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Characterization of the Aleutian Disease Virus Genome and Its Intracellular Forms

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Aleutian disease virus (ADV) of mink is a nondefective parvovirus with a single-stranded DNA genome. We characterized the viral DNA forms found in infected cells prepared by a modified Hirt extraction procedure. Double-stranded DNA molecules corresponding in size to 4.8-kilobase-pair duplex monomers and 9.6-kilobase-pair duplex dimers were identified in agarose gels by blot hybridization to ^{32}P-labeled ADV DNA. A rapidly reannealing ADV duplex monomer was isolated on a preparative scale and physically mapped with a series of restriction endonucleases. The map derived was similar to one derived from double-stranded ADV DNA produced by self-primed synthesis on virion DNA, but differed from restriction endonuclease maps reported for other parvovirus DNAs. The purified duplex monomer could be labeled with [^{32}P]dCTP by nick translation and used as a probe in blot hybridization to detect ADV sequences in DNA from small numbers of infected cells. Additional studies indicated that double-stranded ADV DNA could first be detected at 24 h after infection.

Nondefective parvoviruses are small, mammalian viruses containing linear single-stranded (SS) DNA genomes approximately 1.4×10^6 to 1.6×10^6 daltons in size (3, 7, 20, 25). During virus replication, the SS DNA is converted into a variety of double stranded (DS) replicative forms (RFs) that function both as intermediates in progeny SS DNA strand production and as templates for viral RNA synthesis (3, 7, 18, 24, 25). One species of RF DNA is a duplex monomer (DM) that rapidly reanneals after denaturation in "snap-back" fashion and has the structure of a hairpin (3, 7, 14, 24, 25); this snap-back form is thought to result from self-primed synthesis initiating at the 3′ terminus of virion SS DNA (3, 7, 18, 24, 25). The hairpin RF can be isolated from infected cells by the Hirt extraction procedure and can serve as a suitable substrate for restriction endonuclease mapping (14, 15, 25). We recently showed that Aleutian disease virus (ADV) is a nondefective parvovirus containing an SS DNA genome (5). In this paper, we describe the isolation and characterization of intracellular forms of ADV DNA and present a physical map of the hairpin RF of ADV. We further show that RF DNA labeled with ^{32}P by nick translation can be used to detect ADV sequences in DNA from small numbers of infected cells.

MATERIALS AND METHODS

Materials. Restriction endonucleases, mung bean nuclease, and the large "Klenow" fragment of *Escherichia coli* DNA polymerase purchased from either Bethesda Research Laboratories Inc. (Gaithersburg, Md.), P/L Biochemicals (Milwaukee, Wis.), or New England Biolabs (Beverly, Mass.) were used according to the suppliers' suggestions or published procedures. Phenol (Fisher Scientific Co., Pittsburg, Pa.) was redistilled, stabilized with 0.2% 8-hydroxyquinolone, and saturated with TE buffer (0.02 M Tris-hydrochloride–0.001 M EDTA, pH 7.5) at 4°C. [α-^{32}P]dCTP (400 to 1,000 Ci/mmol) and carrier-free ^{32}P were supplied by New England Nuclear Corp. (Boston, Mass.). RNA (P/L Biochemicals) was prepared at a concentration of 10 mg/ml and stored at −70°C after heating at 70°C for 30 min. Proteinase K was purchased from Boehringer Mannheim Corp. (Indianapolis, Ind.). Formamide purchased from Matheson, Coleman and Bell (Norwood, Ohio) was used without additional purification.

Preparation of virus and virion DNA. The ADV-G isolate of ADV was propagated and assayed on Crandall feline kidney (CRFK) cells (8) as previously detailed (5). For labeling with ^{32}P, infected cells in 150-cm^2 Corning flasks were grown for 40 h in phosphate-free minimal essential medium supplemented with 10% dialyzed fetal calf serum, 1% complete medium (all from GIBCO Laboratories, Grand Island, N.Y.), and 50 μg of gentamycin sulfate (Schering Corp., Bloomfield, N.J.) per ml. At 40 h 1 mCi of carrier-free ^{32}P$_i$

per flask was added, and incubation was continued until 90 h. SS virion DNA was extracted from purified virions by sodium dodecyl sulfate-proteinase K digestion and phenol extraction (5). DNA prepared by this method was of a size and purity comparable to that of DNA isolated from alkaline sucrose density gradients (5).

