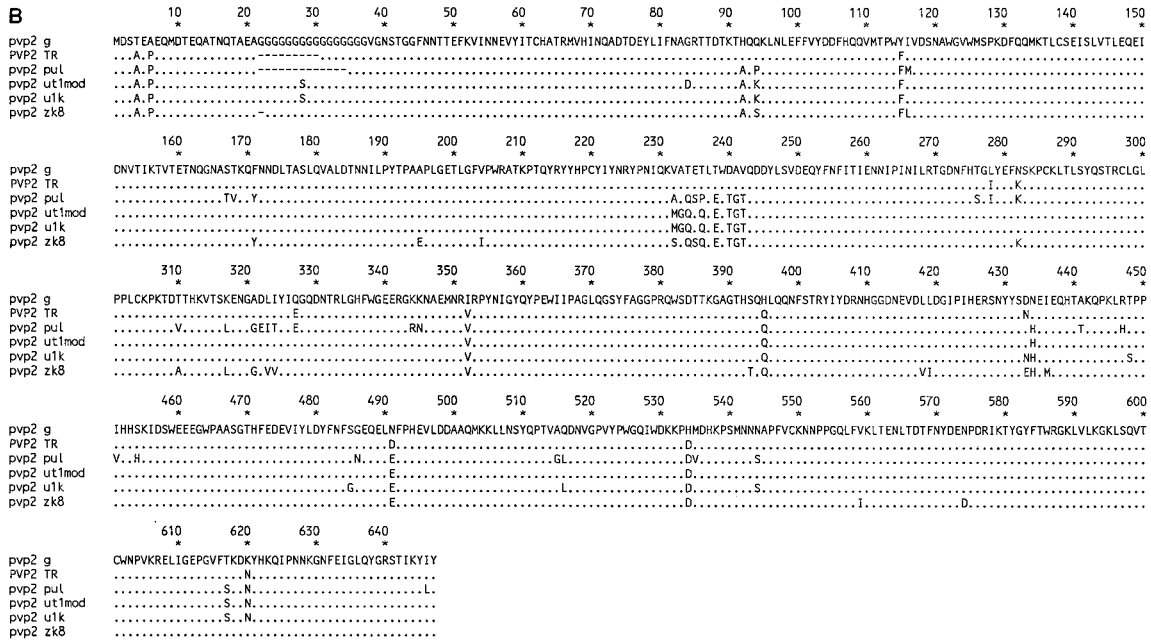

FIG. 2—*Continued.*

mink and 90 days for raccoons) (20, 23, 33, 55, 64), and the presence of ADV DNA in mesenteric lymph node as assayed by PCR (5, 22, 26).

Control group (PBS). None of the control animals showed any evidence of ADV infection or pathologic changes of AD. Furthermore, ADV DNA was not detected in serum or in mesenteric lymph node at the time of necropsy (Fig. 3A and Table 1).

ADV-G group. None of the mink or raccoons inoculated with the molecularly cloned type 1 ADV-G (19) developed anti-ADV antibodies or had pathologic changes at necropsy. In addition, none had a positive serum PCR at the times exam-

