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Molecular Comparisons of In Vivo- and In Vitro-Derived Strains of Aleutian Disease of Mink Parvovirus

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DNA from one cell culture-adapted and two pathogenic strains of Aleutian disease of mink parvovirus (ADV) was molecularly cloned into the vectors pUC18 and pUC19. The DNA from the two pathogenic strains (ADV-Utah I and ADV-Pullman) was obtained from virus purified directly from the organs of infected mink, whereas the DNA from the nonpathogenic ADV-G was derived from cell culture material. The cloned segment from all three viruses represented a 3.55-kilobase-pair *Bam*HI (15 map units) to *Hin*dIII (88 map units) fragment. Detailed physical mapping studies indicated that all three viruses shared 29 of 46 restriction endonuclease recognition sites but that 6 sites unique to the pathogenic strains and 5 sites unique to ADV-G were clustered in the portion of the genome expected to code for structural proteins. Clones from all three viruses directed the synthesis of two ADV-specific polypeptides with molecular weights of approximately 57 and 34 kilodaltons. Both species reacted with sera from infected mink as well as with a monoclonal antibody specific for ADV structural proteins. Because production of these ADV antigens was detected in both pUC18 and pUC19 and was not influenced by isopropyl-β-D-thiogalactopyranoside (IPTG) induction, their expression was not regulated by the *lac* promoter of the pUC vector, but presumably by promoterlike sequences found within the ADV DNA. The proteins specified by the clones of ADV-G were 2 to 3 kilodaltons smaller than those of the two pathogenic strains, although the DNA segments were identical in size. This difference in protein molecular weights may correlate with pathogenicity, because capsid proteins of pathogenic and nonpathogenic strains of ADV exhibit a similar difference.

Aleutian disease of mink (AD) is an acquired disorder of immune function characterized in adult mink by plasma cell hyperplasia, hypergammaglobulinemia, autoimmunity, and fatal immune complex disease (1, 11, 12, 16, 34). This disease results from a persistent infection by the AD parvovirus (ADV) (10, 13), but strains of virus differ greatly in their capacity to produce the full-blown clinical picture of AD (1, 12, 17, 34). Specifically, the highly virulent ADV-Utah I strain causes typical AD in mink of both Aleutian and non-Aleutian genotypes, whereas the ADV-Pullman strain produces AD only in mink of the Aleutian genotype. Non-Aleutian mink when infected with ADV-Pullman develop an inapparent, often nonpersistent infection (12, 17). These in vivo patterns of disease production between strains are highly reproducible and, thus, have implied that genomic features of ADV influence pathogenicity.

Comparison of ADV strains has been limited to date but has suggested that the various strains are very similar (1, 9, 35, 37). A major problem in direct molecular comparison relates to the difficulty in growing virulent virus isolates in cell culture. Although ADV may replicate in mink lymphocytes in vitro (22) and obviously grows in mink cells in vivo, reliable propagation in vitro has been demonstrated only in feline cells at reduced incubation temperatures (32°C) (13, 18, 35). Virulent strains grow poorly or not at all in cell culture (13, 18, 34), and ADV-G, which was derived from the highly virulent ADV-Utah I, lost pathogenicity during adaptation to in vitro growth (13). These results indicate that the pathogenicity or virulence of viruses isolated in cell culture may significantly differ from that of the strain from which they were derived. Thus, to make comparative analyses more meaningful, it would be desirable to avoid possible adverse selection factors exerted by cell culture isolation.

In recent years, considerable information has been gained about the organization of the parvovirus genome, and certain features seem to be common to genomes of all the nondefective parvoviruses. The 5′ or left half of the duplex replicative form contains coding sequences for the nonstructural genes, whereas the right half codes for the virion structural proteins (Fig. 1) (5, 8, 33, 38, 39). The molecular analysis of ADV DNA may help to define what regions of the genome differ among strains and thus give some insight into which viral genes influence pathogenicity.

