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SHORT COMMUNICATION

DNA Polymerase δ-Dependent Formation of a Hairpin Structure at the 5′ Terminal Palindrome of the Minute Virus of Mice Genome

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The parvovirus the minute virus of mice (MVM) has a linear single-stranded DNA genome with unique palindromic sequences at both termini which enable it to fold back on itself and form hairpin or cruciform-type structures. The purpose of this study was to examine the primary events occurring during MVM replication mediated solely by the host cell replication machinery. In an *in vitro* DNA replication system using HeLa cell extracts, we found that there was a distinct activity that utilized the 5′ terminal palindrome sequence of MVM to produce a secondary structure from a duplex extended form, in a time-dependent fashion. The secondary structure was due to the formation of a hairpin rather than a stem-plus-arms type structure and was associated with initiation of DNA synthesis, performed specifically by DNA polymerase δ. Inhibition of DNA polymerase α had no effect upon this activity. Removal of all but 13 base pairs of the hairpin arm abolished the synthesis of DNA, indicating that there is a minimal length requirement for the duplex region of DNA or that this region contains regulatory genetic elements. These data are consistent both with the role of DNA polymerase δ in extending the synthesis of DNA from a DNA primer and with unidirectional continuous DNA synthesis, initiating from a hairpin, as a mode of replication for MVM.

The parvovirus minute virus of mice (MVM) has a linear, single-stranded DNA genome (the minus strand), approximately 5000 bp in size, with unique palindromic sequences at both termini which are capable of folding back on themselves to form hairpin (Fig. 1A (i), (iii), and (iv)) or cruciform-type structures (Fig. 1A (ii); *1*). One of the most widely accepted models for MVM DNA replication is the modified rolling hairpin model (*2*), which proposes that the initial step in replication is the formation of a hairpin at the left end, which is used to initiate the synthesis of a monomer duplex viral replicative-form (RF), covalently closed at the left end. This RF is amplified by subsequent rounds of DNA strand displacement synthesis, resulting in the production of dimeric and tetrameric RF intermediates, DNA synthesis being initiated from 3′-hydroxyl (3′-OH) ends generated by site-specific nicking. Subsequent resolution of concatemer junctions and packaging events result in a progeny genome with two alternate sequence orientations at the right end and no sequence inversion at the left end (*2–4*). The virus is entirely dependent upon host cell proteins for DNA replication, except for one essential virally encoded protein, NS-1 (*5, 6*). In this study we examined the primary DNA replication events dependent upon host cellular proteins alone using an *in vitro* DNA replication system (*7, 8*), utilizing HeLa cell extracts together with a full-length, fully infectious clone of the MVM genome (*9*) or with plasmids containing terminal regions or deletions of the MVM genome (Fig. 1B). To duplicate the linear nature of the genome as closely as possible, the input DNA was linearized by digesting pMM984 with *Bam*HI prior to the reaction, releasing the MVM coding sequence from the plasmid (Fig. 1B, pMM984).

To examine the site of initiation of DNA replication, a time course (5, 10, 15, 30, 45, and 60 min incubation) of *in vitro* replication was performed and the DNA products were digested with *Pst*I (Fig. 2). *Bam*HI-digested pMM984 yielded two radiolabeled DNA fragments, the top (5155-bp) band corresponding to the MVM coding sequence, and the bottom (3740-bp) band corresponding to the vector coding sequence (Fig. 2, *Bam*HI). All fragments were labeled to some extent due to the random incorporation of nucleotides during repair synthesis. With an increase in the length of incubation time, there was an increase in the intensity of labeling of all fragments. After an apparent lag time of approximately 15 min, a novel DNA species was produced with a rate of migration