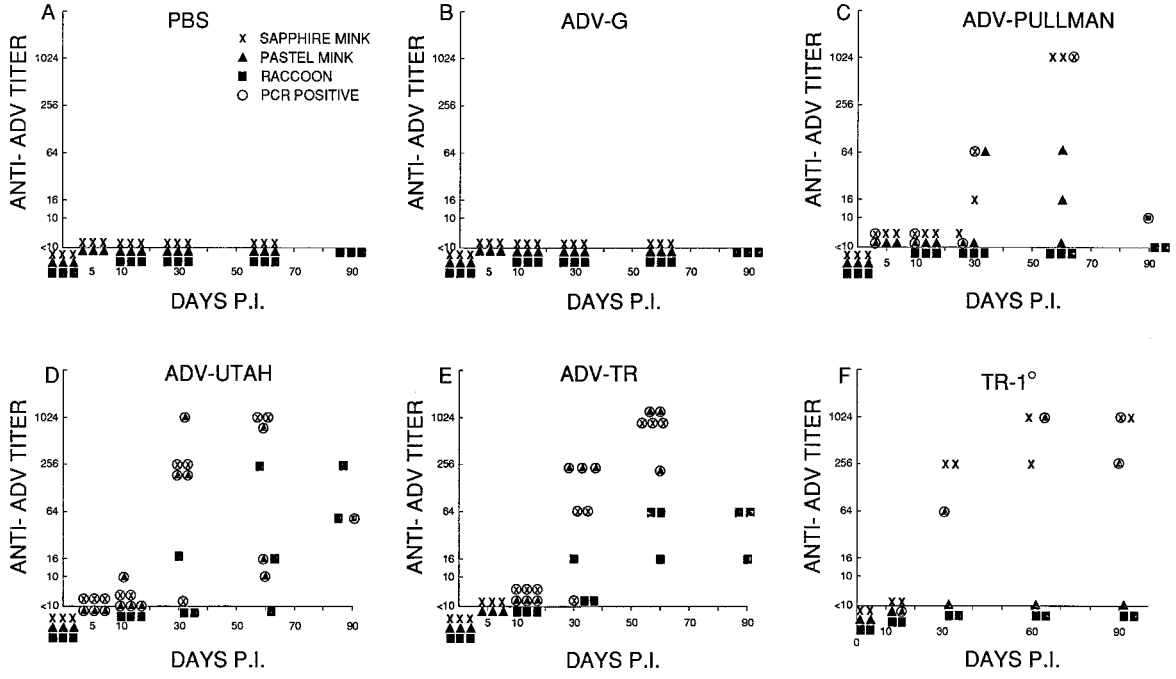

FIG. 3. Responses of mink and raccoons to infection with ADV. Groups of three Aleutian (sapphire) mink, three non-Aleutian (pastel) mink, and three raccoons were inoculated intraperitoneally with PBS (A) or the indicated virus (B to E). At the indicated times, serum samples were collected and subjected to analysis for anti-ADV antibodies by CEP and viremia by direct PCR. Serum samples testing positive by PCR are noted by circled CEP values. (F) Groups of two sapphire mink, two pastel mink, and two raccoons were inoculated intraperitoneally with ADV-TR-1°. At the indicated times, serum samples were obtained and assayed for anti-ADV antibodies and viremia.

ined or ADV DNA in the mesenteric lymph node at necropsy (Fig. 3B and Table 1). These findings indicated that the type 1 ADV-G was noninfectious for either adult mink or raccoons (16, 19).

ADV-Utah group. The sapphire mink inoculated with ADV-Utah developed typical progressive AD and a positive serum PCR as early as day 5 postinfection (p.i.), prior to the appearance of antiviral antibody. ADV DNA persisted in serum until necropsy (Fig. 3C and Table 1).

The pastel mink from this treatment group also developed progressive disease and a persistently positive serum PCR detectable as early as day 5 p.i.

All three raccoons inoculated with ADV-Utah developed increasing titers of antibody to ADV, but its appearance was delayed and the titers were lower than those for the mink. Furthermore, only one raccoon had a positive serum PCR at any time during the course of the experiment. ADV DNA was detected in the mesenteric lymph node DNA from a different raccoon that did not have a positive serum PCR. Thus, although ADV-Utah was replicating in the raccoons, pathologic changes of AD were not induced, at least within the time frame of these experiments.

ADV-Pullman. By the time of necropsy at day 60 p.i., two of the three sapphire mink had typical progressive AD. One of these two developed positive serum PCR and had viral sequences demonstrable in the mesenteric lymph node as assayed by PCR. The third sapphire mink did not develop antibody, have ADV DNA in serum, or show any lesions of AD at necropsy; this animal likely failed to become infected after inoculation (Fig. 3D and Table 1).

In contrast, none of the pastel mink exhibited signs of progressive disease, and only two of the three developed antiviral antibodies. Persistently positive serum PCRs were not detected, although one mink was transiently positive at days 5 and 10 p.i. Interestingly, viral sequences were detected in mesenteric lymph node DNA from two animals in this group. The mink with the transient positive serum PCR had minimal hepatic lesions of subclinical disease typical of pastels infected with this strain of virus. These findings were concordant with previous observations on ADV-Pullman in sapphire and pastel mink (23, 41–43).