In this communication, we report molecular cloning of the *Bam*HI-to-*Hin*dIII (15 to 88 map unit [m.u.]) fragments from two in vivo-passaged ADV strains (ADV-Utah I and ADV-Pullman) and their comparison with a similar clone derived from ADV-G. The cloned DNAs were compared by restriction enzyme mapping, and differences could be detected between in vivo- and in vitro-passaged ADV. Furthermore, ADV-specific polypeptides were demonstrated by Western blot (immunoblot) analysis of lysates of *Escherichia coli* cells harboring these recombinant plasmids. The polypeptides expressed by clones derived from the two in vivo-derived viruses were consistently 2 to 3 kilodaltons larger than those derived from ADV-G.

MATERIALS AND METHODS

Viruses and viral DNA. The propagation of the pathogenic strains ADV-Utah I (12, 15) and ADV-Pullman (12, 16, 37) in infected mink and the preparation of partially purified virus from organs were done by published procedures (2, 12, 37). The preparation of single-stranded virion DNA from partially purified strains ADV-Utah I and ADV-Pullman was

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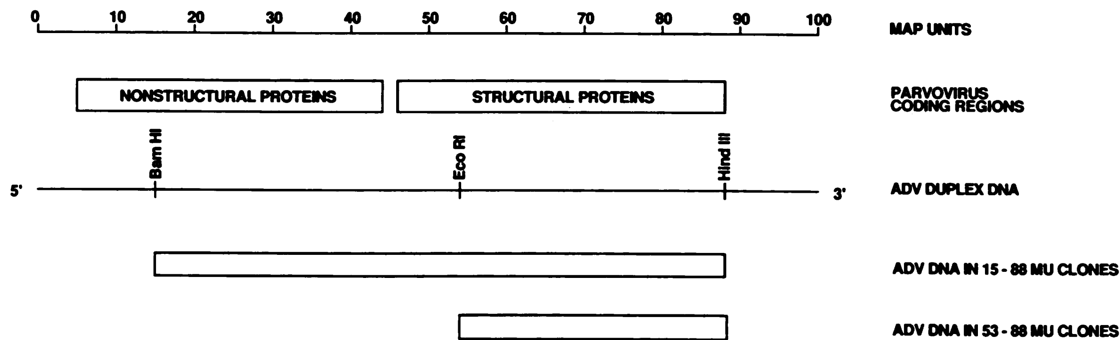

FIG. 1. Schematic representation of molecular clones of ADV DNA. A map representing 0 to 100 arbitrary m.u. of a composite parvovirus genome and showing the location of coding regions for the structural and nonstructural proteins (8, 39) is depicted. In addition, a map of duplex ADV DNA showing the locations of the *Bam*HI, *Eco*RI, and *Hin*dIII sites (10, 29) is also shown. The 15 to 88 m.u. clones described in this paper as well as the 53 to 88 m.u. clone, pBM1, are aligned on this map. The 5′ end of the duplex ADV genome is at the left, and the presumed direction of transcription is from left to right (33).

performed as previously described (29). The growth of ADV-G in Crandell feline kidney cells (CRFK) (13, 35) and the purification of double-stranded ADV-G replicative form DNA by a modified Hirt procedure have also been reported previously (10).

Molecular cloning of ADV DNA. For molecular cloning, single-stranded DNA from ADV-Pullman and ADV-Utah I purified from the organs of infected mink (29) was converted to full-length double-stranded DNA by 3′ self-primed DNA synthesis with the Klenow fragment of *E. coli* DNA polymerase (10). ADV-G DNA used for cloning was isolated from a near full-length molecular clone inserted into pEMBL8$^+$ (M. E. Bloom and B. C. Chesebro, unpublished experiments). All three ADV DNAs were digested with *Hin*dIII and *Bam*HI (10, 29), and the *Hin*dIII-*Bam*HI fragments were prepared by two consecutive DNA electrophoreses in 1% low-melting-point agarose followed by purification on NACS-52 columns (Bethesda Research Laboratories, Inc., Rockville, Md.). These DNA samples were ligated with similarly digested pUC18 or pUC19 DNA (44) and transformed into *E. coli* JM109 (19, 44). The transformants were plated onto NZY agar plates containing 250 μg of carbenicillin per ml, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and 200 μg of 5-bromo-4-chloro-3-indoyl-β-galactoside (X-gal) per ml (29, 43, 44) and screened for ADV-specific DNA sequences by colony hybridization (28) with nick-translated molecularly cloned fragments of ADV-G DNA (10, 11, 29). Insert size was verified by restriction endonuclease cleavage of plasmid DNA made from 2-ml minipreparations (20, 28) grown in NZY broth (29) containing 250 μg of carbenicillin per ml (NZY/Carb). Large-scale purifications of plasmid DNA were prepared essentially as described previously (28, 29). These preparations were stored in convenient aliquots as ethanol precipitates at −20°C. All enzymes and cloning vectors used in these studies were from either Bethesda Research Laboratories or New England BioLabs, Inc. (Beverly, Mass.) except as noted. Isotopes were purchased from Du Pont-New England Nuclear Corp. (Boston, Mass.).