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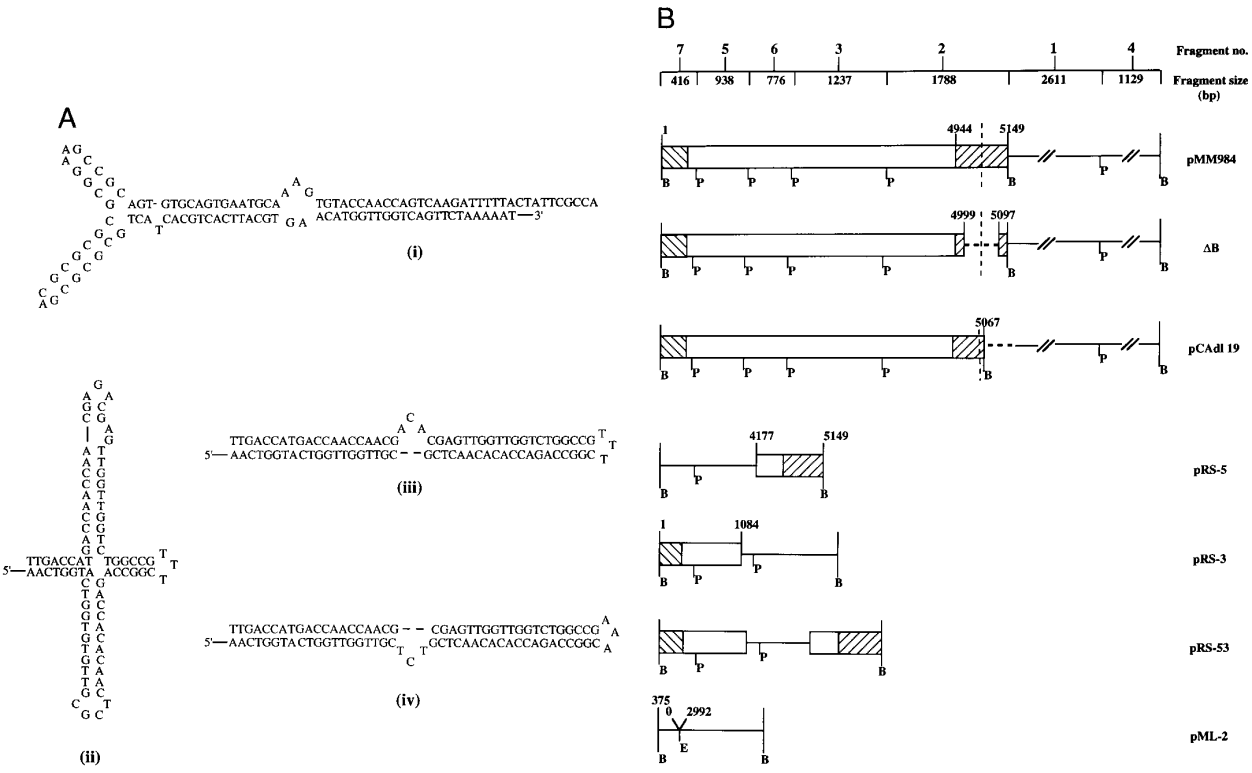

FIG. 1. (A) Alternate conformations of the 3′ and 5′ palindromic sequences at the termini of MVM. In addition to the lineform (extended) form of the palindromic sequences at the termini, the 3′ terminus can also form a rabbit-earred structure (i) while the 5′ terminus can form a stem-plus-arms structure (ii), or a hairpin structure in either the flip (iii) or the flop (iv) sequence orientation. (B) Diagram of plasmids containing different terminal regions or deletions of the MVM genome. Solid white blocks represent MVM sequences, with the terminal palindromes depicted in stripes. Thin horizontal lines represent vector sequences and deleted areas of the plasmid are shown as dashed horizontal lines. The dashed vertical line indicates the axis of symmetry of the 5′ terminal palindrome. Numbers above the diagram correspond to the MVM nucleotide sequence. Restriction enzyme sites are as follows: P, *Pst*I; B, *Bam*HI; E, *Eco*RI. The DNA fragments obtained from a *Pst*I, *Bam*HI digest of pMM984 are shown at the top. Fragment sizes are indicated beneath. The plasmid pMM984 is a fully infectious clone, containing the entire MVM coding sequence in a pBR322 background (*9*). The plasmids pRS-5, pRS-3, and pRS-53 contain portions of the 3′ and 5′ terminal regions of the MVM coding sequence in a pML-2 background (*12*). The plasmid ΔB contains a deletion around the axis of symmetry of the 5′ terminal palindrome and was subcloned into a pAT153 (*29*) vector. Plasmids were extracted by alkaline lysis of transformed bacterial SURE cells and purified by two cycles of isopycnic caesium chloride equilibrium density centrifugation (*11*).

