Initiation of DNA Replication at Palindromic Telomeres Is Mediated by a Duplex-to-Hairpin Transition Induced by the Minute Virus of Mice Nonstructural Protein NS1*

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The linear single-stranded DNA genome of the minute virus of mice (MVM) is replicated via a double-stranded replicative form (RF) intermediate. Amplification of this RF is initiated by the folding-back of palindromic sequences serving as primers for strand-displacement synthesis and formation of dimeric RF DNA. Using an in vitro replication assay and a cloned MVM DNA template, we observed hairpin-primed DNA replication at both MVM DNA termini, with a bias toward right-end initiation. Initiation of DNA replication is favored by nuclear components of A9 cell extract and highly stimulated by the MVM nonstructural protein NS1. Hairpin-primed DNA replication is also observed in the presence of NS1 and the Klenow fragment of the Escherichia coli DNA polymerase I. Addition of ATPγS (adenosine 5′-O-(thiotriphosphate)) blocks the initiation of DNA replication but not the extension of pre-existing hairpin primers formed in the presence of NS1 only. The NS1-mediated unwinding of the right-end palindromic may account for the recently reported capacity of NS1 for driving dimer RF synthesis in vitro.

DNA polymerases are unable to copy unprimed DNA templates. Various mechanisms have therefore evolved in different biological systems to provide the template strand to be replicated with a free 3′-hydroxyl end that can be extended. These mechanisms include RNA priming in the case of pro- and eucaryotes as well as some viruses (1), priming through a DNA-bound protein in the case of adenovirus, some bacteriophages and various linear plasmids (2), as well as self-priming at hairpins created by the folding-back of terminal palindromic sequences (3). Palindromic termini are present in poxvirus (4, 5) and parvovirus (6, 7) telomeres, paramecium mitochondrial DNA (8), and tetrahymena rDNA (9). The terminal palindromes of pox- and parvovirus genomes play an essential role in various biological mechanisms, in particular on the level of DNA replication and transcription (26–31). Factor(s) interacting with specific DNA elements may be necessary for this structural transition.

Minute virus of mice (MVM), a prototype member of the autonomously replicating parvoviridae (17), makes use of a hairpin-priming mechanism to replicate its linear, 5149-nucleotide (nt) (18) single-stranded (ss) DNA genome. MVM DNA replication starts with complementary strand synthesis primed at the genomic left-hand (3′-terminal) hairpin, producing a double-stranded (ds) replicative form (RF) DNA (19, 20). As demonstrated recently in vitro for the majority of processed DNA molecules, complementary strand synthesis stops when reaching the folded-back right-hand (5′-terminal) hairpin, and is followed by ligation of the newly synthesized and parental strands. This results in a molecule covalently closed at both ends (cRF) (14). Such closed forms were also detected in parvovirus-infected cells (21, 22). Further processing of cRF DNA in vitro requires the MVM nonstructural protein NS1 (14, 15). When added as a purified polypeptide expressed from baculovirus vectors, NS1 was found to nick the MVM complementary strand 21 nt inboard of the folded-back genomic 5′ terminus, followed by initiation of strand-displacement synthesis and copying of the hairpin to yield a molecule that is extended at its right end (15). Rearrangement of the copied right-hand palindrome into hairpin structures (formation of the so-called rabbit-eared configuration) provides a primer for reinitiation of replication in a strand-displacement manner leading to the formation of concatenated molecules, in particular dimer-length RF DNA (14, 23).

Restoration of hairpin structures at the duplex right-hand telomere of MVM dsDNA templates (14, 15, 24, 25) and formation of dimer-length RF DNA (14) were recently achieved in vitro. Interestingly, dimer formation was found to be stimulated to a great extent by the NS1 protein. Since the mechanism of this stimulation remained elusive, the present study was undertaken to determine whether NS1 promoted hairpin refolding, extension of the hairpin primer, or both. The present data argue for a role of NS1 in the transition of the extended terminal palindrome into hairpin structures. In this respect, parvoviruses provide a model for the involvement of protein-DNA interactions in the structural transition of DNA known to take part in various biological mechanisms, in particular on the level of DNA replication and transcription (26–31).