Preparation and analysis of *E. coli* cultures for protein expression. For analysis of protein expression, overnight cultures in NZY/Carb were diluted into fresh medium and grown to the mid-log phase (200 Klett units). Samples were removed and centrifuged to pellet the cells, and the cell pellets were suspended in Laemmli sample buffer (14) (100 μl of sample buffer per ml of original culture) before boiling for 5 to 10 min. When noted, the remaining culture was incubated for an additional 60 min in 1 mM IPTG before processing. These boiled samples were stored at −20°C. Immediately before use, the samples were reboiled, centrifuged for 1 min, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% polyacrylamide in the resolving gel (14). Molecular weights of proteins were estimated by comparison of mobilities with those of a set of prestained molecular weight markers (Bethesda Research Laboratories). These prestained markers also provided a convenient means of assessing efficiency of protein transfer onto nitrocellulose in Western blot experiments. In some cases, duplicate gels were prepared and stained with Coomassie blue to examine total protein profiles.

Western blot analysis was done either with a serum pool from ADV-infected mink (14, 29) or with a monoclonal antibody (MAb 47) directed against ADV structural poylpeptides (23, 37). When mink sera were used, the proteins were transferred onto nitrocellulose membranes exactly as previously described (29) with 50 mM phosphate buffer as the transfer buffer. All incubations and washes were performed in 5% nonfat dry milk in phosphate-buffered saline (PBS) (21). The polypeptides recognized by the mink sera were detected with ^{125}I-labeled protein A. To reduce nonspecific background, we absorbed the serum pool before use with a lysate of boiled *E. coli* JM109.

Western blot analysis with MAb 47 (23) utilized transfer of the separated proteins to nitrocellulose in Towbin buffer (42). In this case, the membrane was blocked with PBS containing 0.01% Tween 20 (PBS-T) (6, 29), incubated overnight in undiluted MAb supernatant, washed three times for 15 min each in PBS-T, and reacted with a 1/500 dilution of horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G (Organon Teknika, Malvern, Pa.). After five 10-min washes in PBS-T and 4 rinses in PBS, the peroxidase signal was developed for 30 s; the developer was prepared by dissolving 15 mg of 4-chloro-1-naphthol in 5 ml of methanol, diluting to 30 ml with PBS, and adding 10 μl of H_2O_2 (23). After approximately 30 s, the developer was replaced with PBS and the filter was photographed. For these experiments, a mini-SDS-PAGE and mini-Western transfer unit were used (Bio-Rad Laboratories, Richmond, Calif.).

Comparative physical mapping of cloned ADV DNA. The cloned viral inserts were physically mapped by partial restriction enzyme digestion of asymmetrically end-labeled DNA as described previously (9, 28, 40). Briefly, selected clone inserts were linearized with *Hin*dIII, and the 5′ termini

