The monomer covalently closed linear replicative form DNA is an intermediate of Aleutian disease parvovirus DNA replication

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In this report we present data indicating that the recently detected monomer covalently closed linear replicative form DNA (Mcl RF DNA) is an intermediate of Aleutian disease virus (ADV) DNA replication. This DNA molecule is characterized by covalently closed terminal hairpins on either end of the linear ds genomic molecule. Its first detection early after infection in vitro, the association with ADV-specific replication complexes and the de novo synthesis of Mcl RF DNA by isolated replication complexes point to an important role in parvoviral DNA replication. The presence of the Mcl RF DNA in extracts of virus-producing bone marrow cells of naturally infected mink rules out the possibility that this DNA form represents a mere cell culture artefact. The detection of the ADV non-structural protein 1 (NS1) in replication complexes supports the view that this protein is involved in ADV DNA replication.

Introduction

Aleutian disease virus (ADV) is an autonomously replicating parvovirus (Bloom et al., 1980) that induces a persistent infection in mink of certain genotypes. The targets in vivo are lymphoid cells in mesenteric and peripheral lymph nodes, spleen, bone marrow and peripheral blood (Bloom et al., 1985; Roth et al., 1984; Kaaden et al., 1986; Haas et al., 1988) and alveolar type II cells in mink kits infected neonatally (Alexandersen & Bloom, 1987).

The nuclear replication of autonomous parvoviruses is initiated by the conversion of the ss genome into ds replicative form (RF) DNA of genomic length (monomer RF DNA). This process relies on a self-priming DNA synthesis, starting at the 3'-terminal hairpin of the genome. At present it has not been resolved whether the DNA polymerase stops at the second 5'-terminal hairpin during monomer RF DNA synthesis or whether the hairpin sequence is fully replicated via displacement synthesis (Astell et al., 1983, 1985). A recently published replication model based on findings for the prototype parvovirus minute virus of mice (MVM) (Cotmore & Tattersall, 1987) predicts the first mechanism. It is then suggested that a DNA ligase seals the nick between the newly synthesized cDNA strand and the viral 5' hairpin, resulting in a self-complementary circular DNA of twice the genomic length. This molecule, designated covalently closed linear RF DNA (Mcl RF DNA) should subsequently serve as the substrate for a site-specific nickase, providing a new 3' hydroxyl end for the complete regeneration of the genome termini. Mcl RF DNA molecules were recently detected in the RF DNA pool of cell cultures productively infected with ADV (Löchelt et al., 1989) or MVM (Cotmore et al., 1989).

In this paper we present data indicating that the ADV Mcl RF DNA is a true intermediate in parvoviral DNA replication, as implied by its appearance early after infection in vitro and its presence in the RF DNA pool derived from bone marrow cells of mink naturally infected with ADV. Additionally, the analysis of nuclear extracts revealed that the Mcl RF DNA is present in parvoviral 40S replication complexes and is synthesized by these structures in vitro. The association of the non-structural protein (NS1) with the replication complexes suggests its involvement in DNA replication, possibly as the so far hypothetical nickase.

Methods

Cells, ADV propagation and purification. The propagation of ADV SL3 clone E10 (Löchelt et al., 1989, kindly provided by B. Stolze) in cell cultures of feline kidney CCC clone 81 cells at 32 °C was done as described (van Dawen et al., 1983). ADV RF DNA was Hirt-extracted, as described (Hirt, 1967; Löchelt & Kaaden, 1987). Infectious ADV virions free of contaminating ADV RF DNA were prepared according to the method of Löchelt & Kaaden (1987).
DNA techniques. The isolation of ADV RF DNA from infected cell cultures (Löchelt & Kaaden, 1987) and organs of naturally infected mink (Haas et al., 1988) and the release of viral ssDNA from infectious virions (Löchelt & Kaaden, 1987) have been described. DNA electrophoresis on neutral or alkaline agarose gels was performed as published (Löchelt et al., 1989). Southern blot hybridizations using a cloned central 73% genome fragment of ADV SL3 as a 32P-labelled probe were done as described by Löchelt & Kaaden (1987). Agarose gels were dried on Gel-fix (Serva), with several changes of absorbent paper.