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1 The abbreviations used are: MVM, minute virus of mice; ss, single-stranded; ds, double-stranded; RF, replicative form; nt, nucleotide(s); bp, base pair(s); d, duplex; h, hairpin; ATPγS, adenosine 5′-O-(thiotriphosphate).
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**EXPERIMENTAL PROCEDURES**

Preparation of Cell Extracts and Production of NS1 from Recombinant Baculoviruses—The cultivation of A9 cells in suspension and the preparation of cytosolic extracts were carried out as described previously (14). Nuclear extracts were prepared according to the method of Dignam (32). Cultivation of S9 insect cells, infection with recombinant baculovirus expressing either wild type NS1 or the NS1 mutant K405R (33), extract preparation, and NS1 purification by affinity chromatography were performed as reported (14). The NS1 mutant K405R producing recombinant baculovirus was generously provided by Jesper Christensen (The Royal and Agricultural University of Copenhagen, Frederiksborg, Denmark).

DNA Templates—The MVM-specific insert (nt 1–5068) of the original MVM p98 plasmid (34) was recloned into the pGEM5Zf vector (Promega) (35). Purified plasmid DNA was digested with SalI restriction enzyme and used as template DNA in the in vitro DNA replication assay.

In Vitro DNA Replication and Analysis of Product DNA—In vitro DNA replication was carried out as described previously (14, 15). Standard reaction mixtures (50 µl) consisted of 40 mM HEPES-KOH (pH 7.6), 8 mM MgCl₂, 0.5 mM dithiothreitol, 100 µM each dATP, dGTP, and dTPP, and 5 µCi of [α-32P]dCTP (4 Ci/mmol), 100 µM each CTP, GTP, and UTP, 3 mM ATP, 40 mM phosphocreatine, 20 µg/ml creatine phosphokinase, baculovirus-produced NS1 (100 ng), and 0.5 µg/ml affinity-purified NS1 (10 µg/ml). The MVM-specific insert flanked by SalI left- and right-hand terminal repeat (nucleotides 1–115) and the truncated right terminal repeat (nucleotides 4028–5068) of MVM DNA, at 37 °C and 100 mM NaCl. Restriction-digested replication products were analyzed on 5% polyacrylamide gels using a Tris borate-EDTA as running buffer.

Formation of terminally closed DNA from cloned MVM DNA in A9 cell extract—The infectious clone p98 comprises the MVM DNA sequence extending from nt 1 to 5068, including the entire left-hand and more than half of the right-hand inverted repeat (34). Digestion of p98 DNA with the restriction enzyme SalI releases the MVM-specific insert flanked by SalI linker sequences (depicted in Fig. 1). SalI-digested p98 DNA was used as a template in an in vitro replication reaction containing a mixture of cytosolic and nuclear extracts from A9 murine fibroblasts. The reaction products were digested with the restriction enzyme PshAI at nt 670 and 4916 (see Fig. 1) and first analyzed on a native polyacrylamide gel. Gel-purified DNA products were recovered by electroelution and analyzed under denaturing conditions on 6% polyacrylamide/urea gels using the same running buffer as above.

Thermodynamic Calculations—Thermodynamic calculations were performed using the Mulfold computer program (36–38). Free energy values were calculated for the hairpin and duplex forms of the entire left terminal repeat (nucleotides 1–115) and the truncated right terminal repeat (nucleotides 4028–5068) of MVM DNA, at 37 °C and 100 mM NaCl. The equilibrium constant for the transition: duplex = 2 hairpins was calculated by K = exp ΔG/RT.

Western Blotting—Proteins were separated by discontinuous SDS-polyacrylamide gel electrophoresis (39) and transferred to nitrocellulose membranes using a semidry blotting system (Bio-Rad). Filters were incubated for 1 h at room temperature in blocking buffer (4% nonfat dry milk in phosphate-buffered saline) and then for 2 h with an antiserum raised against the C-terminal part of NS1 (40) at a 1:1000 dilution. Protein-bound antibodies were detected with peroxidase-conjugated specific antibodies and revealed by using the ECL system (Amersham).

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)—Affinity-purified NS1 (10 µg in a volume of 300 µl) was layered onto a 15–40% glycerol gradient (1.4 ml) in 10 mM Hepes-KOH, pH 7.5, 5 mM MgCl₂, 0.1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol. After centrifugation in a Beckman 55 rotor at 50,000 rpm for 18 h at 4 °C, 100-µl fractions were collected by pipetting from the top of the tube.

