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Molecular Cloning of the Aleutian Disease Virus Genome: Expression of Aleutian Disease Virus Antigens by a Recombinant Plasmid

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Three nonoverlapping segments representing ~80% of the 4.8-kilobase pair Aleutian disease virus (ADV-G) duplex genome were molecularly cloned into either bacteriophage M13mp9 (M13bm2 = 0.07 to 0.15 map unit; M13bm1 = 0.15 to 0.54 map unit) or plasmid pUC8 (pBM1 = 0.54 to 0.88 map units). In addition, the 0.54- to 0.88-map unit segment of a Danish isolate of ADV (DK ADV) was also cloned into pUC8 (pBM2). The recombinant plasmids pBM1 and pBM2 induced expression of several polypeptides in *Escherichia coli* JM103 that were specifically recognized by sera from mink infected with ADV. The same three proteins with approximate molecular weights of 55,000, 34,000, and 27,000 were detected both by immune blotting and by immunoprecipitation of [^{35}S]methionine-labeled JM103 (pBM1). None of these proteins were recognized in JM103 or JM103 (pUC8), nor were they detected by sera from normal mink. Purified pBM1 and pBM2 DNA appeared identical in size by gel analysis and contour length measurement, and electron microscopic heteroduplex mapping revealed no visible areas of heterology. However, restriction endonuclease mapping showed that pBM2 was different from pBM1, indicating that this segment of the ADV genome was similar but not identical for two strains of ADV (ADV-G and DK ADV). Furthermore, when cloned DNA from ADV-G was labeled with [^{32}P]dCTP by nick translation, DNA relatedness to several field strains of ADV (Utah I, Pullman, and DK), but not to mink enteritis virus or cellular DNA, was shown by Southern blot hybridization.

In recent years, nondefective parvoviruses have gained much attention. Although several of these viruses (feline panleukopenia virus [8, 19, 22], canine parvovirus [22, 28], and mink enteritis virus [MEV; 19, 26]) cause significant disease in the respective host, none causes as unusual a pathological picture as does the Aleutian disease virus (ADV) of mink (4, 12, 29, 32, 33). Mink inoculated with virulent strains of ADV (32, 33) develop a persistent infection accompanied by severe immune complex disease (12, 29, 31–33) and high titers of antiviral antibodies (1, 4, 29, 33) reacting with both virion and nonvirion ADV proteins (6). Many of the accompanying hallmarks of ADV infection, such as hypergammaglobulinemia and plasmacytosis (4, 12, 29, 30, 33), are consistent with disordered immune regulation, but the exact role of ADV in the genesis of this disorder is not entirely clear. The presence of large amounts of antiviral antibody and of immune complexes containing infectious ADV (31) and ADV antigens (9, 29, 35) indicates a role for ADV in immune complex formation, but the mechanism by which ADV induces the severe hypergammaglobulinemia and plasmacytosis is unknown.

We recently began the study of the ADV genome (3) to analyze pathogenetic mechanisms at the cellular and molecular level and also to compare isolates of ADV which antigenically are very similar but which differ dramatically in virulence for mink (1, 4, 12, 29, 33). In the present report, we describe molecular cloning of much of the ADV genome and expression of ADV antigens in *Escherichia coli* by two recombinant plasmids (pBM1 and pBM2). In addition, we describe the use of cloned ADV-G DNA to demonstrate homology among several field strains of ADV but not to MEV.

MATERIALS AND METHODS

Materials. Restriction endonucleases and DNA ligase were obtained from either New England Biolabs,

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Beverly, Mass., or Bethesda Research Laboratories (BRL), Rockville, Md. Replicative form (RF) DNA for M13mp9 (24), and plasmid pUC8 DNA (40) were purchased from P-L Biochemicals, Inc., Milwaukee, Wis. Isopropyl β-D-thiogalactopyranoside (IPTG), ampicillin, and chloramphenicol were from Sigma Chemical Co., St. Louis, Mo., and the indicator 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) was bought from BRL. Isotopes and the materials for nick translation (38) were obtained from New England Nuclear Corp., Boston, Mass.