ADV DNA replication kinetics. Subconfluent CCC clone 81 cells (in 25 cm² plastic flasks) were infected with purified virions at an m.o.i. of 10. After 2 h at 32 °C non-adsorbed virus was washed off, fresh medium added and the cultures were further incubated. Samples were withdrawn at regular intervals for DNA extraction and analysed on neutral and alkaline gels. ADV DNA was detected by Southern blot hybridization.

Preparation and analysis of nuclear lysates. CCC clone 81 cells were infected with ADV SL3 at an m.o.i. of 10 and mock-infected cells were used as controls. After 28 or 42 h cell were harvested and lysed with 0.1% NP40. Nuclei were then sedimented at a low speed and leached for 3 h at 0°C in 10 mM-HEPES/KOH pH 7.8, 5 mM-KCl, 1 mM-EDTA, 0.1% NP40 and 0.2 M-NaCl. Chromatin was sedimented at 8000 g (10 min, 4 °C). The nuclear lysate was then collected and centrifuged through 5 to 20% sucrose in leaching buffer without NP40 at 164000g (65 min, 4 °C), according to the method of Doerig et al. (1986).

The fractions (18 or 19) obtained after bottom puncture of the tube were assayed by the following four methods.

ADV infectivity was measured by titration of a sample from each fraction for viral infectivity (van Dawen et al., 1983) and the infectivity was expressed as f.f.u./ml gradient fraction.

ADV RF DNA was assayed using proteinase K digestion (Boehringer, 400 µg/ml) for 1 h at 50°C. The DNA was ethanol-precipitated, resuspended in water and analysed on neutral and alkaline gels.

Samples of the gradient fractions were precipitated with 5 vol 99% ethanol and analysed by Western blotting, as described (Löchelt & Kaaden, 1987), except that 20% (v/v) methanol was included in the transfer buffer (Towbin et al., 1979). ADV-specific proteins were identified by immune detection using either a polyclonal serum or monoclonal antibodies that recognized the virion proteins (VP1, 85K and VP2, 75K) or the non-structural proteins (NS1, 71K and p143, 143K; Porter et al., 1984; Kierek-Jaszczuk & Kaaden, 1986).

DNA polymerase activity was assayed by the method of Doerig et al. (1986) with minor modifications. The labelled nucleotide used was [32P]dCTP (400 Ci/mmol; Amersham). The reactions (in a total volume of 60 µl) were incubated for 1 h at 37 °C and stopped by the addition of EDTA, to a final concentration of 90 mM. As indicated, purified viral ssDNA (1 ng) or ATP (1 mM) were added. The samples were digested with proteinase K and precipitated in the presence of carrier DNA. Reaction products were separated on neutral and alkaline agarose gels, transferred onto nitrocellulose membranes and directly exposed. ADV RF DNA separated in parallel and detected by Southern blot hybridization was used as an internal marker.

Results

ADV DNA replication kinetics

All experiments were performed using CCC clone 81 cells incubated at the permissive temperature of 32 °C. The cells were infected with purified virions, free of ADV RF DNA species as demonstrated by Southern blot analysis. At regular intervals post-infection (p.i.) low Mv DNA was extracted and purified by standard techniques. The DNA was analysed on either neutral or alkaline gels and detected by Southern blot hybridization. Neutral gel electrophoresis was used to monitor the kinetics of ADV RF DNA synthesis. Alkaline gels showed the extent of covalent cross-linkage between ss genome equivalents (v DNA), giving rise to oligomeric forms (e.g. 2*v, 3*v DNA) and the ccl RF DNA species.

According to these criteria, ADV DNA replication started at about 12 h p.i. (not shown). The first ADV RF DNA species detected corresponded to the monomer and dimer RF DNA, as well as the virion ssDNA originating from the inoculum. The concentration of all ADV DNA species increased constantly during the incubation period up to 96 h. From 48 h p.i. the increment was retarded.

Under alkaline electrophoresis conditions multimers of up to four genome equivalents (2*v, 3*v and 4*v) were first detected 16 h p.i. (not shown). Using Southern blot hybridization the Mccl RF DNA was also detectable as a faint band and on longer incubation the concentrations of all DNA species and higher oligomeric forms increased. This rise was more pronounced for the ccl molecules than for the other DNA forms (2*v, 3*v DNA etc.).