**RESULTS**

Formation of terminally closed DNA from cloned MVM DNA in A9 cell extract—The infectious clone p98 comprises the MVM DNA sequence extending from nt 1 to 5068, including the entire left-hand and more than half of the right-hand inverted repeat (34). Digestion of p98 DNA with the restriction enzyme SalI releases the MVM-specific insert flanked by SalI linker sequences (depicted in Fig. 1). SalI-digested p98 DNA was used as a template in an in vitro replication reaction containing a mixture of cytosolic and nuclear extracts from A9 murine fibroblasts. The reaction products were digested with the restriction enzyme PshAI at nt 670 and 4916 (see Fig. 1) and first analyzed on a native polyacrylamide gel. As apparent from Fig. 2A (lane 1), doublet bands were detected around the anticipated positions of PshAI left- and right-hand terminal fragments produced from the p98 MVM DNA insert. When the individual bands were gel-purified and further analyzed under denaturing conditions, structural differences became evident (Fig. 2B). The species forming the upper doublet bands in Fig. 2A gave rise to denaturation products expected from the duplex forms (d) of the left-hand (670 bp) and right-hand (152 bp) MVM PshAI fragments of SalI-digested p98 DNA (Fig. 2B, lanes 1 and 3). In contrast, the species forming the lower doublet bands in Fig. 2A were much more retarded under denaturing conditions (Fig. 2B, lanes 2 and 4). This is in line with the assumption that the lower doublet bands represent in vitro replication products with covalently closed termini (hairpin forms, h).
As described recently for a small fraction of *in vitro* processed MVM DNA (14), the extended RF right-hand telomere undergoes a conformational transition from the duplex into the hairpin form, providing a primer for the reinitiation of DNA synthesis. According to Fig. 2 (A and B), this conformational transition appears to take place at both the MVM left and right-hand telomeres of *Sal*I-digested p98 DNA, followed by extension of the primers created in this manner. Therefore, together with our recent findings (14, 15) and the data of others (24, 25), the above results demonstrate hairpin-primed initiation of DNA replication at both the left- and right-hand inverted repeats of double-stranded MVM DNA, as depicted in Fig. 2C.

To gather information about the cellular factors involved in this terminal duplex-to-hairpin transition and primer extension, the initiation of DNA replication at *Sal*I-digested p98 termini was further analyzed in the presence of varying amounts of cytosolic and nuclear extracts (Fig. 2A). Increasing the portion of nuclear extract within the reaction mixture was marked by a dose-dependent enhancement of radioactive precursor incorporation into the component identified as the left-terminal hairpin form (*band h, lanes 1–3*) and by a concomitant decrease of the labeling of the left-terminal p98 duplex fragments (*band d, lanes 1–3*). Similarly, a stimulation of right terminal hairpin formation and extension was induced by nuclear extract components (compare *lanes 1* and *4*) although no dose-dependent increase was observed (*lanes 1–3*). In the presence of cytoplasmic extract alone, left-end initiation was hardly detectable, and only a slight stimulation of right-end initiation was noted (*lanes 4–6*).

**FIG. 2.** Hairpin-primed MVM DNA replication in extract of uninfected A9 cells. A, *Sal*I-digested p98 DNA was incubated with 20 μg of cytosolic proteins plus increasing amounts (5, 10, or 20 μg) of nuclear proteins (*lanes 1–3*, respectively), or with increasing amounts (25, 30, or 35 μg) of cytosolic proteins alone (*lanes 4–6*, respectively). Product DNA was digested with *Psh*AI and analyzed on a 5% polyacrylamide gel. *B*, individual DNA species obtained as in panel *A* were recovered by electroelution and further analyzed on a 6% polyacrylamide/urea gel. *Hpa*II-digested pGEM5Zf DNA was used as molecular weight marker. *C*, schematic representation of hairpin-primed DNA replication, including primer generation by a structural transition at the terminal palindromes and subsequent extension (*solid lines*). *d*, duplex; *h*, hairpin; *v*, viral strand; *c*, complementary strand; *small arrowheads* indicate DNA strand 3′-ends.
FIG. 3. NS1-mediated induction of hairpin-primed MVM DNA replication. A, SalI-digested p98 DNA (100 ng) was incubated with cytosolic (lanes 1 and 2) or cytosolic plus nuclear (lanes 3 and 4) proteins from A9 cell extracts in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of baculovirus-produced NS1. Samples were digested with PshAI prior to analysis on a 5% polyacrylamide gel. B, DNA products obtained in the presence of cytosolic extract and NS1 were digested with PshAI (lane 1), SspI (lane 2), or XbaI (lane 3) and analyzed on a 5% polyacrylamide gel. HpaII-digested pGEM5Zf DNA was used as molecular weight markers. C, schematic representation of the putative repair reaction leading to the completion of the right-hand telomere of MVM p98 DNA as revealed by fragment E (right-hand portion). The release of free hairpins (H) as a result of NS1-induced nicking and strand-displacement synthesis at the right telomere is shown on the left. Nucleotide numbering is according to Refs. 15 and 73. d, duplex; h, PshAI-generated hairpin fragment; E, completed duplex fragment; H, segregated hairpin; shaded ovals, NS1; small arrowheads indicate DNA strand 3' ends; solid lines delineate newly synthesized DNA.
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NS1 led to a dramatic increase in the amount of nucleotide precursors incorporated into DNA products terminating in a turnaround configuration (designated h), whereas the extended duplex products d became hardly detectable. This effect was irrespective of whether the reaction was performed in cytosolic (lanes 1 and 2) or cytosolic plus nuclear (lanes 3 and 4) extract. Thus, the viral NS1 protein proved able to stimulate hairpin-primed DNA replication at both the left and right ends of MVM RF DNA supplied as terminally extended substrate.

