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DNA replication in the autonomous parvoviruses

*Susan F. Cotmore and Peter Tattersall**

Both ends of the linear single-stranded parvoviral DNA genome contain short palindromic sequences which form duplex hairpins containing cis*-acting information required for replication and encapsidation. DNA synthesis is primed directly by the 3' end, and genomes are replicated through multimeric duplex intermediates by unidirectional, leading-strand synthesis. Unit-length genomes are excised from these concatemers, and their telomeres replicated, by the viral NS1 protein, which introduces a single-strand nick into specific origin sequences, becoming covalently attached to the 5' end at the nick and providing a 3' hydroxyl which primes synthesis of a new copy of the telomere. Progeny DNA synthesis requires ongoing replication and is dependent upon packaging.*

Key words: hairpin / telomere / palindrome / nicking / unidirectional

THE AUTONOMOUS PARVOVIRUSES form a group of physically similar viruses, replicating in the nuclei of both vertebrate and invertebrate hosts. Members of the family Parvoviridae are unique among all known viruses in having linear, single-stranded DNA genomes, and similarities of genome structure, organization and coding sequence throughout the family suggest that they all make use of essentially the same, 'rolling hairpin' DNA replication strategy.[1] This closely resembles the rolling circle mechanisms previously described in prokaryotes, and, as such, involves several manipulations which are rarely encountered in eukaryotic systems. However, their small size and limited coding potential necessitates that they mediate this unusual mode of synthesis by subverting the replication machinery of their eukaryotic host for their own preferential amplification. In this review we will focus predominantly on the replication of the widely-disseminated murine parvovirus MVM, (minute virus of mice), and allude briefly to known disparities between this and other members of the family. Although a comprehensive description of the helper-dependent, adeno-associated virus (AAV) genus is outside the scope of this volume, we will refer to AAV-2 in some detail because much of our understanding of one mode of viral telomere regeneration, called 'terminal resolution', has come from work on this virus.

*From the Departments of Laboratory Medicine and *Genetics, Yale University School of Medicine, 333, Cedar Street, New Haven, CT 06510, USA*

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Structure of the viral genome

Infecting parvoviral particles contain a single copy of the linear, non-permuted DNA genome in which a relatively long single-stranded coding region (4–6 kb) is bracketed by short (121–421 base) palindromic terminal sequences capable of folding into hairpin duplexes (Figure 1). While many parvoviruses encapsidate DNA strands of either sense, others, such as MVM, selectively encapsidate strands which are minus sense with regard to transcription.[2] The viral telomeres contain most of the *cis*-acting information required for both DNA replication and encapsidation [reviewed in ref 3], and while MVM and other members of the parvovirus and contravirus genera have unique sequences at each end of their genomes, members of the erythro-, denso- and iteravirus genera contain inverted terminal repeats.[2] Although central to the process of replication, the complexity and remarkable diversity of these telomeres suggest that they also serve other functions in the viral life cycle. In particular, the left-end palindromes of many parvoviruses appear to represent a compromise between sequences required for replication and upstream elements required to modulate an adjacent 'early' transcriptional promoter, referred to as P4 in MVM (Figure 1).

The MVM genome is 5172 nucleotides long, with 4805 nucleotides of single-stranded DNA bracketed between terminal palindromes of 121 and 246 nucleotides at the 3' and 5' ends, respectively.[4] As depicted in Figure 1, these termini are capable of folding into Y-shaped and cruciformed hairpin structures, respectively, although the 5' palindrome may also be arranged as a simple duplex with a single mismatched

bubble. As will be discussed below, replication origins associated with these two palindromes are activated by different initiation strategies and result in the synthesis of telomeres in either a single orientation, designated 'flip', or in two complementary sequence orientations, dubbed flip and 'flop'.[5,6]

The non-structural polypeptides

Parvoviruses encode two separate gene complexes, with transcripts from one half of the genome, designated by convention the right-hand side, programming synthesis of an overlapping set of capsid polypeptides, while the left half gives rise to a small number of non-structural proteins which are involved in DNA replication. In MVM, the pleiotropic 83kd NS1 polypeptide, an abundant and long-lived nuclear phosphoprotein, is the only NS protein which is essential for productive replication in all cell types.[7,8] What is currently known about the domain structure of NS1 is presented in Figure 1. It is a site-specific DNA binding protein, recognizing the sequence $(ACCA)_{1-3}$ present in the viral origins,[9] a sequence which is also reiterated in more or less degenerate forms at multiple sites throughout the viral genome.[10,11] Association and dissociation of NS1:DNA complexes is markedly dynamic under physiological conditions. Experimentally this interaction can only be demonstrated in the presence of ATP or by cross-linking the NS1 molecules with antibodies directed against their amino or carboxytermini.[11] Since the ability of NS1 to form multimers *in vivo* is known to be dependent upon the integrity of its ATP-binding site,[12] it seems probable that ATP, like the aforementioned antibodies, induces NS1 to oligomerize,