A single raccoon inoculated with ADV-Pullman developed anti-ADV antibody by day 90 p.i. and had a positive serum PCR at the same time point. ADV DNA was also amplified from the mesenteric lymph node from this animal. None of the raccoons had pathologic changes of AD. Thus, the response of the raccoons to ADV-Pullman was even more attenuated than that of the pastel mink.

ADV-TR group. The response of the sapphire and pastel mink inoculated with ADV-TR was virtually identical with that of the ADV-Utah-infected sapphire mink. All developed an early positive serum PCR and progressively rising antiviral antibody titers. At necropsy, lesions of progressive AD were apparent, and ADV DNA sequences were present in the mesenteric lymph node DNA. (Fig. 3E and Table 1). Thus, this isolate was highly pathogenic for mink.

All three raccoons inoculated with ADV-TR developed increasing titers of antiviral antibody by necropsy at day 90 p.i., but the titers were lower than in the mink. None had ADV DNA detectable in serum or the mesenteric lymph node, and none had pathologic changes of AD. These results strongly indicated that ADV infected these animals but did not induce disease in them.

Serial passage of ADV-TR from experimentally infected raccoons to raccoons and mink. The results presented above clearly indicated that raccoons could be infected with several of the ADV isolates, although typical pathological findings of ADV infection were not noted. Furthermore, because reports from the ADV outbreak in Utah suggested that raccoons might be involved with the transmission of infection on the mink ranches, we wanted to assess a possible role for raccoons in animal-to-animal transmission.

A 10% (wt/vol) suspension made from the mesenteric lymph nodes of the ADV-TR-infected raccoons (ADV-TR-1°) was injected into mink and raccoons. Both sapphire mink and one of the pastel mink responded to ADV-TR-1° by developing elevated titers of antiviral antibodies (Fig. 3F). A positive serum PCR was detected in one sapphire and one pastel mink. Pathologic changes of AD were observed in the two infected sapphire mink and in the antibody-positive infected pastel mink. The ADV-TR-1°-inoculated raccoons failed to develop antiviral antibodies, a positive serum PCR (Fig. 3F), or any characteristic pathologic changes.

For a control, a similar suspension was made from the PBS-inoculated raccoons and injected into naive mink and raccoons. None of these animals showed evidence of infection or disease.

These results indicated that it was possible to transmit AD to mink with virus from infected raccoons. Furthermore, infectious virus was present in the mesenteric lymph nodes from the raccoons used to prepare the ADV-TR-1°, even though ADV DNA could not be detected in DNA from these tissues. However, the findings also suggested that raccoon-to-raccoon transmission did not occur.

DISCUSSION

The combination of molecular and in vivo studies described here has yielded new information concerning ADV as well as ADV infections in animals. A new isolate of virus, ADV-TR, was obtained from naturally infected ranch mink in Utah and identified as the likely cause of a recent outbreak of AD there. Under controlled experimental conditions, ADV-TR produced persistent viremia and typical progressive disease in both Aleutian and non-Aleutian genotype mink and was as pathogenic as ADV-Utah (20, 42, 64). In contrast, ADV-Pullman, as previously described (23, 41, 42), was nonpathogenic for the non-Aleutian mink. The molecularly cloned, cell culture-adapted ADV-G strain (16, 19) was noninfectious for mink.

Raccoons were implicated as having a potential role in the Utah outbreak. Therefore, to evaluate their susceptibility, we also infected raccoons with ADV-G, ADV-TR, ADV-Utah, and ADV-Pullman. Although none of the raccoons had pathologic changes of progressive disease, all animals infected with ADV-TR and ADV-Utah developed antibody titers of >1:16 against ADV, and one infected with ADV-Utah had transient viremia. Thus, raccoons can definitely become infected with highly pathogenic strains of virus. Furthermore, a suspension made from ADV-TR-infected raccoon lymphoid tissue induced AD in naive mink of both genotypes, suggesting that infected raccoons can transmit disease to mink. However, the same material did not infect additional, naive raccoons, suggesting that serial transmission among raccoons may not occur. It was not possible to conclude that the Utah outbreak originated from free-ranging raccoons because the serum and tissue specimens available for study were collected on ranches already experiencing ADV infections. Nevertheless, these results suggested that raccoons may play a role in the transmission of ADV (44).