As shown in our previous reports (14, 15), NS1 is able to induce secondary rounds of in vitro nicking and extension of the right-hand telomere of MVM RF DNA in vitro, resulting in the release of the right-terminal hairpin DNA in a free form (depicted in Fig. 3C, left-hand part; species H). This segregated hairpin species migrated in polyacrylamide gels at a similar position as the right-end turnaround MVM RF PshAl fragment (14, 15). It was thus necessary to ascertain that the in vitro labeled species h, derived from the right end of p98 MVM DNA, was the telomeric fragment of an RF molecule that underwent a conformational transition from duplex to hairpin (Fig. 3C, right-hand part), rather than a free hairpin displaced from the NS1 nick site. To this end, p98 replication products obtained in the presence of NS1 were digested in parallel with the restriction enzymes PshAI, XbaI, or SspI, cleaving MVM DNA at different positions close to the right terminus (see Fig. 1). As apparent from Fig. 3B, the restriction digestion generated products migrating at the anticipated positions of right-hand XbaI (706 bp), SspI (423 bp), and PshAI (132 bp) turnaround fragments. The size dependence of the low molecular weight DNA species on the restriction enzyme used (rather than on NS1 nicking at a unique telomeric site) indicates that the fast migrating h band from the PshAI-digested sample (Fig. 3A, lanes 2 and 4) consists, at least for its major part, of the right-hand turnaround terminus of RF DNA, with little contribution of displaced free hairpins.

In addition to h, another fragment, designated E, is visible in Fig. 3A (lane 4). The apparent size of species E is in line with the assumption that it represents an extended right-hand PshAI fragment of full-length MVM DNA, in keeping with its comigration with this fragment in a neutral polyacrylamide gel (data not shown). Since p98 harbors a truncated palindrome at its right end, the question arises how this palindrome can be repaired to generate a full-length telomere. As schematized in Fig. 3C, this can be assumed to result from right-end hairpin refolding and copying, followed by NS1-induced nicking and extension.

Stimulation of Duplex-to-Hairpin Transition in the Presence of NS1—The NS1 protein used in these experiments was expressed from a baculovirus vector in Sf9 insect cells and purified by affinity chromatography. Although giving a single NS1 band in Coomassie Blue-stained polyacrylamide gels (data not shown), this preparation may contain minor amounts of contaminating Sf9 proteins. To confirm that the above-mentioned stimulation of replication initiation was indeed due to NS1 rather than to a contaminating cellular factor, the affinity-purified NS1 was subjected to a second purification step by centrifugation through a glycerol gradient. Individual fractions of this gradient were tested for their NS1 content and their presence (lane 2) of NS1–deficient for ATPase and helicase function, completely abol-
ished hairpin-primed DNA replication (data not shown), confirming the requirement for NS1 in this reaction.

