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# Nucleotide Sequence Analysis of Aleutian Mink Disease Parvovirus Shows that Multiple Virus Types Are Present in Infected Mink

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**Different isolates of Aleutian mink disease parvovirus (ADV) were cloned and nucleotide sequenced. Analysis of individual clones from two in vivo-derived isolates of high virulence indicated that more than one type of ADV DNA were present in each of these isolates. Analysis of several clones from two preparations of a cell culture-adapted isolate of low virulence showed the presence of only one type of ADV DNA. We also describe the nucleotide sequence from map units 44 to 88 of a new type of ADV DNA. The new type of ADV DNA is compared with the previously published ADV sequences, to which it shows 95% homology. These findings indicate that ADV, a single-stranded DNA virus, has a considerable degree of variability and that several virus types can be present simultaneously in an infected animal.**

It has become evident that autonomous parvoviruses can cause complex disease patterns. The human B19 parvovirus infection is usually acute, although the virus has been shown to persist in some immunocompromised patients (32). Two strains of minute virus of mice (MVM) exist; the MVMi strain inhibits several T-cell-mediated functions (35), while this immunosuppressive property is not associated with the prototype MVMp. Different isolates of Aleutian mink disease parvovirus (ADV) have been described and found to vary in pathogenicity (1, 3, 8, 9, 16, 22, 26, 33). The highly virulent isolates of ADV cause an acute pneumonia with nearly 100% mortality in mink kits from seronegative dams. In mink infected as adults, these virulent isolates cause a slowly progressing, immune complex-mediated, usually fatal disease. In contrast, low-virulence isolates cause mortality due to pneumonia in only a few infected kits from seronegative dams (3, 33) and cause disease only in adult mink of the Aleutian genotype (24, 27). Although previous work showed serological differences between different isolates of ADV (41), these differences were difficult to correlate with the virulence of the isolate.

ADV, an autonomous parvovirus of mink, has a single-stranded DNA genome of approximately 4,800 nucleotides (12, 13). More than 90% of the ADV virions contain the minus-sense strand (4, 6, 7, 18). The termini of the ADV genome have palindromic sequences and can be arranged as hairpins of about 150 nucleotides (12, 13). The 3′ hairpin is probably responsible for the self-priming of parvoviral DNA replication (23).

The entire genome of the cell culture-adapted ADV G isolate and a 15- to 88-map-unit (MU) sequence of the highly virulent ADV Utah I isolate have been DNA nucleotide sequenced (12, 13). The sequence similarity between these two isolates was calculated to be 97.5%. This degree of similarity resembles what is found for the two strains of MVM (MVMp and MVMi), which are 96.6% homologous (11, 45). Also, the host-range-variant parvoviruses of feline, mink enteritis, raccoon, and canine parvoviruses (CPV) were shown to be more than 98% related to each other in the right open reading frame (ORF) coding for the two structural proteins (37). These findings show that minor changes in the nucleotide sequence of closely related parvoviruses may have a major impact on the biological properties of the virus.

We analyzed different isolates of ADV by cloning and nucleotide sequencing. We present the partial nucleotide sequence of a new type of ADV DNA which had only 95% homology to the previously published ADV sequences. In addition, detailed analysis of several clones indicated the coexistence of more than one type of ADV DNA in highly virulent in vivo-derived isolates. In contrast, analysis of clones from two preparations of a cell culture-adapted isolate of low virulence showed the presence of only one type of ADV DNA. These findings indicated that ADV, a single-stranded DNA virus, has a considerable degree of variability and that several virus types could be present simultaneously in an infected animal.

## MATERIALS AND METHODS

**Materials.** All enzymes were obtained from Bethesda Research Laboratories, Inc., Boehringer Mannheim, or United States Biochemical. The enzymes were used according to the recommendations of the manufacturers. Plasmid vector DNAs were purchased from either Boehringer Mannheim (pUC12 and pUC18) or Promega (pGEM3Z). If not otherwise stated, the vector used was pGEM3Z. $^{35}$S-ATP was obtained from Dupont, New England Nuclear Corp. Synthetic oligonucleotide primers specific for plasmid DNA were from either Boehringer Mannheim or Promega. ADV-specific oligonucleotides were synthesized and used as described previously (13).