approximately 100 bp faster than the 1788-bp 5′ terminus-containing *Bam*HI–*Pst*I fragment (Fig. 2, fragment 2). This novel DNA fragment was not visible on an ethidium bromide-stained gel (not shown) and by comparison to the other MVM fragments showed a marked increase in intensity with increased time of incubation (data not shown), indicating that it was labeled to a high specific activity. For comparison, a duplicate 60-min sample was incubated with 30 μM aphidicolin (Fig. 2, aphidicolin). The formation of the novel DNA was completely inhibited at this concentration of the drug, implying the involvement of DNA polymerases α and/or δ in the formation of the novel DNA species.

The fact that the novel DNA species migrated closely with that of the 5′ fragment and that there was an overall decrease in nucleotide incorporation of the 5′ terminus-containing fragment with a concomitant increase in that of the novel DNA suggested that the two DNA molecules were related to one another and that DNA synthesis *in vitro* might be initiating near the 5′ terminus of the viral genome. This finding is consistent with the observation made by Rhode and Klaassen (*10*) that the replication origin for the RF form of H-1 was localized to the 5′ terminus. To further test this, the MVM coding sequence was purified from the plasmid and tested alone in the *in vitro* replication system. The results (Fig. 3A) demonstrate that the novel DNA (asterisk) was produced from the MVM genome specifically. To confirm whether this activity was associated with either of the termini, plasmids containing just the 5′ and/or 3′ terminal regions of MVM were tested (pRS-5, pRS-3, pRS-53, see Fig. 1B). In the plasmids containing just the 5′ or both the 5′ and 3′ terminal regions (Fig. 3B, pRS-5, pRS-53) the novel DNA was produced, but in neither the plasmid containing just the 3′ terminal region (Fig. 3B, pRS-3) nor the control vector sequence (Fig. 3B, pML-2) was there any evidence for the production of the novel DNA, confirming the association of the novel band with the 5′ terminus. It is noteworthy that the HeLa cell extracts failed to catalyze rearrangement of the 3′ terminal palindrome. This may

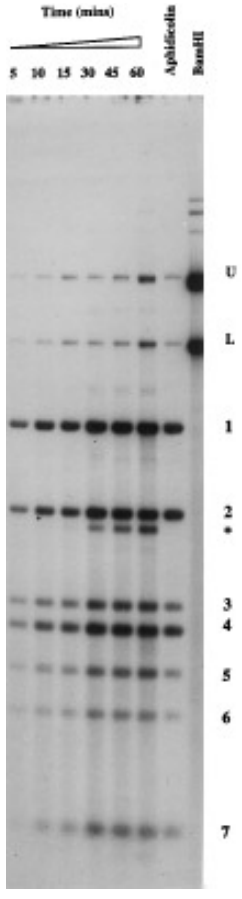

FIG. 2. *In vitro* DNA replication of the plasmid pMM984. The plasmid was linearized prior to the reaction with *Bam*HI and then used as a substrate in an *in vitro* DNA replication reaction. Cell extracts for the *in vitro* replication reaction were prepared from log phase HeLa S3 cells, grown in suspension, as described previously (*7*). The *in vitro* replication assay was performed as detailed elsewhere (*7*) with the following exceptions: 200 ng of template DNA was used for each reaction; the final concentration of dTTP and dCTP was 10 μM; and the reaction was supplemented with 10 μCi of both [α-^{32}P]dCTP and [α-^{32}P]dTTP, specific activity, 3000 Ci/mmol. The products of the reaction were digested with *Pst*I and separated by electrophoresis on a 1.5% agarose gel, fragment sizes as indicated in Fig. 1B. Lanes 1 to 6 represent increased length of incubation time in the *in vitro* reaction. The labeled fragments correspond to the following: 1, 2611 bp (vector sequence); 2, 1788 bp; 3, 1237 bp; 4, 1129 bp (vector sequence); 5, 938 bp; 6, 776 bp; 7, 416 bp; *, 1650-bp novel fragment. Lane 7 shows the effect of adding 30 μM aphidicolin to a sample incubated for 60 min. Lane 8 shows *Bam*HI-digested pMM984. U, upper 5155-bp fragment (MVM sequence); L, lower 3740-bp fragment (vector sequence).

