Nucleotide Sequence of the 5'-Terminal Palindrome of Aleutian Mink Disease Parvovirus and Construction of an Infectious Molecular Clone

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The 5'-terminal palindrome of the ADV-G strain of Aleutian mink disease parvovirus (ADV) was molecularly cloned and sequenced. A full-length molecular clone of ADV-G, denoted pXVB, was then constructed. When this clone was transfected into cell cultures, infectious ADV could be rescued. Virus derived from pXVB was nonpathogenic for adult mink, as is the parent ADV-G strain.

Strains of Aleutian mink disease parvovirus (ADV) differ reproducibly in their pathogenicity for mink (20). The virulent ADV Utah 1 strain, which replicates abortively in cell culture (21, 30), produces a persistent infection associated with severe dysfunction of the immune system in adult mink (4, 9, 20). On the other hand, the ADV-G strain (10) is nonpathogenic for adult mink (10) but replicates permissively in cell culture (7, 10, 12). These findings imply that structural features contained within the genomic sequence may govern pathogenicity and the ability of ADV to replicate in cell culture (5, 6). It would be of great interest to identify such presumed determinants of pathogenicity and to try to determine exactly how such structural features are translated into dramatic differences in biological behavior.

One means of pursuing this goal is to prepare full-length molecular clones from which infectious virus may be rescued (26, 34). The viral DNA in such clones can be manipulated, and segments can be exchanged between different virus strains to produce chimeras. Thus, the functions of discreet portions of the genome can be examined. This approach has made it possible to map host range and pathogenicity determinants of minute virus of mice (3, 22, 33) and canine parvovirus (27, 28) with exquisite precision. The viral host range of canine parvovirus is governed by only a few amino acids in the capsid gene segment (27).

The parvovirus genomes are small (approximately 5,000 nucleotide [nt]), single-stranded DNAs with palindromic sequences at both ends (reviewed in reference 15). A variety of different duplex replicative-form (RF) DNA molecules necessarily exist as replicative intermediates (7, 8, 15). Obviously, the development of full-length molecular clones of parvoviruses requires that palindromic sequences at both the 3' and 5' ends be preserved in a stable form.

We recently described the DNA sequence of the nonpathogenic ADV-G strain, but failed to obtain the 5' palindromic hairpin sequence (5). We also analyzed a portion of the pathogenic ADV Utah 1 strain; a small number of sequence differences between the two strains were observed, primarily in the segment of the genome representing the capsid protein sequences (5). Because similar sequence variation in other parvovirus systems can greatly influence pathogenicity and viral tropism, we speculated that some of these sequence differences may similarly govern the pathogenicity of ADV. However, to test this hypothesis, it will first be necessary to obtain molecular clones containing the right palindromic sequences and to develop an infectious molecular clone of ADV. In this brief report, we detail experiments describing the development and characterization of an infectious molecular clone of ADV-G.

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To develop constructs extending to the extreme 5' end of the molecule, we modified an alternative strategy that was successful for minute virus of mice (13, 26). We produced a clone that extended from the unique EcoRI site at nt 2553 to the extreme right-hand or 5' end at nt 4748, adding 155 nt to the 4,592 bases of sequence obtained previously (Fig. 1). Analysis indicated that the terminal 241 nt could now be arranged into a hairpin configuration similar to the flop orientation of the minute virus of mice 5' palindrome (15) (Fig. 2). The level of homology between the 5' palindromes of the two viruses was only 43%, reinforcing the concept that it is the configuration that is important, not the actual sequence (15). We therefore concluded that this right-half clone contained sequences representing the complete 5'terminal palindrome of ADV-G.

A full-length clone of ADV-G, designated pXVB, was then constructed by combining the right-half clone with a left-half clone (Fig. 1). The continued presence of the 5' terminus was verified by DNA sequencing (5), and the clone was stable upon large-scale propagation in *Escherichia coli* JC8111 (5, 13). The completed sequence of the ADV-G strain is presented in Fig. 3. This sequence has been assigned the GenBank accession number M20036.