**Virus isolates and preparation of DNA for cloning.** The

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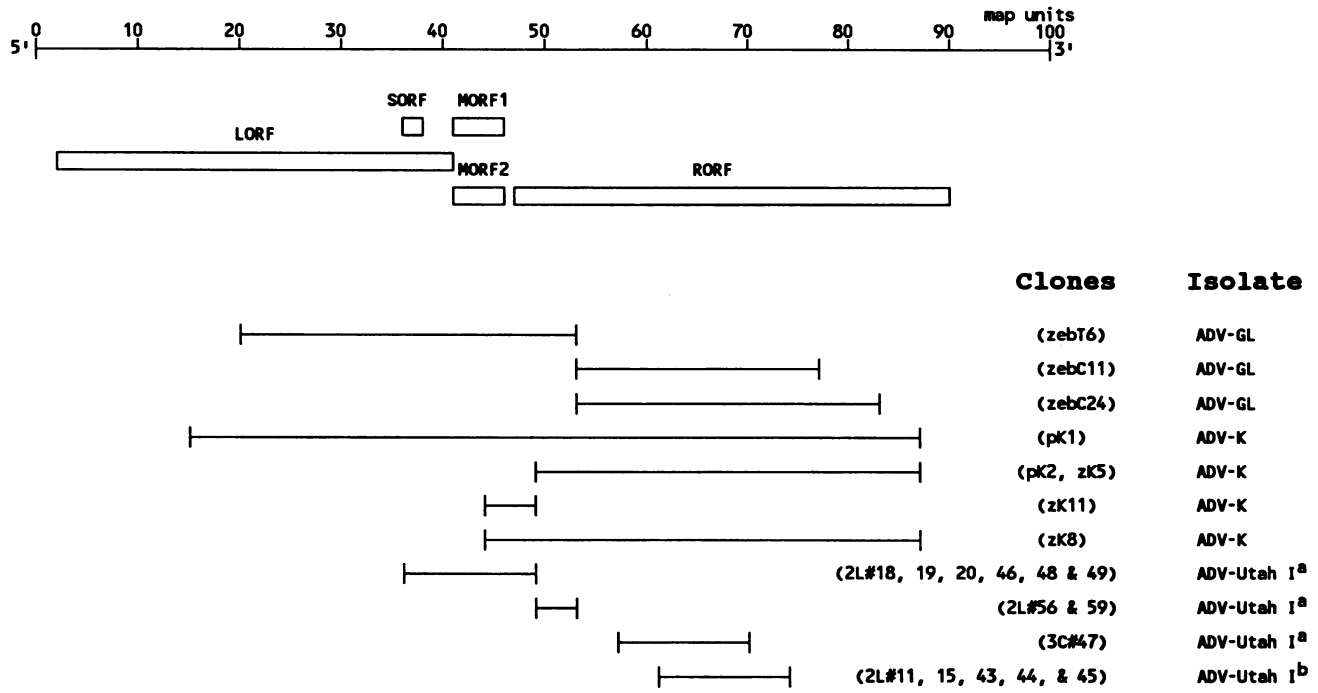


FIG. 1. Map of ADV inserts in the clones described in the present work. The ORFs in the ADV genome are shown at the top of the figure, and the different clones sequenced are depicted below. The isolates from which the clones originated are shown at the right. Clones designated zeb, pK, or zK were obtained by conventional cloning and clones designated 2L# or 3C# were obtained by PCR-amplified cloning by using four different sets of ADV-specific oligonucleotide primers. a, clones derived from an ADV Utah I-infected mink kit. b, clones derived from an ADV Utah I-infected adult mink. LORF, left ORF; SORF, small ORF; RORF, right ORF; MORF, middle ORF.

highly virulent ADV K isolate used for inoculation of mink has been described previously (2, 3, 8, 9, 33). Newborn mink kits were inoculated with $10^6$ adult mink 50% infective doses and killed 10 to 20 days later. Virions were partly purified from lung, liver, and spleen tissues from the mink kits by isopycnic centrifugation in CsCl gradients, and DNA was extracted by the sodium dodecyl sulfate-proteinase K procedure (4, 14).

One adult mink and one newborn mink kit were infected with ADV Utah I as described previously (4, 6, 7, 9). Ten days postinfection, DNA was prepared from the serum of the adult mink as described previously (6), and ADV DNA was amplified and cloned as described below. The mink kit was killed 10 days after infection, and total RNA was isolated from the lung as described previously (5). This RNA preparation was then poly(A)$^+$ selected (mRNA) and converted to double-stranded cDNA by using oligo(dT) priming as described previously (5). The Utah I virus stock used for inoculation of these mink was the same Utah I stock as used previously (6, 13, 14). The Danish cell culture-adapted isolate, designated ADV GL (3), was kindly provided by Ebba Lund, Royal Veterinary University of Copenhagen, Copenhagen, Denmark. DNA was partly purified from cell cultures infected with this virus according to the procedure of Bloom et al. (16) or from a modified Hirt extraction (15, 35).

**Conventional cloning.** The purified single-stranded virion DNA preparations were made double stranded by self-primed extension by using the large Klenow fragment of DNA polymerase I (13, 15, 34). After digestion with selected restriction endonucleases, the DNAs were ligated into similarly digested pUC12, pUC18, or pGEM3Z plasmid vectors. The plasmids were transformed into *Escherichia coli* JM105 or JM109, and white colonies were checked for ADV DNA sequences by hybridization as described previously (5, 13).

ADV DNA was purified from the ADV K isolate, cut with *Bam*HI and *Hin*dIII, and cloned into pUC12. This resulted in one clone (pK1) with a 3.6-kb insert, which was sequenced. Cloning with *Pst*I and *Hin*dIII of DNA from ADV K into pUC18 or pGEM3Z gave several clones with approximately 1.8 kb of insert and one clone with a 2.1-kb insert. Three of these clones (pK2, zK5, and zK8) were sequenced. When ADV K DNA was cut with *Pst*I, the resulting clones had 267-bp inserts, and one clone (zK11) was sequenced. The ADV DNA purified from the ADV GL isolate was made double stranded by self-primed extension. The DNA was then digested with *Eco*RI and ligated into a vector cut with the endonucleases *Sma*I and *Eco*RI. Three clones (zebT6, zebC11, and zebC24) together spanning from nucleotides 957 to 3994 (by ADV G numbering [13]) were analyzed. A schematic map of all of the clones analyzed and sequenced in the present work is shown in Fig. 1.

**DNA amplification techniques.** One- to ten-nanogram aliquots of extracted total DNA containing ADV-specific DNA or cDNA were amplified by the polymerase chain reaction (PCR). All reactions were performed in a volume of 100 μl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.4 at 20°C), 1.5 mM $MgCl_2$, 20 μg of gelatin, each dNTP at 0.2 mM, 5 U of *Taq* DNA polymerase (Perkin Elmer Cetus, Norwalk, Conn.), and about 200 ng of ADV-specific primers. Amplifications were done for 30 cycles at an annealing temperature of 47°C for 2 min, a polymerization temperature of 72°C for 1.5 min, and a heat denaturation temperature of 94°C for 1

min with an automated thermocycler (JSP El-Service APS, Glostrup, Denmark).