have significance for MVM replication *in vivo,* as well, since current replication models postulate that only the 5′ end of MVM undergoes hairpin transfer to generate flip and flop conformations of the 5′ palindrome, whereas the 3′ end has a unique sequence (*1*). This implies that the 3′ ends of monomer RF molecules do not generate rabbit-earred intermediates *in vivo,* consistent with our *in vitro* observations.

The observations made so far indicated a time-dependent appearance of a novel DNA band from a linear, duplex molecule, associated with the 5′ terminus-containing fragment and migrating faster with a difference of approximately 100 bp. This behavior is consistent with an alternate conformation of the 5′ terminus, arising as

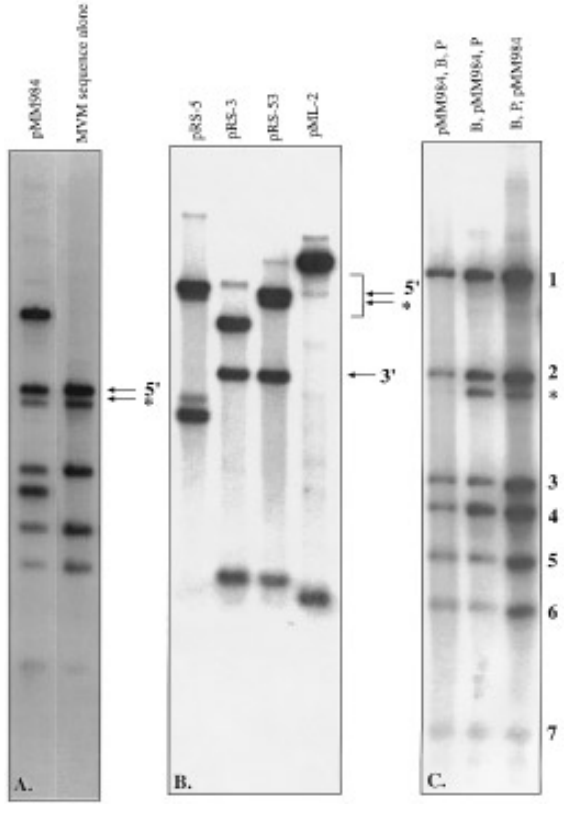

FIG. 3. *In vitro* DNA replication products of different terminal regions of MVM. (A) Lane 1 (pMM984) shows the typical pattern obtained after *Bam*HI-linearized pMM984 has been digested with *Pst*I. Lane 2 (MVM sequence alone) shows the MVM coding sequence purified from pMM984 and subsequently digested with *Pst*I. The MVM insert was purified from pMM984 by subjecting the products of a *Bam*HI digestion to electrophoresis on a 1.5% agarose gel, cutting out the appropriate band and purifying the DNA by isotachophoresis (*30*). The fragment containing the 5′ terminal palindrome sequence (5′) and the novel fragment (*) are indicated with arrows. (B) *In vitro* reaction products with plasmids containing either or both of the 5′ or 3′ termini of MVM. pRS-5 (lane 3) contains just the 5′ terminal region, pRS-3 (lane 4) contains just the 3′ terminal region, pRS-53 (lane 5) contains both the terminal 5′ and 3′ regions, with no intervening MVM coding sequences, and pML-2 (lane 6) contains no MVM sequences. All pRS plasmids were digested prior to the *in vitro* reaction with *Bam*HI, which linearizes the plasmid with the palindromic region(s) located at the ends (Fig. 1A). After the reaction, the plasmids were subjected to digestion with *Pst*I to separate the terminal regions from one another. pML-2 was digested with *Bam*HI prior to the *in vitro* reaction and afterwards with *Eco*RI. The appropriate digestion fragments containing the 5′ or 3′ terminal palindromes are indicated by an arrow. The novel fragment is indicated by an asterisk (*). (C) Comparison of linear to circular input DNA. In lane 1, the pMM984 plasmid was replicated *in vitro* as a circular molecule and digested afterwards with both *Bam*HI and *Pst*I. Lane 2, pMM984 digested prior to the *in vitro* reaction with *Bam*HI and then after the reaction with *Pst*I. Lane 3, pMM984 digested prior to the *in vitro* reaction with both *Bam*HI and *Pst*I. The novel fragment is indicated with an asterisk (*). B, *Bam*HI; P, *Pst*I.