When the 241-nt 5' palindrome was arranged in hairpin form, the presumed 120-base-pair duplex should have been visible by electron-microscopic analysis (18). A structure consistent with this hairpin was readily observed (Fig. 4A); its size and location on the linear molecule matched the predicted values (Fig. 4B). The 3' hairpin was not visualized, probably because it forms a Y-shaped configuration whose

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FIG. 1. Schematic development of a full-length molecular clone of ADV-G. A full-length clone of ADV-G was constructed by joining together a left half and a right half. The left half was developed from a previously described clone (5). Briefly, duplex monomeric replicative form DNA was purified from ADV-G-infected CRFK cells as described previously (5, 24), dC tailed (23), annealed to *PstI*-digested, dG-tailed (23) pEMBL8 (16), and transformed into *E. coli* JM109 (35) to produce a near-full-length clone, pADVG1-5 (5). The 0- to 53-map-unit (mu) fragment was released from purified plasmid by double digestion with *Eco*RI and *Hind*III, ligated into similarly digested pGEM3 (25), and transformed into JM109, thus completing the left-half clone, pXIVQ-20. The right half was made by preparing synthetic duplex monomer from single-stranded virion DNA with Klenow fragment (6, 7, 26), adding *Eco*RI linkers to the 100-map-unit end (23), and isolating the 53- to 100-map-unit piece. This fragment was ligated into *Eco*RI-digested pGEM3 and transformed into the *recBc sbcB recF E. coli* JC8111 (13) to yield the right-half clone, pXIVQ-20 prior to transformation in JC8111.

duplex portion would be too short for visualization (5, 15, 18).

We next performed a series of transfection (19) experiments to verify that the full-length construct was infectious in permissive cell culture. Crandell feline kidney (CRFK) cells (1.5×10^5) were seeded into wells of a 24-well culture plate and incubated overnight at 37°C. The following plasmid DNAs were purified by two cycles of cesium chlorideethidium bromide density ultracentrifugation (23, 24): pUC19 (35) as a negative control, the left-half clone pXIVQ-20, and the full-length clone pXVB. ADV-G RF DNA was purified from Hirt extracts of infected CRFK cells (7, 23). DNA-calcium phosphate precipitates (19) were prepared with the various DNAs and added to replicate wells in a 50- μ l volume. After a 4-h incubation at 31.8°C, the monolayers were treated with 0.5 ml of 15% glycerol for 60 s, washed with media, fed, and incubated at 31.8°C. Five days after transfection, monolayers were fixed with 80% acetone in phosphate-buffered saline and examined for the presence of ADV antigens by direct immunofluorescence. The results of a representative experiment are summarized in Table 1. Both RF DNA and pXVB DNA induced typical ADV intracellular antigen in CRFK cells (2, 32) by 5 days after transfection, and the ability to induce the antigen was similar



FIG. 2. DNA sequence of the 5' palindrome of ADV-G. The extreme 5' termini of both the right-half clone, pXIIIW-13, and the full-length pXVB were sequenced (5). The sequence of the minus or virion strand was arranged into a hairpin form and compared with a similar depiction of the flop orientation of minute virus of mice (MVM) (15).

