A Novel Replicative Form DNA of Aleutian Disease Virus: the Covalently Closed Linear DNA of the Paroviruses

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SUMMARY

The analysis of replicative form (RF) DNA of Aleutian disease virus (ADV) by alkaline gel electrophoresis revealed that all RF DNA species segregate into DNA single strands which represent integral multiples of a genome equivalent. This demonstrates that as with other autonomous paroviruses, the virion and complementary DNA strands are frequently linked by hairpin structures and that also, nicks are present at subterminal sites. Approximately 50% of the 5'-terminal hairpins contain a subterminal nick whereas no nick is detectable in the 3'-terminal hairpin. This finding together with the presence of nicks in the 3' palindrome sequence of the dimer RF DNA (D RF DNA) bridge fragment is the first experimental proof for the so far hypothetical substrate specificity of a nickase. A novel DNA structure was identified in the monomer (M) RF DNA population. This molecule, designated 'monomer covalently closed linear RF DNA' (Mccl RF DNA), consists of a continuous, self-complementary, circular polynucleotide chain of twice the genome length. It was directly visualized by electron microscopy that denatured ADV M RF DNA is a single-stranded circular molecule of twice the genome length with covalently closed terminal hairpins on either end. Alkaline gradient centrifugations, enzymic assays and electrophoretic techniques confirmed the proposed structure. Moreover, evidence was obtained that the D RF DNA species contains an analogous Dccl RF DNA. It is suggested that the newly described Mccl RF DNA form is an important intermediate common to the DNA replication of all autonomously replicating paroviruses.

INTRODUCTION

Aleutian disease virus (ADV), the causative agent of Aleutian disease in mink is an autonomously replicating parovirus (Bloom et al., 1980). During productive infection in mink (Alexandersen & Bloom, 1987; Haas et al., 1988) or cell cultures (Bloom et al., 1983; Löchelt & Kaaden, 1987) ds replicative form (RF) DNA and ss virion DNA are detectable in cellular lysates. Due to the configuration of the terminal hairpins in either the extended (palindrome) or foldback (hairpin), forms, the ADV monomer (M) RF DNA displays a length heterogeneity of about 200 bp. This length heterogeneity is restricted to the genome termini and amounts to 70 bp for the 3' terminus and 160 bp for the 5' terminus. Both configurations appear to reflect distinct stages in the replication of the genome termini. According to current replication schemes for paroviruses (Tattersall & Ward, 1976; Astell et al., 1983a, 1985; Hauswirth, 1984; Cotmore & Tattersall, 1987) the 3' hairpin of the incoming virion DNA is used as a primer for a DNA polymerase converting the genome to ds M RF DNA. There are then two alternatives when the polymerase reaches the 5'-terminal hairpin: either the synthesis continues with displacement and replication of the hairpin sequence, which leads directly to the extended form of M RF DNA (Astell et al., 1985), or the DNA synthesis stops at this point followed by a ligation which seals the nick between the newly synthesized complementary strand (c-strand) and the viral 5' hairpin. This should result in a linear duplex molecule with covalently closed ends, known as the
'collapsed closed circular intermediate' (Cotmore & Tattersall, 1987). Our experimental data presented in this paper support the second mechanism. To describe the structure of the intermediate more precisely we propose the term 'covalently closed linear RF DNA' (ccl RF DNA). According to the model suggested for the minute virus of mice (MVM) (Cotmore & Tattersall, 1987) the monomer (M) ccl RF DNA is the substrate for a site-specific nickase introducing a ss break upstream from the viral 5' hairpin structure. The newly generated 3'-hydroxyl end would then serve as the primer for the replication of the complete viral hairpin sequence extended by 18 bp. An analogous nicking mechanism is postulated for the replication of the original 3'-terminal sequences in the bridge fragment of the dimer (D) RF DNA (Astell et al., 1983a, 1985).