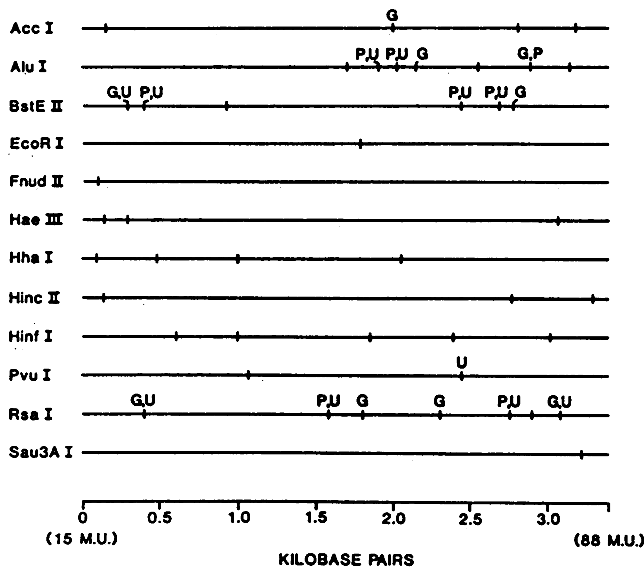

FIG. 2. Comparative physical map of molecularly cloned ADV DNA. The 3.55-kbp *Bam*HI-*Hin*dIII fragments, asymmetrically labeled at the *Hin*dIII end with ^{32}P, were prepared as described in Materials and Methods. The DNAs were subjected to partial digestion with the indicated restriction endonucleases in the presence of an excess of unlabeled bacteriophage lambda DNA (9, 28, 40). The partial products were resolved by electrophoresis in a 2% agarose gel and autoradiography of the dried gel. The molecular weights of the labeled fragments were estimated by comparison with mobilities of fragments of bacteriophage lambda or ϕX174 digested with *Hin*dIII and *Hae*III, respectively. The fragment sizes indicated the distance from the labeled *Hin*dIII site (88 m.u.) to a recognition site for that particular enzyme. The sites for each enzyme are indicated by a slash. Sites which were present but not shared by all three viruses are indicated with superscripts: G, ADV-G; P, ADV-Pullman; U, ADV-Utah I.

were dephosphorylated with calf intestinal phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The 5′ ends were labeled with [γ-^{32}P]ATP by using polynucleotide kinase, and the viral DNA, asymmetrically labeled at the *Hin*dIII end, was released from vector sequences by *Bam*HI digestion. The fragments were subjected to partial restriction enzyme digestion in the presence of an excess of unlabeled bacteriophage lambda DNA. Finally, the partial products were identified by electrophoresis in 2% agarose gels and autoradiography of the dried gels (9).

RESULTS

Development of molecular clones from in vivo- and in vitro-grown ADV strains. The major goal of this work was to generate and to analyze molecular clones of DNA from strains of ADV that do not grow in tissue culture, specifically the pathogenic strains ADV-Utah I and ADV-Pullman. The 3.5-kilobase-pair (kbp) *Bam*HI-to-*Hin*dIII fragment from all three strains, representing 15 to 88 m.u., was molecularly cloned into pUC18 and pUC19 (44). The ADV-specific inserts of clones from all three virus strains were the same size as the equivalent fragment of ADV-G duplex monomer replicative form (data not shown). The alignment of these clones on the ADV genome and their relationship to consensus coding regions for the other nondefective parvoviruses is depicted schematically in Fig. 1. The 15 to 88 m.u. fragment represented approximately 73% of the viral genome and, by analogy, should have contained portions of the genes for both structural and nonstructural proteins (8, 39).

Comparative restriction mapping of molecular clones derived from in vivo- and in vitro-grown ADV strains. All restriction maps published previously for ADV have utilized DNA derived from either in vitro-passaged virus (10) or from molecular clones derived from infected cell cultures (9). Because viruses selected by in vitro cell culture passage may not be representative for the strain from which they are derived, analysis of the molecular clones reported in the previous section provided a way to compare virus strains without the intervention of cell culture. Consequently, we developed detailed restriction maps using the sensitive technique of partial digestion of end-labeled DNA fragments (9, 40) (Fig. 2). If the *Bam*HI and the *Hin*dIII sites at the 5′ and 3′ ends are included, a total of 46 restriction enzyme recognition sites were identified by this analysis, of which 29 were shared by all three strains. The shared sites were found relatively uniformly along the length of the segment analyzed, further suggesting that the cloned inserts were all the same length and that no internal deletions were present within any of the clones. There were seven sites unique to the two in vivo-passaged strains, and six of the seven were found near or to the right of (on the 3′ side with respect to transcription of) the shared *Eco*RI site at 53 m.u. The five sites unique to the cell culture-adapted ADV-G also were found to the right of the *Eco*RI site. Thus, the region to the right of (or 3′ to) the *Eco*RI site seemed to have the greatest heterogeneity. This portion of the genome should contain the coding sequences for the virion structural proteins (8, 39).