1	3' terminal palindrome ATTAATTCTCAACCAATATTCGTTAGCAACCAACACCAGCTCGCTTCGCTCGC	2401	start RORF AGGAAATGGATTCTACTGAAGCTGAACAAATGGACACTGAGCAAGCA
61	CGGCGCTGGTGTTGGGCGCCTCGCGCTTGCTAACTTCATATTGGTTGAGAATTAATCCGT	2461	CTGAAGCTGGTGGTGGGGGGGGGGGGGGGGGGGGGGGGG
121	p3 promoter GTCTTTCCTGTGGAATGAGGAAGTAGTAGTGGTTAGCAGAGGGTTGCTTGGAGCAAAG	2521	ECO RI 53mu poly A GTAACAGCACTGGCGGCTTTAATAACACAACA GAATTC AAAGT AATAA CAATGAAGTGT
181	CACAGACCGGTTACAGCAAAGTAACATGGCTCAAGGCTCAAATTGATGAGCAGAGGAGAGCT	2581	ATATTACTTGTCACGCTACTAGAATGGTACACATTAACCAAGCTGACACAGACGAATACT
241	GCAGGACCTGTATGTGCAGTTGAAGAAGGAGATTAACGACGGTGAAGGAGTTGCCTGGTT	2641	TGATATTTAATGCTGGTAGAACTACTGATACCAAAACACATCAGCAAAAACTAAACTTAG
301	GTTCCAACAAAAGACCTACACCGACAAGGACAACAAACCAACC	2701	AATTTTTTGTATATGATGATTTTCACCAACAAGTAATGACACCTTGGTATATAGTAGATA
361	GAGGACAACCTCTTCTGACCTAAGGTTAGCTTTTGACTCTATTGAAGAGAATTTAACAGC	2761	GCAACGCTTGGGGTGTATGGATGAGTCCTAAAGACTTTCAACAAATGAAAACACTGTGTA
421	TTCTAATGAACACTTAACTAACAATGAGATAAACTTTTGTAAACTAACCTTGGGGAAGAC	2821	GTGAAATTAGTTTGGTTACTTTGGAACAAGAAATAGACAATGTAACCATAAAAACTGTAA
481	GTTGCTGTTAATTGATAAGCATGTAAAAAGCCACAGATGGGATAGTAACAAAGTTAACTT	2881	CAGAAACCAACCAAGGTAACGCATCTACCAAGCAATTCAACAATGACTTAACTGCGTCGT
541	AATTTGGCAAATAGAAAAAGGAAAAACTCAGCAATTTCATATTCACTGTTGCTTAGGTTA	2941	TACAGGTTGCTTTAGATACTAACAACATACTGCCATATACTCCAGCTGCGCCGTTGGGGG
601	CTTTGATAAGAATGAAGATCCTAAGGATGTTCAAAAAATCCTTAGGTTGGTT	3001	AAACACTGGGCTTTGTTCCTTGGAGAGCAACCAAACCAA
661	ACTAAATAAAGACCTAGCAGTTATCTATAGTAACCATCATTGTGACATACAAGATATTAA	3061	CATGTTACATTTACAACAGATATCCTAACATTCAAAAAGTTGCAACAGAAACACTAACCT
721	GGATCCTGAAGATAGAGCTAAGAACCTAAAAGTGTGGATTGAAGATGGACCTACTAAGCC	3121	GGGATGCAGTACAAGATGATTACCTTAGTGTGGATGAACAGTACTTTAACTTTATTACTA
781	TTACAAATATTTTAACAAACAAACCAAACAAGACTACAATAAACCAGTTCACTTGAGAGA	3181	TAGAGAACAACATACCTATTAACATTCTCAGAACGGGAGATAACTTTCATACAGGCTTGT
841	CTATACATTCATATACCTGTTTAACAAAGATAAGATAAATACAGATAGTATGGATGG	3241	ATGAGTTTAACAGTAAACCATGTAAACTAACCTTAAGCTATCAAAGTACACGTTGCTTGG
901	CTTTGCTGCTGGTAACGGTGGCATTGTTGACAACCTAACTAA	3301	GGCTACCTCCTCTGCAAACCAAAGACAGATACAACACAAAGTAACCTCAAAAGAAA
961	AAGAAAAATGTACTTAGATGAGCAGAGTTCAGATATAATGGATGCTAATATAGACTGGGA	3361	ACGGAGCTGACCTAATTTACATACAAGGACAAGATAATACCAGACTAGGTCACTTTTGGG
1021	AGATGGCCAAGACGCGCCAAAAGTAACTGACCAAACTGACTCAGCAACCACAAAAACAGG	3421	GTGAGGAAAGAGGTAAGAAAAACGCAGAGATGAACAGAATTAGACCTTACAACATAGGTT
1081	AACTAGTTTGATTTGGAAATCATGTGCTACTAAAGTAACCTCAAAAAAAA	3481	ACCAATATCCTGAATGGATAATACCAGCAGGGTTACAGGGTAGTTACTTTGCTGGAGGAC
1141	TCCAGTTCAGCAACCTTCTAAAAAACTGTACTCAGCTCAAAGTACTTTAGATGCATTGTT	3541	CAAGACAGTGGAGTGACACAAACCAAAAGGTGCAGGTACACACAGTCAACACTTACAACAGA
