JOURNAL OF VIROLOGY, Oct. 1993, p. 5976–5988
0022-538X/93/105976-13$02.00/0

Vol. 67, No. 10

Characterization of Chimeric Full-Length Molecular Clones of Aleutian Mink Disease Parvovirus (ADV): Identification of a Determinant Governing Replication of ADV in Cell Culture

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Received 22 March 1993/Accepted 1 July 1993

The ADV-G strain of Aleutian mink disease parvovirus (ADV) is nonpathogenic for mink but replicates permissively in cell culture, whereas the ADV-Utah 1 strain is highly pathogenic for mink but replicates poorly in cell culture. In order to relate these phenotypic differences to primary genomic features, we constructed a series of chimeric plasmids between a full-length replication-competent molecular clone of ADV-G and subgenomic clones of ADV-Utah 1 representing map units (MU) 15 to 88. After transfection of the plasmids into cell culture and serial passage of cell lysates, we determined that substitution of several segments of the ADV-Utah 1 genome (MU 15 to 54 and 65 to 73) within an infectious ADV-G plasmid did not impair the ability of these constructs to yield infectious virus in vitro. Like ADV-G, the viruses derived from these replication-competent clones caused neither detectable viremia 10 days after inoculation nor any evidence of Aleutian disease in adult mink. On the other hand, other chimeric plasmids were incapable of yielding infectious virus and were therefore replication defective in vitro. The MU 54 to 65 *Eco*RI-*Eco*RV fragment of ADV-Utah 1 was the minimal segment capable of rendering ADV-G replication defective. Substitution of the ADV-G *Eco*RI-*Eco*RV fragment into a replication-defective clone restored replication competence, indicating that this 0.53-kb portion of the genome, wholly located within shared coding sequences for the capsid proteins VP1 and VP2, contained a determinant that governs replication in cell culture. When cultures of cells were studied 5 days after transfection with replication-defective clones, rescue of dimeric replicative form DNA and single-stranded progeny DNA could not be demonstrated. This defect could not be complemented by cotransfection with a replication-competent construction.

Strains of the nondefective Aleutian mink disease parvovirus (ADV) differ dramatically in a variety of ways (2, 19, 40). Pathogenic strains of ADV, such as ADV-Utah 1, cause severe disease in mink that is influenced by the age of the animals at the time of infection (2, 13). In response to ADV-Utah 1 infection, newborn mink uniformly succumb to a fulminant interstitial pneumonitis accompanied by permissive viral replication in alveolar type 2 cells (2, 3, 6), but adult mink infected with the same virus develop a fatal chronic disease characterized by a persistent but restricted infection (2, 5, 13, 19, 60). This adult or classical form of disease is associated with a variety of immunological aberrations, including immune complex nephritis, hypergammaglobulinemia, and plasmacytosis (2, 13, 34, 60). On the other hand, the cell culture-adapted ADV-G strain shows little evidence of replication in vivo and does not cause disease in adult mink even after injection of large amounts of virus (2, 12, 20). In addition, injection of newborn mink kits with ADV-G produces only limited replication, very mild pulmonary lesions, and no clinical disease (2). Whereas ADV-G replicates permissively in susceptible cell cultures (18), attempts to propagate the ADV-Utah 1 strain serially have been generally unsuccessful (20, 41). These findings indicate that differences in the primary sequence of ADV must control both the ability of a particular ADV strain to replicate in vivo and its pathogenicity.

The complete sequence and transcription map of the nonpathogenic ADV-G strain are known (12, 14), and a full-length molecular clone has been developed (12). When this construct, denoted pXV-B-4, is transfected into susceptible CRFK cells, infectious ADV can be rescued, and this rescued virus faithfully recapitulates the parent ADV-G in that it productively infects CRFK cells but is nonpathogenic for adult mink (12). The sequence of ADV-G has been compared with the partial sequence of several other viruses, notably the pathogenic ADV-Utah 1 (14, 37). These molecular clones, corresponding to approximately 76% of the genome, were derived directly from infected mink organs, avoiding any in vitro selection. The comparisons suggest that ADV-G is greater than 97% related at the DNA level to these viruses, and fewer than 75 nucleotide differences were found between these ADV strains.

In order to hypothesize how a particular sequence variation might influence replication and pathogenicity, it is necessary to understand genomic organization. Nondefective parvoviruses such as ADV are small, nonenveloped icosahedral viruses with single-stranded DNA genomes approximately 5,000 nucleotides (nt) in length (30). The genetic organization of these viruses is relatively simple, and that of ADV resembles that of the others (4, 12, 14). There are major left and right open reading frames and several smaller open reading frames in the mid-genome. Two overlapping capsid proteins (VP1 and VP2) are specified by the right open reading frame, and expression of these proteins is controlled by a promoter located at map units (MU) 36 to 38 (P36) (4, 14, 28). A large nonstructural protein (NS1), coded for by the left open reading frame (14, 29), is driven by a

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constitutively active promoter at MU 4 (P4) (4, 14) and serves several functions. NS1 is absolutely required for genomic replication and also *trans* activates the P36 promoter (32, 42, 47, 67, 82). In addition, NS1 is required for the excision and replication of parvoviral genomes contained in molecular clones (68). Smaller nonstructural proteins, such as NS2, arise by splicing of the amino-terminal portion of NS1 with smaller open reading frames in the middle of the genome (4, 14, 29). The role of NS2 in replication is less clear, but it appears to regulate replication and virus production in a cell type-specific fashion (24, 48, 55).

In recent years, detailed studies on other parvoviruses have also found that apparently minor sequence differences can be correlated with pronounced biological differences. For example, although canine parvovirus (CPV) and feline panleukopenia virus (FPV) are 98% related by DNA sequence and highly cross-reactive antigenically (25, 56–58, 76), they exhibit distinct patterns of host cell tropisms in vitro (76) and, in addition, differ in the ability to replicate and cause disease in dogs and cats, respectively (76). Allotropic strains of minute virus of mice (MVM) provide another example of closely related parvoviruses having different properties. The prototypic MVM(p) and immunosuppressive MVM(i) are serologically indistinguishable and exhibit 97% DNA sequence identity, yet each strain is restricted for viral growth in the reciprocal host cell at some stage in viral replication subsequent to initial entry (9, 70, 73). These examples clearly demonstrate that parvoviruses as closely related as ADV-G and ADV-Utah 1 can exhibit markedly different biological characteristics.

Experiments to assign apparently minor differences in genome structure to observed variability in viral replication and host cell tropism have been performed with the MVM and CPV/FPV models. By the construction of viruses chimeric for selected segments of the genome, it is apparent that differences in host range, cell tropism, and pathogenicity can be accounted for by very limited amino acid changes (9–11, 25, 35, 56, 76). Surprisingly, perhaps, for MVM and CPV, the segment of the viral genome responsible for this phenomenon seems to residue in the portion of the genome coding for the virion capsid proteins (11, 25, 56). Similar studies with porcine parvovirus have suggested that determinants in the nonstructural coding regions may also play a role in some instances (79).

In the experiments reported in this article, we replaced segments of an infectious ADV-G molecular clone with the corresponding portions of clones from pathogenic strains. These chimeric constructions were assayed after transfection into cell cultures for the capacity to yield infectious virus. Virus derived from such replication-competent clones was found to be nonpathogenic for adult mink. Replacement of a 531-nt segment in the capsid protein gene of ADV-G was sufficient to render full-length molecular clones of ADV incapable of generating infectious virus subsequent to transfection. When cultures transfected with these replication-defective plasmids were studied, it was apparent that the levels of DNA replicative form DNA, progeny strand DNA, and, in some cases, capsid protein were greatly reduced.

MATERIALS AND METHODS

Cells, sera, and viruses. ADV-G and viruses derived from infectious molecular clones of ADV were propagated and assayed in Crandell feline kidney cells (CRFK) as reported before (12, 20, 31, 62). The preparation and assay of ADV-Utah 1 have been described before (19, 26).