Recent molecular analysis of the hypervariable regions indicates that several distinct sequence types of ADV exist (18,

TABLE 1. Results of studies performed at necropsy on infected mink and raccoons[a]

Inoculum	Animal group	No. of animals/no. tested			
		Anti-ADV titer[b]	Pathologic changes	Viremia	MLN DNA PCR
PBS	Sapphire	0/3 (<10)	0/3	0/3	0/3
	Pastel	0/3	0/3	0/3	0/3
	Raccoon	0/3	0/3	0/3	0/3
ADV-G	Sapphire	0/3	0/3	0/3	0/3
	Pastel	0/3	0/3	0/3	0/3
	Raccoon	0/3	0/3	0/3	0/3
ADV-Pullman	Sapphire	3/3 (4,096, 256 × 2)	2/3	1/3	1/3
	Pastel	1/3 (16)	1/3[c]	1/3[c]	2/3
	Raccoon	1/3 (4)	0/3	0/3	1/3
ADV-TR	Sapphire	3/3 (1,024 × 3)	3/3	3/3	3/3
	Pastel	3/3 (4,096, 256 × 2)	3/3	3/3	3/3
	Raccoon	3/3 (16, 64 × 2)	0/3	0/3	0/3
ADV-Utah	Sapphire	3/3 (256 × 2, 1,024)	3/3	3/3	2/2
	Pastel	3/3 (256 × 3)	3/3	3/3	2/3
	Raccoon	3/3 (64 × 2, 256)	0/3	1/3	1/3

[a] Animals inoculated with the specified preparations were necropsied (60 and 90 days p.i., respectively, for mink and raccoons). Sera were collected and assayed for anti-ADV antibody by CEP and viremia by PCR as detailed in the text. Whole-cell DNA was extracted from a portion from the mesenteric lymph node (MLN) of each animal, and the presence of ADV DNA was sought by PCR. The development of typical ADV-induced pathologic changes was assessed by gross and microscopic examination.

[b] Numbers in parentheses indicate the reciprocal values for individual animals of the anti-ADV antibody titer determined by CEP. Values indicated as, for example, 256 × 2 indicate that a reciprocal value of 256 was found for two animals.

[c] A single mink of this group, positive for viremia at days 5 and 10, had minimal hepatic lesions.

37, 38). The sequences from mink and raccoons from the current Utah outbreak were all type 1, very similar to ADV-G in this region of the genome (Fig. 1). Thus, these results indicated that ADV-TR was a type 1 ADV and that the same virus was present in both mink and raccoons. The uniformity among the individual samples from the Utah outbreak confirmed previous speculation that the sequence of this region is, in fact, stable within sequence types (37, 57, 58, 62).

The function of the hypervariable region remains a conundrum. Published studies with chimeric ADV constructions suggest that the hypervariable region does not act as a pathogenicity determinant because substitution of the ADV-Utah (type 2) region into an ADV-G (type 1) virus did not confer pathogenicity or alter its host range in vitro (19). The findings from the present experiments confirmed that the hypervariable region is not inextricably linked to pathogenicity, because the type 1 ADV-TR was as pathogenic as the type 2 ADV-Utah. As previously observed (18, 37, 38), the intense variability noted in this short sequence of the various ADV isolates far exceeds that seen among the viruses of the CPV/FPV/MEV group of viruses. The ADV hypervariable region resides at map units 64 to 65 in the coding region for capsid protein VP2 and aligns with surface loop 2 of the CPV crystal structure (19), which has been defined as an antigenic determinant for the FPV/CPV/MEV family (29, 70). Prokaryotic expression studies are under way to determine if the hypervariable region of ADV may serve as a specific epitope.