ADV-specific activities in nuclear extracts from infected cells

Nuclear extracts were prepared from ADV- and mock-infected cells harvested at 28 and 42 h p.i. The extracts were subsequently separated by sucrose gradient centrifugation and each fraction was analysed by different criteria.

(i) Viral infectivity. The infectivity profile of a representative gradient run with nuclear extracts harvested at 42 h p.i. is shown in Fig. 1. The maximum infectivity was detected in fraction 7 at about 110S (determined according to Doerig et al., 1986) and a minor peak of infectivity was in the range of about 70S. A background of infectivity was present throughout the gradient, probably reflecting the mild extraction conditions. The infectivity in extracts harvested 28 h p.i. had a lower titre by about 0.5 log,10.

(ii) ADV-specific proteins. Samples of each gradient fraction were assayed for the presence of ADV-specific proteins by Western blot analysis. Fig. 2 presents the data from an extract harvested 42 h p.i. and assayed with a serum pool from mink naturally infected with ADV. SDS extracts from ADV-infected cells (Kierek-Jaszczuk
Fig. 1. Distribution of ADV-specific infectivity of fractionated nuclear extracts harvested 42 h p.i. The titre corresponding to each fraction is given as log10 f.f.u./ml gradient fraction.

Fig. 2. Western blot analysis of ADV-specific proteins of a fractionated nuclear extract prepared 28 h p.i. Samples of 45 µl of each gradient fraction (beginning at the left with the bottom) were separated on a 10% polyacrylamide gel. In lane E 50 µl of an SDS-extracted sample of ADV-infected clone 81 cells was analysed as a marker. An ADV-specific pooled serum from naturally infected mink was used for the immune detection. The positions of viral proteins are given at the right.

et al., 1986) served as a control for the specificity of the reaction.

The capsid components VP1 and VP2 always appeared together, with a maximum in fraction 10. Traces were also present up to fraction 6 and at higher concentrations at the top of the gradient. NS1 was present in fractions 14 to 18, with a prominent maximum in fraction 16. In fractions 15 to 17 p143, the putative dimeric form of NS1 (Porter et al., 1984), was detected using the NS1-specific monoclonal antibodies ADV-Hyl and ADV-Hy2 (Kierek-Jaszczuk et al., 1986) after SDS-PAGE had been done under reducing conditions. Extracts harvested 28 h p.i. contained significantly lower amounts of the ADV capsid components.

(iii) ADV-specific DNA. DNA samples of fractionated nuclear extracts from ADV-infected cells harvested 42 h p.i. were analysed on neutral and alkaline gels, as mentioned. The neutral DNA electrophoresis (not shown) revealed the presence of viral ssDNA in fractions 6 to 8 and at the top of the gradient. ADV monomer RF DNA reached high concentrations in fractions 6 to 8, 14 and 15. The latter two fractions contained additional ADV dimer RF DNA, but other ADV-specific DNA molecules were not detectable. Samples harvested at either 28 or 42 h p.i. differed mainly in the higher DNA content late in infection.

The alkaline gel electrophoresis of specimens harvested 42 h p.i. (Fig. 3) displayed a complex pattern of ADV-specific DNA forms. ADV v DNA reached its maximum in fractions 7 and 8 and at the top of the gradient. The sub-v DNA, lacking the 5'-terminal hairpin sequence (Löchelt et al., 1989), was present in fractions 12 to 15. This part of the gradient contained 2*v and Mccl RF DNA, although traces of 2*v DNA also appeared from fractions 6 to 18. The 3*v and 4*v DNA were detectable in low concentrations in fractions 11 and 12 and 13, respectively. Significant differences from extracts harvested 28 h p.i. were the absence of molecules larger than Mccl RF DNA and the reduced DNA content early in infection.