Expression of ADV antigens by molecular clones derived from in vivo and in vitro strains of ADV. Experiments previously reported from this laboratory indicated that a molecular clone (pBM1) containing the 53 to 88 m.u. (*Eco*RI-to-*Hin*dIII) segment of the ADV-G genome directed the synthesis of ADV antigens (Fig. 1) and that these antigens reacted with a serum specific for viral structural proteins (14, 29). Because the genomic fragments cloned in the present experiments were substantially larger than those from earlier experiments and represented segments of in vivo- as well as cell culture-derived ADV, we also examined these new clones for expression. Although no ADV-specific proteins could be observed on Coomassie blue-stained gels, molecular clones from all three viruses directed the synthesis of two major ADV antigens with approximate molecular sizes of 57 and 34 kilodaltons (kDa) when analyzed by Western blot analysis (Fig. 3). Both the 57- and 34-kDa proteins reacted with sera from infected mink (14, 29) as well as with an MAb (MAb 47) specific for virion structural proteins (23) (Fig. 4). These findings demonstrated that molecular clones from all three virus strains specified the synthesis of two major polypeptides and that both the 57- and 34-kDa proteins represented products of the viral structural protein genes.

The proteins specified by the ADV-G clones were consistently 2 to 3 kDa smaller than those observed for the corresponding construct of ADV-Pullman or ADV-Utah I (Fig. 3 and 4). This difference was observed for inserts in both pUC18 and pUC19, although for each virus the molecular weights of the proteins from the pUC19 clone were slightly higher than those from the pUC18 clone (Fig. 3 and 5). Interestingly, the sizes of the proteins specified by the 3.55-kbp 15 to 88 m.u. ADV-G clones were almost identical to those expressed by pBM1, the 1.55-kbp 53 to 88 m.u. segment of ADV-G cloned into pUC8 (29, 43) (Fig. 1, 4, and 6). Because the polypeptides from pBM1 and the 15 to 88

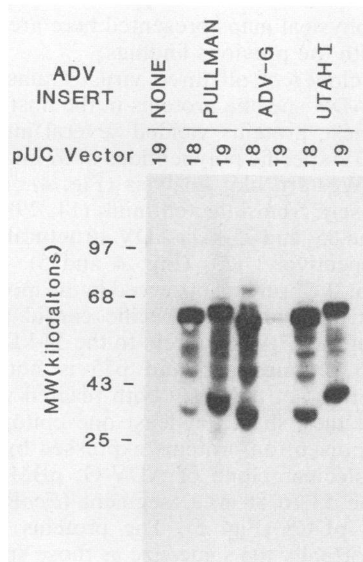

FIG. 3. Detection of proteins expressed by molecularly cloned ADV DNA with sera from ADV-infected mink. Small-scale cultures of *E. coli* JM109 harboring pUC19 or the 15 to 88 m.u. *Bam*HI-*Hin*dIII segment of ADV-G, ADV-Pullman, or ADV-Utah I molecularly cloned into pUC18 or pUC19 (44) were grown to 200 Klett units in NZY/Carb. IPTG was added to 1 mM (29, 43, 44), and the culture was incubated for 60 min. The samples were centrifuged, and the cell pellets were suspended by boiling in Laemmli sample buffer (14) at a ratio of 100 μl/ml of starting culture. Samples were subjected to SDS-PAGE (13, 14) and Western transfer (6, 21, 29) to nitrocellulose with 50 mM phosphate buffer as the transfer buffer. The transferred proteins were incubated with a serum pool from ADV-infected mink (14) followed by ^{125}I-labeled protein A as detailed in Materials and Methods. Molecular weights were estimated by mobility comparisons with a set of prestained molecular weight (MW) markers (Bethesda Research Laboratories).

m.u. clones (pADV-G) both reacted with MAb 47 (Fig. 4), they must have shared at least one epitope.