1201	TAACGTTGGTTGCTTTACTCCAGAAGATATGATTATAAAGCAAAGTGACAAATACCTTGA	3601	ACTITAGTACTAGGTACATCTATGACAGAAACCACGGTGGAGACAACGAGGTAGACCTAT
1261	ACTATCTTTAGAACCAAACGGGCCTCAAAAAATTAACACTTTACTTCACATGAACCAAGT	3661	TAGATGGAATACCCATTCATGAAAGAAGTAACTACTACTCAGACAATGAGATAGAGCAAC
1321	AAAGACATCAACCATGATTACTGCTTTTGATTGTATTATAAAATTTAATGAAGAGGAAGA	3721	ATACAGCAAAGCAACCAAAGTTACGTACACCACCCATTCACCACTCAAAAAATAGACTCGT
1381	TGACAAACCTTTGCTAGCAACTATAAAAGACATGGGACTTAATGAACAATACCTTAAGAA	3781	GGGAAGAAGAAGGTTGGCCTGCTGCTTCAGGCACACACTTTGAAGATGAGGTTATATACC
1441	GGTACTATGTACCATCCTAACCAAGCAAGGTGGAAAGAGAGGTTGTATTTGGTTCTATGG	3841	TAGACTACTTTAACTTTAGTGGTGAACAGGAGCTAAACTTTCCACATGAAGTATTAGATG
1501	ACCGGGGGGCACTGGAAAAACCTTGCTAGCATCTTTAATATGTAAAGCAACAGTAAACTA	3901	ATGCTGCTCAGATGAAAAAGCTACTTAACTCATACCAACCA
1561	TGGTATGGTTACTACAAGCAATCCAAACTTTCCATGGACTGACT	3961	TTGGTCCTGTATACCCGTGGGGACAGATATGGGACAAGAAACCTCATATGGATCACAAAC
1621	CATTTGGGCTGAAGAGTGTGGTAACTTGGTAACTGGGTTGAAGACTTTAAAGCCATTAC	4021	CTAGCATGAACAACGACGCTCCATTTGTATGTAAAAACAACCCTCCAGGTCAACTCTTTG
1681	TGGAGGTGGTGATGTAAAAGTAGACACCAAGAACAAGCAACCTCAATC TATTAAA GGCTG	4081	TTAAACTAACAGAAAACCTCACTGATACATTTAACTATGATGAAAAATCCAGACAGA
1741	TGTGATTGTAACAAGCAACACCAACATAACCAAAGTAACTGTTGGATGTGTGGAAACAAA	4141	AAACCTATGGTTACTTTACTTGGAGAGGC AAGCTT GTACTAAAAGGCAAACTAAGCCAAG
1801	CGCTCACGCAGAGCCACTTAAACAGAGGATGATTAAGATACGTTGCATGAAAACCATCAA	4201	TAACATGCTGGAATCCTGTTAAGAGAGAACTCATAGGAGAACCTGGTGTATTTACTAAAG
1861	CCCTAAAACTAAAATAACACCAGGCATGTTAAAAAGATGGCTAAATACCTGGGATAGACA	4261	ACAAGTATCACAAACAGATACCAAACAAAGGTAACTTTGAAATAGGGTTACAATATG end RORF
1921	end ACCAATTCAACTAAGCCATGAGATGCCTGAACTGTACTTAGGTAAGTGCCGTTGG TAA GT	4321	GAAGAAGTACTATCAAATATATCTAC
1981	LORF AACACATTTTAAATGCCAACTTTAAACCAACATCAATTTATGAGGTTACTTTACTTTACA	4381	ATGATAATATCTC
2041	GAGACTACTGGACCAAACTCGAGTGCCACAACTGCCACGAAGAATACTGGCAACTCACAA	4441	TTATTTTACCAGAAAGTGGCGGATTAAAATAAACCTACATTCTATACTATCTAT
2101	CCTACTACTGCAAAGAGTGCAGAAAGTGTGAACACGGAAAACTGCGACACACCAAAAAAGG	4501	CTAACTAACCTATAGGTTACTTGCTTTGCTATACTGATGTAGGAATACAGGATACTAAC
2161	AGTGCGAGCAGTGTGCCTGCAAAGCAGCACAAGAGACCTCGGCATGAGTAAAAGTAAATA	4561	ATTTATATATATACTAACATCTATACTACCTAACCTATGGCCCTATTGTATGCATTGT
2221	ACCTACTTAAAGTAACCTAACACCATAACACTTTACTTT	4621	CGGCTTTGCCGACAATGCATGCATTAATAGGCCATAGTTAGGTTAGTAGTAGTAGATGTTA
2281	CTTTAGTTCCTCAGCACTATCCTGGGAAAAAGAGAAGTGCTCCAAGACACGTGTTTATTC	4681	GTATATATATAAATGTTAGTATCCTGTATTCCTACATCAGTATATCAAAGCAAAGTAACC
2341	AGCAAGCAAAAAAGAAGAAGCAAACTAACCCTGCGGTCTACCACGGAGAGGACACCATAG	4741	TATAGGTT