Viruses, cells, and sera. The propagation and preparation of partially purified virus from organs of mink infected with Utah I ADV (4, 9, 33), DK ADV (1), or Pullman ADV (4, 12) have been described previously (4, 9). ADV-G was grown in cell cultures of Crandell feline kidney cells (CRFK) (10) as previously described (5). DK ADV (1) was adapted to growth in CRFK at 31.8°C and subsequently purified by terminal dilution in CRFK at 31.8°C. A Danish isolate of mink enteritis virus (DK MEV) was adapted to grow in CRFK, purified by serial passage at 37°C in the mink lung cell line CCL-64 (6), and identified by Colin Parrish of Cornell University as a type 2 MEV (28). Pools of terminal mink sera were prepared from animals infected with various strains of ADV or from normal mink.

Molecular cloning of ADV DNA. The techniques of the modified Hirt extraction (21), agarose gel electrophoresis (11), Southern blot hybridization (39), and nick translation (38) were done exactly as described previously (3). For molecular cloning, RF ADV-G DNA or RF DNA from CRFK-adapted DK ADV was isolated from Hirt supernatants as previously noted (3). Restriction fragments of these DNAs were ligated to previously digested plasmid DNA (pUC8) (40) or bacteriophage RF DNA (M13mp9) (24) according to standard procedures (20) and subsequently transformed (13) into competent JM103 *E. coli* cells generously provided by J. Messing. The transformants were plated in the presence of IPTG and X-gal (24, 40) onto YT agar (25) (M13mp9) or YT agar with ampicillin (pUC8). Colorless plaques of recombinant bacteriophages were selected, and the presence of ADV sequences was verified by blot hybridization of phage DNA to nick translated ADV-G RF DNA (3). Insert size was verified by restriction endonuclease digestion of phage RF DNA (24) and gel electrophoresis. Colorless colonies of recombinant plasmids were picked onto YT-ampicillin agar plates and then screened for ADV sequences, using colony hybridization techniques (20). Insert size was verified by restriction endonuclease digestion of plasmid DNA isolated from small cultures grown in liquid broth (18) and resolution of fragments by gel electrophoresis. Batch preparation of DNA from plasmids or bacteriophages was done essentially as described previously (11, 18).

Isolation of DNA from ADV isolates. The preparation of DNA from partially purified Utah I, Pullman, and DK ADV was done as follows. Several milliliters of virus (or 0.5 ml of ADV-G virus stock) was ultracentrifuged in an SW50.1 rotor at 50,000 rpm for 60 min (4, 9). The pelleted virus was digested with 1 mg of proteinase K per ml in 0.05 M Tris (pH 8.0)–0.01 M EDTA–0.3% sodium dodecyl sulfate (SDS) at 65°C for 30 min and then for 3 h at 37°C (3, 7). The samples were extracted with redistilled phenol and chloroform and then were ethanol precipitated with yeast RNA as carrier. After centrifugation, DNA was dissolved in a small volume of TE buffer (0.01 M Tris–0.001 M EDTA, pH 8.0).

Preparation of bacterial cultures for protein expression. Cultures of bacteria were prepared for protein expression analysis according to the chloramphenicol (CAM) amplification method of Neidhardt et al. (27). Briefly, cultures initiated from an isolated plaque or colony were grown to approximately one-half saturation in YT broth. At this time, one-half of the culture was analyzed. The rest was incubated overnight in 200 μg of CAM per ml, washed twice by centrifugation, and resuspended in broth.

For immune blotting, samples of untreated culture or CAM-treated cultures after washing were treated with 10^{-3} M IPTG (15) for 1 h and then were centrifuged at 8,000 rpm for 10 min. The cell pellets were suspended by boiling in SDS-polyacrylamide gel electrophoresis sample buffer for 10 min at a ratio of 150 μl per ml of culture. Samples (100 μl) were applied to wells, and SDS-polyacrylamide gel electrophoresis was done as described previously (6). After electrophoresis, transfer of proteins onto nitrocellulose paper (HA filter, 0.45-μm pore size, Millipore Corp., Bedford, Mass.) was performed by using 50 mM sodium phosphate buffer, pH 7.5 (2). Transfer was accomplished in a chamber filled with transfer buffer at approximately 30 V (1.6 A) for 2 h. The nitrocellulose filter was then saturated for 1 h at 37°C in 0.004 M KH_2PO_4–0.016 M Na_2HPO_4, 0.115 M NaCl buffer (pH 7.3) containing 0.05% Tween 20 according to Batteiger et al. (2). Filters were then incubated for 2 h with 1:100 dilutions of either a normal mink serum pool or sera from ADV-infected mink followed by washing three times and incubation for 1 h with 10^6 cpm of ^{125}I-labeled staphylococcal protein A (specific activity, 24 μCi/μg), kindly provided by L. Evans. After extensive washings, the filter was dried and autoradiographed at −70°C with Lightning-Plus screens (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.). The transfer of protein bands onto the nitrocellulose paper was verified by amido black staining (2).