a result of the palindromic nature of the sequences (Fig. 1B (ii)–(iv); *1*). If the novel band represented an alternate conformation of the 5′ terminus (which was subsequently involved in the initiation of DNA synthesis), then this reaction would be severely inhibited if either the terminus were not free to form a secondary structure or a terminal 3′-OH group were not free to allow initiation of DNA synthesis. This hypothesis was tested by using the undigested circular plasmid pMM984 as a template for DNA synthesis. In the sample which was digested only after the *in vitro* replication reaction (Fig. 3C, lane 1), no novel DNA was produced, while in samples in which the DNA was digested such that the right end of the plasmid was released, freeing the 3′-OH group (Fig. 3C, lanes 2 and 3), the putative novel structure was formed and DNA synthesis initiated. These data suggest that there is a requirement for a free 3′-OH group at the 5′ terminus of the duplex, that the sequences within the 5′ terminal 1788-bp *Bam*HI–*Pst*I fragment are sufficient, and that this is associated with initiation of synthesis and secondary structure formation.

To further examine the possibility that the novel DNA band represented a DNA molecule with secondary structure (Fig. 1A), the products of an *in vitro* replication reaction of plasmid pMM984 were subjected to electrophoresis on a two-dimensional (2D) neutral/alkaline gel (*11*). The results (Fig. 4A, top) showed that the novel DNA migrated approximately 100 bp faster than its duplex counterpart in the first dimension (as seen previously, Fig. 2), while in the second dimension, the duplex forms migrated on a smooth arc, and the novel band was clearly retarded, consistent with it being a secondary structure. A smaller retarded fragment, comigrating with the third largest *Pst*I fragment in the first dimension, was also detectable in the second dimension. The identity of this fragment is not known.

In order to differentiate between a potential hairpin and a cruciform structure, a mutant (ΔB) of pMM984 was utilized, which has a deletion around the axis of symmetry of the 5′ terminal palindrome (Fig. 1B). This deletion of 97 bp would not permit the formation of a stem-plus-arms cruciform structure but would allow the formation of a truncated hairpin (*1, 12;* Fig. 4B, bottom). The pattern obtained by 2D gel analysis (Fig. 4B, top) was similar to that obtained for the full-length clone (Fig. 4A, top). The difference in mobility of the novel DNA species seen for the ΔB mutant was consistent with the size of the deletion. The results, therefore, indicated that the novel DNA is most likely a hairpin and not a stem-plus-arms (cruciform) structure. The higher background (smearing) of products migrating on the second arc with plasmid ΔB (Fig. 4B), by comparison to the wild-type pMM984-derived MVM genome (Fig. 4A), was consistently seen with ΔB, regardless of the state of the DNA or the method of its preparation. It is possible that the deletion in ΔB alters replication initiation and elongation, resulting in a