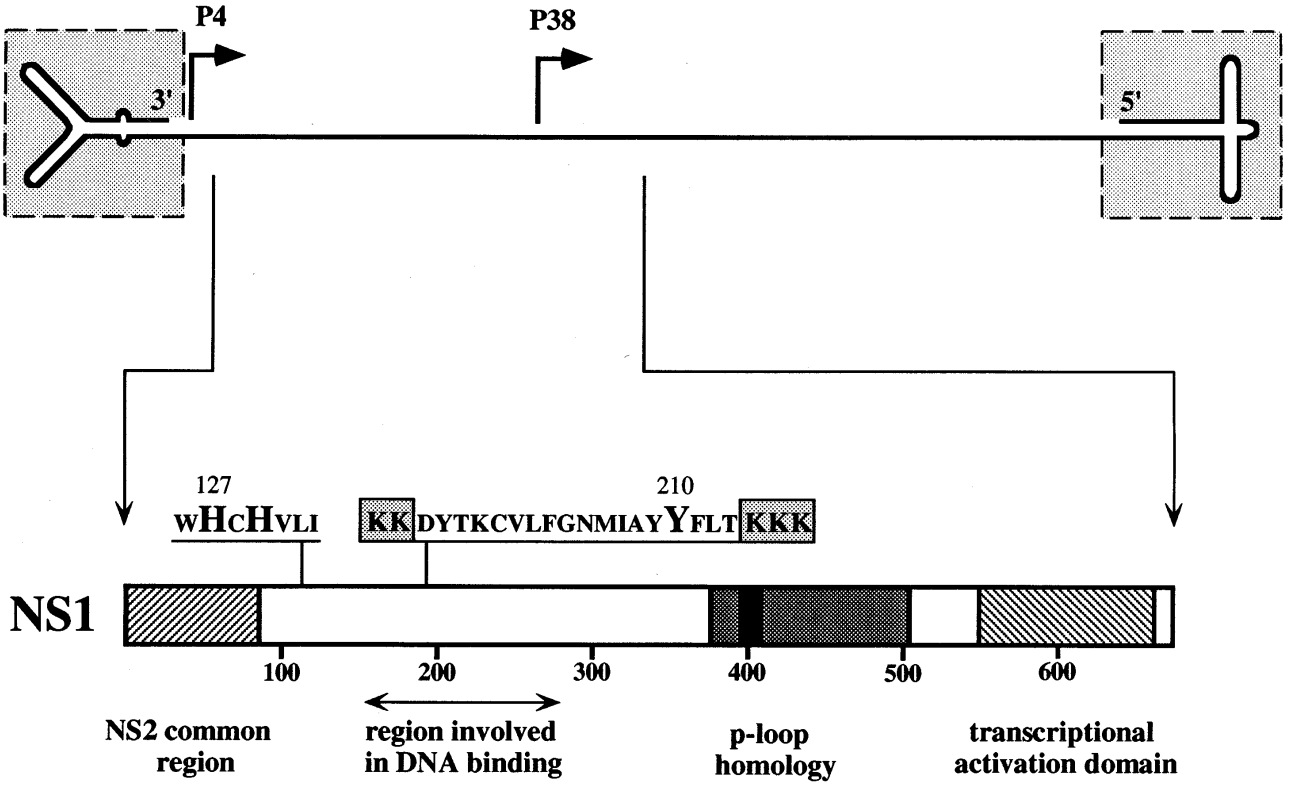

Figure 1. Genetic strategy of MVM. The line diagram of the MVM genome shows the non-structural gene promoter (P4) and the capsid gene promoter (P38). The terminal hairpins are shown, in the shaded, dashed boxes, scaled up approximately 20-fold. Below this is a block diagram of the NS1 protein encoded in the left half of the genome, indicating the positions of domains involved in DNA binding, ATP binding ■, helicase p-loop homology and transcription activation. Above this the rolling circle initiator protein homologies are detailed, with the putative metal coordination site involving histidines 127 and 129, and the DNA-linking tyrosine at residue 210. The two lysine clusters of the bipartite nuclear localization signal which straddle this residue are boxed.