Isolation of intracellular ADV DNA. A modified Hirt extraction was used to prepare intracellular DNA (13, 14). Flasks of CRFK cells (150 cm^2) infected for 40 to 48 h were washed once with chilled phosphate-buffered saline before the addition of 3 ml of 0.6% sodium dodecyl sulfate–0.01 M Tris-hydrochloride–0.01 M EDTA (pH 7.5) per flask (14). After 20 min at room temperature the lysate was poured into 40-ml polycarbonate tubes (5 flasks per tube). Proteinase K (75 μl per flask, 20 mg/ml in TE) was added, and the tubes were gently rocked and then incubated at 37°C for 2 to 6 h. Then 5 M NaCl (0.75 ml per flask) was added, and the tubes were incubated at 4°C in an ice bath overnight. The Hirt supernatant was recovered after centrifugation at 4°C in a Sorvall SS34 rotor at 15,000 rpm for 40 min and precipitated overnight with 2.5 volumes of 95% ethanol at −20°C. DNA was recovered by centrifugation at 10,000 rpm for 30 min at −5°C and dissolved in 0.05 M Tris–0.005 M EDTA (pH 8.5) (14) at a volume ratio of 0.3 ml per flask.

For preparative purification of rapidly reannealing RF (14), the Hirt supernatants were incubated at 37°C for 30 min with 30 μl of RNase A (10 mg/ml) per flask and denatured for 5 min at 100°C after the addition of another 0.3 ml of buffer per flask. These samples were quickly cooled in ice-water, and 0.1 volume 5 M NaCl was added. The DNA was extracted first with 2 volumes of phenol saturated with 0.5 M NaCl and then 2 volumes of chloroform. The final aqueous phase was precipitated with 2.5 volumes of 95% ethanol (21), after centrifugation the DNA was dissolved in a small volume of TE buffer. The samples were electrophoresed in a 1% agarose gel (see below) with a preparative comb, and the position of the DNA band was identified by staining a parallel marker track. The portion of the gel containing the desired fraction was excised, and the DNA was recovered by electroelution (22). Recoveries were 50 to 75%.

Agarose electrophoresis and blot hybridization. Samples for electrophoresis were mixed with 0.25 volume of Southern stop buffer (0.2% Orange G–10% Ficoll–0.01 M Tris-hydrochloride–0.025 M EDTA, pH 8.0) (23) and electrophoresed in buffer E (0.04 M Tris acetate-0.002 M EDTA, pH 8.0) into agarose gels (usually 1%) at 40 mA constant current (9, 23). Samples to be denatured were either boiled for 5 min or made 0.3 M NaOH for 10 min before stop buffer addition and electrophoresis at 60 mA constant current (5, 25). Gels were stained with ethidium bromide (0.5 μg/ml) for 15 min and destained for 30 min before viewing and photography. Bacteriophage λ DNA digested with *Hin*dIII (Bethesda Research Laboratories) was utilized as a marker on agarose gels. For Southern blot hybridization, gels were soaked first in 0.5 M NaOH–1.5 M NaCl for 30 min and then in 2.0 M NaCl–1.0 M Tris-hydrochloride (pH 7.5) for 30 min, and the DNA in the gel was blot transferred overnight to nitrocellulose filters with 20× SSC (1× SSC is 0.15 NaCl–0.015 M Na citrate) by using a Bethesda Research Laboratories horizontal blot-transfer system (23). The filters were baked at 80°C for 2 h in a vacuum oven and prehybridized with Denhardt solution (0.02% each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin in 3× SSC) (9) at 65°C for 2 h in a sealable plastic bag. The Denhardt solution was then replaced with the appropriate ^{32}P-labeled denatured probe in 3× SSC–0.2% sodium dodecyl sulfate and incubated in a shaking water bath at 65°C for 24 h. The probe was then aspirated, and the filter was extensively washed at 65°C with 2× SSC. Finally, the filter was removed, dried, and exposed to X-ray film at −70°C in the presence of a Du Pont Cronex Lightning-Plus intensifying screen. For direct autoradiography, gels were dried after staining and viewing and exposed at −70°C with an intensifying screen.