NS1 may trigger replication from the duplex ends of MVM RF DNA by stimulating terminal hairpin refolding, elongation of the hairpin primer, or both. Should NS1 promote terminal hairpin formation, it was reasoned that the viral protein may induce the initiation of Sal I-digested p98 DNA replication in a defined reconstituted system supplemented with a purified DNA polymerase. In a first step, it was ascertained that without cell extract, no labeling of the DNA substrate took place in the presence of NS1 alone (data not shown), indicating that the NS1 preparation used was free of detectable DNA polymerase activity. Furthermore, the initiation of MVM dsDNA replication (as revealed by the nonappearance of labeled h bands) was not achieved with the Klenow fragment of E. coli DNA polymerase I in the absence of NS1 (Fig. 4C, lane 1), confirming the inability of the Klenow enzyme to trigger the duplex-to-hairpin transition assumed to generate a primer-template structure. The labeling of duplex PshAI terminal fragments occurring under these conditions presumably resulted from the Klenow-mediated filling of substrate DNA recessed ends. In contrast, when the DNA substrate was incubated with both NS1 and Klenow polymerase, the turnaround telomeric fragments (h bands) became labeled (Fig. 4C, lane 2), pointing to the formation and further extension of terminal hairpins. This was verified by performing control experiments similar to those shown in Figs. 2B and 3B for the cell extract-mediated reaction, confirming that the newly synthesized DNA species visible in Fig. 4C (lane 2) are indeed hairpin forms arising from structural transitions of the MVM RF duplex telomeres (data not shown).

Equilibrium constants of 2.7 × 10⁻⁶⁴ M⁻¹ and 2.2 × 10⁻¹⁴ M⁻¹ can be calculated for the duplex-to-hairpin conformational transitions at the left and truncated right end of p98 MVM DNA, respectively (36–38). This makes it most unlikely that denaturation and refolding of the telomeres took place spontaneously, and NS1 only facilitated Klenow-driven elongation of the hairpin primers generated in this way. Consistently, pre-incubation of the DNA substrate in replication buffer at 37 °C failed to increase the yield of hairpin-primed replication upon subsequent addition of NS1 and Klenow polymerase (data not shown). Altogether, these observations argue for a direct role of NS1 in inducing the rearrangement of MVM RF duplex telomeres into hairpin structures that provide a primer for strand-displacement synthesis.

Terminal Hairpin Formation Is ATP-dependent—The structural transformation of MVM DNA termini is likely to consume energy. Therefore, we were interested to determine whether hairpin-primed MVM DNA replication was ATP-dependent and performed competition experiments with ATPγS. This analogue is known to compete with ATP for the binding to helicase and kinases while not being hydrolyzed (41), acting as an analogue is known to compete with ATP for the binding to helicase and kinases while not being hydrolyzed (41), acting as an inhibitor. As mentioned above, incubation of Sal I-digested p98 DNA with Klenow polymerase only resulted in the labeling of duplex PshAI terminal fragments (Fig. 5A, lane 1). Supplementing the replication reaction with NS1 led to a limited hairpin-primed initiation of replication in the absence of added ATP, as revealed by the appearance of labeled h species (lane 2). Addition of ATP highly increased the efficiency of replication initiation at both termini (lane 3). Replication was reduced by supplying an equimolar amount of ATPγS (lane 4) and completely abolished by a 3-fold excess of this analogue (lane 5). We therefore conclude that the initiation of hairpin-primed DNA replication at the termini of MVM duplex DNA is an ATP-consuming process. The low level of replication initiation observed in the absence of added ATP (Fig. 5A, lane 2) may be due to residual ATP bound to NS1. Indeed, ATP binding and processing have been shown to occur as part of the helicase function of NS1 (42).

To confirm the role of NS1 in the duplex-to-hairpin transition, we tested whether pretreatment of Sal I-digested p98 DNA with NS1 and ATP allowed subsequent primer elongation in the presence of Klenow polymerase and an excess of ATPγS. To this end, MVM dsDNA was incubated with NS1 and ATP for various times prior to the addition of Klenow polymerase and radioactive nucleotides in the presence of ATPγS. As illustrated in Fig. 5B, ATPγS prevented the formation of a hairpin replication product when supplied at the same time as NS1 and Klenow polymerase (lane 1), while this reaction took place, at least to some extent, when the template DNA was first exposed to NS1 and ATP before the addition of DNA polymerase in the presence of ATPγS (lanes 3, 5, and 7). These data confirm that NS1 drives a step preceding primer extension, most likely hairpin formation, in an ATP-dependent way. However, the efficiency of hairpin-primed replication occurring under these conditions was low as compared with the reaction measured in the absence of ATPγS (lanes 2, 4, and 6). This suggests that NS1 may stimulate Klenow-mediated strand-displacement synthesis besides hairpin primer formation. It is also worth noting that hairpin formation was maximal at the right end after a 10-min incubation in the presence of NS1, whereas it increased continuously at the left end within the time interval studied. This difference may reflect the higher complexity (characterized by a higher ΔG for the duplex-to-hairpin transition) of the left- versus the right-hand terminal palindrome.