To study the process of ADV DNA replication, we analysed the terminal linkage between the viral and the c-strand of ADV M RF DNA. Here we demonstrate that 5 to 10% of the ADV M RF DNA molecules consist of a continuous, self-complementary, circular DNA chain of twice the genome length. The possible role of this novel intermediate in parvoviral DNA replication is discussed.

METHODS

Cells and virus. Cell culture techniques, infection with the cloned ADV SL3 isolate E10 (kindly provided by Birgit Stolze) and purification of ADV RF DNA (Hirt extraction), M RF DNA and virion DNA were done as previously described (Löchelt & Kaaden, 1987).

DNA separation techniques. Alkaline sucrose gradient centrifugation of ADV RF DNA was performed according to Straus et al. (1976) after dialysis of the DNA (Carter & Khoury, 1975). Gradients were fractionated in 300 μl aliquots and precipitated with ethanol after neutralization with an equal volume 1 M-Tris HCl pH 7.0. Five to 30% neutral sucrose gradients were run according to Straus et al. (1976). The gradients were fractionated in 360 μl aliquots, diluted 1:2 and ethanol-precipitated in the presence of 0.3 M-sodium acetate pH 5.2. This method was also used to purify ADV M RF DNA. Alkaline agarose gels were run as described (Maniatis et al., 1982). Two-dimensional (2D) DNA analysis (Favaloro et al., 1980) was performed in 0.9% gels. HindIII-digested λ DNA (Bethesda Research Laboratories, BRL) and ADV M RF DNA served as markers for the first and second dimensions. Southern blot hybridizations (with partial depurination of the DNA) using 35S-labelled pSL18 insert DNA as a probe, were performed as described (Löchelt & Kaaden, 1987).

Enzymic techniques. SI nuclease treatment of 0.4 μg ADV M RF DNA and 6 μg denatured carrier DNA was done in 120 μl samples with 30 units (U) SI nuclease (BRL) at 37 °C as recommended by the supplier. The reaction was terminated by mixing gel-loading buffer with 10 μl aliquots taken at regular intervals. Restriction endonuclease digests with BamHI and HindIII (Boehringer) were performed as previously described (Löchelt & Kaaden, 1987). Digests with exonuclease III and λ exonuclease (ExoIII and λ-Exo, BRL) were done as recommended by the supplier. The ExoIII digest contained 0.1 μg ADV M RF DNA and 3.5 μg HindIII-digested λ DNA (BRL) in 80 μl with 130 U ExoIII. λ-Exo assays were performed in 100 μl aliquots with 1 μg ADV RF DNA, 3.5 μg HindIII-digested λ DNA and 8 U enzyme.

Electron microscopy of ADV M RF DNA. ADV M RF DNA was purified by sedimentation through a neutral sucrose gradient. Native DNA was analysed after adsorption to mica (Kiss et al., 1980). For denaturation the DNA was precipitated with ethanol and taken up in 2 mM- or 6 mM-sodium phosphate pH 6.5, containing 1.0 or 1.2% formaldehyde, and incubated at 80 or 85 °C, respectively, as described in the figure legends. The samples were spread with cytochrome c from 30% formamide, 0.1 M-Tris-HCl (Davis et al., 1971) onto a hypophase of 0.005% octyl-glucopyranoside. The samples were stained with uranyl acetate and rotary-shadowed with platinum. Pictures were taken at ×5000 magnification.

RESULTS

Electrophoresis of ADV DNAs under alkaline conditions

The ADV Hirt extract containing all virus-specific DNA species (ADV M RF DNA, purified virion DNA and DNA from mock-infected cells) was fractionated under denaturing conditions on 0.9% agarose gels. After Southern blot hybridization using as a probe 35S-labelled pSL18 insert DNA, the following bands were detected (Fig. 1). The virion DNA (lane 2) migrated as a band of 4800 nucleotides (nt, used here only for ssDNA). The M RF DNA (lane 3), migrating in neutral agarose gels as a double band of 4.8 and 4.6 kb dsDNA, respectively (shown in Fig. 3b),
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Fig. 1. Detection of Mccl RF DNA in the RF DNA pool of ADV-infected cells. DNA from mock-infected cells (lane 1), virion DNA (lane 2), ADV M RF DNA (lane 3) and Hirt-extracted DNA from ADV-infected cells (lane 4) were run on a 0.9% alkaline agarose gel. ADV-specific DNA was detected by Southern blot hybridization. The bands of more than 40000, 29000 and 24000 nt are indicated by a square, a circle and an asterisk, respectively.