Nick translation of RF DNA molecules. RF DNA (0.2 to 0.5 μg) was labeled with [$\alpha^{32}P$]dCTP by nick translation (19) with a commercially available kit from New England Nuclear Corp. The specific activities, determined as described previously (9), were ~10^7 cpm/μg.

Self-primed synthesis of DS ADV DNA from virion DNA. SS virion DNA (0.1 μg) in 30 μl was treated for 10 min at 25°C in 0.1 N NaOH and neutralized with HCl in the presence of 0.1 M Tris. The reaction mixture was adjusted to 0.15 M Tris, 0.005 M $MgCl_2$, 0.01 M 2-mercaptoethanol, 0.05 mg of bovine serum albumin per ml, and 20 × 10^6 M each dATP, dCTP, dTTP, and dGTP, including 0.5 μCi of [α-^{32}P]dCTP (7, 9). Five units of the Klenow fragment of DNA polymerase was added in a final total volume of 80 μl. The reaction was incubated 37°C for 90 min, and the enzyme was inactivated at 70°C for 5 min (7). These conditions yielded stable full-length products suitable for restriction endonuclease digestion and gel electrophoresis.

Electron microscopy. DNA molecules were denatured with 0.1 M NaOH at room temperature for 10 min, renatured for 5 min in 50% (vol/vol) formamide–0.1 M Tris-hydrochloride (pH 8.5)–0.001 M EDTA, and mounted for electron microscopy by the Kleinschmidt aqueous procedure as previously described (11). To reduce protein concentration, samples digested by restriction endonucleases were extracted with phenol and ether before study. Grids were rotary shadowed with platinum-palladium and examined in a Hitachi Hu-11E at 40 kV accelerating voltage. Electron micrographs were taken on Kodak electron image plates at magnifications of ×11,000. Magnification was calibrated with a grating replica (E. F. Fullam, Latham, N.Y.)

RESULTS

Identification of ADV DNA forms in Hirt supernatants. To identify intracellular forms of ADV DNA, fractionation of infected CRFK cells was done by a modified Hirt procedure (13, 14). The DNA from the Hirt supernatant was electrophoresed in neutral agarose gels either in native form or after denaturation (0.3 M NaOH), and ADV-specific forms were identified by blot hybridization to SS DNA extracted from ^{32}P-labeled virions. Native DNA forms of 4.8 and 9.6 kilobase pairs (kbp) pairs and DNA comigrating with SS DNA were observed (see Fig.