Surprisingly, ADV gene products were observed independently of whether the presumed direction of viral transcription (8, 39) was colinear with (pUC18) or opposite to (pUC19) *lac* gene transcription in the vector (43, 44) (Fig. 3 and 5). The level of expression was similar in both orientations for ADV-Pullman and ADV-Utah, but an effect of orientation was noted for ADV-G, because expression was greater in pUC18 than in pUC19. Nevertheless, detection of ADV antigens in pUC19 suggested that expression of the 15 to 88 m.u. fragment was not controlled by the *lac* gene promoter in the vector. This independence of *lac* gene promotion was further implied by the finding that expression in the 15 to 88 m.u. clones was relatively unaffected by IPTG induction in both orientations (Fig. 5). In contrast, expression by the 53 to 88 m.u. ADV-G clone pBM1 clearly was influenced by IPTG (Fig. 6). Taken together, these findings implied that expression of ADV polypeptides by the 15 to 88 m.u. clones was controlled in a procaryotic host by an element to the left (5′ side) of the *Eco*RI site at 53 m.u.

DISCUSSION

In this study, we molecularly cloned DNA from two pathogenic strains of ADV (ADV-Utah I and ADV-Pullman) into the expression vector pUC and compared them with an analogous clone of the cell culture-adapted ADV-G. Previ-

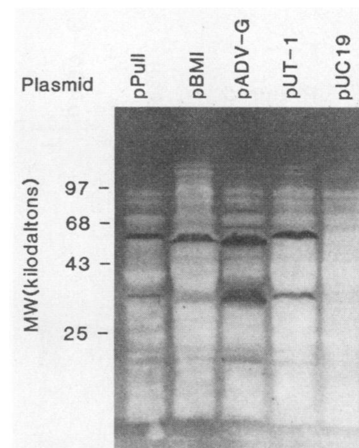

FIG. 4. Detection of proteins expressed by molecularly cloned ADV DNA with an MAb specific for ADV structural proteins. Samples from the cultures of pUC19 or the ADV clones in pUC18 described in the legend to Fig. 3 were subjected to SDS-PAGE. Another sample was prepared from a similar culture of JM103 containing pBM1 (53 to 88 m.u. segment of ADV-G cloned into pUC8 [29]) and also analyzed. The proteins were transferred to nitrocellulose with Towbin buffer (42), reacted with an MAb (MAb 47) specific for ADV structural proteins (24), and detected with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G (24). MW, Molecular weight.

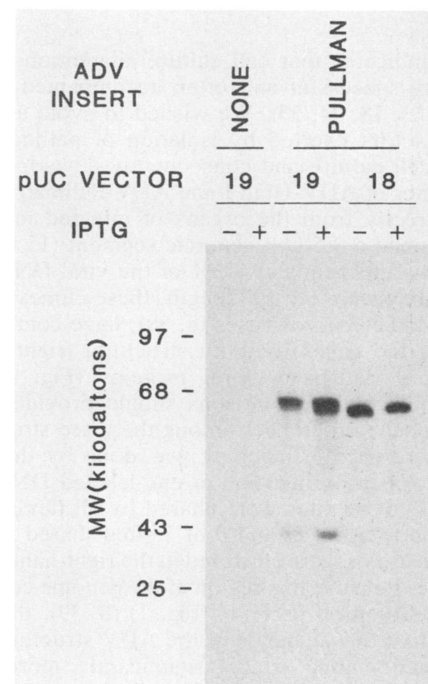

FIG. 5. Effect of IPTG on expression of proteins specified by molecularly cloned ADV DNA. Small-scale cultures of JM109 harboring pUC19 or the 15 to 88 m.u. segment of ADV-Pullman cloned into either pUC18 or pUC19 were grown to 200 Klett units. A portion was removed and processed for analysis; the remaining culture was adjusted to 1 mM IPTG and incubated for an additional 60 min before processing. Sample preparation, SDS-PAGE, and Western blotting with sera from ADV were performed as described in the legend to Fig. 3. MW, Molecular weight.