To test whether hairpin formation at the right terminus also varied as a function of time, Sal I-digested p98 DNA was pre-incubated with NS1 and ATP for shorter intervals from 2 to 8 min before the addition of Klenow polymerase. As apparent from Fig. 5C, the yield of hairpin-primed DNA replication at the right end also showed a time dependence under these conditions, reaching a peak after a few minutes of DNA pre-incubation with NS1 and ATP. Since, in this experiment, Klenow polymerase-catalyzed primer extension was carried out in the absence of ATPγS and therefore in the presence of functional NS1, it is somewhat surprising that a few minutes difference in the time of preincubation led to a pronounced variation in the overall labeling of the hairpin reaction product. A possible explanation for this lies in the fact that Klenow polymerase, besides extending the hairpin primer, may fill the recessed ends of Sal I-digested p98 MVM DNA. Filling before hairpin formation results in a 3'-terminal mismatch at the subsequently folded-back palindrome (compare with Figs. 1 and 2C). Such molecules cannot be extended. The preincubation with NS1, therefore, favors hairpin formation plus extension over terminal filling.

DISCUSSION

Initiation of DNA Replication at the Terminals of Cloned MVM DNA—Replication of the parvovirus ssDNA genome involves its initial conversion into a dsRF by extension of the left-hand (3') palindrome folded-back into a terminal hairpin structure that serves as primer for the synthesis of a complementary strand (14, 19, 20). The newly synthesized strand is ligated to the right-terminal hairpin, the processing of which is driven by the parvovirus protein NS1, as was recently characterized in our laboratory by means of an in vitro replication assay (14, 15). Besides mediating the site-specific nicking of the right-end hairpin and its extension into a duplex structure, NS1 was found to stimulate strand-displacement synthesis, which starts from the duplex right telomere and eventually gives rise to a dimer-length RF intermediate (14, 15). The present work was undertaken to investigate the mechanism by which NS1 promotes DNA synthesis initiated at a duplex telomere. To this
Fig. 5. Inhibition of hairpin-primed DNA replication by ATPγS. A. SalI-digested p98 DNA was incubated with E. coli DNA polymerase I (Klenow fragment) in the absence (lane 1) or presence (lanes 2–5) of NS1 and other components indicated on top of lanes. ATPγS was added in equimolar amount (lane 4) or 3-fold excess (lane 5) relative to ATP, or was not included (lanes 1–3). B. SalI-digested p98 DNA was incubated with NS1 and ATP for the indicated times before the addition of radioactive precursors plus Klenow polymerase in the presence (lanes 1, 3, 5, and 7) or absence (lanes 2, 4, and 6) of a 3-fold molar excess of ATPγS. Product DNA was digested with PshAI and analyzed on a 5% polyacrylamide gel. C. SalI-digested p98 DNA (50 ng) was preincubated with NS1 and ATP for the indicated times before the addition of radioactive precursors and Klenow polymerase. d, duplex; h, hairpin.
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end, we made use of an MVM DNA clone (p98) from which the MVM part can be excised, to give an RF molecule terminating in telomeres of full (left-hand) or partial (right-hand) size. The results presented in this report show that DNA replication is initiated at both duplex DNA ends in a process that includes unwinding of viral and complementary strands, hairpin refolding, and primer extension. This process is driven by nuclear components and highly stimulated by NS1. It should be stated that, although truncated, the right telomere of p98-derived MVM DNA has more than half the length of the original palindromic sequence and therefore is still able to fold back into a hairpin primer. Consistently, when p98 DNA was replaced by a nondimeric sequence and therefore is still able to fold back into a hairpin primer. Consistently, when p98 DNA was replaced by a natural MVM RF template terminating in full-size duplex telomeres on both sides, similar results were obtained (data not shown). The MVM insert of plasmid p98 is bracketed by SalI linker sequences. As shown in Fig. 1, the 3'-hydroxyl terminus on the right-hand side of MVM DNA excised by SalI digestion, corresponds to the last nucleotide of the viral sequence and can thus base pair with its complement upon folding-back of the hairpin. On the left-hand side, the free hydroxyl group is carried by the first flanking nucleotide of the linker sequence, G, which is complementary to the C present immediately downstream from the terminal palindrome on the MVM viral strand. Therefore, the presence of linkers does not result in mismatches that would interfere with full pairing of the free end of either terminal palindromic sequence and with its use as a primer for strand elongation.