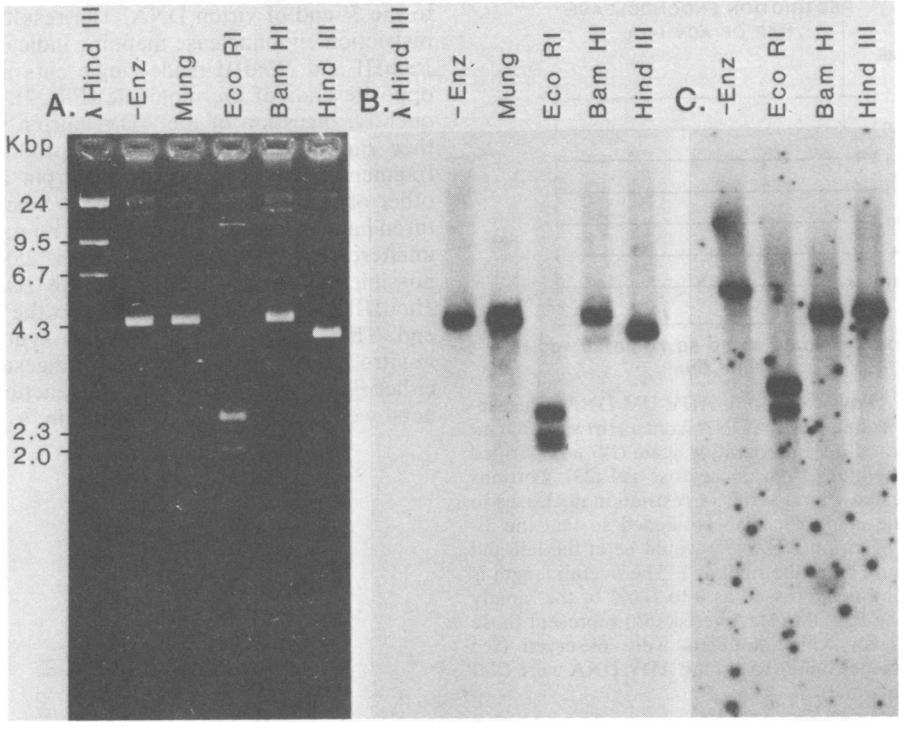

FIG. 1. Analysis of DM forms of ADV DNA. (A and B) The rapidly reannealing fraction of the Hirt supernatant was isolated from ADV-infected cells (13, 14). DNA from this fraction was recovered by ethanol precipitation and digested with SS DNA-specific mung bean nuclease (Mung) (2) or the indicated restriction endonuclease. The products were electrophoresed in 1% agarose gel and analyzed either by staining with ethidium bromide (A) or by blot hybridization (23) (B). The digestion with *Bam*HI in panels A and B was incomplete in this experiment. λ-*Hin*dIII is a parallel track of bacteriophage λ DNA digested with *Hin*dIII. The molecular weights of λ-*Hin*dIII fragments in kbp (9) are given to the left. (C) DS ADV DNA was prepared in vitro by incubating SS ADV virion DNA (5, 6) with the Klenow fragment of DNA polymerase I in a reaction containing [α-^{32}P]dCTP for 60 min at 37°C (7). After inactivation at 70°C for 5 min, portions of this reaction were digested with the indicated restriction endonuclease and electrophoresed in a 1% agarose gel. After staining with ethidium bromide to determine migration of λ-*Hin*dIII fragments in a parallel track, the gel was dried, and an autoradiogram was prepared.

5A). The 4.8- and 9.6-kbp species corresponded in size to expected weights of DM and duplex dimer forms of ADV DNA (5). Upon denaturation (5, 25), essentially all of the ADV DNA migrated with mobilities either of DM DNA or of SS DNA of genome length (see Fig. 5B). The observation that some ADV DNA migrated as DM even after denaturation indicated that some of the ADV DM molecules existed in a rapidly reannealing snap-back configuration, as has been reported for RF DNA from other parvoviruses (3, 14, 15, 18, 24, 25). Thus, selective extraction of these RF molecules could be effected on a preparative scale (14), and these purified molecules could then be analyzed by restriction endonuclease mapping. Blot hybridization (23) with purified RF DNA labeled with [α^{32}P]dCTP by nick translation (9) gave identical results (data not shown). In some Hirt preparations, rapidly reannealing ADV DNA forms of 9.6 kbp were detected (most easily seen in the mung bean nuclease-treated RF in Fig. 1B). These probably are analogous to the concatameric forms observed with other parvoviruses (3, 14, 24, 25).

Physical map of ADV DM DNA. The derivation of a physical map of ADV DNA is essential to further study and analysis. Therefore, RF molecules of ADV DNA were isolated and digested with a series of restriction endonucleases as well as the SS DNA-specific mung bean nuclease (2). The reaction products were electrophoresed and blot hybridized (Fig. 1A and B). A physical map for the ADV genome was thus derived (Fig. 2). It may be pointed out that although the RF existed in a rapidly reannealing form, this property apparently did not abrogate its ability to blot transfer on to nitrocellulose

