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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 *Eco*RI 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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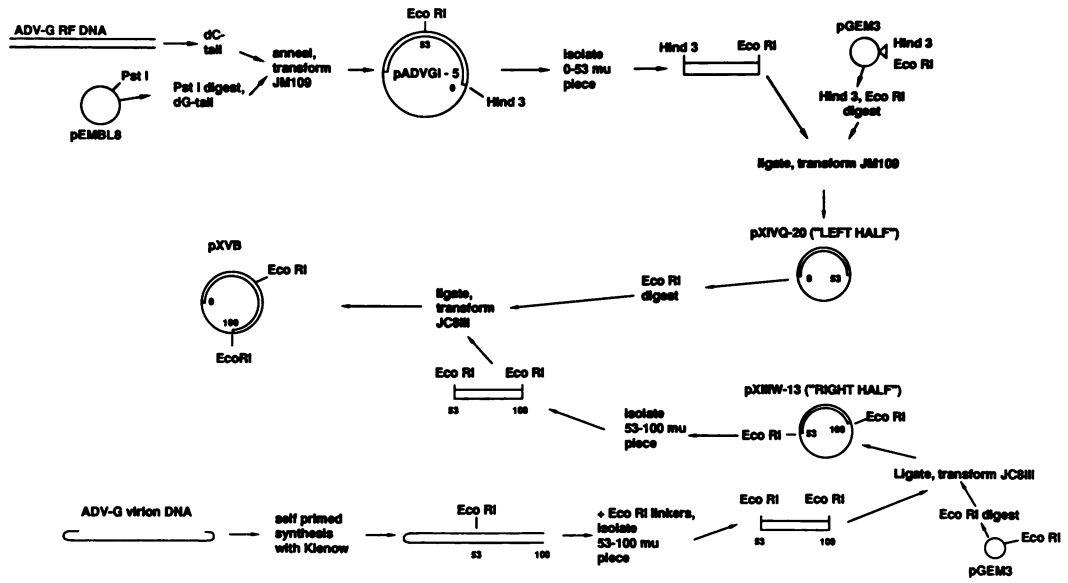

FIG. 1. Schematic development of a full-length molecular clone of ADV-G. A full-length clone of ADV-G was constructed by joining together a left half and a right half. The left half was developed from a previously described clone (5). Briefly, duplex monomeric replicative form DNA was purified from ADV-G-infected CRFK cells as described previously (5, 24), dC tailed (23), annealed to *Pst*I-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 *Hin*dIII, 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, pXIIIW-13. Finally, the full-length clone, pXVB, was developed by inserting the 53- to 100-map-unit *Eco*RI piece from pXIIIW-13 into *Eco*RI-digested 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 chloride-ethidium 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-μl 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

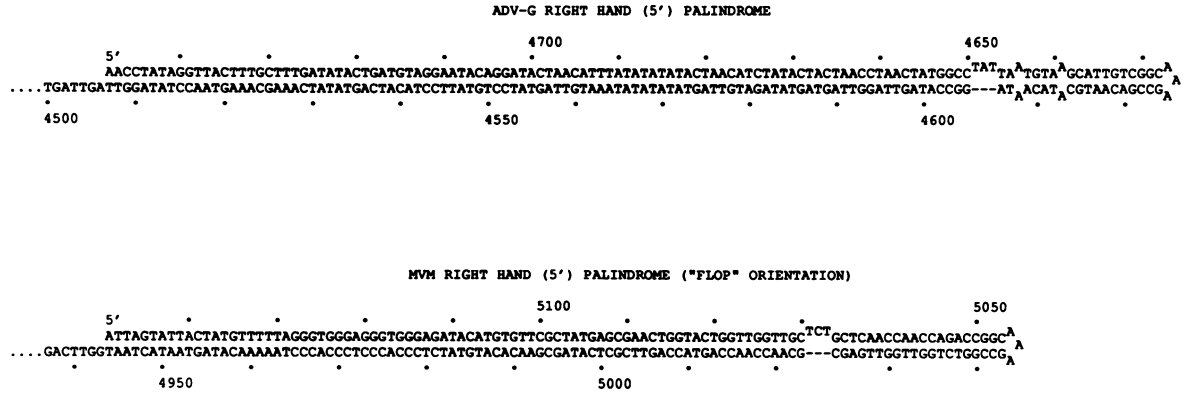

FIG. 2. DNA sequence of the 5′ palindrome of ADV-G. The extreme 5′ termini of both the right-half clone, pXIIIW-13, and the full-length pXVB were sequenced (5). The sequence of the minus or virion strand was arranged into a hairpin form and compared with a similar depiction of the flop orientation of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