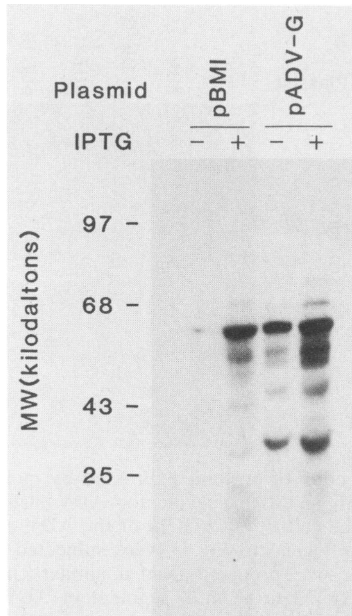

FIG. 6. Comparison of proteins expressed by molecular clones containing either the 15 to 88 m.u. (pADV-G) or the 53 to 88 m.u. (pBM1) segment of ADV-G. Small-scale cultures of either JM103 harboring the 1.55-kbp 53 to 88 m.u. segment of ADV-G in pUC8 (pBM1) or JM109 harboring the 3.55-kbp 15 to 88 m.u. fragment of ADV-G in pUC18 (pADV-G) were prepared. Portions of each culture were analyzed by SDS-PAGE and Western blotting either before or after IPTG treatment. Sera from ADV-infected mink were used to detect ADV-specific proteins. MW, Molecular weight.

ous work indicates that cell culture adaptation of ADV is infrequently successful and often accompanied by loss of virulence (13, 18, 34, 35). We wished to avoid any adverse selection factors exerted by isolation of pathogenic ADV strains in cell culture and consequently developed the molecular clones of ADV-Utah I and ADV-Pullman from DNA isolated directly from the organs of infected mink. These clones spanned a 3.5-kbp genomic segment (15 to 88 m.u.) representing approximately 73% of the viral DNA (10, 29). Although they were not full length, these clones should, by analogy to other parvoviruses (8, 39), have contained large portions of the genes for both structural (right-hand) and nonstructural (left-hand) viral proteins (Fig. 1). Consequently, molecular comparisons should provide important insights into the differences among the three strains.

Detailed restriction mapping was done by the sensitive technique of partial digestion of end-labeled DNA (Fig. 2). Although 29 of 46 sites were shared by all three viruses, 5 sites unique to ADV-G and 6 of 7 sites shared by the two pathogenic strains were clustered in the right-hand portion of the genome. Because this region of the genome codes for the parvoviral structural proteins (Fig. 1) (8, 39), these results suggested that the sequence of the ADV structural proteins had diverged among strains significantly more than the left-hand portion, which codes for nonstructural proteins (8, 39). Nevertheless, data accumulated for other nondefective parvoviruses, such as minute virus of mice (5, 33, 38), indicate that significant differences between closely related viruses may be evident only by direct comparison of the actual DNA sequences. Thus, the three strains of ADV may differ in areas not evident by this analysis. We recently reported a similar analysis of smaller clones from ADV-G and ADV-Utah I representing 53 to 88 m.u. (1.55 kbp) (9, 29), and the physical maps presented here are in very close agreement with the previous findings.

Molecular clones of all three virus strains directed the synthesis of ADV-specific proteins in the host bacteria, and analysis of these proteins yielded several interesting findings. Two ADV-specific polypeptides of 57 and 34 kDa were detected by Western blot analysis (Fig. 4). Both proteins reacted with sera from infected mink (14, 29) and an MAb specific for the 85- and 75-kDa ADV structural proteins (p85 and p75, respectively) (23) (Fig. 4 and 5). We therefore concluded that the proteins observed in the molecular clones represented truncated ADV-specific capsid proteins. The relationship of the 57-kDa protein to the 34-kDa protein and to the virion proteins p85 and p75 is not immediately obvious, but the fact that they both reacted with the MAb indicates that they shared at least one epitope. The same epitope was present on proteins expressed by a previously described molecular clone of ADV-G, pBM1 (29), which represents the 53 to 88 m.u. segment (*Eco*RI to *Hin*dIII) inserted into pUC8 (Fig. 5). The proteins expressed by pBM1 were virtually the same size as those specified by the 15 to 88 m.u. segment (*Bam*HI to *Hin*dIII) in pUC18 (Fig. 5 and 6). The fact that both sets of polypeptides contained the same epitope and were of the same size led us to postulate that the proteins expressed by the 15 to 88 m.u. segment were the same as those expressed by the 53 to 88 m.u. segment. This hypothesis suggested that the bulk of the coding sequences for the proteins expressed by the 15 to 88 m.u. clones must be located 3′ to (i.e., to the right of) the *Eco*RI site (Fig. 1). This would be consistent with the genomic organization reported for the other parvoviruses (8, 39).