and that only multimeric forms of the protein bind efficiently to its recognition sequence. NS1 initiates replication at the viral origins by introducing a site-specific single-strand nick,[13-16] thus liberating a base-paired 3' nucleotide to serve as a primer for strand displacement synthesis. During this process it becomes covalently linked to the 5' end of the DNA at the nick site via a tyrosine residue at amino acid 210[16] and is thought to remain in the replication fork. NS1 molecules remain attached to the 5' end of the genome through the packaging process, and are found on the outside of mature virions, anchored to them by a short region of 5' hairpin sequence, dubbed the 'tether'.[17] Interestingly, removal of this NS1 or cleavage of the tether sequence, which can be performed *in vitro* but also occurs upon entry into the cell, does not affect infectivity, implying that early in the replication process a mechanism exists for the restoration of these nucleotides.[17] NS1 molecules exhibit DNA-dependent ATPase and helicase activity *in vitro*[16,18-20] and inhibit cellular DNA replication by an as yet undefined mechanism.[21] Conserved protein sequence motifs in the catalytic site of this nickase [ref 16, as indicated in Figure 1] suggest that NS1 may have evolved from the initiator endonuclease of a prokaryotic replicon related to the modern pUB110 and pMV158 families of so-called single-strand DNA plasmids.[22]

NS1 molecules also act as a potent transactivators of transcription from the viral promoters, modulate the activity of various heterologous promoters and serve multiple, as yet poorly defined, cytostatic or cytotoxic functions (see Vannacker & Rommelaere, this volume). The 25kd NS2 polypeptides, also coded from the left half of the viral genome, are relatively short-lived, predominantly-cytoplasmic proteins synthesized to high copy number early in infection.[23] They share an 84 amino acid N-terminal sequence with NS1, have a central exon which is common to all forms of NS2 and are then spliced into small, unique terminal exons. NS2 proteins are only required for productive MVM replication in cells of murine origin,[7,8,24] but in these cells they clearly influence multiple steps in the replication cycle, including the extent of DNA amplification, single-strand progeny synthesis and capsid assembly.

Parvoviruses are unable to induce resting cells to enter S-phase,[25] and it is not until infected cells enter S-phase of their own volition, that viral transcription initiates.[26] As most of the initiating P4 promoter sequence, including its SP1-binding site and TATA box, is packaged as single-stranded DNA, synthesis of the complementary strand presumably precedes viral gene expression. As the early gene products NS1 and NS2 accumulate, host cell DNA replication is terminated.[21] Progression through the cell cycle is suspended, and cells remain actively synthesizing viral DNA until lysis or apoptosis occurs, resulting in the release of progeny virus starting around 16–20 hours after entry into S phase.

Rolling hairpin replication

Parvoviruses replicate their DNA through a series of duplex, concatemeric intermediates by the unidirectional, quasi-circular rolling hairpin mechanism[1] illustrated in Figure 2. As shown in step (i), the left-end terminal hairpin of MVM mediates conversion of virion DNA to the first duplex intermediate by pairing its extreme 3' nucleotide with an internal base and thus creating a DNA primer which can be used by a host polymerase to synthesize a complementary DNA strand. This generates a monomer-length, duplex intermediate in which the two strands are covalently-cross-linked at one end via a single copy of the viral left-end telomere. By analogy with AAV2,[27] in viruses which have a single terminal repeat sequence the cross-linked ends of such duplex molecules probably function as new replication origins, undergoing a terminal resolution reaction, discussed below, catalysed by the viral endonuclease NS1. However, in viruses with unique termini, only turn-around structures involving the right terminal palindrome (i.e. derived from positive-sense DNA strands) appear to be cleaved by NS1, while turn-around left-end termini, such as those derived from the negative-sense MVM genome, do not. In any event, such terminal processing is apparently limited at early times in infection, and the major pathway of DNA amplification appears to be through concatemeric duplex intermediates, as shown in Figure 2.

While synthesis of the first monomer-length duplex DNA molecules can occur in the absence of viral gene expression *in vitro*, in MVM their subsequent conversion to the dimer duplex intermediate is greatly facilitated by the addition of purified NS1 (Cotmore & Tattersall, unpublished observations). Exactly how this process occurs is uncertain, but most models suggest that it involves a non-nucleolytic process called 'hairpin transfer', in which the palindrome at the right-end terminal hairpin is first unwound and copied by strand displacement synthesis to create an extended-form terminus containing a single new DNA

strand [Figure 2, step (i)]. These termini are then melted-out and re-formed into hairpinned rabbit-ear structures [step (ii)], so that newly synthesized DNA now creates the hairpin needed to prime synthesis of additional linear sequences [step (iii)].[1] NS1 is known to bind to DNA fragments containing the right-end hairpin (Cotmore & Tattersall, unpublished observations) and, as seen in Figure 3, consensus NS1 binding sites are located both near the cut site and throughout the arms of its potential cruciform structure. It seems likely that NS1 oligomers bound at these sites might mediate hairpin transfer by aiding the unwinding or subsequent formation of rabbit-ear structures.