The anti-ADV murine monoclonal antibodies 30, 68, P-2-1, and 163 have been described and characterized previously (64). The anti-ADV murine monoclonal antibody Y-2-9 was prepared by standard methods (64). All react against both ADV capsid proteins VP1 and VP2. Murine monoclonal antibody 355, reactive against rabies virus glycoprotein, was kindly provided by Don Lodmell.

Molecular cloning techniques. Standard molecular biology techniques were used for cloning, DNA sequencing, restriction enzyme digestion, ligation, linker insertion, agarose gel electrophoresis, and plasmid batch preparation (12, 14, 51, 69).

Full-length molecular clones were successfully transformed into and amplified in *Escherichia coli* JC8111 (*recBC sbcB recF*) (12, 23). Transformation efficiencies and yields of full-length clones were low, typically in the range of 25 to 200 μg of plasmid per liter of culture, even when chloramphenicol amplification was used. Other plasmids were propagated as described previously in *E. coli* JM109 (12, 14).

To facilitate manipulations, the MU 100 *Eco*RI site of pXV-B-4 was deleted and replaced with a unique *Bgl*II site. This construction, pXXI-Q-3-15, behaved exactly the same as pXV-B-4 in all respects.

For the preparation of chimeric virus constructions, the described and sequenced molecular clones representing the 3,455-nt *Bam*HI-*Hin*dIII (MU 15 to 88) segment of ADV-Utah 1 (pUT1-IX-G) (14, 15) or the ADV-Utah 1-like clone derived from the Danish ADV-K strain (pK1) (12, 37) were used. These two clones are identical except for a single-nucleotide deletion in pUT1-IX-G of an A at nt 806, interrupting the left open reading frame. A previously reported C-A difference between these two isolates at nt 3100 was a sequencing error (37). Although one of the chimeric constructions herein described (pXX-B-1) used a segment from pK1, for simplicity we shall refer to all the constructions as having been made between ADV-G and ADV-Utah 1.

Plasmid pXX-B-1 was constructed by replacing the MU 15 to 88 *Bam*HI-*Hin*dIII segment of pXV-B-4 with the corresponding segment from ADV-Utah 1. Plasmids pXVII-T-1-2 and pXVII-T-2-2 contained the *Xho*I-*Eco*RV and the *Eco*RV-*Bst*EII fragments, respectively. Clones pXXI-Q-2-2, pXXIII-S-2, and pXXII-K1 were prepared by exchanging the MU 30 to 69 (*Afl*II), MU 15 to 54 (*Bam*HI-*Eco*RI), and MU 54 to 65 (*Eco*RI-*Eco*RV) fragments, respectively, of the pathogenic strain for those of ADV-G in plasmid pXXI-Q-3-15. The structure of these constructions was verified by restriction enzyme analysis and by DNA sequencing. The maintenance of the unstable right-hand (MU 100) hairpin was confirmed by restriction enzyme analysis.

Plasmids destined for transfection were stored in aliquots as ethanol precipitates. Prior to use, the DNA was pelleted and resuspended in sterile deionized water, and the concentration was verified by agarose gel electrophoresis. Because the circular plasmids migrated with a mobility similar to that of duplex monomeric ADV replicative form DNA, in some experiments DNAs were digested with the enzyme *Bgl*I (which does not cut ADV) prior to use in transfection. This had no effect on the ability of these molecules to transfect cultures (data not shown).

Transfections. DNA transfections (with 5 μg of plasmid DNA, unless noted) were done as described previously in 35-mm 6-well plates for analysis of nucleic acids or proteins (12, 38). The transfected cultures were harvested after 5 days of incubation at 31.8°C.

When DNAs were to be tested for infectivity, transfections were performed in individual 35-mm dishes. At the

time of harvest, cell pellets were collected in 1 ml of medium, freeze-thawed, sonicated, and serially passaged in 25-cm^2 flasks before assay.

When DNAs were to be transfected in vivo (33, 71, 80) into adult sapphire mink, both an intranasal and an intraperitoneal route were used on sedated animals. For intranasal treatment, 5 μl of precipitate containing 10 μg of plasmid DNA, 6.5 μg of DNA prepared from an ADV-Utah 1-infected mink kit lung, or 2.7 μg of normal lung DNA was instilled into each nostril (i.e., 10 μl per mink). For intraperitoneal treatment, 10 μl of the same precipitates was diluted to 100 μl with sterile phosphate-buffered saline and injected.

To verify the number of fluorescence-forming units (FFU) per microgram of DNA for each experiment, transfections of 50-μl samples were performed in four-well Labtek chambers or the wells of 24-well culture dishes containing coverslips. At the time of harvest, these specimens were acetone fixed and stained for ADV antigens with a direct fluorescein isothiocyanate conjugate prepared from infected mink sera (20). The number of cells displaying ADV antigens per chamber was counted and used to estimate the FFU per microgram for the various DNA samples.

SDS-PAGE and immunoblot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting for ADV proteins were performed as detailed in previous publications (1, 15, 21, 53), but the signal was developed with a chemiluminescent substrate (ECL; Amersham, Chicago, Ill.). This method amplified the sensitivity by at least a factor of 10 over standard chromogenic substrates.

Nucleic acid isolation and Southern blot analysis. For preparation of whole-cell DNA (72), cell pellets or tissue samples were lysed in 0.01 M Tris (pH 8.0) containing 1% Sarkosyl and 100 μg of RNase (boiled to inactivate extraneous nucleases) per ml and incubated for 3 h at 37°C. Following addition of proteinase K to 200 μg/ml, cellular DNA in the samples was sheared through a 27-gauge needle, and the incubation was allowed to proceed for another 3 h at 37°C. DNA was ethanol precipitated in the presence of oyster glycogen (74) after phenol-chloroform extraction. The DNA was collected by centrifugation and resuspended in 30 μl of 0.01 M Tris (pH 8.0)–0.001 M EDTA (TE buffer).

For preparation of DNase-resistant, single-stranded (SS) virion DNA (17, 20), the cell pellets were freeze-thawed three times and sonicated before a 3-h incubation at 37°C in 0.01 M $MgCl_2$–20 μg of RNase per ml–200 μg of DNase per ml. Following inactivation of the DNase with 0.02 M EDTA and the addition of 1 μg of salmon sperm DNA as the carrier, the samples were incubated at 37°C with 1% Sarkosyl and 500 μg of proteinase K per ml for 3 h. DNA was recovered after phenol-chloroform extraction and ethanol precipitation in the presence of oyster glycogen by dissolution in 30 μl of TE.

Whole-cell DNA was isolated from a sapphire mink kit lung 12 days after injection with ADV-Utah 1 or from a normal mink lung. The ADV genome content was determined by Southern blot hybridization with a known standard DNA preparation and found to be approximately 5×10^8 genomes per μg (18, 22). This DNA contained both replicative form and SS DNA (data not shown). The normal lung DNA did not contain detectable ADV DNA sequences.

Samples containing equivalent amounts of DNA were electrophoresed in 0.7% agarose gels, alkaline blotted onto Hybond-N membranes, and hybridized as before with ^{32}P-labeled positive-sense ADV probes (22).

Hybridization with ADV type-specific oligonucleotides end labeled with [γ-^{32}P]ATP was performed as reported in previous publications (4, 16, 51). The type-specific oligonucleotides were based on the sequences reported (14) for the positive-sense strand of the ADV-G and ADV-Utah 1 "hypervariable regions." The ADV-G oligonucleotide sequence, spanning nt 3093 to 3131, was 5′-GAAAAAGTTGCAACA GAAACACTAACCTGGGATGCAGTA-3′. The corresponding ADV-Utah 1 oligonucleotide sequence was 5′-CAAAAG ATGGGGCAGGAGAGCAATTAGAATGGACTGGTA CA-3′.