Fig. 2. Sedimentation of ADV RF DNA through neutral sucrose gradients. ADV Hirt extract was centrifuged through a neutral sucrose (5 to 30%) gradient. Selected fractions were analysed on a neutral gel (a). ADV RF DNA (far right lane) served as a size marker, displaying the bands of ADV D, P and M RF DNA and virion DNA. The same gradient fractions were additionally run on an alkaline gel (b). ADV M RF DNA (far right lane) was used as a size marker, showing bands of v, 2v and Mccl RF DNA. Hirt extract (far left lane) displayed additionally Dccl RF DNA (indicated by an asterisk in fraction 18) and 3v and 4v DNA. In both autoradiographs, sedimentation is represented from right to left.

displayed under alkaline conditions additional bands of 4500, 9600 and approx. 13000 nt. All were ADV-specific as proven by their absence in mock-infected cells (lane 1). Hirt lysates (lane 4) showed additional bands with approximate sizes 14000, 19000, 24000 and 29000 nt. A faint band above 40000 nt was also detected. The relative electrophoretic mobilities of the bands representing 13000 nt and approx. 40000 nt varied slightly depending on the agarose concentration used.

Summarizing the results of alkaline gel electrophoresis of ADV RF DNA, we conclude that the bands of 9600, 14000, 19000 and 24000 nt are regular multiples (2v, 3v, 4v and 5v) of one viral genome equivalent (v) with a size of 4800 nt. They probably originate from either the covalent interstrand linkage of viral and c-strands via terminal hairpins, or from the intrastrand linkage of alternate strands in D and higher oligomeric RF DNAs, or from both processes. The 4500 nt molecule detected under denaturing conditions was designated sub-v DNA. In the course of the study the DNA band of about 13000 nt was shown to represent the so far hypothetical Mccl RF DNA. The band of about 40000 nt is very likely to be an analogous form derived from the D RF DNA and was thus designated Dccl RF DNA.
**Sedimentation of ADV RF DNA through neutral sucrose gradients**

ADV RF DNA was centrifuged through neutral sucrose (5 to 30%) gradients. The DNA was analysed either on neutral or alkaline 0.9% agarose gels and detected by Southern blot hybridization. The neutral gel (Fig. 2a) revealed that the ADV M RF DNA appeared as a broad peak with a maximum value in fraction 21. The virion DNA reached a maximum in fractions 15 and 16. The highest concentration of D RF DNA corresponded to fraction 18. The partially replicated dimer RF DNA (P RF DNA; Löchelt & Kaaden, 1987) was at its maximum in fraction 16. Higher oligomeric forms were only poorly separated and not further analysed. Under alkaline electrophoresis conditions (Fig. 2b), v DNA was detected up to fraction 23, derived either from the virion DNA (the minor peak in fraction 16) or denatured M RF DNA (the major peak in fraction 21) or other RF DNA species. 2v DNA reached its maximum concentration at fraction 21, but appeared also in all fractions with higher sedimentation values. The Mccl RF DNA appeared predominantly in fraction 21, hence was obviously derived from ADV M RF DNA. The Decl RF DNA was present exclusively in fraction 18. The 3v and 4v ADV DNAs appeared in fraction 18 and were consistently detectable in all other fractions with higher sedimentation values.