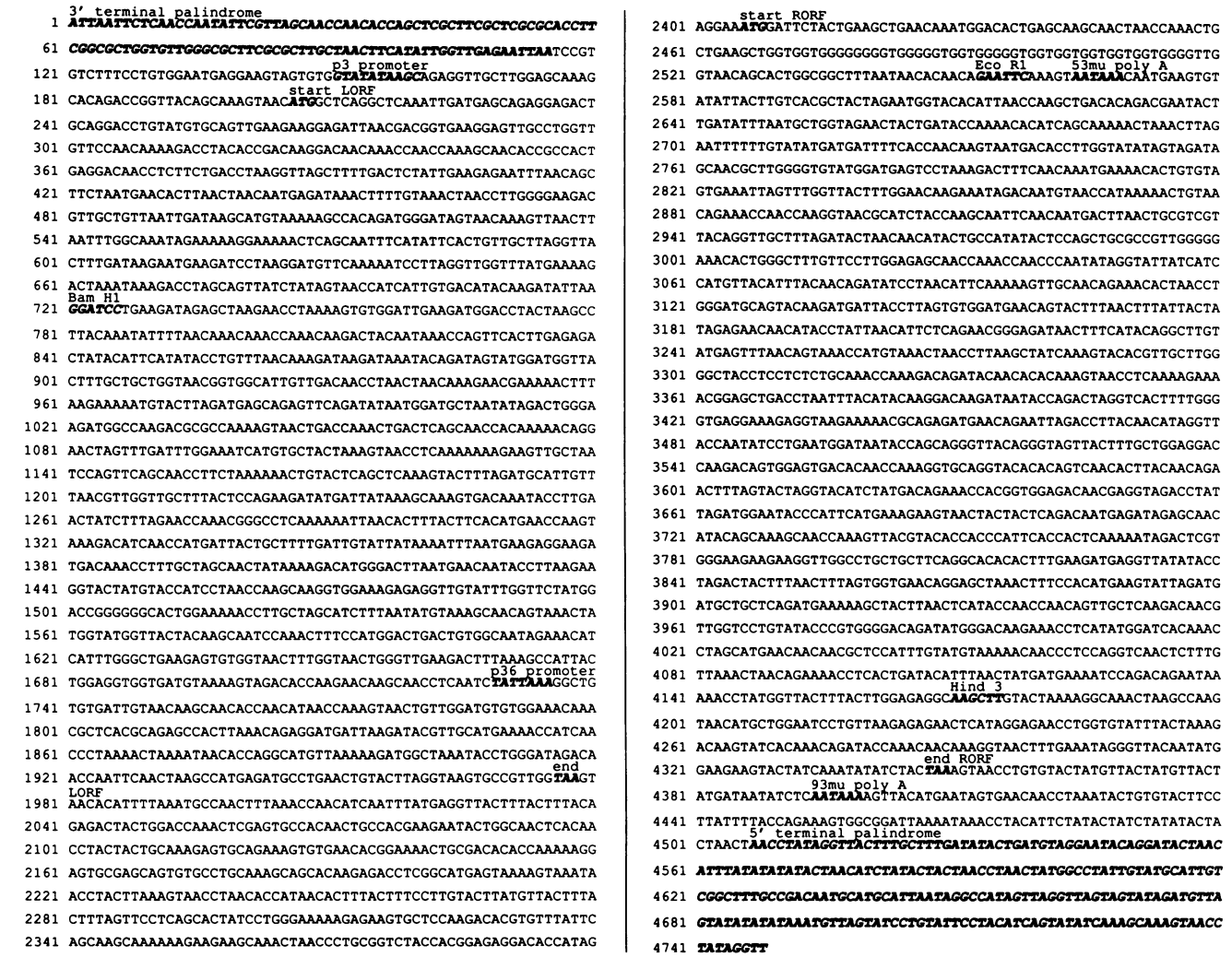

FIG. 3. DNA sequence of ADV-G. The newly obtained sequence for the 5′-terminal palindrome was joined to the 4,592-nt sequence previously reported (5) to produce a completed ADV-G sequence of 4,748 nt. The extent of the 3′- and 5′-terminal palindromes, the location of the p3 and p36 promoters, and the 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 *Bam*HI, *Eco*RI, and *Hin*dIII 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)[b]
Mock	ND	ND
pUC19	ND	ND
pXIVQ-20	ND	ND
ADV-G RF	$(27.5 \pm 4.5) \times 10^3$	1.8×10^6
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/μg 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.