NS1-induced Duplex-to-Hairpin Transition—The ability of NS1 to stimulate hairpin-primed strand-displacement synthesis may take place at the level of hairpin formation, primer extension, or both. To test the role of NS1 in hairpin folding, replication was analyzed in the absence of cellular factors, which may also contribute to this process, by incubating the DNA substrate with NS1 and the Klenow fragment of E. coli DNA polymerase I. Hairpin formation was revealed through the known capacity of the polymerase for strand displacement synthesis (43), leading to the extension of the hairpin primer. Whereas the Klenow fragment was unable by itself to induce hairpin formation, hairpin-primed strand elongation was observed when p98 MVM DNA was incubated with both NS1 and the bacterial polymerase. This result strongly argues for an inducing effect of NS1 on hairpin formation, although it does not rule out that NS1 facilitates in addition the strand-displacement synthesis reaction. The involvement of NS1 in the remodeling of MVM telomeres is consistent with the fact that the viral enzyme is endowed with an ATP-dependent helicase function (42), and that hairpin-primed replication could be fully inhibited by an excess of ATPγS competitor. Furthermore, preincubation of the DNA substrate with NS1 plus ATP alone allowed subsequent hairpin-primed DNA replication in the presence of ATPγS upon addition of DNA polymerase. Therefore, NS1 appears able to drive a strand-dependent event that takes place in the absence of DNA synthesis and primes ensuing elongation, most likely consisting in a duplex-to-hairpin transition at both palindromic termini of MVM DNA. This function of NS1 may account for the recent finding that the viral protein stimulates the in vitro formation of dimer-length DNA from a monomeric MVM RF substructure terminating in a turnaround (left-hand side) and a duplex (right-hand side) conformation (14).

The present in vitro data indicate that hairpin-primed DNA synthesis can start from both left- and right-hand telomeres in a MVM DNA template having duplex structures at each end. While strand-displacement synthesis from the left terminus is part of the current AAV DNA replication model (12), its occurrence in vivo is questionable for paroviruses of the MVM type in which the DNA strand of plus polarity is hardly packaged (20). It is indeed assumed that the asymmetrical encapsidation of the minus (viral) strand reflects the predominant initiation of DNA synthesis at the right end of RF DNA, leading to the preferential displacement of the viral over complementary strand (20, 44–46). In keeping with this assumption, it is worth noting that NS1-induced hairpin formation, as measured in the present in vitro assay, is more efficient for the right telomere than for the left one. This bias of replication initiation from the duplex right end might be further enhanced in vivo, due to the lack of resolution of the turnaround left-hand palindrome in monomeric RF (14) or the interaction of the left telomere with capsid proteins (35, 46, 47).

A cellular factor named PIF (for parovirus initiation factor) was recently reported to be required for the NS1-mediated nicking involved in the initiation of DNA replication at the junction sites of MVM head-to-head concatamers (48). As shown in the present work, the initiation of hairpin-primed DNA replication at the termini of monomeric MVM DNA occurs in the absence of cellular factors, consistent with a role of PIF as a cofactor in nicking. Notwithstanding their dispensability for the NS1-mediated terminal initiation of MVM DNA replication, cellular factors may enhance this process. This is suggested by the asymmetric distribution of replication activities across the glycerol gradient, as apparent in Fig. 4. Moreover, the efficiency of NS1-mediated left-end initiation was consistently higher in reactions driven by cell extract components compared with Klenow enzyme. Finally, cellular nuclear components were found to mediate hairpin-primed replication of MVM DNA independently of NS1 at a low but significant level, indicating that the telomeric duplex to hairpin transition can be sustained to a limited extent by cellular factors, in agreement with recent reports (24, 25).

Mechanism of Hairpin Folding—Alternative mechanisms, designated “indirect” and “direct” slippage, have been proposed for the formation of hairpins from duplex palindromes (49). As illustrated in Fig. 6A, the direct process (numbered 1) consists in whole duplex unwinding followed by rapid intrastrand reannealing to form hairpins, whereas the indirect mechanism (numbered 2) involves the initial formation of a cruciform structure around the axis of symmetry of the palindrome, followed by branch migration and hairpin formation. Kinetic studies with short synthetic palindromes suggest that one or the other process may occur in different systems, depending on the palindromic size and nucleotide sequence as well as on the salt concentration and pH (50–52).