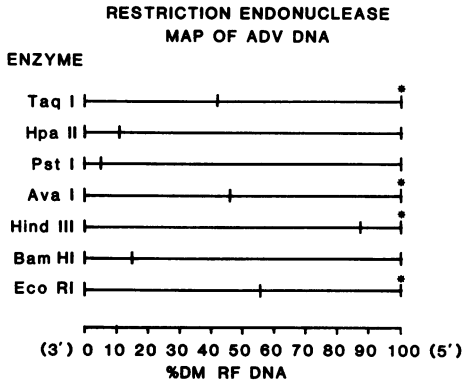

FIG. 2. Physical map of ADV DM DNA. The rapidly reannealing RF DM DNA from a Hirt supernatant was prepared on a preparative scale (14) and purified by electroelution from an agarose gel (22). Portions were digested with a series of restriction nucleases to deduce the map. The map is aligned so that the 3'-terminus of virion DNA (3') would be at the left and the 5' would be at the right (5'). The overall length is 4.8 kbp and is expressed as 0 to 100% of the rapidly reannealing RF DM (1). Asterisks (*) represent those fragments for which doublets were observed (see text). Enzymes failing to cut the ADV DNA were *Cla*I and *Hpa*I.

paper and to hybridize with the radioactive probe. Nevertheless, the hybridization signal from mung bean nuclease-treated RF DNA was stronger, and this may reflect nicking of RF DNA by the mung bean nuclease. The fact that mung bean nuclease did not appreciably alter the migration suggested that the DM DNA was in fact fully DS. Several of the enzymes (*Eco*RI, *Hin*dIII, *Ava*I, and *Taq*I) produced a large fragment and, in addition, two small fragments differing by approximately 100 base pairs. A series of double digestions indicated that this heterogeneity was due to variation at the extreme right end of the RF molecule. Similar terminal heterogeneity has been demonstrated for RFs of other parvoviruses (14, 15, 25). The digestion of RF by *Bam*HI in Fig. 1A and B was incomplete.

Since virion DNAs of parvoviruses contain 3' termini capable of allowing self-primed DNA synthesis in vitro (7), we compared the RF DNA from the Hirt supernatant to DS DNA derived from ^{32}P-labeled, in vitro-synthesized ADV DNA. It was evident that these maps were very similar (Fig. 1B and C). The size of the undigested species was 4.8 kbp, the same as for the in vivo DM RF. Electrophoresis after denaturation revealed that the synthetic DM was in fact a hairpin structure since denaturation did not affect the mobility (5, 25) (Fig. 3).

Assignment of the hairpin end of the RF molecule. We next oriented this physical map relative to the 3' end of virion DNA. The results of the restriction endonuclease mapping indicated that *Bam*HI and *Hin*dIII made single cuts near the opposite ends of the molecule (Fig. 2). Consequently, digestion of the ADV hairpin with either *Bam*HI or *Hin*dIII would produce two fragments, one of which is a hairpin and the other of which is a simple duplex. Since denatured hairpins rapidly reanneal and migrate with unaltered mobility in neutral gels (5, 25), it was possible to determine which enzyme (*Bam*HI or *Hin*dIII) cleaved the RF closest to the hairpin end. Therefore, DM hairpin forms synthesized in vitro from virion SS DNA were digested with either *Bam*HI or *Hin*dIII, and the reaction products were then electrophoresed in a neutral