FIG. 3. DNA sequence of ADV-G. The newly obtained sequence for the 5'-terminal palindrome was joined to the 4,592-nt sequence previously reported (5) to produce a completed ADV-G sequence of 4,748 nt. The extent of the 3'- and 5'-terminal palindromes, the location of the p3 and p36 promoters, and the positions of the functional polyadenylation sites are indicated (1, 5). The probable initiation (start) and termination (end) codons of the major left (LORF) and right (RORF) open reading frames, as well as the location of the unique BamHI, EcoRI, and HindIII restriction enzyme recognition sites are also identified (1, 5).

for both DNAs. Furthermore, when cell lysates were blind passaged three times, only these two samples were capable of producing infectious ADV. In some transfection experiments, the left-hand clone, pXIVQ-20, induced a very faint pattern of granular, exclusively nuclear antigen; however, infectious virus could not be identified on repeated passage. This result may have reflected transient synthesis of nonstructural protein components (27) because that clone contains coding information for the ADV nonstructural proteins (1, 14, 15). The results of these experiments indicated that the full-length clone, pXVB, was capable of generating infectious ADV in CRFK cells.

A virus stock (XVB), derived from a pXVB transfection, was then used to compare selected properties with those of a standard ADV-G preparation. When studied 72 h after infection by Western immunoblot (6, 24), CRFK cells infected with either ADV-G or the molecularly cloned XVB virus contained the same ADV proteins. The two virion proteins, p85 and p75, as well as the major nonstructural protein, p71, were readily visualized (6, 11, 24) (Fig. 5A) and were identical in apparent size from both viruses.

TABLE 1. Transfection of CRFK cells

DNA tested	Infectivity of DNA (FFU/μg) ^a	Virus titer after third passage (FFU/ml) [*]	
Mock	ND	ND	
pUC19	ND	ND	
pXIVO-20	ND	ND	
ADV-G RF	$(27.5 \pm 4.5) \times 10^3$	$1.8 imes10^6$	
pXVB	$(7.4 \pm 1.3) \times 10^3$	7.3×10^{6}	

^{*a*} CRFK cells were transfected, glycerol boosted, and incubated as described in the text. The lower limit of detection was estimated to be 0.4 FFU/ μ g of DNA transfected.

^b CRFK cells were transfected, glycerol boosted, and incubated as detailed in the text. After 5 days, the cell pellets were collected, freeze-thawed, sonicated, and then blind passaged three times in CRFK cells at 31.8°C. Samples from third-passage material were assayed for the presence of infectious ADV. The lower limit of detection was 10 FFU/ml.



FIG. 4. Electron microscopy of the 5' (right-hand) palindrome of ADV-G. Purified pXVB plasmid was linearized at the unique *Bam*HI site located at ADV-G nt 721 (5). (A) the DNA was denatured in alkali, allowed to reanneal under conditions that favored intrastrand duplex formation, and analyzed by electron microscopy (18). Numerous unit-length, single-stranded molecules contained a short panhandle structure, consistent with a short palindromic hairpin (arrows). (B) Length measurements (18) were made on 25 molecules, and the location of the panhandle was aligned on a schematic of *Bam*HI-digested pXVB. The extent of the ADV-G and vector sequences is indicated.