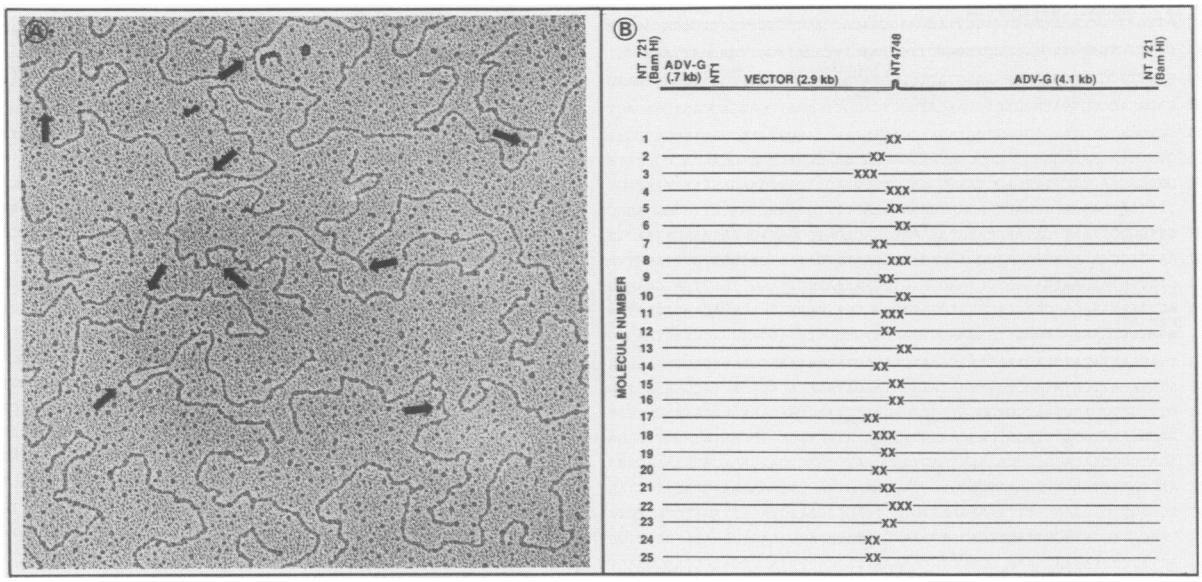

FIG. 4. Electron microscopy of the 5′ (right-hand) palindrome of ADV-G. Purified pXVB plasmid was linearized at the unique *Bam*HI site located at ADV-G nt 721 (5). (A) the DNA was denatured in alkali, allowed to reanneal under conditions that favored intrastrand duplex formation, and analyzed by electron 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.

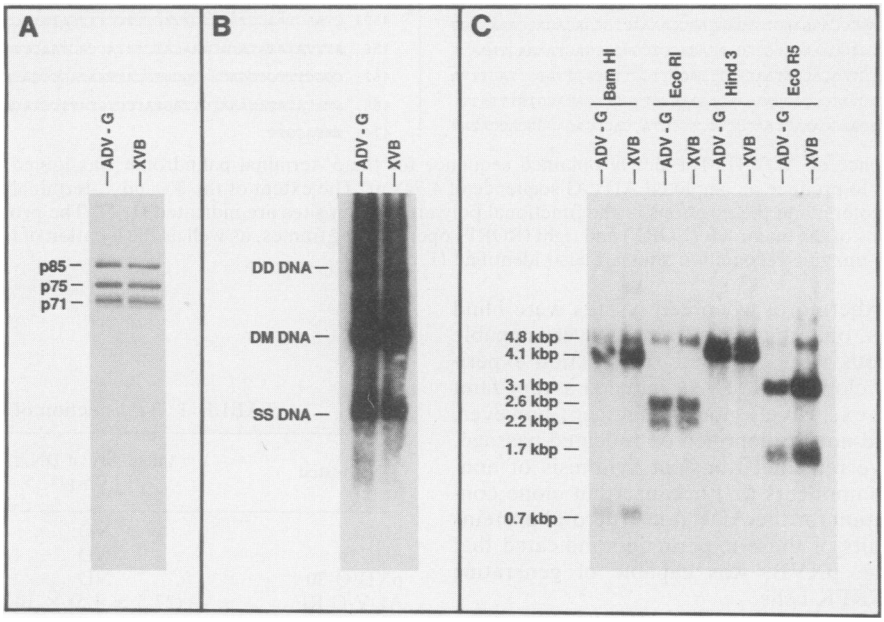

FIG. 5. Comparison of ADV-G with virus derived from molecularly cloned pXVB. A virus stock (XVB) derived by serial passage of lysates from pXVB-transfected CRFK cells was infected in parallel with a standard ADV-G stock into CRFK cells for 72 h (10). (A) A sample from each culture was lysed in sample 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: *Bam*HI (nt 721), *Eco*RI (nt 2553), *Hin*dIII (nt 4169), and *Eco*RV (nt 3069) (5, 7). Digestion of purified RF DNA was incomplete, and undigested DM DNA was visible in all lanes.