For analysis of protein expression by immunoprecipitation, untreated or CAM-treated cultures were washed once in chilled 1× M9 salts (27) and suspended in one-fourth volume chilled labeling medium. This medium was prepared by adding to 30 ml of 1× M9 salts the following reagents: 3 μl of 1% thiamine, 30 μl of 0.1 M $CaCl_2$, 52 μl of 1.2 M $MgCl_2$ and 1.2 ml of 10% maltose (25). IPTG to 10^{-3} M (15) and [^{35}S]methionine to 50 μCi/ml were then added. Incubation was at 37°C for 1 h, and after pelleting, the cells were suspended by sonication in 1 ml of lysing buffer per 5 ml of starting culture (6). The lysates were precleared, immunoprecipitated, and analyzed on SDS-polyacrylamide gels as detailed previously (6).

Electron microscopy. Purified plasmid DNAs (pBM1 and pBM2) were digested with *Eco*RI and *Hin*dIII for 60 min and mounted for electron microscopy as described previously (3, 14). Grids were examined in a JEOL 100B electron microscope at 40 kV. Micrographs were taken on Kodak Electron Image Plates (Eastman Kodak) at a magnification of ×8,000. The pUC8 portion of the plasmids (2.7 kilobase pairs [kbp]) (40) was used as an internal calibration standard for each set of measurements. Contour lengths were mea-

sured with a Numonics Graphics Calculator. Heteroduplexes were formed by mixing 1 μg each of *Eco*RI-*Hin*dIII double-digested pBM1 DNA and pBM2 DNA. The mixture was denatured with alkali, renatured for 1 h at 30°C in 50% formamide containing 0.1 M Tris (pH 8.5)–0.05 M NaCl–0.001 M EDTA and mounted for study, using the formamide technique (14).

RESULTS

Molecular cloning of ADV DNA. We recently published a physical map of the ADV-G genome based on restriction endonuclease digestion of duplex ADV DNA from Hirt supernatants (3). The orientation of several of these sites (Fig. 1A) suggested that segments of ADV DNA could be conveniently cloned by "forced" cloning into several vectors. Consequently, three fragments of the ADV genome representing approximately 80% of the duplex genome length were cloned into either bacteriophage M13mp9 (24) or the plasmid pUC8 (40). Recombinant phages (M13bm1, M13bm2) and plasmid (pBM1) were constructed as schematized in Fig. 1B, C, and D.

All potential insertions were screened by colony hybridization (20) to ADV-G DNA labeled by nick translation with [^{32}P]dCTP (3), and insert size was verified further by restriction endonuclease digestion of either bacteriophage RF DNA or form I plasmid DNA made by small-scale screening methods (data not shown). Representative clones were selected and prepared for additional studies as described below.

In addition to the molecular cloning of these ADV-G fragments, an *Eco*RI-*Hin*dIII fragment (Fig 1D) from a Danish isolate of ADV (DK ADV) (1) was also cloned into pUC8. This recombinant plasmid, denoted pBM2, appeared identical in size to pBM1 by both agarose gel electrophoresis (data not shown) and electron microscopic analysis (Fig. 2). Mean contour lengths (50 molecules ± standard deviation) were 1.63 ± 0.054 kbp for pBM1 and 1.630 ± 0.055 kbp for pBM2 (Fig. 2A). Heteroduplex comparison of the two inserts revealed no detectable regions of extended heterology (Fig. 2B). Comparison by restriction endonuclease digestion, however, indicated that the ADV insert in pBM2 (DK ADV) contained two *Acc*I sites corresponding to 0.67 and 0.84 map unit of the ADV RF, but that pBM1 (ADV-G) contained only a single *Acc*I site at 0.84 map unit. Neither pBM1 nor pBM2 was cleaved by *Xma*I or *Sac*I. These results suggest that although the segments of viral genome represented were very similar for the two virus strains ADV-G and DK-ADV, discreet differences existed.