Summarizing these results, in comparison to the data from Doerig et al. (1986) and known structural features of parvoviruses, the following conclusions can be drawn. Infectious mature virions have a sedimentation value of about 110S. These gradient fractions (6 to 8) are characterized by high infectivity, the maximum amount of viral ssDNA and the presence of both capsid proteins. Fractions containing empty capsids (10 and 11, corresponding to 70S) are characterized by a low level of infectivity, an almost complete absence of viral ssDNA, but large amounts of both structural proteins. The
putative replication complexes with about 30S to 40S (fractions 13 to 16) contain Mccl RF DNA, monomer and dimer RF DNA and low concentrations of viral ssDNA. Low amounts of all viral proteins are also present.

**Detection of DNA polymerase activity in ADV-specific replication complexes**

All gradient fractions were analysed for DNA polymerase activity by the synthesis of labelled high $M_r$ DNA. Nuclear extracts from ADV- and mock-infected cells were prepared at both 42 and 28 h p.i. and all fractions were assayed for DNA polymerase activity using endogenous templates.

Specimens derived from ADV-infected cells harvested 42 h p.i. showed incorporation of radioactivity in fractions 12 to 16. The majority of the DNA products had a length varying from 0.3 to about 2 kb and appeared as a broad smear after analysis on neutral gels. Additionally, distinct DNA bands comigrating with ADV monomer RF DNA (M in Fig. 4) appeared in fractions 12 to 16 and a band equivalent to dimer RF DNA (D in Fig. 4) was found in fraction 14. The maximum synthesis of these ADV-specific DNA species was seen in fraction 14. Analogous results were obtained with ADV-infected cells harvested 28 h p.i.

The gradient fractions capable of DNA synthesis correspond well with the assumed position of ADV replication complexes in fractionated nuclear extracts from ADV-infected cells. Samples from mock-infected cells showed no accumulation of DNA polymerase activity throughout the gradient. After electrophoretic separation of these samples and autoradiography of the dried gel a very faint smear of label was visible in all fractions, but specific DNA bands could not be detected.

In **vitro ADV DNA synthesis by replication complexes in the presence of ATP and exogenous viral ssDNA**

Owing to the absence of ADV-specific DNA bands in extracts from mock-infected cells the reaction products of monomer and dimer RF DNA were considered to be
Fig. 6. Alkaline gel electrophoresis of a DNA polymerase assay of ADV-specific replication complexes harvested 42 h p.i. The samples correspond to fraction 14 of the material analysed in Fig. 4. They were incubated without (a) or with (b) ATP. The asterisk in (b) indicates the position of ADV Mccl RF DNA.

virus-specific. The synthesis products with a lower $M_r$ of about 0.3K to 2K appeared to be either virus-specific, or at least virus-induced and after addition of viral ssDNA the concentration of these products was significantly increased. Thus this material probably represented viral premature termination products rather than resulting from the extension synthesis of repair processes on cellular DNA templates.

Replication complexes derived from cells harvested 28 h p.i. were analysed for DNA polymerase activity in either the presence or absence of 1 mM-ATP or exogenous viral ssDNA (to a final concentration of 1 ng/60 µl). The addition of ATP alone had no obvious effect, as judged by neutral gel electrophoresis. The inclusion of viral ssDNA as an exogenous template resulted in an increase in overall DNA synthesis. However, the application of both components together yielded an additional enhancement of polymerase activity (Fig. 5b) and, as well as the known synthesis products (monomer and dimer RF DNA), a band corresponding to viral ssDNA was also labelled. This labelling began at the viral ssDNA band and extended up to the higher $M_r$ range.

Analysis of in vitro DNA synthesis products under alkaline conditions and detection of newly synthesized Mccl RF DNA

To demonstrate that the Mccl RF DNA is not only an inert constituent of the replication complexes, the in vitro synthesis products described above were also analysed on alkaline gels.

In the absence of ATP, labelled ADV DNA bands corresponded to $v$ and $2^*v$ DNA as well as the high amount of low $M_r$ reaction products, irrespective of the time of harvest. DNA synthesis in the presence of 1 mM-ATP yielded additional ADV-specific bands, as verified by their electrophoretic mobility. Mccl RF DNA was consistently synthesized by replication complexes harvested 42 h p.i., and was detectable as a faint but distinguishable band against the background (Fig. 6b). Higher oligomeric forms of $3^*v$ and possibly $4^*v$ DNA were only synthesized by replication complexes harvested at 42 h p.i. and appeared exclusively in replication complex-containing fractions with a slightly higher $S$ value.