**Cloning of PCR-amplified DNA.** The PCR products were purified by phenol extraction, and the resulting DNA was 5′-phosphorylated by T4 polynucleotide kinase. The DNA samples were blunt end ligated into an *Sma*I-cut pGEM3Z vector and transformed into *E. coli* JM109. This strategy of cloning was done with DNA isolated from the serum of a Utah I-infected adult mink. The resulting five clones (2L#11, 2L#15, 2L#43, 2L#44, and 2L#45) (Fig. 1) were sequenced. From the lung of an ADV Utah I-infected mink kit, mRNA was isolated and converted to cDNA as described previously (5). The ADV-specific cDNA was amplified and cloned, resulting in several clones, of which nine (2L#18, 2L#19, 2L#20, 2L#46, 2L#48, 2L#49, 2L#56, 2L#59, and 3C#47) (Fig. 1) with inserts of lengths between 96 to 376 bp were sequenced. Some cDNA clones had spliced out introns similar to the B and C introns described previously for ADV G grown in cell culture (5). A detailed analysis of the splice pattern of our cDNA clones obtained from in vivo material will be given elsewhere. The positions of the cloned segments in relation to the ADV genome are depicted in Fig. 1.

**DNA nucleotide sequencing techniques.** All sequencing was performed by the dideoxy-chain termination method (46) with $^{35}$S-ATP as the label as described previously (5, 12, 13). Templates for sequencing were double-stranded plasmid DNA prepared as described previously (21). The primers used were either commercially available or ADV-specific oligonucleotides. All regions were sequenced at least twice in both directions.

**DNA sequence analysis.** All DNA sequences were analyzed by using the Microgenie sequence analysis program (Beckman Instruments). The sequences of ADV G and the 15- to 88-MU segment of ADV Utah I were published previously (12, 13). The nucleotide numbering for ADV G (13) will be used in the present article.

**Nucleotide sequence accession numbers.** GenBank accession numbers for the sequences reported here are M63039 to M63051.

## RESULTS

The main purpose of the present study was to determine, at the DNA level, differences among and within several isolates of ADV. In the following, ADV types are defined as sequence types having major nucleotide differences compared with those of other ADVs. ADV DNAs with only minor sequence differences are considered to be of the same type. Thus, ADV G and ADV Utah I, which are only 97.5% related at the DNA level, are two different types. As will be evident in the following studies, even well-defined isolates of ADV may contain more than one type of ADV sequence, making the old term "strain" imprecise. For the purpose of this article, we will refer to sequences highly similar to ADV G as type 1 ADV sequences and sequences highly similar to ADV Utah I as type 2 ADV sequences.

**Comparisons of DNA sequences from ADV G-like isolates of ADV.** The best-characterized ADV is the cell culture-adapted ADV G isolate. The American ADV G isolate does not induce acute pneumonia in newborn mink kits, nor is it pathogenic in adult mink (3, 16). A Danish cell culture isolate (ADV GL) is also nonpathogenic in adult mink but does cause pneumonia in kits, although with a reduced frequency compared with isolates of high virulence (3, 33). Although similar conditions were attempted, these differences in virulence may have been due to differences in mink genotypes or virus dosage rather than the actual virus itself (3).

TABLE 1. Differences of type 1 ADV sequences between nucleotides 2558 and 3994[a]

| Nucleotide no. | Isolate or clone | | | | Codon change |
|---|---|---|---|---|---|
| | ADV G | ADV GL | | ADV Utah I[b] (2L#11) | |
| | | zebC11 | zebC24 | | |
| 3371 | A | A | G | A | Asp to Gly |
| 3460 | A | A | A | G | Ile to Val |
| 3591 | C | T | G | ND | None (C to T) or His to Gln (C to G) |
| 3600 | G | A | G | ND | None |
| 3703 | G | G | A | ND | Asp to Arg |
| 3860 | G | ND | T | ND | Ser to Ile |
| 3978 | G | ND | A | ND | None |

[a] DNA sequence differences are listed, with the nucleotide coordinates of ADV G as a reference point (13). In addition, amino acid changes are also described. ND, the particular nucleotide was not determined (not included in the particular clone).

[b] A single type 1 ADV clone derived from an ADV Utah I-infected adult mink. This clone was sequenced from nucleotides 2938 to 3562 and had only one sequence difference compared with ADV G.

In order to assess genetic variation between the two cell culture-adapted ADVs available, we compared the sequence of the American ADV G isolate characterized at Rocky Mountain laboratories (16) and that of the Danish ADV GL isolate grown in the laboratory of Ebba Lund (3). It should be pointed out that both ADV G and ADV GL were derived from the same primary cell culture isolate which was previously adapted to in vitro growth from ADV Utah I-infected adult mink tissues (3, 16, 39).

Several clones were obtained from purified virion DNA from ADV GL-infected cell cultures. The extent of the resulting sequence was from nucleotides 957 to 3994, and all three clones analyzed (zebT6, zebC11, and zebC24) (Fig. 1) represented type 1 ADV sequences. When compared with the corresponding published ADV G sequence (13), only nine nucleotide substitutions were observed, suggesting an overall similarity at the DNA level of 99.7% for the segment of the genome studied (approximately 64% of the total genome length). Seven of the nucleotide changes were located in the right ORF (Table 1). Three of these were silent substitutions and four changed the amino acid sequence (Table 1). The single clone zebC6 (Fig. 1), spanning into the left ORF, had two nucleotide substitutions, and both gave rise to conserved amino acid changes (data not shown).