By virtue of its helicase activity NS1 may contribute to duplex denaturation. This would be in line with our observation that the baculovirus-produced mutant form of NS1, K405R, which is deficient in the helicase function (33), failed to support hairpin-primed DNA synthesis on SalI-digested p98 template DNA (data not shown). Melting of the whole duplex starting from the genomic end (process 1) is likely to require stabilization of the single-stranded DNA portions so as to prevent their reannealing. This could be achieved through the association of single-strands with cellular ssDNA-binding proteins such as RPA (53). However, hairpin refolding in vitro was achieved in the sole presence of NS1 and Klenow polymerase, without the need of additional cellular factors. Moreover, the single-stranded DNA-binding protein SSB, a bacterial polypeptide that can be substituted for RPA in the unwinding of the simian virus 40 origin of replication (54), had no detectable effect on the efficiency of hairpin formation in the present assay (data not shown). These data argue against a structural transition according to mechanism 1, unless it is assumed that stabilization of single-stranded DNA regions is also achieved.
by NS1. The latter possibility deserves to be considered since NS1 is able to oligomerize and may bind to DNA in the form of larger complexes (55, 56).

Involvement of NS1 in hairpin formation through mechanism 2 may be envisioned as the result of NS1 binding to the palindrome close to the axis of symmetry, followed by local DNA unwinding, branch migration, and cruciform extrusion (Fig. 6B). This process would have a significantly lower activation energy than a mechanism involving prior strand separation, and would not require stabilization of long single-stranded DNA stretches. It is pertinent to note that the sequence ACCAACCA, recently reported to constitute an NS1 binding motif (57), is present in the vicinity and on both sides of the axis of symmetry of the right-hand inverted repeat (Fig. 6B). Thus, NS1 may indeed bind to the right terminal palindrome, initiating the structural transition described above. Similarly, cruciformation was shown to occur in vivo within the terminal inverted repeats of poxvirus DNA, and was suggested to rely on specific telomere-protein interactions, since inverted copies of a 58–76-bp core target sequence are required for the duplex-to-hairpin transition (16). The left-hand palindrome also contains a pair of [ACCA]$_2$ sequences that are located on both sides of the axis of symmetry but are further apart in comparison with the right telomere (35/40 versus 8 nt from the axis). It may be speculated that this greater spacing of potential NS1-binding sites is less favorable to cruciform induction and possibly accounts for the observed reduced efficiency of hairpin-primed initiation of DNA replication at the left versus right genomic end.

**Conclusion**—In general, significant energetic barriers restrain duplex palindromes from folding into hairpins (29, 50–52), as exemplified by the above mentioned equilibrium constants calculated for the MVM DNA termini. Studies with synthetic palindromes have shown that the transition energetic extensively varies with environmental conditions such as salt concentration and pH (29, 50–52). In cells, changes in local pH may be brought about by the interaction of the palindrome with electrically charged side chains of proteins (29), facilitating the structural transition. This is in keeping with the possibility that NS1 may induce the conformational changes at the MVM RF DNA ends through a direct interaction with the terminal palindromes.

The interaction of specific protein(s) with distinct DNA regions, referred to as origins of replication, is a prerequisite for the induction of DNA replication in many viral, procaryotic and eucaryotic systems (1, 27, 28, 58–60). As in the parvovirus model, several origins contain a palindromic core sequence, e.g. those of mitochondrial DNA from yeast to men (61). Origin recognizing proteins take part in local DNA unwinding or influence replication in other not yet defined ways (62–67). As mentioned above, NS1 may interact with [ACCA]$_2$ motifs present in the center of the terminal palindromes (57). Mutational analyses should indicate whether these sides mediate the NS1-induced generation of hairpin primers, besides their known contribution to the NS1-dependent processing of bridge junctions within MVM multimeric RF intermediates (68, 69).

The rearrangement of inverted terminal repeats into hairpins, proven to be involved in pox- and parvovirus-DNA replication (10–13), has also been proposed as an essential step in the amplification of the rRNA gene of *Tetrahymena ther-
mophila (70) and during gene amplification in mammalian cells (for a review, see Ref. 26). Furthermore, palindromic sequences have been found in the regulatory regions of eukaryotic genes (29, 30). Theoretical (71) and experimental (29, 30, 72) data from these systems indicate a role of palindromes in determining biologically relevant DNA secondary structures. Proteins involved in the conformational transition of palindromes may thus be ubiquitously distributed. The limited rearrangement of MVM DNA left- and right-hand palindromes into hairpins, as induced by nuclear extract in the absence of data from these systems indicate a role of palindromes in genes (29, 30). Theoretical (71) and experimental (29, 30, 72)

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