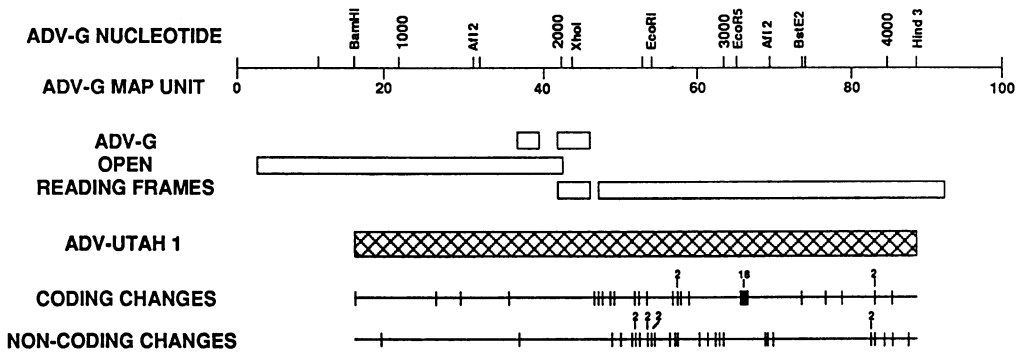
In vivo infectivity and pathogenicity. For determination of the in vivo infectivity and pathogenicity of chimeric viruses, adult female sapphire mink, housed and maintained under standard conditions, were injected intraperitoneally with 10^5 FFU of the indicated virus or with the indicated cell lysate (18, 26). Serum samples were collected 10, 30, and 60 days after infection and assayed for anti-ADV antibodies by counterimmunoelectrophoresis (18). At the time of necropsy, animals were inspected for the typical lesions of Aleutian disease (18, 34).

Viremia at 10 days after infection was used as a surrogate for in vivo viral replication in some experiments (18, 22, 34, 63). Virus was concentrated from 200 μl of serum by ultracentrifugation for 30 min at 80,000 rpm in a Beckman Airfuge. The pellet was suspended by sonication in 10 μl of sterile TE buffer, denatured for 30 min by the addition of 190 μl of 1.5 M NaCl–0.5 M NaOH, and applied as 100-μl duplicates to Hybond-N membranes under vacuum with a hybridization manifold with a 96-well dot-blot format. As standards, serial twofold dilutions from 20 to 0.15 pg of a nearly full-length segment of recombinant ADV DNA were prepared in TE containing salmon sperm DNA, denatured, and applied to manifold wells in parallel with the unknown samples. The wells were allowed to fill and empty twice with 1.5 M NaCl–0.5 M NaOH. Filters were removed from the manifold, rinsed in 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), baked at 80°C under vacuum, and reacted with a ^{32}P-labeled positive-sense ADV RNA probe as described before (22). The lower level of detection was 0.15 pg of ADV DNA, which corresponds to 7×10^5 genomes per ml (18, 22).

RESULTS

Construction of full-length chimeric clones. The development of an infectious molecular clone of the nonpathogenic ADV-G strain of ADV, designated pXV-B-4, has already been described (12). In order to prepare chimeric full-length clones, this clone was modified to prepare pXXI-Q-3-15, which had the same characteristics as the parent clone. Segments from clones of the pathogenic ADV-Utah 1 strain (14) were exchanged for the corresponding segment in an infectious ADV-G clone. A series of full-length chimeric constructions were prepared and are depicted schematically in Fig. 1.

Infectivity of chimeric full-length clones for CRFK cells. The infectivity of the constructions was assessed by transfecting CRFK cells with purified plasmid DNA, serially passaging lysates of the infected cells a total of four times, and titering the final material in CRFK cells to see whether infectious ADV had been rescued (Fig. 1). Of the eight clones tested and compared with our initial infectious clone (pXV-B-4), four were able to initiate a productive infection. Lysates from cells transfected with clones pXXI-Q-3-15, pXXIII-S-2, and pXVII-T-2-2 all contained chimeric virus



CONSTRUCTION	INFECTIVITY FOR CRFK CELLS: PRIMARY TRANSFECTION (FFU/μg)	INFECTIVITY FOR CRFK CELLS: FOURTH PASSAGE (FFU/ml)
XXI-Q-3-15	624	12×10^7
XX-B-1	442	< 45
XXI-Q-2-2	571	< 45
XVII-T-1-2	1014	< 45
XXIII-S-2	655	5.2×10^7
XVII-T-2-2	871	0.95×10^7
XXII-K-1	784	< 45
XXIII-Q-5	655	8×10^7

FIG. 1. Schematic of chimeric ADV plasmids. The chimeric ADV plasmids used in this study are aligned under a map of the ADV genome identifying the open reading frames and the restriction enzyme recognition sites used in preparing the chimeric plasmids. The extent of the ADV-Utah 1 sequence used, as well as coding and noncoding sequence differences, are also shown. The infectivity for CRFK cells of each of the clones assayed either 5 days after primary transfection (expressed as FFU per microgram of plasmid) or after four serial passages of cell lysates (expressed as FFU per milliliter of cell lysate) is also indicated in tabular form adjacent to the relevant construction. The initial replication-competent ADV-G clone pXV-B-4 had the same structure as pXXI-Q-3-15 and is not shown.

infectious for CRFK cells and were therefore defined as replication competent. Furthermore, there was no significant difference in the virus titer attained by any of these viruses compared with that by the virus derived from pXV-B-4-transfected cells. Thus, pXV-B-4 was omitted from subsequent studies.

No virus was rescued from lysates of cells transfected with the remaining five clones, which were therefore replication defective. On examination of the structure of these five clones, the minimal common feature appeared to be the presence of the ADV-G MU 54 to 65, *Eco*RI-*Eco*RV segment. In other words, any construct that contained an *Eco*RI-*Eco*RV fragment from ADV-Utah 1 was replication defective. Interestingly, the *Eco*RV-*Bst*EII segment, which contains the hypervariable region, had no such effect. Thus, some determinant located within the MU 54 to 65 segment governed the ability of a full-length clone of ADV to produce infectious virus subsequent to transfection of CRFK cells.

In order to confirm that the MU 54 to 65 segment was controlling the ability of these clones to initiate productive replication, the ADV-G MU 54 to 65 piece was put into the replication-defective construction pXXI-Q-2-2 (Fig. 1). The resulting construction, pXXIII-Q-5, was replication competent.

All of the clones produced ADV antigens in CRFK cells after primary transfection (Fig. 1). There was no obvious correlation between the level of FFU per microgram after primary transfection and the ability of the constructions to generate infectious virus.

Comparison of replication-competent viruses derived from chimeric full-length molecular clones in CRFK cells. The findings described in the previous section indicated that virus infectious for CRFK cells could be derived from several of the chimeric full-length clones. In order to compare several parameters of viral replication in CRFK cells, cultures were infected at equivalent multiplicities of infection with virus stocks derived from these replication-competent clones and subjected to analysis.