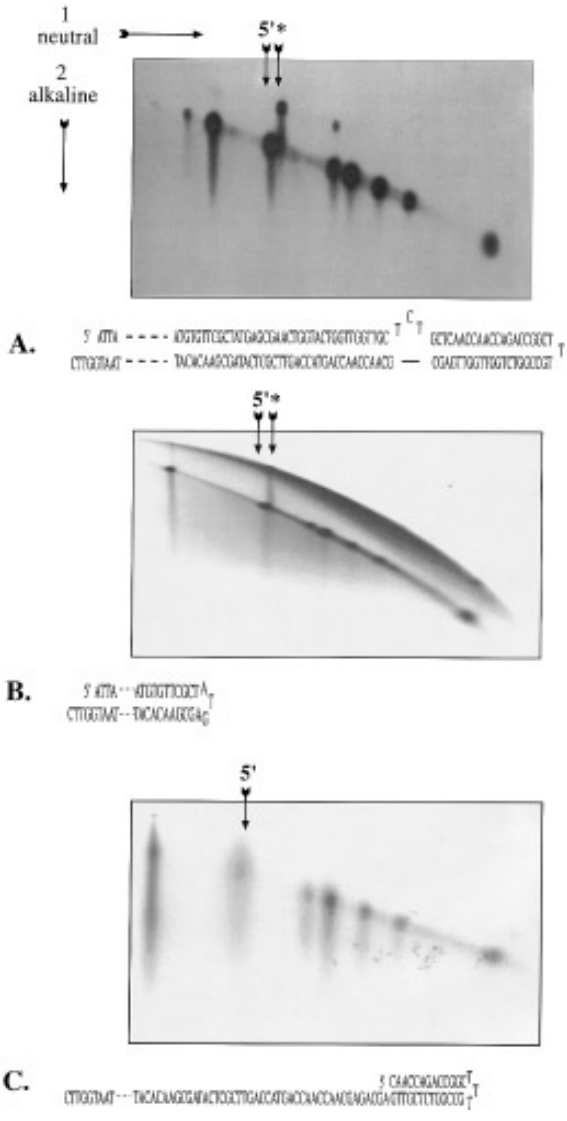

FIG. 4. Anomalous migration patterns in two-dimensional neutral/alkaline gels. (A) Two-dimensional neutral/alkaline gel analysis of the plasmid pMM984 (see Fig. 1B). (B) Two-dimensional neutral/alkaline gel analysis of the plasmid ΔB (see Fig. 1B). (C) Two-dimensional neutral/alkaline gel analysis of the plasmid pCAdI 19 (see Fig. 1B). All plasmids were digested prior to the *in vitro* reaction with *Bam*HI and digested afterwards with *Pst*I. The products from an *in vitro* reaction were subjected to electrophoresis in the first dimension in a neutral 1.5% TAE agarose gel. The gel was stained in ethidium bromide and the appropriate lane cut from the gel using a clean scalpel blade. The gel slide was then equilibrated in alkaline running buffer (*11*) for 30 min, rotated 90° and placed at the top of a second gel support at right angles to the direction of migration. A 1.5% alkaline agarose gel was then poured around the gel slice and electrophoresis carried out at 4° (*11*). The direction of electrophoresis in each dimension is indicated (arrows). The novel fragment is indicated by an asterisk (*). The bottom panel of each figure shows the structure of the 5′ terminus of the plasmid in the hairpin configuration.

population of molecules of differing size. Alternatively, ΔB may simply be prone to degradation. To verify the conclusion that the novel band was due to a hairpin rather than a stem-plus-arms structure, bandshift assays

were performed on purified samples of both the putative hairpin and the duplex *Pst*I fragments, using a monoclonal antibody, 2D3, that specifically recognizes and binds DNA cruciform structures but not linear double-stranded or hairpin DNA (*13*). 2D3 failed to bind the novel DNA (data not shown), confirming the results obtained with the ΔB mutant plasmid above.

Finally, a different mutant, pCAdl 19, was tested in which 82 bp have been deleted from the 5′ terminus of the viral genome (*12;* Fig. 1B). This deletion, which removes almost all of one arm of the palindrome (Fig. 4C, bottom), allows the formation only of a truncated (13-bp) hairpin structure. This mutant failed to support the production of a novel DNA band (Fig. 4C, top) analogous to that seen with the wild-type pMM984-derived MVM genome (Fig. 4A, top) or with the ΔB mutant (Fig. 4B, top). The removal of the majority of one arm of the hairpin at the right (5′) end of MVM removed the ability of MVM to prime DNA synthesis. However, the truncated arm could still form a fully base-paired hairpin, 13 base pairs in length. This implied that there might be a requirement for a minimal hairpin length in order for DNA polymerase δ to be able to bind and initiate DNA synthesis or, alternatively, that there may be regulatory genetic elements in the deleted region which control the initiation of DNA synthesis. Ward and Berns (*15*) recently found that a hairpin primer was necessary for initiation of DNA synthesis in an *in vitro* assay supplemented with AAV rep68 using a linear duplex AAV substrate similar to the one used in this study. However, in this instance, priming was able to initiate from a hairpin which was only 4 bp in size.