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Fig. 3. Two-dimensional electrophoresis of ADV RF DNA and purified ADV M RF DNA. (a) ADV RF DNA was first separated under neutral conditions (from left to right). The position of the major ADV DNA species is indicated at the bottom of the autoradiograph. The migration in the second dimension was from the top to the bottom. ADV M RF DNA served as a marker (far left) showing the v, 2v and Mccl RF DNA species. (b) ADV M RF DNA was first separated under neutral conditions (horizontal arrow) and subsequently under denaturing conditions perpendicularly to the first dimension (vertical arrow). The upper autoradiograph presents M RF DNA run under neutral conditions displaying the parvovirus-specific length heterogeneity of about 0.2 kb. The lower autoradiograph shows the corresponding 2D analysis.
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Fig. 4. Alkaline sedimentation of ADV RF DNA. Selected fractions (indicated on top of the autoradiograph) from a 5 to 20% alkaline gradient, run with ADV Hirt extract were analysed on an alkaline gel. Sedimentation was from right to left. ADV M RF DNA (far right lane) and RF DNA (far left lane) were used as size markers, showing the known pattern of ADV DNA molecules. The position of the Dccl RF DNA in fractions 13 and 14 is indicated by asterisks.

Two-dimensional separation of ADV RF DNA and ADV M RF DNA

The 2D analysis of ADV Hirt extracts was performed to determine more precisely the origin of the molecules detected under alkaline gel conditions. This technique was used as it avoids further manipulations of the DNA (e.g. DNA extraction) and obtains a better resolution of the RF DNA species than seen in the neutral gradients. Hirt extracts were separated on 0.9% agarose 2D gels, blotted onto nitrocellulose membranes and probed. The stained HindIII-digested λ DNA provided a marker for the first dimension; the migration in the second dimension was compared with ADV M RF DNA which was separated in parallel. The virion DNA migrated in the neutral gel with a length of 2.3 kb, whereas under alkaline conditions its real length of 4800 nt became apparent (Fig. 3a). As expected, M RF DNA contained v and 2v DNA. At the faster migrating edge of the M RF DNA double band, Mccl RF DNA was detectable, indicating that this DNA species was predominantly present in the faster migrating band of the M RF DNA (4.6 kb dsDNA). The P RF DNA dissociated upon denaturation into v, 2v and 3v DNA. In addition, the D RF DNA contained 4v DNA and Dccl RF DNA. Similarly, the higher oligomeric forms segregated integral multimers of v DNA length, whereas ccl RF DNA was only detected in the positions described above. In an additional 2D analysis, intended to identify unambiguously the origin of the Mccl RF DNA, only M RF DNA was used. It is obvious from Fig. 3(b) that only the faster migrating band of the M RF DNA contained the Mccl RF DNA, whereas v and 2v DNA were present in both ADV M RF DNA bands.

Alkaline sedimentation of ADV RF DNA

ADV RF DNA was sedimented through 10 to 30% alkaline sucrose gradients and selected fractions were analysed on 0-9% alkaline gels. Fig. 4 represents the corresponding autoradiograph. ADV v and sub-v DNA reached their maximum in fraction 26. The next length class, 2v DNA, was at its maximum in fraction 22, whereas the Mccl RF DNA peaked, corresponding to fractions 19 and 20. The 3v DNA had a slightly higher sedimentation value. The peak of 4v DNA was detectable in fraction 16. Higher oligomeric forms were only poorly resolved. An exception to this is the Dccl RF DNA which appeared exclusively in fractions 13 and 14.

Alkaline gel analysis of ADV M RF DNA restriction fragments

ADV M RF DNA was digested with BamHI, HindIII (cleavage sites at m.u. 0.15 or 0.88, respectively) or both enzymes simultaneously. The generated fragments were separated on 0-9% alkaline gels and detected by Southern blot hybridization. The undigested control DNA (Fig. 5,
Fig. 5. Alkaline electrophoresis of ADV M RF DNA restriction fragments. ADV M RF DNA (lane 1) was digested with BamHI (lane 2), HindIII (lane 3) and both enzymes simultaneously (lane 4) and analysed on an alkaline gel. The position of the ADV M RF DNA species is indicated at the left. On the right is indicated the length of 2v, v DNA and the central restriction fragment after BamHI and HindIII digestion (from top).