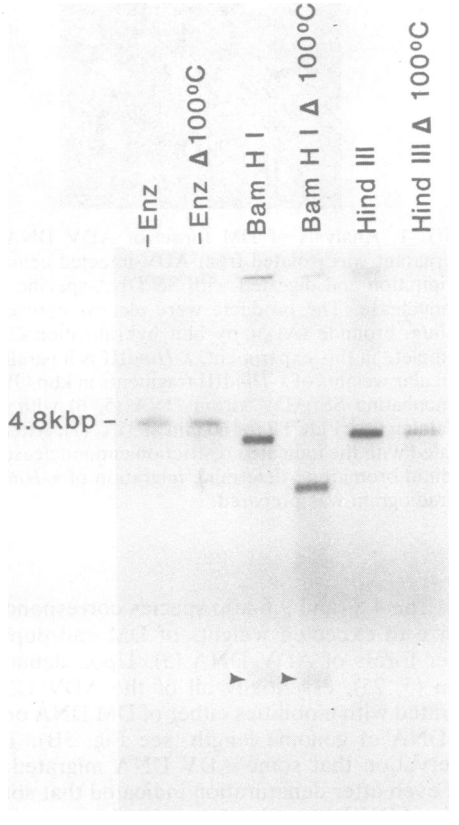

FIG. 3. Assignment of the hairpin end of in vitro-synthesized DS ADV DNA. DS DM DNA labeled with [α-^{32}P]dCTP was prepared from virion DNA as described in the text. Portions were digested with either *Bam*HI or *Hin*dIII or given no treatment (−Enz). Samples were then electrophoresed at 60 mA in a neutral agarose gel either in native form or after denaturation by boiling. An autoradiogram of the dried gel was prepared as detailed in the text. The arrow indicates the small, rapidly reannealing product of the *Bam*HI digestion.

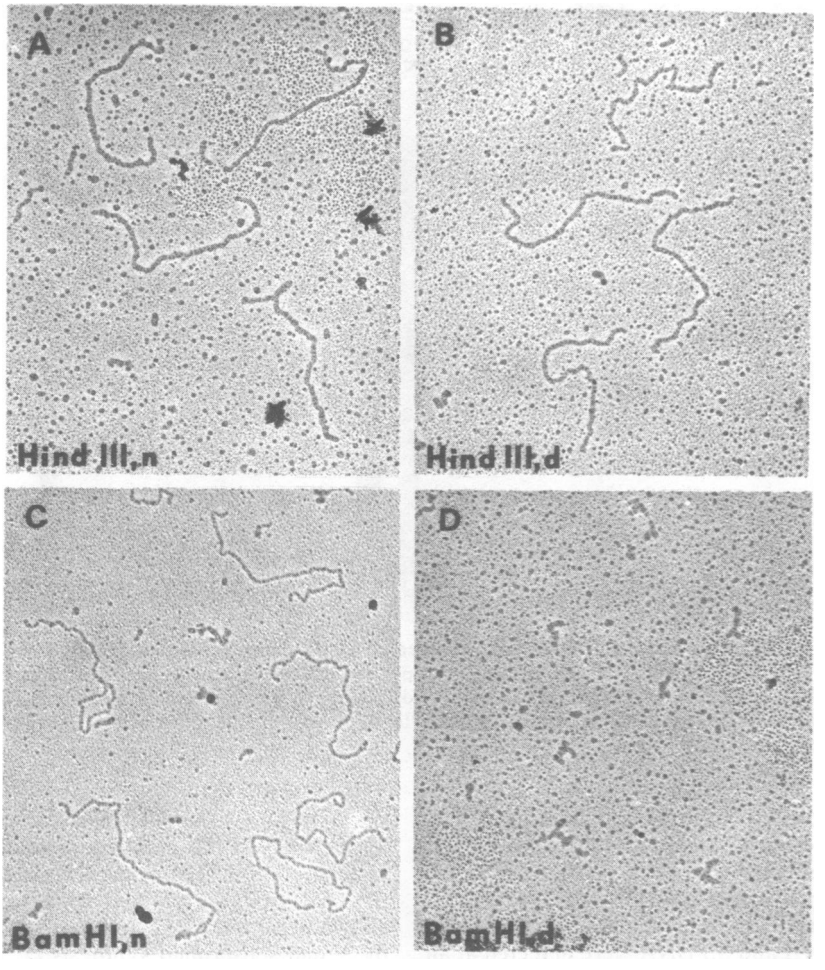

FIG. 4. Assignment of the hairpin end of RF DNA from Hirt supernatants. DS DM RF DNA, purified from a Hirt supernatant, was digested with either *Bam*HI (A and C) or *Hin*dIII (B and D). Samples were then examined by electron microscopy either in native form (n) or after denaturation and rapid reannealing (d). Details are noted in the text.

agarose gel (Fig. 3) either in native configuration or after denaturation. Since migration of the large *Bam*HI fragment was accelerated, but that of the large *Hin*dIII fragment was not, the large *Hin*dIII fragment was a hairpin. Thus, the self-priming 3′ end of virion DNA was nearest the *Bam*HI site. Careful examination of the autoradiogram further indicated that migration of the small *Bam*HI fragment was unaffected by denaturation (Fig. 3), as expected if this fragment were in fact a hairpin.