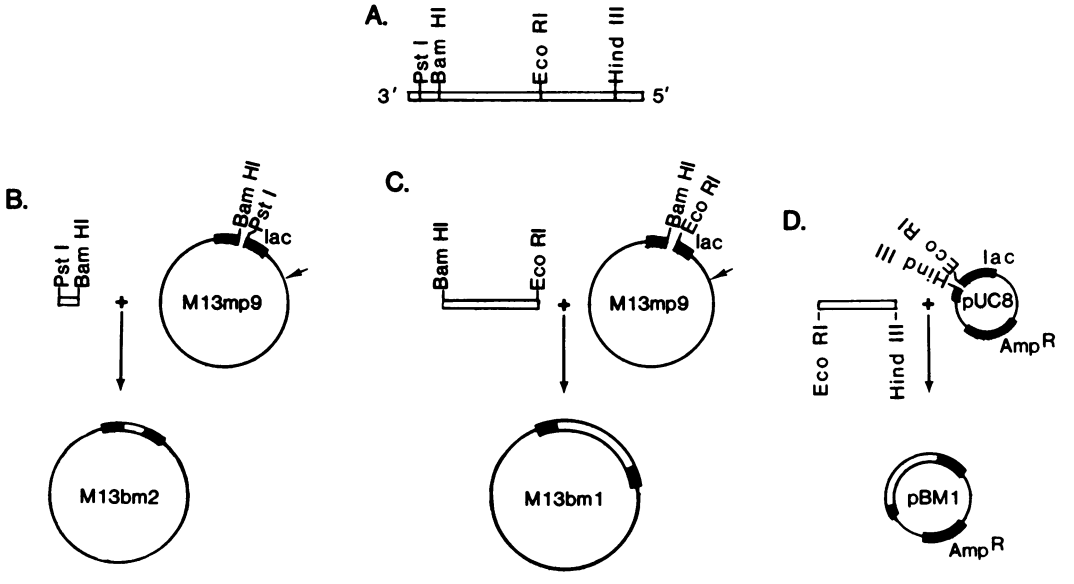

FIG. 1. Schematization for molecular cloning of ADV DNA. (A) Physical map of ADV genome, indicating restriction endonuclease sites used for molecular cloning (3); 3′ and 5′ refer, respectively, to the ends of ADV replicative form corresponding to the 3′ and 5′ ends of the virion DNA. (B) Construction of recombinant M13bm2 from bacteriophage M13mp9 (24) and *Pst*I-*Bam*HI fragments of ADV (0.07 to 0.15 map unit). (C) Construction of recombinant M13bm1 from bacteriophage M13mp9 (24) and *Bam*HI-*Eco*RI fragments of ADV (0.15 to 0.54 map unit). (D) Construction of recombinant pBM1 from plasmid pUC8 (40) and *Eco*RI-*Hin*dIII in fragments of ADV (0.54 to 0.88 map unit). "lac" indicates the portion of β-galactosidase gene in pUC8. Amp^R denotes ampicillin resistance gene in pUC8. The arrow (←) in M13mp9 refers to the *Hpa*I reference site in wild-type M13 (23).