Western immunoblot analysis (Fig. 2A) showed that both capsid proteins VP1 and VP2 as well as the major nonstructural protein NS1 (21, 54) were synthesized by all of these replication-competent chimeric viruses. The molecular masses of NS1 were the same for all the viruses, but the capsid proteins of XVII-T-2 appeared to be 1 to 2 kDa larger than those of the other preparations. The only unique sequence of XVII-T-2 compared with the other replication-competent viruses resides within the *Afl*II-*Bst*EII segment. A similar difference between the capsid proteins of ADV-

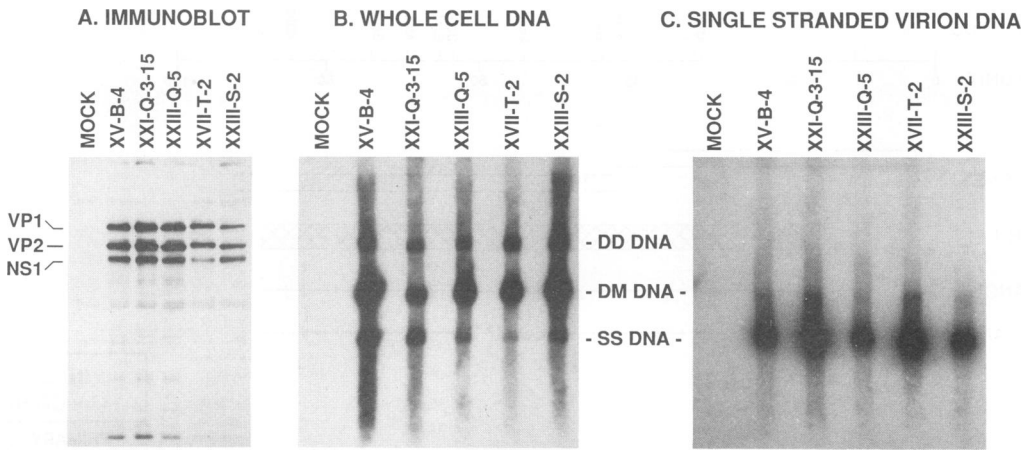


FIG. 2. Analysis of CRFK cells infected with replication-competent chimeric ADV viruses. Cultures of CRFK cells were infected with the indicated replication-competent chimeric virus (fourth passage) at an approximate multiplicity of infection of 1 FFU/cell. After 3 days of incubation at 31.8°C, aliquots of the culture were analyzed. (A) Cell pellets were lysed in Laemmli sample buffer, resolved on SDS–7.5% polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with high-titered mink anti-ADV serum. Specific ADV signals were developed by a chemiluminescent method with peroxidase-conjugated protein A as the reporter molecule. The locations of the capsid proteins VP1 and VP2 and the major nonstructural protein NS1 are indicated. (B and C) Equivalent amounts of whole-cell (B) and SS virion (C) DNA were isolated as detailed in Materials and Methods and electrophoresed in 0.7% agarose gels. Following denaturing, the samples were transferred to Hybond-N membranes by alkaline transfer and hybridized to positive-sense ADV RNA probes labeled with ^{32}P. The locations of duplex monomeric (DM) and duplex dimeric (DD) replicative form DNA as well as SS virion DNA are indicated.

Utah 1 and ADV-G has been noted previously (8, 64), and this finding suggests that some sequence within that region may account for the difference.

Southern blot analysis (Fig. 2B) showed that cells infected by each of the viruses contained both duplex monomeric and duplex dimeric replicative form DNA (17, 18, 22). In addition, SS virion DNA, defined as DNase-resistant DNA, was also present in these cells (Fig. 2C) (20).

Pathogenicity of chimeric viruses for adult mink. The results detailed in the previous sections indicated that we could exchange certain genomic segments between pathogenic and nonpathogenic types of ADV without noticeably affecting replication in CRFK cells. We wished to assess whether the chimeric viruses were nonpathogenic, like ADV-G, or pathogenic for adult mink, like ADV-Utah 1. Virus stocks were prepared from the replication-competent chimeras XXI-Q-3-15, XXIII-S-2, XVII-T-2-2, and XXIII-Q-5 and inoculated into adult sapphire mink. None of these mink exhibited signs of Aleutian disease, nor did they develop detectable anti-ADV antibody (Table 1).

We also looked for direct evidence of in vivo viral replication by assessing viremia 10 days after infection (18, 22). None of the animals injected with either the ADV-G construct XXI-Q-3-15 or any of the chimeric viruses had detectable viremia, whereas mink injected with pathogenic ADV-Utah 1 did (Table 1).

The failure of these animals to mount an anti-ADV antibody response or to develop viremia at 10 days strongly suggested that in vivo viral replication is extremely limited or does not occur. This result was identical to that noted previously for pXV-B-4 (12). Because one of these chimeric viruses, XVII-T-2, was chimeric for the hypervariable region (Fig. 1), this result also suggested that the mere presence of the ADV-Utah 1 hypervariable region in an ADV-G virus was not sufficient to render the virus pathogenic.

Comparison of replication-defective full-length molecular clones in CRFK cells. A number of the full-length molecular clones described above and depicted in Fig. 1 were replication defective, as they were unable to generate infectious virus in CRFK cells. To try to elucidate the block to replication, we prepared cultures of cells transfected with several of these plasmids and compared selected parameters of replication with those in cells transfected with the replication-competent XXI-Q-3-15.

On close examination, the pattern of immunofluorescence of the replication-defective clones differed slightly from that of the replication-competent clone (data not shown). Cells transfected with replication-competent pXXI-Q-3-15 exhibited a pattern typical of ADV-G and the replication-competent clones; that is, both nuclear and cytoplasmic antigen signals, with some of the cytoplasmic signal being of a finely granular nature (54). On the other hand, the replication-defective clones induced primarily a nuclear signal. Only pXX-B-1 DNA yielded a readily observable cytoplasmic signal. Although similar numbers of cells contained ADV

TABLE 1. In vivo analysis of viruses derived from replication-competent ADV chimeric molecular clones[a]

Virus inoculum	No. of animals positive/no. tested		
	Anti-ADV antibodies	Viremia	Aleutian disease
XV-B-4	0/2	0/2	0/2
XXI-Q-3-15	0/2	0/2	0/2
XXIII-S-2	0/2	0/1	0/2
XVII-T-2-2	0/2	0/2	0/2
XXIII-Q-5	0/2	0/2	0/2
ADV-Utah 1	2/2	2/2	2/2
None	0/2	0/2	0/2

[a] Adult sapphire mink were inoculated intraperitoneally with 10^5 FFU of the indicated virus preparation or with 10^5 50% mink infectious doses of ADV-Utah 1. The development of anti-ADV antibodies within 60 days after infection was assessed by counterimmunoelectrophoresis. The presence of viremia was determined 10 days after infection by dot-blot hybridization as described in Materials and Methods. The presence of typical lesions of Aleutian disease was sought at necropsy 60 to 90 days after infection.

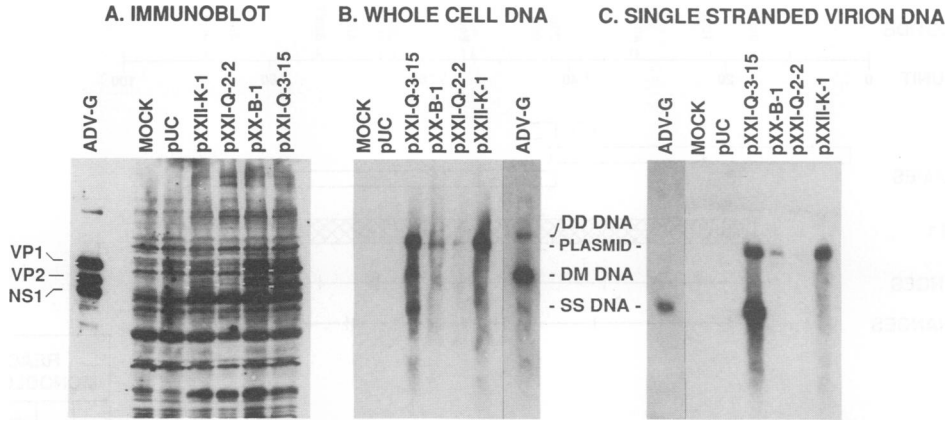


FIG. 3. Analysis of CRFK cells transfected with replication-competent and replication-defective chimeric ADV plasmids. Cultures of CRFK cells in 35-mm culture wells were transfected with 5 μg of the indicated replication-competent and replication-defective plasmids (previously digested with *BglI* to linearize the supercoiled plasmids), glycerol boosted after 4 h, and incubated at 31.8°C for 5 days. Replicate samples from each transfection were processed as described in Materials and Methods and the legend to Fig. 2 for (A) immunoblot, (B) whole-cell DNA, and (C) SS virion DNA. The location of the 7.6-kb linearized residual input plasmid (B and C) and the locations of VP1 and VP2 (A) are indicated.

antigen after transfection with pXXI-Q-2-2 and pXXII-K-1, the signal was weaker and localized primarily to the nucleus.