It was also necessary to confirm that the covalent hairpin end of the RF corresponded to that of self-primed in vitro produced DS DNA. A similar protocol of digestion with either *Bam*HI or *Hin*dIII was utilized with RF DNA, but the products were analyzed by electron microscopy (11) (Fig. 4). Examination of the molecules after digestion revealed long and short species corresponding to large and small fragments for both *Hin*dIII (Fig. 4A) and *Bam*HI (Fig. 4B). After denaturation, however, only the large fragments from the *Hin*dIII reaction (Fig. 4C) rapidly reannealed to duplex configuration; the large *Bam*HI fragments (Fig. 4D) collapsed into typical SS "bushes" (11), indicating that the hairpin was at the end closest to the *Bam*HI site. Thus, the covalent end of the RF hairpin corresponded to the 3′ end of the virion DNA. No forms consistent with a hairpin at the *Hin*dIII end were observed.

Detection of ADV DNA from cells infected with ADV at different multiplicities of infection. Since a major goal of this work was to look for viral DNA in tissues which might contain only small amounts of virus, we wanted to gain information

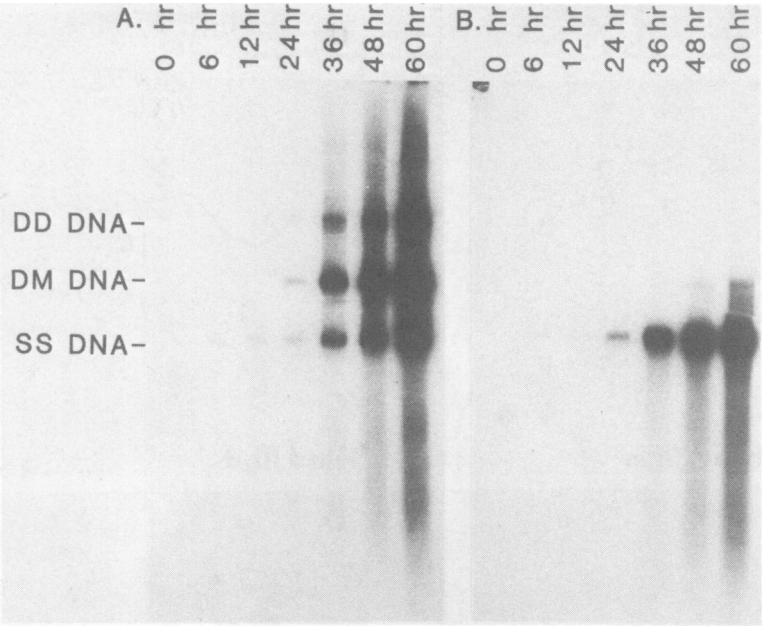

FIG. 5. Kinetics of appearance of ADV DNA in infected CRFK cells. A series of 25-cm^2 flasks was infected with ADV at a multiplicity of ~10 focus-forming units per cell. At the indicated times after infection, the DNA from Hirt supernatants (13, 14) was prepared and electrophoresed in 1% agarose gels either in native form (A) or after denaturation for 10 min in 0.3 M NaOH (B). ADV DNA forms were identified by blot hybridizing the gel to nick-translated, purified RF DNA.

about the sensitivity of blot hybridization in this system. Therefore, a series of flasks of CRFK cells were infected with serial dilutions of ADV. At 48 h, Hirt supernatant DNA was prepared, blot transferred, and hybridized with ^{32}P-labeled RF. DM DNA and SS DNA were detected at an input multiplicity as low as 0.01 focus-forming unit per cell (data not shown). This corresponded to the Hirt supernatant DNA from 6,000 cells positive for ADV antigens by immunofluorescence (0.8% of 7.5 $\times$ 10^5 cells), assuming that only fluorescence-positive cells contained viral DNA. This result suggested that detection of DNA in samples with a small number of infected cells should be possible.