It should be noted that a hairpin formed from the 5′ palindromic region of the MVM sequence derived from pMM984 DNA would be expected to have a 3′ tail consisting of eight unpaired nucleotides. This is due to the *Bam*HI linkers used in the construction of the pMM984 clone (*9*). It is conceivable that this tail may have been removed by the intrinsic 3′–5′ exonuclease associated with DNA polymerase δ (*16*). Alternatively, the 3′ tail may have primed DNA synthesis by transiently base-pairing to the template strand just inboard of the hairpin stem. Our results do not allow us to distinguish between these possibilities since the electrophoretic mobility of the hairpin synthesised *in vitro* would not be significantly different in each of these two cases.

Preliminary analysis of the effect of inhibitors on the appearance of the hairpin structure (Fig. 2, aphidicolin) showed that a concentration of 30 μM aphidicolin was sufficient to completely inhibit its formation. Aphidicolin selectively inhibits the replicative DNA polymerases α and δ (as well as ϵ), but has no effect on the DNA polymerases β and γ (*17*), whereas the inhibitor butylphenyl-deoxyguanosine triphosphate (BuPdGTP) inhibits DNA polymerase α at low concentrations (<1 μM), and DNA polymerase δ only at high concentrations (>100 μM; *18*).

Figure 5 shows the results obtained when the *in vitro* replication reaction was carried out in the presence of

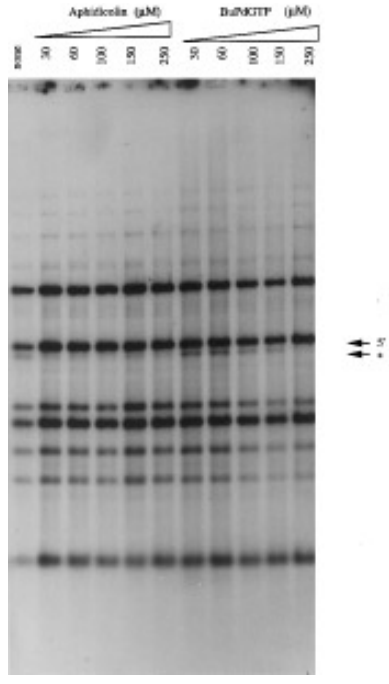

FIG. 5. The effect of DNA polymerase inhibitors on the formation of a DNA secondary structure. Increasing concentrations of the inhibitor aphidicolin (lanes 2 to 6) or butylphenyl-deoxyguanosine triphosphate, BuPdGTP (lanes 7 to 11), were used to inhibit the *in vitro* replication of the linearized form of pMM984, digested afterwards with *Pst*I (lane 1). The cell extracts were preincubated with the inhibitor (aphidicolin, Boehringer-Mannheim, 30–250 μM, or butylphenyl-deoxyguanosine triphosphate, a gift from Dr. George Wright, 30–250 μM) at 30° for 15 min prior to adding the remainder of the reaction components and continuing the incubation for an hour.

increasing concentrations of the above two inhibitors. The presence of just 30 μM aphidicolin was sufficient to completely inhibit the formation of the hairpin structure. There was no effect on the other DNA species, confirming our previous findings that some labeling is due to repair synthesis (*7*). In the presence of BuPdGTP, however, no inhibitory effect was seen until the concentration reached 100 μM. The extent of inhibition increased thereafter until, at a level of 250 μM, the formation of the novel DNA was completely inhibited. These data suggest that the formation of the hairpin structure, coupled with the initiation of DNA synthesis, is dependent upon DNA polymerase δ specifically.