FIG. 5. Comparison of ADV-G with virus derived from molecularly cloned pXVB. A virus stock (XVB) derived by serial passage of lysates from pXVB-transfected CRFK cells was infected in parallel with a standard ADV-G stock into CRFK cells for 72 h (10). (A) A sample from each culture was lysed in sample buffer (11) and analyzed by Western blot with serum from ADV-infected mink and peroxidase-protein A (6, 24). The structural (p85 and p75) and nonstructural (p71) proteins were observed in both instances (6, 11, 24). (B) Low-molecular-weight DNA, isolated from other samples of infected cells (5, 24), was analyzed by Southern blot hybridization (5, 7, 12). A 9.6-kilobase-pair (kbp) duplex dimer RF DNA (DD DNA), a 4.8-kilobase-pair duplex monomer RF DNA (DM DNA), and a 4.8-kilobase single-stranded virion DNA (SS DNA) were readily visualized (5, 7, 12). (C) DM DNA was purified (5, 24) and physically mapped with several restriction enzymes, each having a single recognition site: BamHI (nt 721), EcoRI (nt 2553), HindIII (nt 4169), and EcoRV (nt 3069) (5, 7). Digestion of purified RF DNA was incomplete, and undigested DM DNA was visible in all lanes.

5034

Mink no.	Virus inoculum (FFU) ^a	Anti-ADV antibody ^b	Viremia (pg/ml) ^b	Gamma globulin ^b	ADV pathol- ogy ^c					
5025	$2.9 \times 10^{6} (XVB)$	<1/10	<20	Normal	Absent					
5026	$2.9 \times 10^6 (\text{XVB})$	<1/10	<20	Normal	Absent					
5027	2.9×10^4 (XVB)	<1/10	<20	Normal	Absent					
5028	$2.9 \times 10^4 (\text{XVB})$	<1/10	<20	Normal	Absent					
5043	7.2×10^6 (ADV-G)	<1/10	<20	Normal	Absent					
5044	7.2×10^6 (ADV-G)	<1/10	<20	Normal	Absent					
5041	7.2×10^4 (ADV-G)	<1/10	<20	Normal	Absent					
5042	$7.2 \times 10^4 \text{ (ADV-G)}$	<1/10	<20	Normal	_d					
5033	Mock	<1/10	<20	Normal	Absent					

TABLE 2. Inoculation of adult mink with molecularly cloned ADV-G

^a Adult sapphire (Aleutian genotype) mink were inoculated with the indicated doses of virus stock derived from pXVB (XVB), inoculated with a standard stock of ADV-G (10), or mock infected.

< 1/10

Normal

<20

Absent

At 6 to 8 weeks after infection, the mink were bled and their sera were assayed for the presence of anti-ADV antibodies and elevated gamma globulin levels characteristic of progressive ADV infection (4, 9, 29). Serum samples were also obtained 8 to 10 days after infection and the amount of ADV DNA was determined to be <20 pg/ml ($<2.5 \times 10^4$ mink infectious doses/ml [12]); this value indicates the absence of acute viremia (12).

Mink were necropsied at 113 (XVB and MOCK) or 77 (ADV-G) days after infection and examined for the presence of pathological lesions diagnostic of progressive ADV infection (4, 17, 29).

^d Mink no. 5042 was not necropsied.

Mock

In addition, when low-molecular-weight DNA was purified from cells infected with both viruses and compared (7, 8,12) (Fig. 5B), the sizes and relative ratios of the various intracellular ADV DNA forms were the same. Furthermore, physical mapping of purified RF DNAs confirmed this result because the restriction fragments generated for several restriction endonucleases were the same for both viruses (Fig. 5C). Thus, XVB virus derived from the molecular clone appeared to replicate productively in CRFK cells identically to the parent ADV-G strain.

Finally, to assess the pathogenicity of the molecularly cloned virus, we inoculated adult Aleutian mink with either XVB virus or the parent ADV-G strain (Table 2). Even after inoculation with more than 10⁶ fluorescence-forming units (FFU), no mink developed an acute viremia (17), high antibody titers (9, 29), elevated gamma globulin levels (9, 17, 29) or the pathological lesions characteristic of progressive Aleutian disease (17, 29, 31). This pattern of response was identical to that of mink inoculated with the parental virus, ADV-G. Thus, the molecularly cloned XVB, like the parent ADV-G, is nonpathogenic for adult mink.

The work related in this note has described the development of a full-length molecular clone of ADV-G. This clone generated complete and infectious ADV when transfected into CRFK cells, and the virus rescued from this clone retained the biological properties of the parent ADV-G strain. This molecular construct has provided us with the means of preparing chimeric viruses that contain segments of the pathogenic ADV Utah 1 strain. Such chimeras will prove useful in determining which regions of the genome govern the pathogenicity of ADV. These experiments are currently in progress.

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