Western blot analysis (Fig. 3A) of the transfected cells revealed that the levels of NS1 directed by the replication-defective clones were similar to that of the replication-competent clone, but that the relative levels of capsid proteins VP1 and VP2 were reduced (21, 54), although VP1 and VP2 could be detected in lysates of pXX-B-1-transfected cells and the molecular masses were greater than for the corresponding proteins from the ADV-G clone (8, 64). The capsid proteins from pXXI-Q-2-2 and pXXII-K-1 were present at a very reduced level compared with those from pXXI-Q-3-15 and pXX-B-1. Because the capsid proteins were noted in cells transfected with pXX-B-1, these results suggested that the replication defect could not be linked solely to impaired capsid protein synthesis.

Southern blot analysis of DNA isolated from cells transfected 5 days earlier with replication-competent or -defective plasmids also revealed differences (Fig. 3B and C). Cells transfected with the replication-competent pXXI-Q-3-15 contained DNA species that comigrated with replicative form duplex monomeric DNA and SS virion DNA (Fig. 3B) (17, 18, 22); thus, there was evidence that the ADV genome had been rescued and amplified from the plasmid. Furthermore, although 9.6-kb duplex dimeric replicative form DNA could not be identified, a band corresponding to residual linearized plasmid (7.6 kb) was evident. In contrast, only the residual plasmid was present in cells transfected with the replication-defective constructs. Neither duplex monomeric nor SS DNA could be clearly identified in whole-cell DNA samples prepared from the several replication-defective chimeric clones, and a smear of signal from DNA representing residual input plasmid made it impossible to judge whether a small amount of duplex monomeric DNA was present.

Analysis of DNase-resistant SS virion DNA (Fig. 3C) (20) indicated that virion DNA was readily apparent in pXXI-Q-3-15-transfected cells but not in parallel samples from the replication-defective clones. Interestingly, even though these samples had been digested with DNase, some of the input plasmid was still present. These findings suggested that the rescue and amplification of ADV replicative form DNA as well as the generation of encapsidated virion DNA were severely impaired in cells transfected with the replication-defective clones.

Taken together, these results suggested that there was a severe block to rescue and amplification of ADV sequences from these replication-defective clones and that levels of capsid proteins were also reduced. The facts that ADV antigen was induced and that NS1 was synthesized implied that these plasmids were capable of entering transfected cells and arriving at a cellular site where they could serve as initial transcriptional templates.

Inoculation of adult sapphire mink with viral and plasmid DNA. The results described in the previous sections revealed that full-length chimeric molecular clones containing the MU 54 to 65 segment of ADV-Utah 1 were replication defective in CRFK cells. Thus, we were unable to propagate virus from these clones in vitro and test the pathogenicity of these constructions for mink. Work with a variety of other virus systems reveals that either viral DNA or full-length molecular plasmid clones can be introduced directly into animals by several methods and infections can be initiated thereby (33, 71, 80). We also showed previously that purified replicative forms of ADV-G were infectious in cell culture (12). In order to determine whether pathogenic virus might be rescued from replication-defective ADV clones, we prepared DNA precipitates and inoculated them directly into adult sapphire mink. As a control, we also injected a precipitate from whole-cell DNA from an ADV-Utah 1-infected mink kit lung. This DNA sample contained 5×10^8 ADV genomes per μg of whole-cell DNA and consisted of both virion and replicative form DNA (18, 22). None of the animals injected with any of the DNA preparations developed any signs of Aleutian disease or any anti-ADV antibody during the 90-day period of observation (data not shown). As a result, we concluded that these DNAs were not infectious for adult sapphire mink under the conditions used. The fact that the DNA from the infected mink lung was also apparently noninfectious makes it impossible to draw any inferences about the plasmids tested.

Reactivity of chimeric constructions with anti-ADV monoclonal antibodies. Previous work with murine monoclonal antibodies against ADV capsid proteins indicates that there are distinct antigenic differences between strains of ADV,

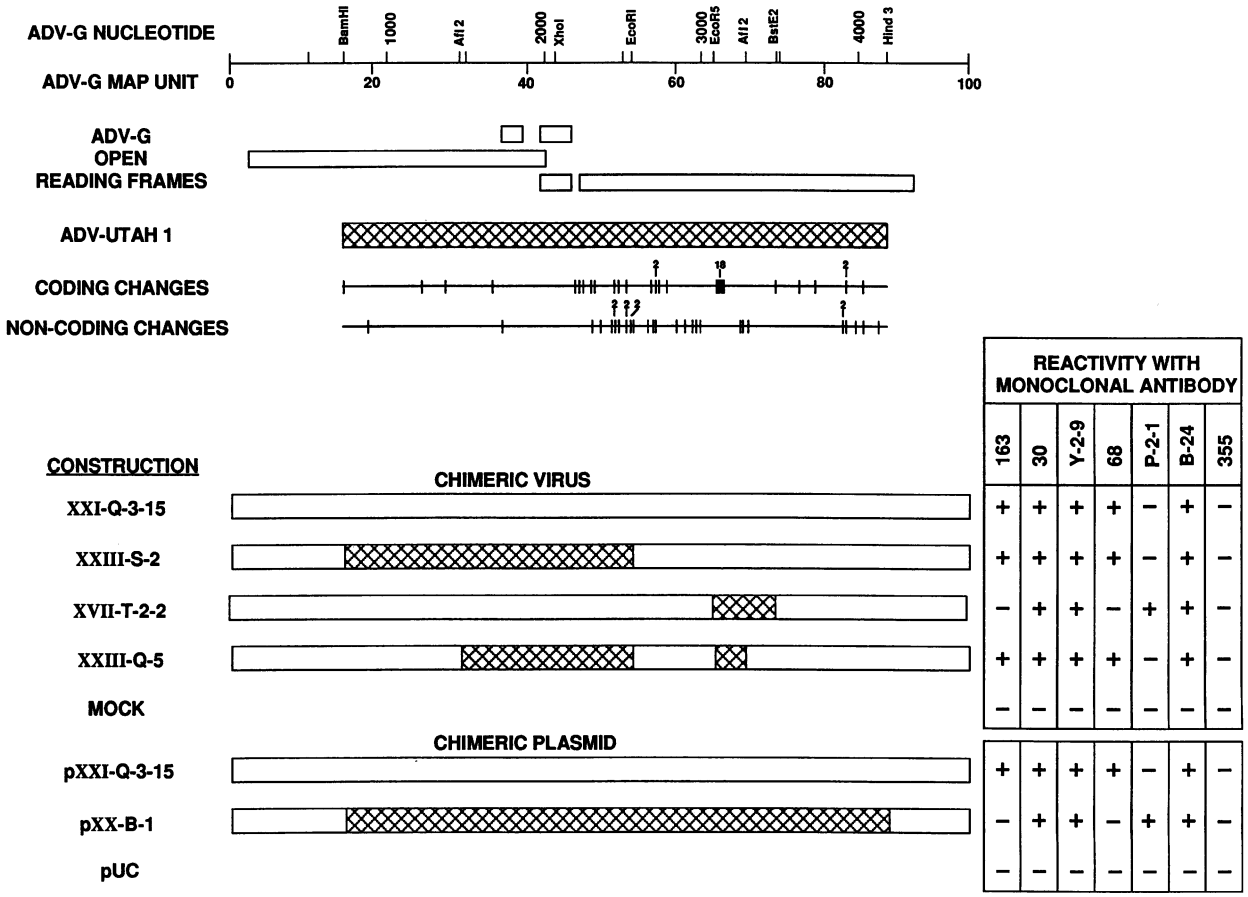


FIG. 4. Reactivities of chimeric ADV viruses and plasmids with anti-capsid protein monoclonal antibodies. Cultures of CRFK cells in eight-chamber Labtek slides were either infected with the indicated chimeric ADV virus for 3 days or transfected with the indicated ADV chimeric plasmid for 5 days. The cultures were fixed with acetone, reacted with the various monoclonal antibody supernatants, and developed with fluorescein-conjugated protein A. For convenience, the structures of the several viruses and plasmids are represented and related to the ADV-G and ADV-Utah 1 genomes.