Fig. 6. S1 nuclease digestion of ADV M RF DNA. ADV M RF DNA was digested with S1 nuclease and samples were withdrawn after regular intervals (0 min, lane 1; 2-5 min, lane 2; 5 min, lane 3; 10 min, lane 4; 30 min, lane 5; 90 min, lane 6). The fragments generated were analysed on an alkaline gel. The position of the undigested ADV M RF DNA species is indicated on the right.

lane 1) displayed the known pattern of sub-v, v, 2v and Mccl RF DNA. In none of the enzyme-treated samples were the original bands still present. BamHI digestion (lane 2) generated fragments of 7900, 4080 and 3770 nt and HindIII (lane 3) gave rise to bands of 8250 and 4200 nt. The largest HindIII fragment bearing a covalently closed hairpin and thus migrating with double length was present in twice the concentration of the corresponding BamHI fragment. The double digestion (lane 4) resulted in a single band of 3500 nt. Terminal DNA fragments ranging from map units 0-00 to 0-15 and 0-88 to 1-00 were not detected with the hybridization probe used. The detection of DNA forms longer than v DNA in both of the restricted DNA samples demonstrates that covalently closed hairpin structures are present at both termini of M RF DNA.

**S1 nuclease digestion of ADV M RF DNA**

ADV M RF DNA was digested with S1 nuclease; at regular intervals, samples were taken and the fragments were analysed on 0-9% alkaline agarose gels. ADV-specific DNA was visualized by hybridization (Fig. 6). During the incubation the concentration of the Mccl RF DNA and the 2v DNA decreased whereas the amount of v ADV DNA increased concomitantly. The Mccl RF DNA was most sensitive to S1 nuclease. After 90 min, only traces of this DNA species were left, whereas a strong hybridization signal was obtained with the 2v DNA. Nonspecific degradation
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Fig. 7. Digestion of ADV M RF DNA with ExoIII. ADV M RF DNA was incubated with ExoIII for different times (0 min, lane 1; 20 min, lane 2; 40 min, lane 3; 80 min, lane 4). The digestion products were analysed on a neutral (a) and an alkaline (b) gel. For the neutral gel, the size of the major fragments is given. The position of the different ADV DNA species is shown for the alkaline gel.

Fig. 8. Length distribution of native ADV M RF DNA. ADV M RF DNA was purified by sucrose gradient centrifugation and prepared by mica adsorption for electron microscopy. The peak at 10 kb represents the circular PM2 molecules which were used as an internal standard.

leading to a faster migrating smear was observed only in front of the v DNA band. The primary targets of S1 nuclease are evidently the terminal hairpins of the RF DNA. Their cleavage by S1 nuclease leads to the fast conversion of Mccl RF DNA to 2v DNA and further conversion to v DNA.

ExoIII and λ-Exo digestion of ADV M RF DNA
ADV RF DNA was digested with λ-Exo for 0, 20, 40 and 80 min at 37 °C. The ADV DNA reaction products were analysed either on neutral or alkaline gels and detected by Southern blot hybridization. λ-Exo degraded only the viral ssDNA resulting in a loss of approx. 200 bp. In order to rule out possible enzyme inhibition, HindIII-digested λ DNA was included and the assay repeated. Again, ADV RF DNA was not affected by the enzyme, whereas the λ DNA
Fig. 9. Electron micrographs of partially or completely denatured ADV M RF DNA. ADV M RF DNA purified by neutral sucrose gradient fractionation was denatured with formaldehyde and prepared by the cytochrome spreading procedure. (a) Partially denatured molecules were obtained by incubation for 5 min at 80 °C in 6 mM-sodium phosphate in the presence of 1.2% formaldehyde, and spreading from 30% formamide. (b) Fully denatured DNA was prepared by incubation for 10 min at 85 °C in 2 mM-sodium phosphate in the presence of 1.0% formaldehyde, and spreading from 50% formamide. The molecule to the right is an ADV Mcl RF molecule as a completely denatured covalently closed ssDNA circle. The molecule to the left is ds PM2 DNA added as a length reference. Bar marker represents 1 μm.

fragments were hydrolysed. This result indicated that ADV RF DNA contained no free 5' termini. In contrast, the virion ssDNA had a free 5' terminus.