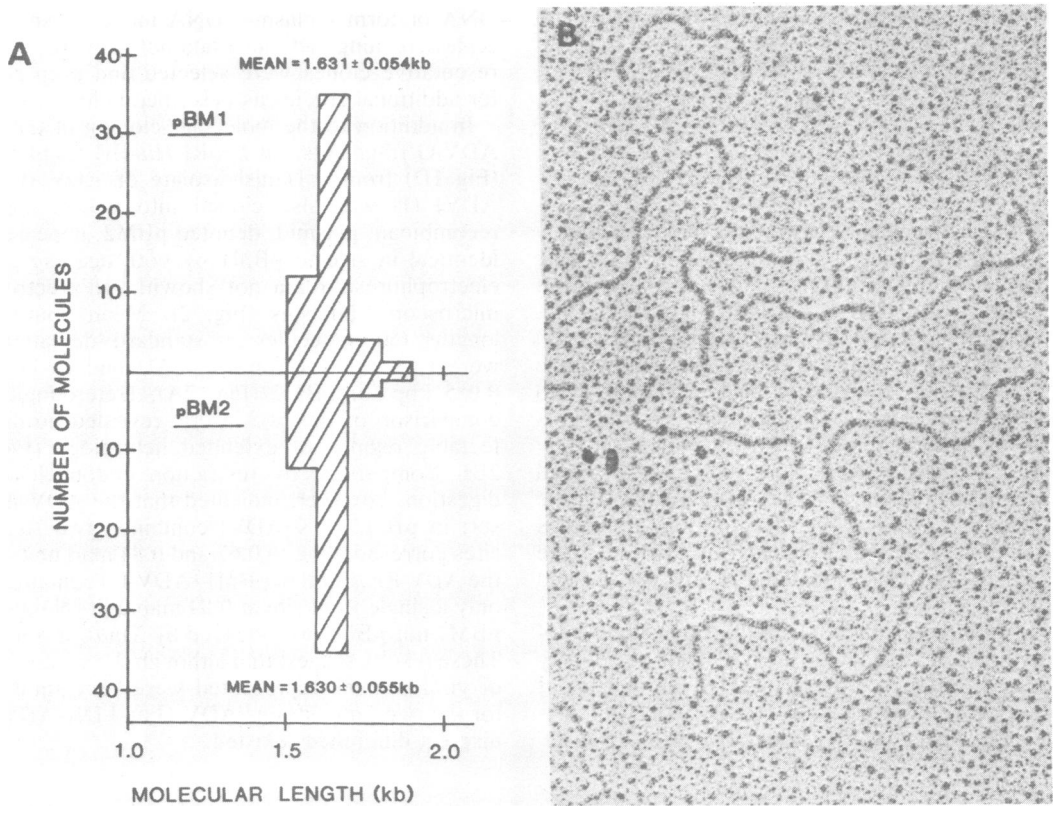

FIG. 2. Electron microscopic comparisons of pBM1 and pBM2 DNA. (A) Contour lengths of the ADV inserts in pBM1 and pBM2 DNA were performed as detailed in the text after digestion of *Eco*RI and *Hin*dIII. The pUC8 portion of pBM1 (2.7 kbp) was used as an internal standard (40). (B) Heteroduplex comparison of pooled pBM1 and pBM2 DNA after *Eco*RI-*Hin*dIII digestion was done as described in the text. The longer pieces of DNA are homoduplexes of the pUC8 portions of pBM1 and pBM2, and the shorter fragments are the heteroduplexes of the ADV inserts.

Expression of an ADV-determined protein by the recombinant plasmid pBM1. Successful cloning in the vectors used in these studies was detected by insertional inactivation of the β-galactosidase (*lac*) gene fragment in M13 or pUC8 (23, 24, 40). Since the ADV insert could potentially be in an appropriate orientation and reading frame for protein expression, we analyzed molecular clones for protein expression. JM103 (pBM1) as well as uninfected JM103 cells and JM103 (pUC8) were analyzed in detail by immune blotting as well as by immunoprecipitating [^{35}S]methionine-labeled lysates of bacterial cells. The results (Fig. 3) indicated several polypeptides specifically recognized in JM103 (pBM1) by sera from mink infected with ADV. Proteins with similar masses were detected by both methods; the most abundant species was ~34,000 daltons, but a 55,000-dalton and a 27,000-dalton polypeptide were also identified. Synthesis of these proteins was clearly amplified by the CAM treatment described by Neidhardt et al. (27), and sera from mink inoculated with several different ADV strains (Pullman, Utah I, ADV-G, and DK-ADV) all reacted with these proteins (1, 6), whereas normal mink sera did not. Taken together, these data indicated that the recombinant plasmid pBM1 contained a gene with sequences capable of directing synthesis of ADV antigenic determinants in JM103. Similar polypeptides were observed when pBM2 was studied (data not shown). Analysis of the two M13 clones revealed no evidence of ADV-specific protein production (data not shown).