Thus, the results suggested that these two ADV isolates of low virulence, although passaged in different parts of the world for an extended period of time, were very similar in DNA sequence. Interestingly, the nucleotide substitution at position 3591 changed the codon from a histidine in ADV G and in ADV GL clone zebC11 to a glutamine in the ADV GL clone zebC24, similar to what is found in the highly virulent isolates of ADV (Fig. 2 and 3). However, it is yet unknown whether this amino acid change or any of the other coding changes have any effect on the possible biological difference in virulence in mink kits observed between ADV G and ADV GL (3).

**Analysis of DNA from a virulent Danish isolate of ADV.** The Danish ADV K isolate (2, 3, 33) is highly virulent for mink of all ages and in that regard is biologically similar to the ADV Utah I isolate. We have studied several clones derived from

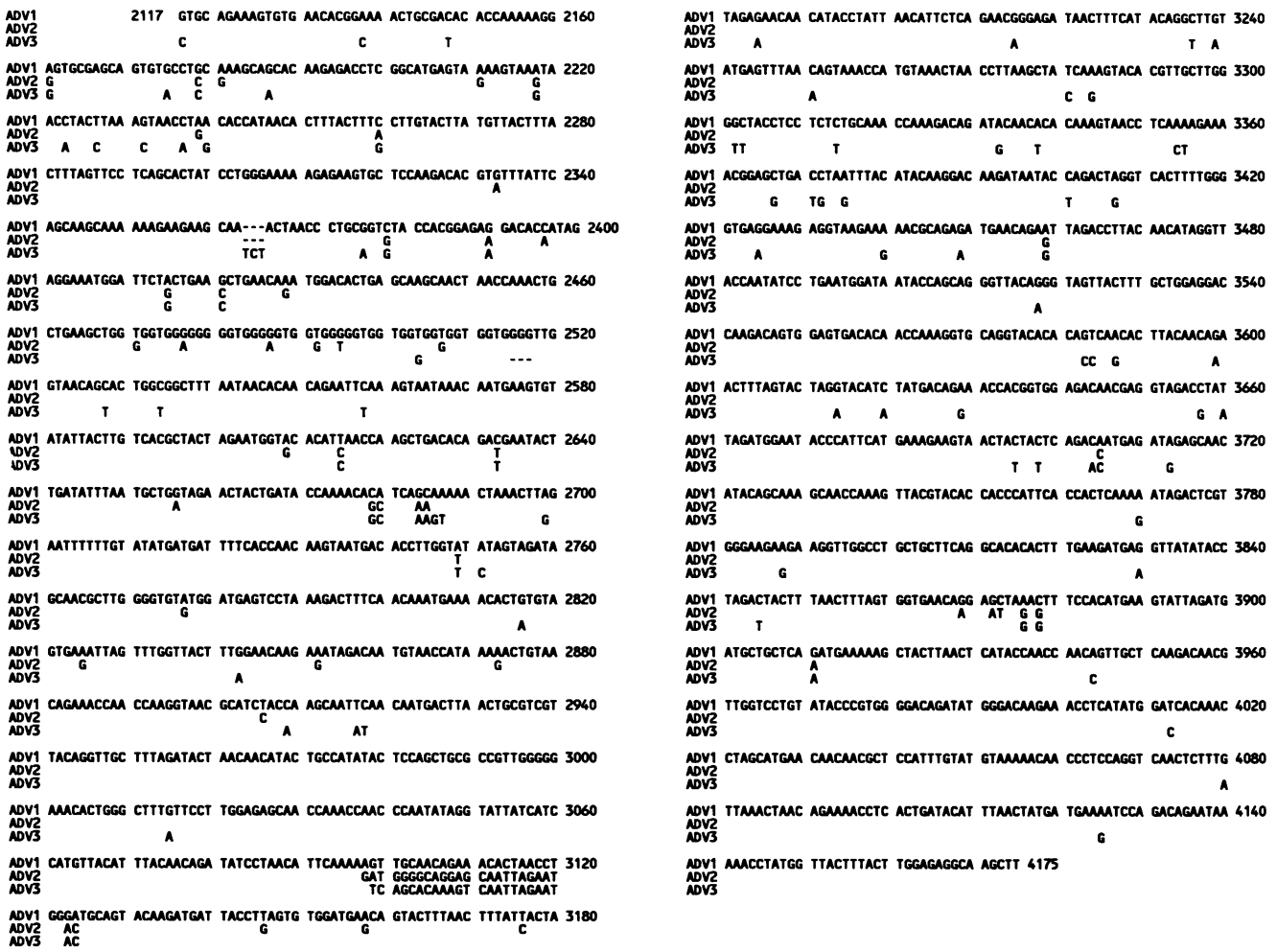

FIG. 2. Comparison of the DNA sequences of the three types of ADV from 44 MU (nucleotide 2117) to 88 MU (nucleotide 4175). Only the differences among the types are marked, with the new nucleotide beneath the type 1 ADV sequence (13). -, the nucleotides are deleted in the given sequence. The type 2 and 3 ADVs shown are the sequences of clones pK1 and zK8, respectively, both determined in the present study. ADV1, ADV2, and ADV3 are ADV types 1, 2, and 3, respectively.

ADV K and made sequence comparisons to ADV G (ADV type 1) and ADV Utah I (ADV type 2).