although the location of the specific epitopes defined by these reagents is unclear (64). Because several of the chimeric viruses and plasmids described in previous sections and depicted in Fig. 1 were chimeric for segments of the capsid coding region, we tested a panel of these monoclonal antibodies by immunofluorescence to see whether we could map their reactivities to specific regions (Fig. 4). Several of the monoclonal antibodies (30, Y-2-9, and B-24) reacted with all the chimeric viruses; however, three (68, 163, and P-2-1) exhibited specific patterns of staining. Antibody 68 was positive only against viruses containing an *Eco*RV-*Afl*II (MU 65 to 69) segment derived from ADV-G; this piece of the genome contains the hypervariable region of the right open reading frame. Antibody 163 reacted only with viruses containing the ADV-G *Afl*II-*Bst*EII (MU 69 to 73) segment, and antibody P-2-1 reacted specifically with viruses having the ADV-Utah 1 *Afl*II-*Bst*EII piece. The control antibody 355, reactive against the rabies virus glycoprotein, was uniformly negative.

The same panel of monoclonal antibodies were tested against CRFK cells transfected 5 days earlier with pUC, pXXI-Q-3-15, and pXX-B-1 (Fig. 4). The staining pattern for cells transfected with the ADV-G plasmid pXXI-Q-3-15 was identical to that of the derivative virus; all the monoclonal antibodies except MAb P-2-1 and the control MAb 355 reacted. Furthermore, the ADV-G-reactive antibodies 163 and 68 did not stain positive cells in pXX-B-1-transfected cells, whereas P-2-1 did.

Attempted rescue of replication-defective plasmid by replication-competent plasmid. In a final set of experiments, we wished to determine whether virus from a replication-defective construction (pXX-B-1) could be rescued by cotransfection with a replication-competent one (pXXI-Q-3-15). CRFK cells were transfected with a mixture of the two plasmids, and lysates of the transfected cells were serially passaged four times. CRFK cells were then infected with a virus stock prepared from this material and analyzed for the presence of ADV-Utah 1 DNA sequences by performing Southern blots with oligonucleotides specific for either ADV-G or ADV-Utah 1 (Fig. 5). As a control for the presence of the ADV-Utah 1 sequences, a parallel sample was prepared from cells infected with the replication-competent XVII-T-2-2 virus, which contains the ADV-Utah 1 target sequence (Fig. 1). No ADV-Utah 1 signal was detected in DNA from cells infected with virus derived from the cotransfection but was readily apparent in DNA from XVII-T-2-2-infected cells. ADV-G sequences were present in cells infected with either XXI-Q-3-15 virus or virus derived from the cotransfection.

We also looked for the presence of ADV-Utah 1 capsid

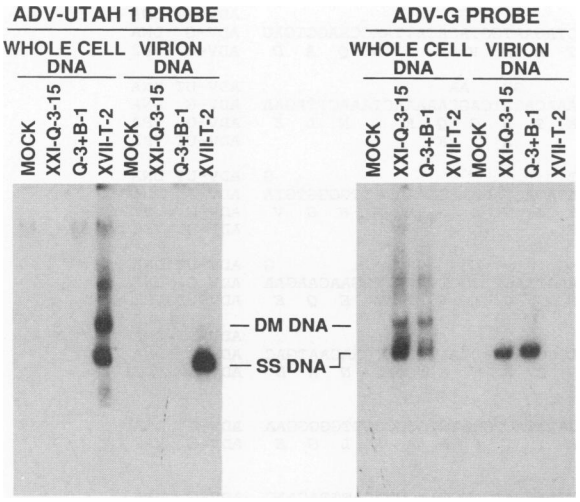


FIG. 5. Hybridization of DNA from ADV-infected cells with ADV-G- and ADV-Utah 1-specific oligonucleotides. Individual 35-mm dishes of CRFK cells were transfected with 5 μg (total) of the replication-competent plasmid pXXI-Q-3-15 DNA, a mixture of pXXI-Q-3-15 and replication-defective pXX-B-1 DNA, or replication-competent pXVII-T-2 DNA as described in previous figure legends. Cell pellets were collected, lysed, and serially passaged a total of four times. Following titration of the final passage material, cultures were either mock infected or infected at a multiplicity of 1 for 3 days, at which time whole-cell and SS virion DNA was prepared. Equivalent amounts of DNA were resolved on 0.7% neutral agarose gels and alkaline blotted onto Hybond-N membranes. Duplicate membranes were hybridized with oligonucleotide probes specific for either the ADV-G or the ADV-Utah 1 hypervariable region end labeled with [γ-^{32}P]ATP.

antigens in the same preparations with the type-specific monoclonal antibodies detailed in a previous section. There was no evidence of ADV-Utah 1 antigen in cells infected with virus derived from the cotransfected cells (data not shown).

These experiments suggested that virus from a replication-defective construction could not be rescued by the presence of a replication-competent virus.

DISCUSSION

In this study, we characterized full-length molecular chimeric clones of ADV. Specifically, we replaced segments of a full-length infectious molecular clone of the nonpathogenic ADV-G (12) with the corresponding pieces from a pathogenic strain of ADV (14) and examined the ability of these constructions to initiate a productive infection in cell cultures.

Several of the chimeric constructions containing ADV-Utah 1 sequences were replication competent in CRFK cells (Fig. 1). Included in this group were clones that contained from ADV-Utah 1 all but the 172 amino-terminal residues of NS1 (XXIII-S-2), the VP1-specific region of the capsid proteins (XXIII-S-2 and XXIII-Q-5), and the unique portions of NS2 and the putative NS3 (XXIII-S-2 and XXIII-Q-5). None of these replication-competent constructions were pathogenic for adult mink, nor did they cause detectable viremia at 10 days after infection (Table 1). Thus, it appeared that substantial portions of the ADV-Utah 1 genome did not confer pathogenic potential or the ability to replicate to high titer in vivo in adult mink. Analysis of infection by these viruses in highly susceptible mink kits (3, 26) is in progress.

A number of other clones could not initiate in vitro infection and were replication defective in CRFK cells. This replication defect made it impossible to inject mink with virus stocks prepared from these constructions. Attempts to bypass in vitro replication in CRFK cells and deliver plasmid DNA directly in vivo were unsuccessful (data not shown). Furthermore, this block to replication in vitro could not be overcome by cotransfection with a replication-competent clone, suggesting that the defect could not be complemented in *trans* in vitro (Fig. 5).

The experiments indicated that a determinant governing the ability of ADV to replicate in CRFK cells mapped to a 531-bp *Eco*RI-*Eco*RV fragment spanning nt 2553 to 3084 (Fig. 1) (12, 14, 15). No construction containing this MU 54 to 65 piece from ADV-Utah 1 was replication competent in CRFK cells. Comparison of ADV-G and ADV-Utah 1 (Fig. 6) reveals only 14 nucleotide differences in this segment, and thus some change resulting from one or more of these differences likely controls replication in CRFK cells.