Kinetics of appearance of ADV DNA in infected CRFK cells. Finally, we determined at what point after infection ADV DNA could be detected with nick-translated RF DNA as a ^{32}P-labeled probe (Fig. 5). DS DNA (DM and duplex dimer) could be observed at 24 h after infection, and the amount detected by blot hybridization increased throughout the period of observation (60 h). As in previous results, the bulk of the DNA was converted to SS DNA upon denaturation, although the rapidly reannealing RF DM was noted after 36 h. The appearance of the DS DNA thus correlates with the onset of detectable viral protein synthesis (6). A signal from SS DNA could be detected as early as 6 h after infection.

DISCUSSION

Our analysis has identified a number of intracellular ADV DNA forms. All other nondefective parvoviruses studied to date have been shown to have DS RFs derived from virion DNA by 3′ end self-primed synthesis (2, 3, 7, 14, 15, 24, 25). That ADV had similar forms was a further indication that ADV is a parvovirus. One form of parvovirus RF is a DM with the complementary strands attached by a stable hairpin linkage at the end corresponding to the 3′ end of virion DNA (2, 3, 7, 14, 15, 18, 24, 25). Our data indicated that the ADV RF also had a hairpin located at the 3′ end (Fig. 3 and 4). Taken together, this information suggests that ADV genome structure and strategy replication are similar to those of other parvoviruses.

The physical map presented here (Fig. 2) for ADV DNA differed completely from those reported for other parvoviruses (adenovirus-associated virus [3], minute virus of mice [7, 14, 25], and another pathogenic parvovirus of mink, mink enteritis virus [15]). Although mink enteritis virus is closely related to several other pathogenic parvoviruses [canine parvovirus and feline panleukopenia virus [15]), our data strongly suggest that ADV, on the other hand, is not closely related to mink enteritis virus. Similar conclusions have also been reached with serological analyses (16).

Isolates of ADV have been readily distinguished on the basis of virulence for mink (4, 10, 12, 16, 17). Whereas the highly virulent Utah I isolate causes persistent infection with progressive fatal disease in mink of all genotypes (6, 7), the less virulent Pullman ADV typically causes disease only in Aleutian mink (4, 10). The virus we have used in these studies (ADV-G) (5), although derived from the highly virulent Utah I ADV (17), lost in vivo virulence for mink on serial passage in cell culture (5). Another cell culture-adapted isolate of ADV (ADV-P) (5, 16) is also Utah I ADV derived (17), but does not attain the high titers in cell culture that the ADV-G does (5, 16). Interestingly enough, ADV-P retains the full virulence of the parent Utah I ADV (16). In spite of such pronounced biological differences, studies on isolates of ADV suggest a high degree of antigenic relatedness among viruses (4, 6, 7, 16). This phenomenon is not unique to ADV. Recent studies on other nondefective parvoviruses show that antigenically similar viruses can vary widely in biological characteristics such as host range (14, 15) and that these differences can be correlated with differences in genome structure (14, 15). Now that we have characterized the structure of the ADV-G genome, it will be possible to begin direct comparison of isolates of ADV. Furthermore, localization of viral genomic information within cells of infected tissues will be a sensitive means of defining precise sites of viral replication and persistence.

SS ADV DNA was noted as early as 6 h (Fig. 5) after infection, and this probably represents accumulation of virion DNA from input particles. DS ADV DNA, however, could not be detected in Hirt supernatants by blot hybridization until 24 h after infection (Fig. 5). This is substantially later in infection than for the minute virus of mice system, in which DS viral DNA can be detected between 4 to 8 h (25). This lag between initial infection and the appearance of duplex DNA probably explains why ADV protein synthesis and virus production occur as late as they do (5, 6, 17).

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