The first clone of ADV K DNA we analyzed was derived from virion DNA purified from organs of mink infected with the pathogenic ADV K isolate and cloned by using a strategy similar to that described for cloning ADV Utah I (13). Single-stranded virion DNA was converted in vitro to a duplex, digested with *Bam*HI and *Hin*dIII, ligated into pUC12, and transformed into JM109. A single clone, designated pK1, was obtained and was nucleotide sequenced. This clone, 3,455 nucleotides in length, had only two differences from the published ADV Utah I sequence (99.9% homology), and we therefore defined it as a type 2 ADV.

One difference was a single-base insertion of an A (adenine) at the position corresponding to nucleotide 806, a change that corrected the interrupted left ORF of the reported ADV Utah I sequence. The second sequence difference was a single-base substitution of a C (cytosine) to an A at nucleotide 3100, which caused an amino acid change from leucine to methionine (Table 2) in the hypervariable region of the right ORF (13).

Although we were unable to isolate additional *Bam*HI-to-*Hin*dIII clones from ADV K, molecular cloning of *Pst*I or *Pst*I-*Hin*dIII digests yielded several clones, designated zK11, pK2, zK5, and zK8, schematically depicted in Fig. 1. Sequencing of all the shorter clones indicated that they all were subsets of the longest (2.1 kb) clone, zK8, and that the sequence obtained extended from 44 (nucleotide 2117) to 88 MUs (nucleotide 4175). Endonuclease digest analysis of an additional 13 clones indicated that all of these contained ADV inserts with similar restriction digest patterns (data not shown). Clones corresponding to the left portion of the genome were not obtained by using this strategy. The pattern of the restriction fragments in the clones suggested that the ADV K preparation must have contained ADV DNA with a physical map and sequence substantially different from those of either ADV G or ADV Utah I. These marked differences were corroborated by sequence analysis (see below) which indicated that the clones were only 95% related to either ADV G (type 1) or ADV Utah I (type 2) (Fig. 2). Therefore, we defined this additional variety of ADV DNA to be a type 3 ADV. Furthermore, since we had already showed the presence of type 2 ADV (pK1, see above) in the ADV K isolate, these results indicated that this

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ADV-1 HHNTLLSLYLCYFTLVPQHT PGKKRSAPRHVFIQQAKKKK QTNPAVYHGEDTIEEMDSTE
ADV-2   D   Y                                                     A
ADV-3 DNS   C                                    S                A

ADV-1 AEQMDTEQATNQTAEAGGGG GGGGGGGGGGGGVGNSTGGF NNTTEFKVINNEVYITCHAT
ADV-2 P                     S
ADV-3 P                           *

ADV-1 RMVHINQADTDEYLIFNAGR TTDTKTHQQKLNLEFFVYDD FHQQVMTPWYIVDNSAWGVW
ADV-2                   D       A K                   F
ADV-3                           A S                   FL

ADV-1 MSPKDFQQMKTLCSEISLVT LEQEIDNVTIKTVTETNQGN ASTKQFNNDLTASLQVALDT
ADV-2
ADV-3                                             Y

ADV-1 NNILPYTPAAPLGETLGFVP WRATKPTQYRYYHPCYIYNR YPINQKVATETLTWDAVQDD
ADV-2                                             MGQ Q E TGT
ADV-3                   I                         S QSQ E TGT

ADV-1 YLSVDEQYFNFITIENNIPI NILRTGDNFKTGLYEFNSKP CKLTLSYQSTRCLGLPPLCK
ADV-2
ADV-3                                   K

ADV-1 PKTDTTHKVTSKENGADLIY IQGQDNTRLGHFWGEERGKK NAEMNRIRPYNIGYQYPEWI
ADV-2                                             V
ADV-3     A         L   G VV                       V

ADV-1 IPAGLQGSYFAGGPRQWSDT TKGAGTHSQHLQQNFSTRYI YDRNHGGDNEVDLLDGIPIH
ADV-2                       Q
ADV-3                             T Q                 VI

ADV-1 ERSNYYSDNEIEQHTAKQPK LRTPPIHHSKIDSWEEEGWP AASGTHFEDEVIYLDYFNFS
ADV-2         H
ADV-3       EH M

ADV-1 GEQELNFPHEVLDDAAQMKK LLNSYQPTVAQDNVGPVYPW GQIWDKKPHMDHKPSNNNNA
ADV-2      E                                      D
ADV-3      E                                      D

ADV-1 PFVCKNNPPGQLFVKLTENL TDTFNYDENPDRIKTYGYFT WRGKL
ADV-2
ADV-3               I               D
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FIG. 3. Predicted amino acid sequences of the first 92% of the right ORF of ADV types 1, 2, and 3 from nucleotides 2241 to 4175. Only the amino acid changes are depicted beneath the predicted ADV type 1 protein sequence. S, a serine (S) inserted between the glutamine (Q) and threonine (T). *, a missing amino acid (3 nucleotides deleted). The type 2 sequence shown is from the present work. In the type 2 sequence (Utah I) given by Bloom et al. (13), there is one difference (see Fig. 4). ADV-1, ADV-2, and ADV-3 are ADV types 1, 2, and 3, respectively.

particular isolate of ADV contained more than one genetic type, i.e., both type 2 and type 3 ADVs.

In the 2.1-kb segment of the type 3 DNA from the ADV K isolate sequenced, there was a total of 117 differences between the type 3 and the type 1 ADV G sequences and 111 differences between the type 3 and the type 2 sequences. A comparison of the actual DNA sequences from 44 to 88 MUs of types 1, 2, and 3 is shown in Fig. 2. Most of the differences were distributed throughout the segments, but one short segment at 64 to 65 MU of the right ORF showed marked heterogeneity among the types (see below). Analysis of several independent clones confirmed this type 3 DNA.