Inspection of the ADV sequence (12, 14) and transcription map (4) indicates that the mid-genomic AATAAA polyadenylation motif (nt 2564) and poly(A) addition site (nt 2589) are present at the 5′ end of this fragment (Fig. 6). This polyadenylation site is utilized for production of R2 and RX, which code for NS2 and a putative NS3, respectively (4). NS2 has been shown to regulate MVM permissivity in a cell type-specific fashion (24, 48, 55). Furthermore, the mid-genomic polyadenylation site has been implicated in limiting replication of B19 in nonpermissive cells (50). However, neither the polyadenylation motif (AATAAA) nor the poly(A) addition site differ between ADV-G and ADV-Utah 1, although there are several noncoding nucleotide changes apparent 3′ to the poly(A) site. Poorly defined sequences 3′ to the actual poly(A) addition site have also been implicated in regulating polyadenylation in some systems (46, 52, 81), and detailed comparison of transcription and gene expression of replication-competent and replication-defective molecular clones may allow us to determine whether such noncoding changes are involved in the phenomena that we have reported here.

The MU 54 to 65 portion of the genome is wholly contained within the shared coding sequence for the capsid proteins VP1 and VP2 (4, 12). Furthermore, there are four amino acid differences between ADV-G and ADV-Utah 1 in this region (Fig. 6), and it is possible that one or more of these coding changes are involved in regulating permissivity.

A role for the capsid proteins in governing the ability of ADV to replicate in CRFK cells might be reasonable when recent elegant studies on CPV/FPV (25, 56, 61, 76) and MVM(i)/MVM(p) (9, 10, 24) are considered. In both cases, tropism and permissivity for particular cell types are regulated by short sequences in the capsid genes, and in both cases, two or three amino acids acting coordinately are required for the phenotypic change. Because the crystallographic structure of CPV has been determined (77), it has been possible to map these sequences to short loops of amino acids on the surface of the virus particle near the three-fold axis of symmetry. Although no crystallographic data are available for ADV, when the ADV VP2 sequence is optimally aligned with the CPV VP2 (65) (Fig. 7), the four coding changes observed in the ADV *Eco*RI-*Eco*RV segment appear clustered near surface loop 1 of the CPV sequence, extremely close to the critical CPV residues N-93 and A-103. Thus, it may be reasonable to speculate that for

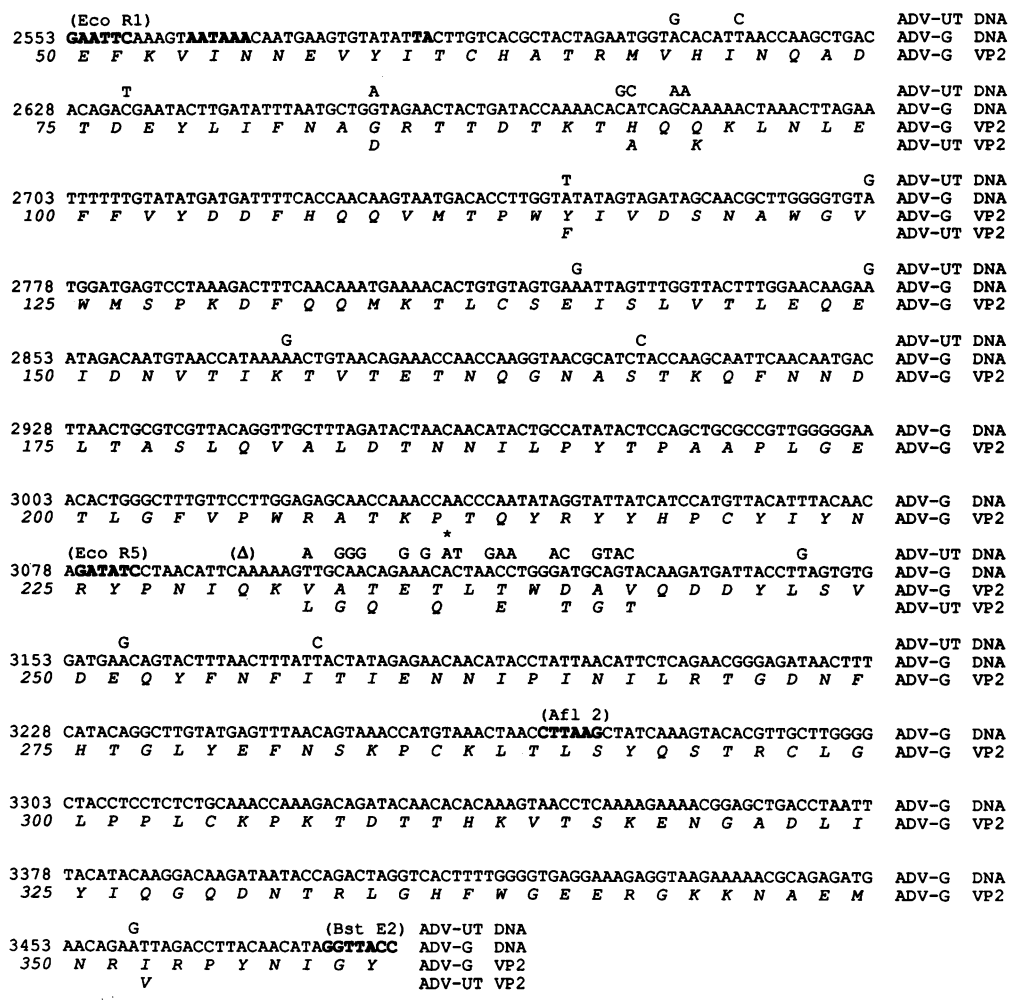


FIG. 6. Comparison of ADV-G and ADV-Utah 1 DNA and amino acid sequences between the *Eco*RI and *Bst*EII recognition sites. The DNA sequence and single-letter amino acid translation of ADV-G and ADV-Utah 1 (ADV-UT) were compared for the span between the *Eco*RI and *Bst*EII recognition sites. Simple sequence differences are noted with the appropriate letter; the deletion (Δ) and addition (*) of A residues in the ADV-Utah 1 sequence corresponding to ADV-G nt 3094 and 3113, respectively, are indicated. The AATAAA polyadenylation signal (nt 2564 to 2569), the site of actual poly(A) addition (TA at nt 2585 to 2586), and the cleavage sites for *Eco*RI, *Eco*RV, *Afl*II, and *Bst*EII are emphasized in boldface.

ADV-G/ADV-Utah 1, these coding changes acting singly or coordinately may regulate permissivity in cell culture. Experiments are under way to mutate these four residues and observe the effect on replication.

Eight of the nine amino acid differences found in the MU 65 to 73, *Eco*RV-*Bst*EII fragment (hypervariable region) appeared clustered in a position likely to correspond with CPV surface loop 2 (Fig. 6 and 7) (77). Although exchange of this region by itself did not render infectious clones replication defective, the pronounced variability noted in this area might still have some undetermined biological importance, perhaps an as yet undefined epitope.

Our results suggested that the block to rescue of the replication-defective plasmids was characterized by a virtual absence of encapsidated progeny strands and a failure of amplification of replicative form DNA (Fig. 3). This result seemed slightly different from the MVM(i)/MVM(p) situation, in which the block to replication can be overcome by transfection of the allotropic genome (36). In the MVM system, the accumulation of progeny SS DNA is dependent on capsid protein production (43, 66, 75), and the genomes are thought to be captured within preformed capsids (39), which can self-assemble in the absence of genome synthesis (28). However, the replication defect reported here cannot be related simply to capsid protein synthesis, because both VP1 and VP2 were produced in cells transfected with pXX-B-1 (Fig. 3A), although virus was not propagated. Perhaps there are subtle differences in cellular localization and transport which we have not yet identified. Alternatively, capsid formation or stability in CRFK cells may be impaired if the proper *cis*-acting sequence is absent. Nevertheless, the mechanism by which a *cis*-acting sequence in the capsid coding sequences might cause this remains obscure.