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

Mink no.	Virus inoculum (FFU)[a]	Anti-ADV antibody[b]	Viremia (pg/ml)[b]	Gamma globulin[b]	ADV pathology[c]
5025	2.9×10^6 (XVB)	<1/10	<20	Normal	Absent
5026	2.9×10^6 (XVB)	<1/10	<20	Normal	Absent
5027	2.9×10^4 (XVB)	<1/10	<20	Normal	Absent
5028	2.9×10^4 (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	7.2×10^4 (ADV-G)	<1/10	<20	Normal	–[d]
5033	Mock	<1/10	<20	Normal	Absent
5034	Mock	<1/10	<20	Normal	Absent

[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.

[b] 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).

[c] 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.

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 10^6 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.

Robert Evans and Gary Hettrick assisted with preparation of the figures, and Lori Lubke assisted with the electron microscopy.

LITERATURE CITED

1. **Alexandersen, S., M. E. Bloom, and S. Perryman.** 1988. Detailed transcription map of Aleutian mink disease parvovirus, J. Virol. **62**:3684–3694.
2. **Alexandersen, S., M. E. Bloom, J. Wolfinbarger, and R. E. Race.** 1987. In situ molecular hybridization for detection of Aleutian mink disease parvovirus DNA by using strand-specific probes: identification of target cells for viral replication in cell cultures and in mink kits with virus-induced interstitial pneumonia. J. Virol. **61**:2407–2419.
3. **Astell, C. R., E. M. Gardiner, and P. Tattersall.** 1986. DNA sequence of the lymphotropic variant of minute virus of mice, MVM(i), and comparison with the DNA sequence of the fibrotropic prototype strain. J. Virol. **57**:656–669.
4. **Bloom, M. E., S. Alexandersen, S. Mori, and J. B. Wolfinbarger.** 1989. Analysis of parvovirus infections using strand-specific hybridization probes. Virus Res. **14**:1–26.
5. **Bloom, M. E., S. Alexandersen, S. Perryman, D. Lechner, and J. B. Wolfinbarger.** 1988. Nucleotide sequence and genomic organization of Aleutian mink disease parvovirus (ADV): sequence comparisons between a nonpathogenic and a pathogenic strain of ADV. J. Virol. **62**:2903–2915.
6. **Bloom, M. E., O.-R. Kaaden, E. Huggans, A. Cohn, and J. B. Wolfinbarger.** 1988. Molecular comparisons of in vivo and in vitro derived strains of Aleutian disease of mink parvovirus. J. Virol. **62**:132–138.
7. **Bloom, M. E., L. W. Mayer, and C. F. Garon.** 1983. Characterization of the Aleutian disease virus genome and its intracellular forms. J. Virol. **45**:977–984.
8. **Bloom, M. E., R. E. Race, B. Aasted, and J. B. Wolfinbarger.** 1985. Analysis of Aleutian disease virus infection in vitro and in vivo: demonstration of Aleutian disease virus DNA in tissues of infected mink. J. Virol. **55**:696–703.
9. **Bloom, M. E., R. E. Race, W. J. Hadlow, and B. Chesebro.** 1975. Aleutian disease of mink: the antibody response of sapphire and pastel mink to Aleutian disease virus. J. Immunol. **115**:1034–1037.
10. **Bloom, M. E., R. E. Race, and J. B. Wolfinbarger.** 1980. Characterization of Aleutian disease virus as a parvovirus. J. Virol. **35**:836–843.
11. **Bloom, M. E., R. E. Race, and J. B. Wolfinbarger.** 1982. Identification of a nonvirion protein of Aleutian disease virus: mink with Aleutian disease have antibody to both virion and nonvirion proteins. J. Virol. **43**:608–616.
12. **Bloom, M. E., R. E. Race, and J. B. Wolfinbarger.** 1987. Analysis of Aleutian disease of mink parvovirus infection using strand specific hybridization probes. Intervirology **27**:102–111.
13. **Boissy, R., and C. Astell.** 1985. An *Escherichia coli* recBCsbc BrecF host permits the deletion-resistant propagation of plasmid clones containing the 5′-terminal palindrome of minute virus of mice. Gene **35**:179–185.
14. **Cotmore, S. F., and P. Tattersall.** 1986. Organization of nonstructural genes of the autonomous parvovirus minute virus of mice. J. Virol. **58**:724–732.
15. **Cotmore, S. F., and P. Tattersall.** 1987. The autonomously replicating parvoviruses. Adv. Virus Res. **33**:91–174.
16. **Dente, L. G., G. Cesareni, and R. Cortese.** 1983. pEMBL: a new family of single-stranded plasmids. Nucleic Acids Res. **11**: 1645–1655.
17. **Eklund, C. M., W. J. Hadlow, R. C. Kennedy, C. C. Boyle, and T. A. Jackson.** 1968. Aleutian disease of mink: properties of the etiologic agent and the host responses. J. Infect. Dis. **118**: 510–516.
18. **Garon, C. F.** 1986. Electron microscopy of nucleic acids, p. 161–182. *In* H. C. Aldrich and W. J. Todd (ed.), Ultrastructure techniques for microorganisms. Plenum Publishing Corp., New York.
19. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
20. **Hadlow, W. J., R. E. Race, and R. C. Kennedy.** 1983. Comparative pathogenicity of four strains of Aleutian disease virus for pastel and sapphire mink. Infect. Immun. **41**:1016–1023.