We also compared the complete VP2 coding regions of ADV-G, ADV-TR, two isolates of ADV-Utah, and ADV-Pullman (Fig. 2). When correlated with our published work on ADV-G/ADV-Utah chimeras (19), the findings provide insights into potential determinants of pathogenicity for mink and of host range for in vitro growth in Crandall feline kidney (CRFK) cells. Exchange of the ADV-G VP2 sequence from amino acids 50 to 226 with that of ADV-Utah renders the resulting virus replication defective for CRFK cells (18, 19). In other words, this region, which aligns with surface loop 1 of the CPV crystal structure (29), contains a viral host range determinant for CRFK cells. ADV-TR differed at a single residue from ADV-G in this segment (F for Y at position 115). Only F-115 was shared by ADV-TR with ADV-Utah, ADV-ZK8, and ADV-Pullman. There was some variation evident among ADV-Utah, ADV-Utah kit, ADV-Pullman, and ADV-ZK8; however, there were three conserved differences (amino acids 92, 94, and 115), and a single amino acid (A-92) was conserved among the non-ADV-TR pathogenic isolates. Thus, even though this segment governs CRFK host range, it seems unlikely that it contains a simple determinant of pathogenicity because ADV-TR is highly pathogenic. In addition, the similarity between the ADV-G and ADV-TR sequences implied that ADV-TR might be replication competent for CRFK cells, a possibility currently under investigation.

The results of VP2 sequence comparisons from amino acid residues 359 to 647 suggested that differences between ADV-G and pathogenic isolates in this area are confined to a fairly small number of residues (Fig. 2). The segment from residues 359 to 647 contains four loci at which all of the pathogenic isolates, including ADV-TR, differed from ADV-G (Q for H at residue 395, N for D at 433/434, D or E for N at 491, and D for H at 534). Although the alignment with the CPV sequence is not as good as for surface loops 1 and 2, CPV surface loop 4 aligns roughly between residues 440 and 500 (19, 29), thus potentially encompassing several of these changes. Furthermore, the host range determinants in the FPV (1, 69) and minute virus of mice (13, 14, 35) systems govern the surface topography of the viral particle on a spike near the threefold axis symmetry formed by the interdigitation of surface loops 3 and 4 in a complex fashion (29, 70). Our previous chimeras (19) did not include any in which VP2 residues 359 to 647 from a pathogenic strain were included in an ADV-G background in the absence of the residue 50 to 226 host range determinant. Consequently, some of the differences between residues 359 to 647 may be involved in ADV pathogenicity and host range. We plan to construct additional ADV chimeras to examine this possibility.

Unfortunately, no skunk samples were available from the Utah outbreak even though the ranchers reported problems with these animals, too. Thus, we could not directly assess a possible role for skunks in the Utah outbreak. Little is known about ADV infections in skunks even though they are reported to have high seroprevalence to ADV (44, 50). Our studies found that sera from 4 of 22 free-ranging skunks from South Dakota contained anti-ADV antibody. Serum samples from one of them and from two seronegative skunks contained detectable ADV DNA. The hypervariable region sequences from the two skunks were definitely not ADV-TR and, in fact, differed the most from all of the other sequences examined (Fig. 1). This finding may mean that skunks have a unique ADV type. On the other hand, it may simply indicate that the ADV from the South Dakota area differs from other viruses. It would be interesting to collect specimens and to examine the sequences of ADV isolates from skunks, raccoons, and mink from different areas of the country.

Finally, although this study has focused on the VP2 capsid

gene sequences of ADV, other portions of the ADV genome might also influence pathogenicity. For example, ADV-Pullman is pathogenic only for non-Aleutian mink (23, 42, 43), although its VP2 sequence is extremely similar to those of ADV-Utah and ADV-ZK8 (Fig. 2). In fact, studies on porcine parvovirus implicate both capsid and nonstructural proteins in porcine parvovirus host range (72). In addition, it was recently found that different types of ADV exhibit an unusual degree of variability in the nonstructural genes (38). Consequently, even though our initial studies with chimeric ADV constructions do not demonstrate a role for the nonstructural proteins in determining host range or pathogenicity (19), this question remains to be conclusively resolved.

In summary, the particular isolate of virus is an important determinant of whether infection with ADV leads to progressive disease. Elucidating the viral genomic sequences responsible for pathogenicity requires sequence comparison of a number of viral isolates. In the present set of experiments, we compared capsid genes of a number of different ADVs. Our analyses indicated that the hypervariable region of VP2 is not an obligate determinant of pathogenicity. Our results also found that a limited number of other amino acid residues in VP2 differ consistently between pathogenic and nonpathogenic isolates. These amino acids may function singly or coordinately in controlling host range and pathogenicity of ADV.

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