ExoIII on the other hand exhibited a specific reaction pattern with ADV M RF DNA. Electrophoresis under neutral conditions revealed (Fig. 7a) that M RF DNA was rapidly degraded, leaving only small amounts of molecules of 4.6 and 4.8 kb intact, which were resistant
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to longer enzyme exposure. Additional bands of approx. 3.2, 3.0 and 2.0 kb appeared as a result
of the enzyme digestion. Separation on alkaline gels showed that only the Mccl RF DNA
remained unaffected compared to the untreated sample (Fig. 7b). The v and 2v DNA bands
were gradually reduced in DNA content. During enzymic digestion, an additional band of about
2300 nt appeared. In Hirt extracts all ADV RF DNA species except for a minor part of the
4.6 kb M and the D RF DNA were susceptible to ExoIII digestion (data not shown).

Electron microscopy of ADV M RF DNA

ADV M RF DNA was purified by preparative sucrose gradient centrifugation and analysed
by electron microscopy. The length distribution of native ds M RF DNA prepared by
adsorption to mica is shown in Fig. 8. The peak at 10 kb (Stüber & Bujard, 1977) represents the
circular PM2 molecules which were used as an internal standard (14 molecules had a size of
3.412 ± 0.024 μm, s.d. 0.7%). The average size of 50 molecules between 4.4 kb and 4.8 kb was
found to be 4.604 ± 0.096 kb, with an s.d. of 2.0%.

In native DNA preparations either derived from ADV M RF DNA or from whole Hirt
extracts, no circular dsDNA molecules were observed as described for MVM (Bratosin et al.,
1979). A small fraction of the non-denatured ADV M RF DNA exhibited Y-shaped ‘rabbit ears’
on one genome end. The length of these dsDNA regions varied between 110 and 180 bp. If the
ADV M RF DNA was denatured with formaldehyde (Inman, 1966) circular structures were
observed (Fig. 9). When partially denatured, many molecules of the M RF size display terminal
ssDNA loops (Fig. 9a). Such terminal loops resist even conditions leading to complete
denaturation of dsDNA. Whereas the majority of the M RF molecules were converted to linear
ssDNA molecules of v and 2v length, approx. 5 to 10% were seen as ssDNA circles of 2v length
(Fig. 9b). To demonstrate that they are of twice the genomic length, selected completely
denatured molecules were measured and compared with the non-denatured PM2 DNA: 36
linear molecules of 4813 ± 488 nt, 17 linear molecules of 9640 ± 566 nt and 17 circles of
9637 ± 477 nt. The values determined for the ssDNA molecules can only be an approximation
because the formaldehyde-treated DNA will have a slightly different number of base pairs per
unit length than the native PM2 DNA.

DISCUSSION

We have demonstrated in this paper that approximately 5 to 10% of the ADV M RF DNA
molecules exist as continuous, self-complementary polynucleotide chains with the virion and
c-strands covalently linked by hairpins at both ends of the duplex structures. This form was
designated ‘monomer covalently closed linear RF DNA’ (Mccl RF DNA). To our knowledge
this is the first time that the Mccl RF DNA has been detected in the intracellular DNA pool of
productively replicating parvoviruses. The Mccl RF DNA was visualized as ssDNA circles of
twice the genomic length by electron microscopy of ADV M RF DNA under denaturing
conditions. The discovery of partially denatured molecules with terminal ssDNA loops rules out
the possibility that the ssDNA circles could be the result of intermolecular reassociation via self-
complementary hairpin structures. These structures are not derived from denatured circular or
lasso-like DNA forms because those forms are not present in whole Hirt extracts from ADV-
infected cells. Bratosin et al. (1979) have described such molecules in MVM-infected cells by
harvesting the DNA with a Triton extraction procedure. The generation and function of the
lasso-like and circular dsDNA in MVM-infected cells remains unresolved. Studies using the
above mentioned Triton extraction procedure for ADV DNA are in progress.