Replication complexes harvested 28 h p.i. yielded Mccl RF DNA only after addition of viral ssDNA. This observation obviously reflects the low concentration of template DNA early in infection.

Detection of Mccl RF DNA in bone marrow samples of naturally infected mink

DNA samples derived from naturally infected mink of non-Aleutian genotype were analysed on alkaline gels and probed by Southern blot hybridization. Mccl RF DNA was detectable in bone marrow samples (Fig. 7) known to contain ADV RF DNA (Haas et al., 1988). In organs with low amounts of RF DNA, such as spleens and the lymph nodes (Haas et al., 1988), Mccl RF DNA could not be demonstrated.

Discussion

In this paper we present data indicating that the Mccl RF DNA of ADV is an intermediate in parvoviral DNA replication. A linear ds molecule of genome length with covalently closed terminal hairpins on either end was postulated to be an intermediate in MVM DNA replication, accounting for the presence of additional bases at the 5' end of MVM monomer RF DNA (Cotmore & Tattersall, 1987). They are complementary to sequences upstream of the 5'-terminal hairpin and should be generated as follows. The newly synthesized c-strand, linked to the 3' terminal hairpin of the incoming viral ssDNA, is covalently sealed to the 5' hairpin. A
to represent the recognition sites of the nickase (Astell et al., 1983). The suggestion that only one nickase is involved in replication is strengthened by the demonstration of a covalent linkage of MVM NS1 to both palindromic ends of MVM RF DNA via the terminal 5' hydroxyl groups (Cotmore & Tattersall, 1988). The nickase may also be able to self-excite from the 5' ends of RF DNA by the concomitant loss of the added bases, thus generating a template of genome length for the displacement synthesis of virion progeny DNA, as outlined by Cotmore & Tattersall (1987). Although the demonstration of additional bases at the 5' end of ADV RF DNA is still lacking we can assume a similar situation for ADV (Löchelt et al., 1989).

The previous detection of the ADV and MVM Mccl RF DNA (Löchelt et al., 1989; Gunther & Tattersall, 1988; Cotmore et al., 1989) and the data presented here support the view (Cotmore & Tattersall, 1987) that Mccl RF DNA is a common intermediate in parvoviral DNA replication. This is indicated by its presence in and synthesis by purified ADV-specific replication complex early and late in infection in vitro. This finding implies that a DNA ligase is a necessary constituent of parvoviral replication complexes, since such an activity is a prerequisite for the generation of Mccl RF DNA. Furthermore, the biological significance of Mccl RF DNA is strengthened by its detection in bone marrow samples derived from mink naturally infected with ADV.

The extensive accumulation of Mccl RF DNA in the ADV system may be related to the use of heterologous cells at a permissive temperature of 32 °C for ADV propagation in vitro. Thus, the presence of Mccl RF DNA in detectable amounts in bone marrow cells of infected mink may likewise reflect suboptimal conditions for virus growth at this site in vivo and may have some implications in ADV persistence (Alexandersen et al., 1988). Preliminary results from the comparison of ADV replication at the permissive and non-permissive temperatures of 32 °C and 37 °C, respectively, showed an enhanced and accelerated RF DNA synthesis and an obvious increase in Mccl RF DNA and DNA forms with a higher linkage degree between viral and c-strands at the non-permissive temperature.

As well as these ADV-specific features the elucidation of the nickase function, assumed to act on the Mccl RF DNA, is of more general interest in parvoviral DNA replication. As pointed out the MVM NS1 may be covalently attached to both 5' ends of the RF and viral ssDNA (Cotmore & Tattersall, 1988). 5'-Terminal bound protein(s) have also been described for H-1 parvovirus (Wobbe & Mitra, 1985) and ADV (Löchelt et al., 1989).

In this study we were able to demonstrate NS1 and its probable dimeric form p143 (Porter et al., 1984) in

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**Fig. 7.** Detection of Mccl RF DNA in bone marrow cell (BMC) Hirt extracts (20 μg in each lane) on an alkaline gel. The ADV-specific DNA was detected by Southern blotting. ADV RF DNA and purified monomer RF DNA (M RF DNA) served as controls.

hypothesized nickase (possibly NS1 of autonomous parvoviruses) then introduces a nick upstream of the original 5' hairpin (18 bp inboard for MVM). Thus a new primer for the complete replication of the 5' end, resulting in the terminal addition of some bases to the palindromic, extended form is provided.