TABLE 2. Differences among type 2 ADV sequences in the region between nucleotides 2930 and 3740[a]

| Nucleotide no. | Isolate or clone | | | | | Codon change |
|---|---|---|---|---|---|---|
| | pK1 | Utah I[b] | 2L#15 | 2L#43 | 2L#44 | |
| 2990 | G | G | G | A | G | None |
| 3100 | A | C | A | A | A | Leu to Met |
| 3333 | A | A | G | ND[c] | A | Ala to Thr |
| 3382 | A | A | A | ND | G | Ile to Val |

[a] Two other clones, 2L#45 and 3C#47, had no differences from the type 2 ADV. In addition to sequence differences, codon changes are listed.
[b] From Bloom et al. (13).
[c] ND, not determined.

Comparison of the putative translation products of the right ORF, encoding the viral structural proteins, was also performed. Since we only had DNA clones of types 2 and 3 to the *Hin*dIII site at MU 87, the 3′ portions of the right ORF were not available for analysis. Thus, the truncated translation products, encompassing 92% of the right ORF, were compared (Fig. 3). The putative structural proteins of type 1 (ADV G) and type 3 (ADV K) are 93.6% related and have 41 amino acid changes. Eight of these residues are found in the 11-amino-acid hypervariable region at MU 64 (13), giving a variability in this small area of 72.7%. The comparison between the putative proteins of type 2 (Utah I) and type 3 (ADV K) showed that these are 95.6% related and that there are 28 amino acid changes, of which 12 are rated as conservative. Three of the 28 amino acid changes are found at the hypervariable region at 64 MUs (a variability of 3 of 11 amino acids [27.3%]), and no other regions could be characterized as hypervariable. The changes in the hypervariable region among ADV types 1, 2, and 3 are highlighted in Fig. 4.

**Comparison of DNA sequences found within preparations of the ADV Utah I isolate.** In the previous sections, we presented evidence that cell culture-adapted ADVs contained only a single sequence type but that more than one sequence type was present in an isolate of in vivo-passaged high-virulence material (ADV K). We next examined samples of the ADV Utah I isolate, which previously was shown to contain the type 2 ADV (13). ADV-specific cDNA or DNA was PCR amplified with four different sets of primers. Nine clones were obtained from PCR-amplified cDNA from an ADV Utah I-infected mink kit (Fig. 1). The nine clones were all of the type 2 ADV; i.e., seven of the clones were identical

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Type 1 (ADV-G)          CAAAAAGTTGCAACAGAAACACTAACCTGGGATGCAGTA
Type 2 (ADV-Utah I*)    -----GC-G-GG-AG--GCA-T--GAA---AC--GTAC-
Type 2 (other clones)   ------A--------------------------------
Type 3 (ADV-K)          -----ATCA-CA--AAGT----------------A---

Type 1 (ADV-G)            Gln Lys Val Ala Thr Glu Thr Leu Thr Trp Asp Ala Val
Type 2 (ADV-Utah I*)        -   - Leu Gly Gln Glu Gln  -  Glu  -  Thr Gly Thr
Type 2 (other clones)     -   - Met  -   -   -   -   -   -   -   -   -   -
Type 3 (ADV-K)            -   - Ser Ala  -  Ser  -   -   -   -   -   -   -
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FIG. 4. The nucleotide and predicted amino acid sequences of the hypervariable region at MUs 64 to 65. −, same nucleotide or amino acid as the sequence above. *, the Utah I sequence given by Bloom et al. (13).

TABLE 3. Differences among type 2 ADV sequences in the region between nucleotides 1750 and 2430[a]

| Nucleotide no. | Isolate or clone | | | Codon change |
|---|---|---|---|---|
| | ADV-Utah I* | 2L#19 | 2L#48 | |
| 1780 | T | ND[b] | C | None |
| 1924 | A | C | A | None |
| 2006 to 2007 | | A | | Frameshift |
| 2017 | T | C | T | F to L (MORF[c] 1), I to T (MORF 2) |
| 2022 | G | A | G | M to I (MORF 1) |
| 2024 | G | A | G | R to K (MORF 1), E to K (MORF 2) |

[a] No differences were found in seven additional clones. In addition to sequence differences, codon changes are listed.
[b] ND, not determined.
[c] MORF, middle ORF.

to type 2 (Utah I) (2L#18, 2L#20, 2L#46, 2L#49, 2L#56, 2L#59, and 3C#47), and one clone (2L#48) had a single substitution. In the last clone (2L#19), representing unspliced mRNA sequences, four substitutions and an insertion between nucleotides 2006 and 2007 leading to a frameshift were observed. The sequence differences of these clones from the previously published Utah I sequence (13) are shown in Table 3.

Further analysis of ADV Utah I clones derived from PCR-amplified DNA isolated from the serum of a single ADV Utah I-infected adult mink revealed that both types 2 and 1 ADV were present in vivo. From such material, five clones of a length between 245 and 803 bp have been sequenced, and one contained DNA of type 1 (2L#11) (Table 1) and four of the clones had DNA of type 2 (2L#15, 2L#43, 2L#44, and 2L#45) (Table 2). The four type 2 clones all had the same change in position 3100 as the type 2 from the K isolate mentioned above. This particular substitution and the other substitutions found in the clones are depicted in Table 2. The single clone from the Utah I isolate with ADV type 1 DNA had one nucleotide substitution compared with the ADV GL sequence (Table 1). The substitution was in position 3460, where an A is substituted with a G. In this position, both types 2 and 3 ADV DNA have a G. This nucleotide substitution gave rise to a codon change from an isoleucine in ADV G to a valine in the other clones (Table 1).