Blot hybridization of pBM1 to DNA from MEV and several strains of ADV. Adaptation of ADV to cell culture has been difficult (5, 17, 20), and as a result, direct comparison of ADV strains has been limited (29). We attempted to show homology between ADV-G and several ADV strains prepared in mink tissues. Partially purified virus preparations were treated according to a modified procedure for virion DNA extraction, and the DNA was analyzed by agarose gel

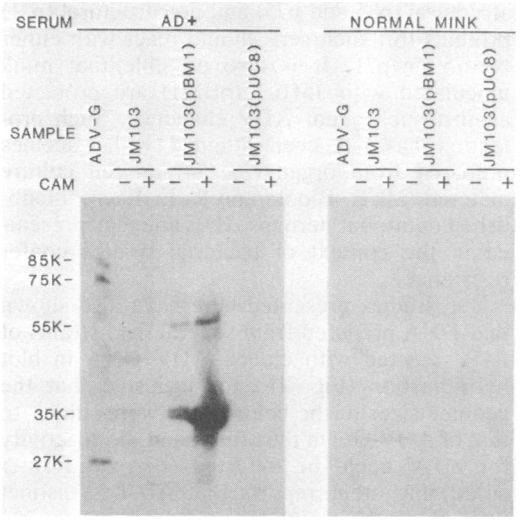

FIG. 3. Immune blotting of ADV proteins expressed by the recombinant plasmid pBM1. Samples of cultures of JM103 or JM103 containing the plasmid pUC8 [JM103 (pUC8)] or the recombinant plasmid pBM1 [JM103 (pBM1)] were electrophoresed on replicate SDS-polyacrylamide gels either before (−) or after (+) treatment with CAM (27) as detailed in the text. After electrophoresis, the proteins were electroblotted onto nitrocellulose paper (2) and reacted with serum either from normal mink or from a pool of mink infected with ADV (AD+). After washing, the filters were developed with ^{125}I-labeled protein A, washed, and autoradiographed. For a positive control, a sample of partially purified ADV-G (5, 6) was included in the first lane. Masses, estimated by comparison with values for ADV-G (5, 6), are indicated in the left-hand margin, with K meaning kilodaltons.

electrophoresis and blot hybridization to a pBM1 probe. As a positive control, a sample of crude ADV-G virus stock was treated in a parallel fashion. The results of these studies (Fig. 4) indicated that homology existed between DNA from the various viruses and the ADV-G-derived plasmid. The reaction of the Pullman ADV DNA was detectable only on prolonged exposure. This may have reflected the lower titer of this virus (12) rather than a difference in homology for the genome segment represented by pBM1, since insufficient DNA was recovered from the Pullman preparation to be visualized by ethidium bromide staining (data not shown). This experiment also suggested that the molecular weights of the single-stranded genomes of the various ADVs were similar.

Finally, we compared the reactivity of nick translated pBM1 DNA and M13bm1 RF DNA with that of uninfected CRFK cell DNA, RF DNA from ADV-G (3), and RF DNA from MEV, another nondefective parvovirus of mink (19, 22, 28) (Fig. 5). The autoradiograms of the Southern blot hybridizations indicated a strong reaction of both ADV clones to ADV-G RF, but no detectable reaction to total cellular DNA or MEV DNA. Both the extended and "fold back" forms of ADV-G RF (3, 21, 22) were resolved in this experiment (Fig. 5A), and the 4.8-kbp RF of ADV-G DNA was clearly different in size from the 5.0-kbp RF of MEV (22).

DISCUSSION

The expression of ADV antigenic determinants by a recombinant plasmid (pBM1) in bacterial cells has been reported in this paper. Antigenic proteins with approximate molecular weights of 55,000, 34,000, and 27,000 were detected with anti-ADV sera (Fig. 3). The ADV fragment in pBM1 is ~1.6 kbp long (Fig. 3A) and could maximally encode a polypeptide with a molecular weight of ~59,000 (20), similar to that observed for the larger protein expressed by pBM1. The smaller proteins could be breakdown or premature termination (41) products of the larger protein. Alternatively, the larger species could be a hybrid protein with both ADV

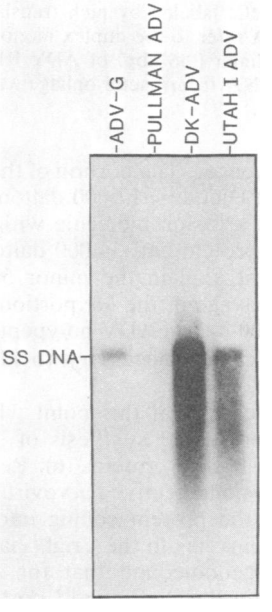

FIG. 4. Comparison of DNA from several strains of ADV. DNA was prepared from partially purified suspensions of the indicated strains of ADV as detailed in the text and electrophoresed into a 1% agarose gel after denaturation in 0.3 M NaOH (3). Blot hybridization was performed with nick translated pBM1 DNA, and the filter was autoradiographed (3). In this figure, all viruses except for ADV-G were prepared from infected mink organs. The ADV-G used was a sample of crude virus stock (5). SS DNA denotes the position of single-stranded virion DNA (3).