The nicking event upstream of the viral 5' hairpin accounts for some additional features of parvoviral DNA replication. Only one nickase is necessary for both termini, as postulated by Astell et al. (1983), both cleaving 5'-terminal configurations and exclusively attacking the 3' palindromic sequence in the dimer RF DNA, as discussed for ADV (Löchelt et al., 1989). Conserved sequence motifs at both termini are assumed
replication complexes isolated from ADV-infected cells. This result is a direct hint that the NS1 (or its dimeric form p143) is indeed involved in DNA replication, as previously suggested for H-1 parvovirus (Rhode & Paradiso, 1989) and MVM (Cotmore & Tattersall, 1987). The kinetics of MVM NS1 transcription (Clemens & Pintel, 1988) supports the view that it is synthesized prior to the burst of RF DNA synthesis to re-initiate the replication of Mccl RF DNA via site-specific nicking. Thus the Mccl RF DNA, provided that the nickase is identical to NS1, should be the first template for NS1 mRNA synthesis. The DNA replication-deficient mutants of H-1 parvovirus (Rhode & Paradiso, 1989) may be characterized by this reinitiation defect due to a lack in nuclear translocation of NS1.

The failure of our kinetic studies to demonstrate the Mccl RF DNA as the initial intermediate may be due to the use of randomly growing cells, the fragility of the Mccl RF DNA, or the sensitivity of the detection system. However, this failure is paralleled by the investigations of Cotmore et al. (1989) and is more likely to be related to the very low concentration of Mccl RF DNA in the initial phase of replication. To establish the intermediate nature of this species, kinetic studies using synchronized clone 81 cells are under way. Furthermore, by transfection experiments the metabolic function of Mccl during the ADV DNA replication will be investigated.

The Mccl RF DNA detected during this study may be created during the dissociation of the dimer RF DNA, as outlined in the modified replication model (Fig. 8, step...
11), based on that postulated by Cotmore & Tattersall (1987). Central points are the existence of Mccl RF DNA, the identity of the nickase with the parvoviral NS1, the specificity of the nickase, as postulated by Astell et al. (1983) and shown for ADV by structural analysis of the RF DNA species (Löchelt et al., 1989) and the fact that both terminal configurations exist in equal amounts. The latter point is fulfilled for ADV (Löchelt & Kaaden, 1987), but in the case of MVM differences were observed (Ward & Dadachanji, 1978). So it seems that despite a large conformity in the replication of ADV and MVM, some differences exist. As depicted in the model, both alternatives for the dissociation of the dimer RF DNA are possible.

Additional observations, not directly concerned with the Mccl RF DNA, but important for parvoviral DNA replication can be summarized as follows. DNA biosynthesis by purified replication complexes can be extensively stimulated by the addition of ATP in the presence of exogenous viral ssDNA (excess of template). This finding, together with the immunodetection of two DNA polymerase alpha cross-reactive proteins of 220K and 230K (antisera provided by F. Grosse, data not shown), parallel the previous findings of Faust et al. (1984). Furthermore, the analysis of reaction products under alkaline electrophoresis conditions showed not only the synthesis of Mccl RF DNA in the presence of ATP, but also that genome-length reaction products are synthesized in the presence and absence of ATP. This was shown by the strong increase of 2*γ DNA after addition of ssDNA to the replication complexes. The synthesis of this DNA form was possible only by the complete generation of a cDNA strand linked to the template ssDNA molecule. The detection of 2*γ DNA molecules synthesized in the absence of ATP makes the involvement of discontinuous RNA-primed DNA synthesis unlikely, since Okazaki fragments could not be ligated under these conditions. Thus, the parvoviral DNA replication should be primarily regarded as a hairpin-primed continuous process.

The genome length reaction products should also be generated by a nicking event that unlinks the viral ssDNA template from the newly synthesized c-strand. To find out whether this process is specific, and possibly mediated by NS1 further investigations using the system described here are necessary.

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