Expression of these proteins from the 15 to 88 m.u. clones were observed in both pUC18 and pUC19 (Fig. 3, 4, and 5) and, unlike the expression of the proteins found in the 53 to 88 m.u. clones, was relatively unaffected by IPTG induction of the *lac* gene promoter (Fig. 5 and 6). These observations indicated that protein expression by the 15 to 88 m.u. clones was independent both of orientation within the vector and of *lac* gene promotion, thus implying that synthesis of the ADV polypeptides was directed by sequences in the ADV insert. The promoter for the structural genes of parvoviruses is found near 40 m.u. (8, 33, 39) and should be contained within the 15 to 88 m.u. clones but not the 53 to 88 m.u. clone (pBM1). It is possible that expression in the 15 to 88 m.u. clones is regulated by the analogous promoter in ADV, but it is unusual for eucaryotic promoters to function in procaryotic hosts (25). It may be more likely that some sequences within the cloned DNA sufficiently resembled bacterial promoters to be active (31, 36). Resolution of this point must await examination of the actual DNA sequence and promoter mapping (33).

In addition, the sizes of the proteins in pUC19 were slightly larger than those in pUC18 (Fig. 4 and 5), although the insert was identical in both orientations. Thus, if it is assumed that the same ADV promoter was utilized in both orientations, the explanation for the difference in molecular weights likely is specified by vector sequences adjacent to the *Hin*dIII site at 88 m.u. at the presumed carboxy end of the cloned ADV proteins.

One point of difference previously observed among strains of ADV is that the structural proteins of ADV-Utah I are 2 to 3 kDa larger than the corresponding proteins of ADV-G (4). The molecular weights of the gene products of cloned ADV-Utah I and ADV-Pullman DNA maintained this difference. There are several possible explanations for the size

differences of the polypeptides. First, ADV-G might code for amino acids of sufficiently different size or charge to alter the observed molecular weight. Support for this notion might be derived from the restriction mapping data which suggested sequence variation in the portion of the genome expected to code for the structural proteins (Fig. 1 and 2). Although we favor this hypothesis, several other mechanisms can be envisioned. For example, the cloned ADV-G insert might have been shorter than that of the two other viruses. However, a 2- to 3-kDa variation in protein molecular weight would correspond to approximately 50 bp of DNA, and because such a difference was not evident in our restriction mapping studies, we considered this possibility to be unlikely. Next, the cell culture adaptation of ADV-G from ADV-Utah I (13) may have been accompanied by mutations which created new termination sequences or stop signals, thus leading to premature termination of protein synthesis (3, 25). Finally, recent experiments (7, 26) provide evidence that secondary structures of DNA in noncoding regions, such as hairpins or stem-and-loop structures, may alter translation of mRNAs. Perhaps ADV-G, but not the in vivo-derived viruses, contains such a sequence that causes altered translation of its primary sequence. Until the sequences of the three viruses are compared directly, it will not be possible to differentiate with certainty among these possibilities.

The host range of parvoviruses can be extremely limited (24, 32, 41), and differences in host range have been suggested as one determinant of pathogenicity (24). It has been recently shown that the host range of canine parvovirus is governed by sequences in the virion structural protein (32). Although differences in host range or structural proteins may not be an obligate correlate with parvovirus pathogenicity (24, 30, 38, 41), the pathogenicity of ADV strains might be governed by such factors. The structural proteins of pathogenic and nonpathogenic strains of ADV differ in size (4), and perhaps these differences play a role in the relative pathogenicity. In this paper, we reported the development of molecular clones of DNA from ADV isolated directly from infected animals, and the proteins expressed by these clones showed a size variation similar to that observed in vivo. We plan to use these clones to produce constructs containing segments of pathogenic and nonpathogenic strains of ADV. In vivo analysis of viruses derived from such hybrid clones will help to define the influence of ADV structural proteins on pathogenicity.

While this article was in preparation, a report from one of our laboratories (O.-R. Kaaden) described the development of a similar 15 to 88 m.u. clone in pUC18 from the ADV isolate SL3 (27). This clone also directed the synthesis in *E. coli* of ADV antigens of similar size to those reported here. Expression of SL3 antigens was also observed in pUC19, but was not reported in that manuscript (O.-R. Kaaden, personal communication).

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Irene Cook Rodriguez and Helen Blahnik assisted in preparation of the manuscript, and Robert Evans and Gary Hettrick constructed the figures. LPVD staff members provided constructive criticism.

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