21. **Hahn, E. C., L. Ramos, and A. J. Kenyon.** 1977. Expression of Aleutian mink disease antigen in cell culture. Infect. Immun. **15**:204–211.
22. **Kimsey, P. B., H. D. Engers, B. Hirt, and C. V. Jongeneel.** 1986. Pathogenicity of fibroblastic- and lymphocyte-specific variants of minute virus of mice. J. Virol. **59**:8–13.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Mayer, L. W., B. Aasted, C. F. Garon, and M. E. Bloom.** 1983. Molecular cloning of the Aleutian disease virus genome: expression of Aleutian disease virus antigens by a recombinant plasmid. J. Virol. **48**:573–579.
25. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, G. Zinn, and M. R. Green.** 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
26. **Merchlinksy, M. J., P. Tattersall, J. J. Leary, S. F. Cotmore, E. M. Gardiner, and D. C. Ward.** 1983. Construction of an infectious molecular clone of the autonomous parvovirus minute virus of mice. J. Virol. **47**:227–232.
27. **Parrish, C. R., C. F. Aquadro, and L. E. Carmichael.** 1988. Canine host range and a specific epitope map along with variant sequences in the capsid protein gene of canine parvovirus and related feline, mink, and raccoon parvoviruses. Virology **166**: 293–307.
28. **Parrish, C. R., and L. E. Carmichael.** 1986. Characterization and recombination mapping of an antigenic and host range mutation of canine parvovirus. Virology **148**:121–132.
29. **Porter, D. D.** 1986. Aleutian disease: a persistent parvovirus infection of mink with a maximal but ineffective host immune response. Prog. Med. Virol. **33**:42–60.
30. **Porter, D. D., A. E. Larsen, N. A. Cook, H. G. Porter, and S. L. Suffin.** 1977. Isolation of Aleutian disease virus of mink in cell culture. Intervirology **8**:129–144.
31. **Porter, D. D., A. E. Larsen, and H. G. Porter.** 1969. The pathogenesis of Aleutian disease of mink. I. In vivo replication and the host antibody response to viral antigen. J. Exp. Med. **130**:575–589.
32. **Race, R. E., B. Chesebro, M. E. Bloom, B. Aasted, and J. Wolfinbarger.** 1986. Monoclonal antibodies against Aleutian disease virus distinguish virus strains and differentiate sites of virus replication from sites of viral antigen sequestration. J. Virol. **57**:285–293.
33. **Sahli, R., G. K. McMaster, and B. Hirt.** 1985. DNA sequence comparison between two tissue-specific variants of the autonomous parvovirus, minute virus of mice. Nucleic Acids Res. **13**:3617–3632.
34. **Shull, B. C., K. C. Chen, M. Lederman, E. R. Stout, and R. C. Bates.** 1988. Genomic clones of bovine parvovirus and effect of deletions and terminal sequence inversions on infectivity. J. Virol. **62**:417–426.
35. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.