**Differences in the restriction endonuclease maps.** A sensitive method to compare closely related viruses utilizes restriction endonuclease mapping of viral DNA. This technique has been applied to both ADV and other parvoviruses and has shown differences between viruses not readily distinguishable by serological means (14, 35, 48). When we compared the DNA of three types of ADV, some remarkable differences in the restriction endonuclease maps were seen. In order to get information on restriction endonuclease sites in the majority of the DNA from the ADV GL, Utah I, and K isolates, we took advantage of the PCR amplification technique, which allowed us to use restriction endonuclease digests directly on the amplified DNA without cloning. DNA from the ADV GL, the Utah I, and the K isolates, representing types 1, 2, and 3 ADV DNA, respectively, were amplified in different regions, i.e., from 2.6 to 20 MUs, 41 to 57 MUs, 57 to 70 MUs, and 85 to 90 MUs. The amplified DNA samples were analyzed for restriction sites for *Acc*I, *Bam*HI, *Eco*RI, *Hin*dIII, and *Pst*I. By combining these data with the sequence data, a partial restriction map of the three types of ADV DNA present in the isolates could be predicted (Fig. 5). As expected from our cloning and sequencing experiments, this map showed extensive variation of the restriction enzyme sites in the DNA of the three different types of ADV. Thus, of eight sites examined, only one site was conserved among all three types of ADV. Also, this analysis confirmed our cloning efforts, indicating that the type 2 ADV was the most prevalent in the ADV Utah I isolate used and that type 3 ADV was the most prevalent in the ADV K isolate used.

## DISCUSSION

Two main topics are presented in this report. The first set of results presents the partial nucleotide sequence of an ADV isolate not published before and divides the ADVs from different isolates into three nucleotide sequence types. This article also reports the interesting observation that more than one ADV sequence type is present in each of the highly virulent isolates analyzed.

The major biological difference between the cell culture-adapted ADVs of low virulence and the in vivo isolates of high virulence is characterized by the ability of the former to grow well in CRFK cells but poorly in mink (16) and the capacity of the latter to grow and cause fulminant disease in

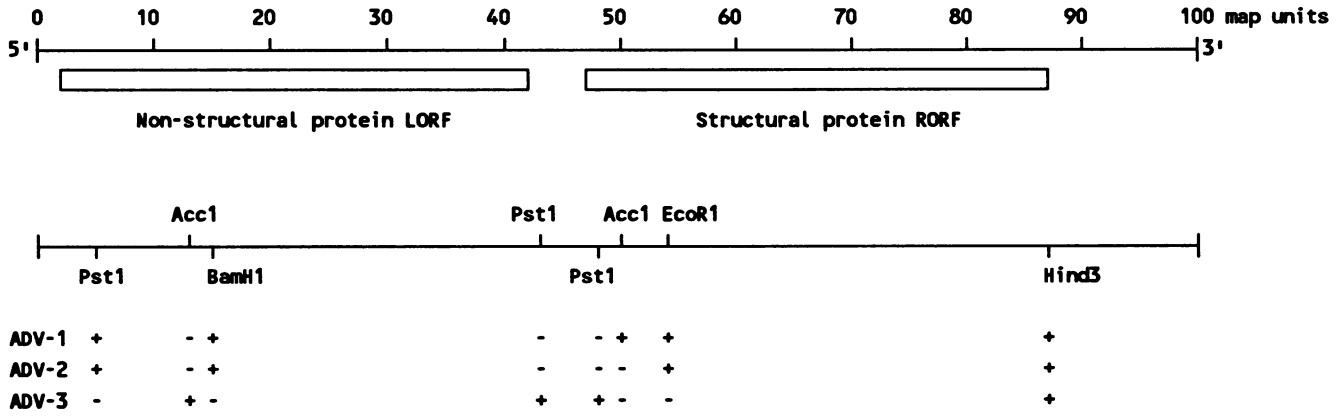


FIG. 5. Partial restriction map of the three different types of ADV DNA. The restriction sites were obtained either from the nucleotide sequence of cloned DNA or from endonuclease digests of amplified DNA. The nucleotide positions of the sites are as follows: *Pst*I at 241, 2119, and 2373; *Acc*I at 719 and 2378; *Bam*HI at 721; *Eco*RI at 2553; and *Hin*dIII at 4175. +, the restriction enzyme site is present in the sequence; −, the restriction enzyme site is not present in the sequence.