The *in vitro* system described here utilizes a linear viral RF molecule as a substrate for DNA replication enzymes in HeLa cell extracts. Although similar in many respects to the monomer RF molecules produced *in vivo,* these molecules differ from natural RF molecules in that they contain *Bam*HI linkers at the ends and their 5′ ends are not covalently bound to NS-1. We note, however, that these molecules are infectious (*9*) and, furthermore, that virus particles in which the 5′ terminal 18 nucleotides of the encapsidated DNA were removed, along with the covalently bound NS-1 molecule, exhibited infectivity

identical to that of virus particles in which the 5′ ends were intact (*14*). Therefore, the rearrangement of the 5′ terminal palindromic sequence from a lineform to a hairpin configuration in the presence of HeLa cell proteins alone, as shown here, suggests that NS-1 may not be required for the isomerization of the 5′ terminal palindromic sequence *in vivo.* The fact that replication of viral DNA proceeds normally even when the infecting parental DNA molecules do not have NS-1 bound to them is also consistent with this suggestion. It should also be noted that, although these data demonstrate that cruciform structure is not necessary for this event, they do not rule out the possibility that cruciform structures do form *in vivo* with the wild-type genome or even that a cruciform structure is forming from the wild-type hairpin *in vitro.*

According to the model for parvovirus DNA replication proposed by Astell *et al.* (*2*), and others (*19*), MVM initiates DNA replication on the infecting single-stranded DNA molecule by utilising the 3′ end of the left-end hairpin as a primer. DNA synthesis from this site leads to the formation of a parental RF molecule in which the 5′ end is in the extended configuration. The next step in all parvovirus replication models is the postulated rearrangement of the 5′ terminal palindrome to a rabbit-earred structure consisting of two arms of the palindrome folded back on themselves. The existence of this intermediate has never been demonstrated experimentally and therefore it was not clear as to how this rearrangement occurred and which cellular and/or viral proteins were involved. The *in vitro* system that we have described here suggests that this rearrangement is carried out entirely by cellular proteins and furthermore that DNA polymerase δ utilizes the end of one of these hairpins in order to prime unidirectional DNA synthesis. Although previous studies have shown that MVM is capable of priming DNA synthesis from DNA primers formed by hairpin loops these studies involved a largely single-stranded DNA template (the viral genome) with hairpins at the ends that were already in a configuration that could be utilized by many different DNA polymerases (*20–23*).

Interestingly, although there was evidence for a structural change in the DNA and initiation of replication, full-length synthesis was not observed. To test the possibility that elongation was being blocked at some point due to the build-up of torsional constraint in the DNA, the *in vitro* reaction was supplemented with calf thymus topoisomerase I (which can replace NF II in adenoviral DNA replication *in vitro; 31*), SV40 T-antigen, or a bacterial source of DNA gyrase. None of these additions had any effect on the arrest of elongation (data not shown), suggesting that arrest was not due to a torsional constraint on the DNA. Theoretically, as all the necessary components are present in the *in vitro* system to support replication (*7*), full-length leading-strand synthesis would be expected to take place. In similar experiments involving the adeno-associated virus (AAV), Ni *et al.* (*32*) also found that uninfected cell extracts were not capable of supporting the replication of AAV *in vitro* and proposed that this was due to a deficiency in the reinitiation or elongation step during strand displacement.

It is possible that virally encoded proteins may alter the interaction of different cellular DNA polymerases with the 5′ terminal palindrome. NS-1, for example, is known to bind specifically to the 5′ terminus of MVM in the extended configuration (*24, 25*) and this may alter the efficiency with which the replication machinery binds to the 3′ end or the efficiency with which hairpin structures are formed. NS-1 is also essential for the correct terminal resolution of MVM *3, 4*). Recent evidence indicated that DNA synthesis carried out during terminal resolution was performed by DNA polymerase δ with no requirement for RNA primer synthesis (*19, 26*), an observation which is in agreement with our data. It is also possible that *in vivo* requirements for replicative DNA polymerases in MVM replication are more complex and that DNA polymerase α/primase is involved at some later phase of replication, possibly due to modifications of the DNA polymerase α/primase complex induced by virus infection (*27, 28*). Since DNA polymerase δ would not be expected to convert a lineform DNA to a hairpin configuration by itself, other proteins must be involved in this process. The HeLa cell extracts can now be fractionated and this factor(s) identified using the *in vitro* assay for DNA polymerase δ-dependent hairpin formation. The results of this study can also be used as a basis for defining a role for the viral DNA replication protein NS-1 and for mapping *cis*-acting genetic elements near the 5′ end of the MVM genome that might be required for initiating viral DNA replication.

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