Before we can conclude that only the capsid genes control ADV permissivity for CRFK cells, it will be necessary to analyze constructions that contain the portions of ADV-Utah 1 not used in these experiments. The preliminary sequence obtained from a nearly full-length clone of ADV-Utah 1 (59) suggests a very limited number of sequence differences in the portion of the genome spanning the left-

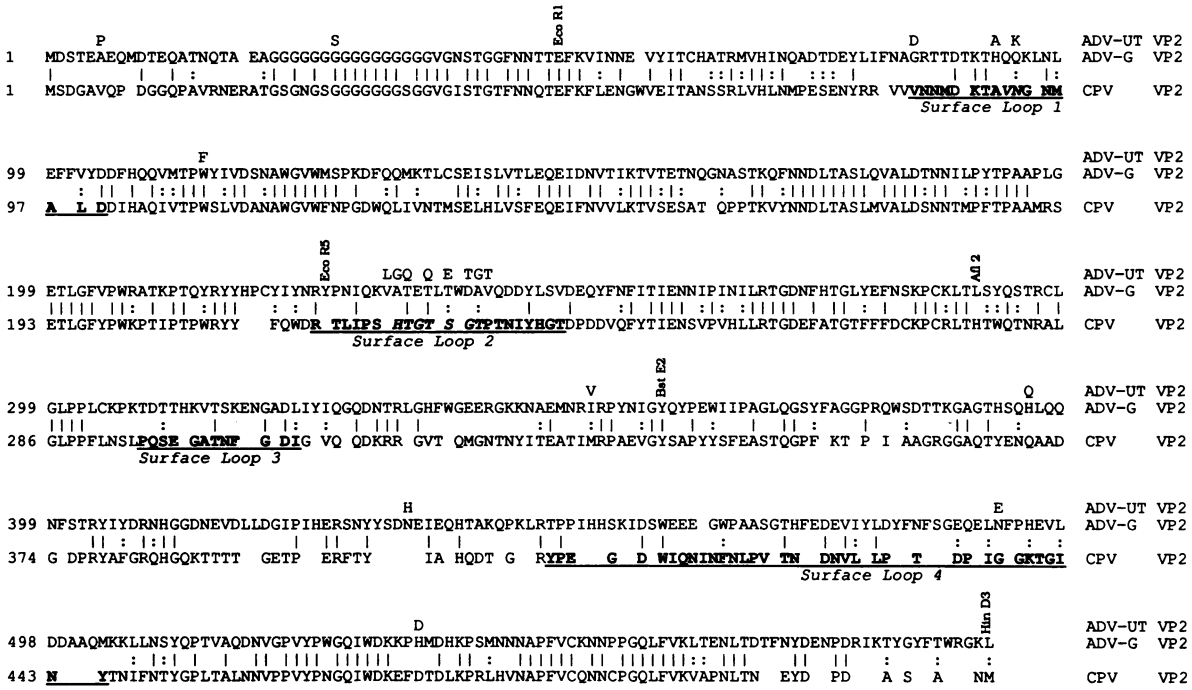


FIG. 7. Comparative alignment of the ADV-G and ADV-Utah 1 VP2 sequences with the CPV VP2 sequence. The VP2 sequence of ADV-G was optimally aligned with that of CPV VP2 from the initial methionine codon through the location of the ADV *Hin*dIII site and compared with the corresponding segment of the ADV-Utah 1 (ADV-UT) VP2. Variations between the ADV-G and ADV-Utah 1 sequences are specified by noting the variant residues. The boundaries of the defined CPV surface loops 1 through 4 are noted and emphasized in boldface, and the apical residues are italicized. The ADV *Eco*RI, *Eco*RV, *Afl*II, *Bst*EII, and *Hin*dIII sites are noted.

hand palindrome inboard to the *Bam*HI site but somewhat more heterogeneity between the *Hin*dIII (MU 88) site and the right-hand end of the genome. Studies are also planned to investigate a potential role for these regions of the ADV-Utah 1 genome. A role for the NS1 protein in the host range of porcine parvovirus has been proposed (79), so it remains a possibility that some of these regions are also involved in influencing the permissivity and pathogenicity of ADV. Furthermore, the nature of the block to replication reported here resembles that seen for NS2 mutant viruses in certain cell types (4, 7, 27, 49). It has recently been reported for H-1 parvovirus that NS2 regulates viral gene expression via a sequence in the 3′ untranslated region of the capsid protein mRNA (49). In fact, the 3′ untranslated region of the ADV capsid protein mRNA (R3) is located to the right of the *Hin*dIII site at MU 88, and thus the ADV-Utah 1 sequence for this region is not represented in the chimeras described in this report. Perhaps, in addition to the determinant we have mapped in the *Eco*RI-*Eco*RV segment, there will also prove to be another one controlled by an NS2-responsive sequence in the 3′ untranslated portion of the genome.

It is tempting to speculate that the same genomic features that constrain these constructions from permissively replicating in cell culture may be related to pathogenicity. Studies that have defined reciprocal determinants for MVM(i)/MVM(p) (10, 35, 36, 73) and CPV/FPV (25, 56, 61, 76) have been facilitated by the availability of cell lines nondiscriminatory to these various features. However, although the pathogenic strains replicate permissively in vivo in the alveolar type 2 cells of newborn mink kits (6), a reliable cell culture system fully permissive for the replication of pathogenic strains of ADV has not been identified (20, 41, 44, 45, 62). The lack of such a cell line has obviously hampered some of our investigations.

Finally, these constructions enabled us to map the reactivity for several anti-ADV monoclonal antibodies (64). Monoclonal antibody 68 reacted specifically with the *Eco*RV-*Afl*II segment. Because the only coding changes in this fragment are in the hypervariable region (Fig. 6) (14) and because this monoclonal antibody reacts in the particle-dependent counterimmunoelectrophoresis (64), the relevant epitope likely resides on the particle surface. These findings may in fact bolster our suggestions that the hypervariable region may be located in the ADV equivalent of surface loop 2 of CPV. Monoclonal antibodies P-2-1 and 163 reacted reciprocally with the *Afl*II-*Bst*EII fragments of ADV-G and ADV-Utah 1. The only coding difference in this segment is replacement of an ADV-G isoleucine with an ADV-Utah 1 valine (Fig. 6). Examination of the ADV-CPV amino acid alignment (Fig. 7) places this residue near CPV surface loop 3, another potential location for an epitope-controlling residue.

Furthermore, capsid proteins from viruses (e.g., XVII-T-2) or plasmids (e.g., XX-B-1) containing the ADV-Utah 1 *Afl*II-*Bst*EII fragment were 1 to 2 kDa larger in molecular mass than those from other constructions (Fig. 2A and 3A). This suggested that the I to V transition in this fragment not only controlled the epitope for antibodies P-2-1 and 163 but also effected a change in apparent molecular mass not accounted for by a change of an I (131.2 Da) to a V (117.1 Da). Perhaps the I to V transition causes a conformational change that yields aberrant migration in the SDS-PAGE system (78).

In summary, the construction of chimeric ADV clones has

enabled us to map a determinant within the capsid protein-coding region that governs replication in cell culture. Although it is as yet unclear how this determinant functions in vitro and whether the same genomic region governs in vivo replication and pathogenicity, these studies have provided information that will facilitate further examination of the structure-function relationships of the ADV genome.

ACKNOWLEDGMENTS

During the course of these studies, Bradley D. Berry was supported by generous grants from the Mink Farmers' Research Foundation. Wu Wei was a Special Volunteer on leave from the Chinese Academy of Agricultural Sciences, Jilin, People's Republic of China.

We thank Gary Hettrick and Robert Evans for assistance in drafting the figures and the scientific staff of LPVD for crucial and illuminating scientific discussions. Animal care was provided by William Anderson and other members of the RML Animal Care Unit under the supervision of Don Dale.

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