When RF DNA was analysed by alkaline gel electrophoresis the Mccl DNA was observed to
migrate more slowly than the 2v ssDNA. The 2D technique revealed that under non-denaturing
conditions the Mccl RF DNA migrates together with the faster M RF DNA band, in which
both terminal hairpin structures are preserved (Löchelt & Kaaden, 1987).

In alkaline gradients the Mccl RF DNA molecule had a sedimentation value about 10% higher than the corresponding linear 2v DNA, a difference which is expected for circular and
linear molecules of the same size (Vinograd & Lebowitz, 1966). This was also observed by
Bourguignon et al. (1976) who synthesized Mccl RF DNA in vitro using the MVM virion ssDNA as a template–primer for Escherichia coli DNA polymerase in the presence of DNA ligase.
The slow migration of the Mccl RF DNA molecule in alkaline gel electrophoresis depends completely on the integrity of the two hairpin termini. Due to its structure, the Mccl RF DNA is unaffected by treatment with either ExoIII or λ-Exo nucleases. The ss-specific cleavage of the termini with S1 nuclease is sufficient to convert the Mccl RF DNA to a linear dsDNA molecule which migrates in alkaline gels corresponding approximately to the size of 2v or v DNA, depending on whether one or both terminal hairpins are digested.

The alkaline gel electrophoresis of the ADV M RF DNA restriction fragments extends these findings and shows that both termini can exist as covalently closed hairpins. This occurs approximately twice as often at the 3' terminus than at the 5' terminus, as estimated by the concentrations of ss double-length restriction fragments after HindIII and BamHI digestion.

The sub-ν DNA molecule, approx. 300 nt (or 160 bp) smaller than the genome length viral ssDNA, is evidently characterized by the loss of the 5' terminal hairpin sequence. This assumption is supported by the analysis of restriction fragments. Only cleavage with BamHI results (apart from molecules with one intact hairpin) in two DNA fragments, one with the complete 5'-terminal sequence (4080 nt) and the other lacking this sequence (3770 nt). The smaller fragment, as discussed by Ward & Dadachanji (1978), is generated by a nick at the end of the 5' hairpin structure. An analogous subfragment for the 3' terminus, lacking about 140 nt (corresponding to the length difference of about 70 bp), was not detected. This finding, together with the result that both terminal configurations on either genome end exist in equal amounts (Löchelt & Kaaden, 1987), indicates that approximately 50% of the 5' hairpins contain a nick whereas none or very few nicks are present in the 3' hairpin. These observations together with the detection of nicks in the 3' palindrome configuration of the D RF DNA (2D analysis) constitute the first experimental evidence for the hypothetical substrate specificity of the nickase (Astell et al., 1983a) which suggested that the 5' hairpin and the 3' palindrome of the D RF DNA bridge fragment are cleaved whereas the 3' hairpin is resistant due to some mismatched bases in the recognition sequence. In this context it is noteworthy that one of the three postulated recognition sites of the MVM nickase is completely conserved and one moderately so in the 3' terminal sequence of ADV (derived from Bloom et al., 1988; Astell et al., 1983a).