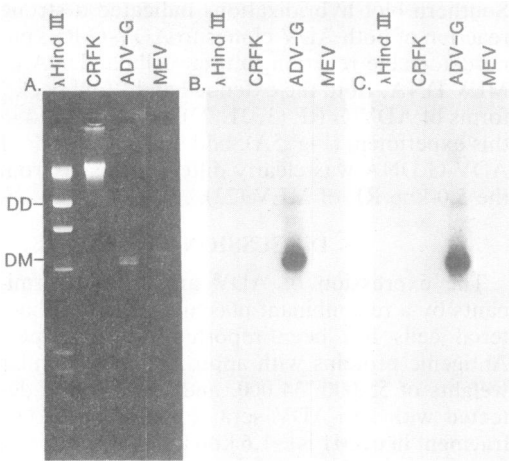

FIG. 5. Blot hybridization of cloned ADV DNA to DNA from MEV, ADV-G, and uninfected CRFK cells. DNA from the following sources was electrophoresed into duplicate 1% agarose gels: ADV-G and MEV refer to RF DNA purified from Hirt supernatants (3). CRFK refers to total cellular DNA from uninfected CRFK cells. The agarose gels were stained with ethidium bromide (A) and autoradiographed after blot hybridization to either M13bm1 RF DNA (B) or pBM1 DNA (C) labeled by nick translation (3). DM and DD DNA refer to the duplex monomer (4.8 kbp) and duplex dimer (9.6 kbp) of ADV RF DNA (3). λ *Hin*dIII = DNA from bacteriophage λ digested with *Hin*dIII (BRL).

and *lac* sequences. The portion of the *lac* gene in pUC8 could encode ~17,000 daltons of protein (16, 23) and a fusion molecule with 17,000 daltons of *lac* protein and 34,000 daltons of ADV protein might explain the minor 59,000-dalton protein. Cleavage of the *lac* portion would then yield a 34,000-dalton ADV polypeptide. Further analysis is clearly necessary to identify these molecules.

It is not certain at this point whether these proteins represented synthesis of structural or nonstructural ADV proteins (6). Recent elegant work on the nondefective parvovirus HI (36, 37) shows that the protein coding information for capsid proteins lies in the "right-hand" portion of the HI genome and that for a noncapsid protein lies in the "left-hand" portion. Assuming that the genetic organization of ADV is similar, expression of ADV capsid protein determinants by the fragment represented in pBM1 would seem a reasonable hypothesis, but to date we have not been able to verify this speculation unequivocally.

We plan to immunize animals with lysates of JM103 (pBM1) and to analyze the sera for antibody to structural and nonstructural viral proteins (6). Based on peptide mapping studies which predict different amino acid sequences for structural (p85 and p75) and nonstructural (p71) proteins (6), such sera should react with either p85/75 or p71. It is also possible that mink inoculated with JM103 (pBM1) are protected against subsequent ADV challenge. Such protection has not been attained with vaccines prepared from organ (29, 34) or cell culture material (M. E. Bloom and R. E. Race, unpublished data), but perhaps ADV antigens presented in the context of bacterial lysates confer resistance.

The studies presented here have also shown that DNA prepared from several field strains of ADV reacted with cloned ADV DNA in blot hybridization (Fig. 4) and suggested that the genome sizes of the field strains were similar to that of ADV-G. On the other hand, no reactivity for MEV could be detected (Fig. 5), further confirming other reports that ADV is distinct from this other mink nondefective parvovirus (29, 32, 33).

Heteroduplex mapping (Fig. 2) and restriction endonuclease mapping of the plasmids pBM1 (ADV-G) and pBM2 (DK ADV) indicated a close relationship between these two strains of ADV, at least for the regions represented by these cloned genome segments. Consequently, it was not surprising that both JM103 (pBM1) and JM103 (pBM2) produced similar ADV antigens.

The availability of nucleic acid probes that specifically react with DNA from field strains of ADV will allow us to study the distribution of viral DNA sequences in mink infected with ADV. The results of such studies may provide further insights into the nature of persistent infection by ADV.

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