mink (2, 16, 17, 27, 38, 40) but not to grow productively in CRFK cells (16, 28, 29, 39). Recently, the complete nucleotide sequences of the cell culture-adapted ADV G isolate and the central region of the highly virulent Utah I isolate were reported (12, 13). The overall similarity between these two isolates was 97.5%. In order to get a better understanding of the variability of highly virulent ADV isolates, we decided to characterize the Danish ADV K isolate. ADV K was isolated from an outbreak of ADV-induced pneumonia among Danish mink in 1982 and is highly virulent for both newborn and adult mink (2, 3, 33). The initial strategy for cloning DNA from this isolate involved use of the two endonuclease sites *Bam*HI at 15 MU and *Hin*dIII at 88 MU because these sites were conserved in the published sequences of ADV G and ADV Utah I (13). By using this strategy, only a single clone was obtained. The insert was analyzed and was shown to be 99.9% homologous to the ADV Utah I sequence reported previously (13). Despite substantial effort, we did not obtain any more clones from the ADV K isolate by using *Bam*HI and *Hin*dIII digests. However, cloning of ADV K DNA digested with the endonucleases *Pst*I and *Hin*dIII resulted in several clones with 1.8-kb fragments (49 to 88 MUs), and cloning with *Pst*I alone resulted in clones with 0.3-kb inserts (44 to 49 MUs). The nucleotide sequences of three of these clones revealed that they represented a new type of ADV, here designated type 3 ADV. The sequenced region (from 44 to 88 MUs) of the new ADV type 3 was only 94.4% related to ADV G (designated type 1) and 94.7% to ADV Utah I (designated type 2). The single type 2 clone obtained from the ADV K isolate was most likely not a cloning artifact, considering the very high similarity, but not identity, to the ADV Utah I clone published previously (13). Analysis of several more clones and of PCR-amplified DNA from the ADV K isolate showed that the majority of the DNA had two *Pst*I sites in the middle of the genome not reported for ADV G or ADV Utah I. However, the *Pst*I site at 5 MU reported for ADV G and the *Bam*HI site at 15 MUs reported for both ADV G and ADV Utah I (13) were not present. These major differences among the isolates stress the importance of not uncritically using previously reported restriction enzyme sites for cloning of new virus isolates. As shown here, the first clone obtained from the ADV K isolate (a type 2 ADV) represented only a minority of the ADV DNA present in the isolate. Further analysis showed the majority of the ADV in this isolate to be of type 3. Thus, the study showed the presence of two different types of ADV to be present in the same isolate.

The existence of significantly different types within the individual parvoviruses has been described for feline parvovirus, CPV, mink enteritis parvovirus, and MVM (35, 37, 47). A recent report described the use of PCR for detecting human parvovirus B19 DNA in sera from patients (31). Two of nine sera tested with three sets of primers failed to react with some primers. Moreover, epidemiologically different isolates of B19 have variations in restriction enzyme sites (see reference 31). These findings, together with ours, indicate that genetic heterogeneity among parvovirus isolates is common, although the biological significance still is unsolved.

Comparisons of the structural genes from the closely related CPV and feline parvovirus showed that these are more than 98% homologous (19, 42, 43). Although the nucleotide differences were distributed throughout the genome, a certain amount of clustering of coding differences was observed (37). Analysis of recombinants between CPV and feline parvovirus showed that the CPV-specific antigenic site, the pH dependence of hemagglutination, and at least part of the canine host range of CPV mapped between 59 and 64 MUs. Those differences were therefore determined by one or more of the three amino acid sequence differences in that region, indicating that the sequence variation around 60 MUs defined an important functional region on the virus capsid (37). A similar variable region between 69 and 85 MUs in the structural genes of MVM also seems to play a pivotal role in the ability of MVMp and MVMi to replicate in fibroblasts and lymphocytes, respectively (10, 11, 45).

Comparisons of the structural genes of the three ADV types showed that although most of the differences are distributed throughout the genes, one small region at 64 MUs has a high degree of divergence. This region is, as proposed by Bloom et al. (13), very heterogeneous between the types 1 and 2, but interestingly, type 3 is also different in this region, having three differences from type 2 ADV. The type 1 ADVs (G and GL) grow productively in CRFK cells, while the highly virulent type 2 (ADV Utah I) and type 3 (ADV K) grow productively only in mink. This suggests that this part of the structural proteins is one of the determinants of cell specificity and pathogenicity, similar to the results reported for both MVM and feline parvovirus and CPV (10, 37). However, any of the other differences found between the ADV G and GL isolates and the in vivo-virulent isolates may also contribute to the significant variation in biological properties observed. Studies using infectious, chimeric clones of ADV are in progress and may shed some light on this interesting aspect of ADV virulence factors.

All the analyzed ADV DNA clones sequenced so far fall into one of the three types described. A few nucleotide substitutions in some clones were observed, and we cannot exclude the possibility that some of the differences present in the PCR-generated clones could be artifacts introduced by the *Taq* DNA polymerase, which has been shown to induce an error rate of $2.1 \times 10^{-4}$ after $10^6$-fold amplification (30). The predominant transitions for *Taq* DNA polymerase have been described as A to G and T to C (30). These transitions were indeed predominant in our clones. However, this was true for clones with inserts that were derived from PCR-amplified ADV DNA or cDNA but also for clones that were derived from conventional cloning. This suggests that the majority of the changes observed are not artifacts introduced by the *Taq* DNA polymerase but represent real differences among ADVs in the isolates. The biological significance of these minor differences, if any, is still obscure.

Further analysis of PCR-derived clones from the ADV Utah I isolate revealed that type 1 ADV also is present in this highly virulent isolate. This might not be so unexpected because the ADV G isolate is a cell culture-adapted derivative of the ADV Utah I isolate that lost pathogenicity for adult mink after serial passage in CRFK cells (16, 39). Interestingly, we have analyzed clones of DNA derived from two different in vivo isolates, and of these clones, a few had DNA that matched the ADV type 1 (data not shown). This suggests that the type 1 ADV is present at low levels in in vivo isolates of different origins and perhaps serve some function in the pathogenesis of ADV-induced disease.

The results presented have interesting implications for the pathogenesis of disease caused by ADV infection. At least two types of ADV were found in the highly virulent isolates, while in contrast, we demonstrated only one, and the same, in the cell culture-adapted ADV G and GL isolates of low virulence. It is tempting to speculate that the presence of two or more ADV types in the same in vivo isolate has importance for the persistence and severity of the infection, a

situation similar to certain retrovirus systems (20, 25, 36, 44). Further experiments are under way to address these important issues.

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