Many features observed in prototype parvovirus MVM DNA replication have also been found in the ADV system so that it seems justifiable to assume a common mechanism of replication for both viruses. In both systems, terminal length heterogeneities of M RF DNA related to the configuration of the hairpins were observed (Chow et al., 1986; Löchelt & Kaaden, 1987). The observed linkage between plus and minus strands by hairpins and the existence of subterminal nicks in MVM DNA (Ward & Dadachanji, 1978) are fully applicable to ADV. As in ADV replication, M, D and higher oligomeric RF DNA forms were found in MVM, which released DNA molecules of genome length and multimers after denaturation (Ward & Dadachanji, 1978). In that study the MVM DNA was heat-denatured and subsequently run on neutral gels. Thus, Mccl RF DNA could not be detected, because the complementary strands of both Mccl RF DNA and 2v DNA renatured immediately under these conditions. Neither were 3v DNA nor higher oligomeric forms of MVM ssDNA detected after denaturation. It is possible that the higher degree of linkage observed in our system may reflect a lower activity of the postulated nickase in relation to the ADV RF DNA, perhaps resulting from the conditions required to propagate ADV in cell culture i.e. heterologous feline cells and incubation at 32 °C (Porter et al., 1977). These conditions may increase the ratio of ligated DNA to nicked DNA in the ADV RF DNA pool and allow even the detection of the Dccl RF structures.

Similar to other parvoviruses (Chow et al., 1986; Wobbe & Mitra, 1985; Cotmore & Tattersall, 1988) ADV appears to have a covalently linked protein at the 5' termini of RF DNA. This is implied by the resistance of RF DNA to λ-Exo digestion. An additional hint is the almost complete absence of M RF molecules with extended hairpins after Hirt extraction without proteinase digestion (M. Löchelt & O.-R. Kaaden, result not shown).

All the results presented here for ADV are consistent with the DNA replication model for MVM (Cotmore & Tattersall, 1987). The major reason for their modification of the rolling hairpin scheme is based mainly on the detection of 18 additional bp at the extended 5' termini of
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MVM M RF DNA (Astell et al., 1983a, b, 1985). Although this finding has yet to be confirmed for ADV, our data support the proposed replication model (Cotmore & Tattersall, 1987) from our experimental results. The incoming virion DNA is converted to ds M RF DNA without displacement of the 5' hairpin. The nick between the newly synthesized c-strand and the 5' hairpin is then sealed by a DNA ligase. The resulting early intermediate, the Mccl RF molecule (designated collapsed closed circular DNA for MVM) serves subsequently as a substrate for the postulated nickase. The nickase should recognize a defined 5'-subterminal site on the Mccl RF DNA, cleave the c-strand and possibly remain covalently attached to the newly created 5' end. This process initiates the displacement synthesis of the foldback hairpins from the new free 3' end resulting in the regeneration of the 5'-terminal sequence elongated by 18 additional bp (MVM). The conversion of the M RF DNA to the D RF DNA, most likely initiated at the 'rabbit ear' structure seen by electron microscopy (Singer & Rhode, 1977), results in the generation of the 3' palindrome sequence which is subsequently cleaved by the nickase. This process initiates the dissociation of the D RF DNA into two M RF DNA molecules via displacement synthesis.

Poxviruses, possessing linear dsDNA with covalently closed hairpin ends as their genome (Baroudy et al., 1982), seem to use related mechanisms for telomere replication. After infection, a vaccinia virus-encoded endonuclease introduces nicks at both termini, destroying the covalent linkage between the DNA strands (Pogo, 1980). Recent experiments revealed that this endonuclease or a similar enzyme requires a special topological conformation of the substrate for its activity (Lakritz et al., 1985). In paroviral DNA replication the non-structural protein NS1 may be a candidate for the postulated nickase. The covalent linkage of the NS1 to both extended termini of MVM RF DNA was recently demonstrated (Cotmore & Tattersall, 1988). The mechanisms discussed for paroviral and poxviral DNA replication may have a more general bearing on the problem of the preservation of terminal sequences, e.g. the telomere replication of eukaryotic chromosomes (Cavalier-Smith, 1974; Cooke, 1987).

To investigate the mechanism of ADV DNA replication further, studies on the kinetics of the Mccl RF DNA and on its association with the different stages of DNA replication in vitro and in vivo are presently being performed.

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