An alternative hypothesis involving ligation has also been suggested (17,21) which is based on the observation that NS1 and its associated tether sequence are removed from the outside of the virion during viral entry. According to this scheme, the advancing polymerase on parental single-stranded virion DNA does not replace the right-end hairpin, rather the 3' end of the newly synthesized complementary strand is ligated to the genomic 5' end. In this way a covalently continuous duplex genome would be synthesized which could now complete step (i) of Figure 2 by undergoing the NS1-mediated nucleolytic process dubbed terminal resolution, discussed later. While this process could occur at any round of right-end replication, it presumably could only occur on parental genomes or other genomes which had lost terminal nucleotides, since the presence of NS1 on all newly-formed 5' ends would preclude the ligation step. Whether such a reaction actually occurs is uncertain, but covalently-continuous duplex, or 'no-end' molecules, have been observed in the infected cell.[28]

The result of rolling hairpin synthesis is that the coding sequences of the virus are copied twice as often as the termini, and palindromic duplex dimeric Figure 2, [step (iv)] and tetrameric [step (vi)] concatemers accumulate in which unit-length genomes are fused in left-end:left-end and right-end:right-end orientations. Individual genomes are ultimately excised from these concatemers, and their telomeres replicated, by the introduction of single-

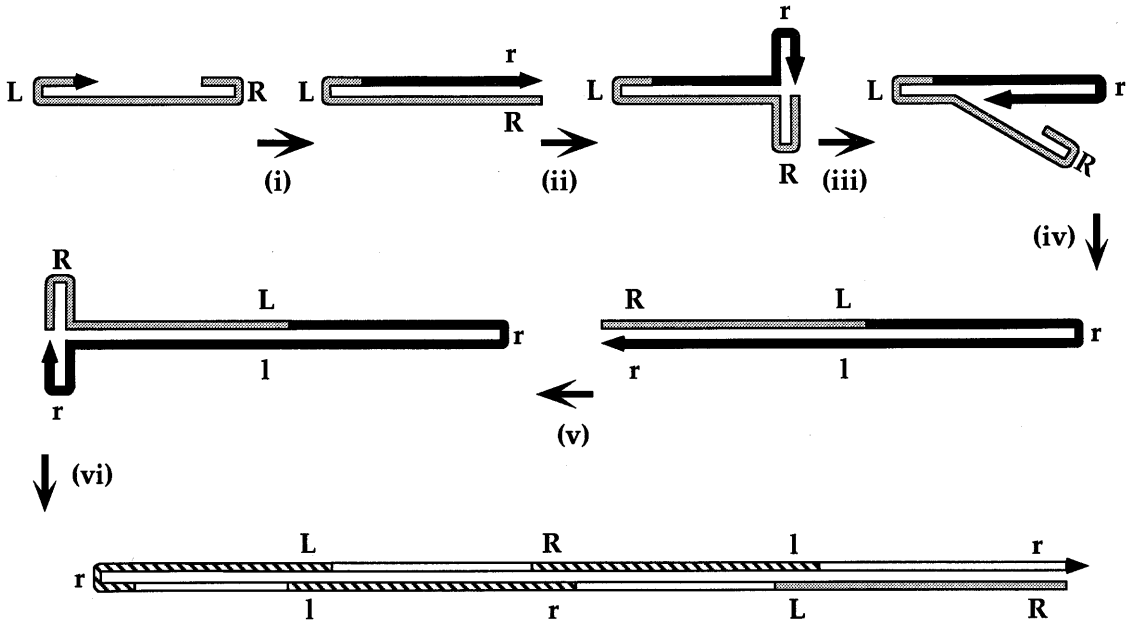

Figure 2. Rolling hairpin replication. The sequence of the parental parvoviral genome is represented by a shaded bar. In steps (i) through (v) newly synthesized DNA is shown as a black bar with an arrow at its 3' end. **L** and **R** depict the palindromic sequences at the left and right termini, with their complements represented by **l** and **r**, respectively. Step (vi) produces a tetramer in which there are three progeny genomes, shown cross-hatched, in addition to the parental sequence. These overlap by their terminal palindromes and are distributed throughout the tetramer molecule on alternate strands.

strand nicks into replication origins generated at either end of the genome during the rolling hairpin process. After serving as the site-specific endonuclease, 5'-linked NS1 molecules are thought to be involved in the establishment and maintenance of a unidirectional replication fork initiating at the nick which allows duplication of the terminal palindrome. Extended form right-end termini created by this process are equivalent to those seen in Figure 2, steps (i) and (iv), and can be melted out and reformed into rabbit ear structures capable of priming additional rounds of DNA synthesis. Subsequent displacement of progeny single-strand DNA appears to be entirely dependent upon the availability of competent capsids and packaging of infectious progeny virus requires ongoing viral DNA synthesis.[29]

The products and kinetics of DNA synthesis, as determined in highly synchronized single rounds of infection,[28,30] support this general model. Between 4 and 12 h after release of infected cells into S-phase, palindromic monomer (mRF) and dimer (dRF) replicative form molecules increase exponentially and at similar rates, such that the ratio of mRF relative to dRF remains constant. During this phase the majority

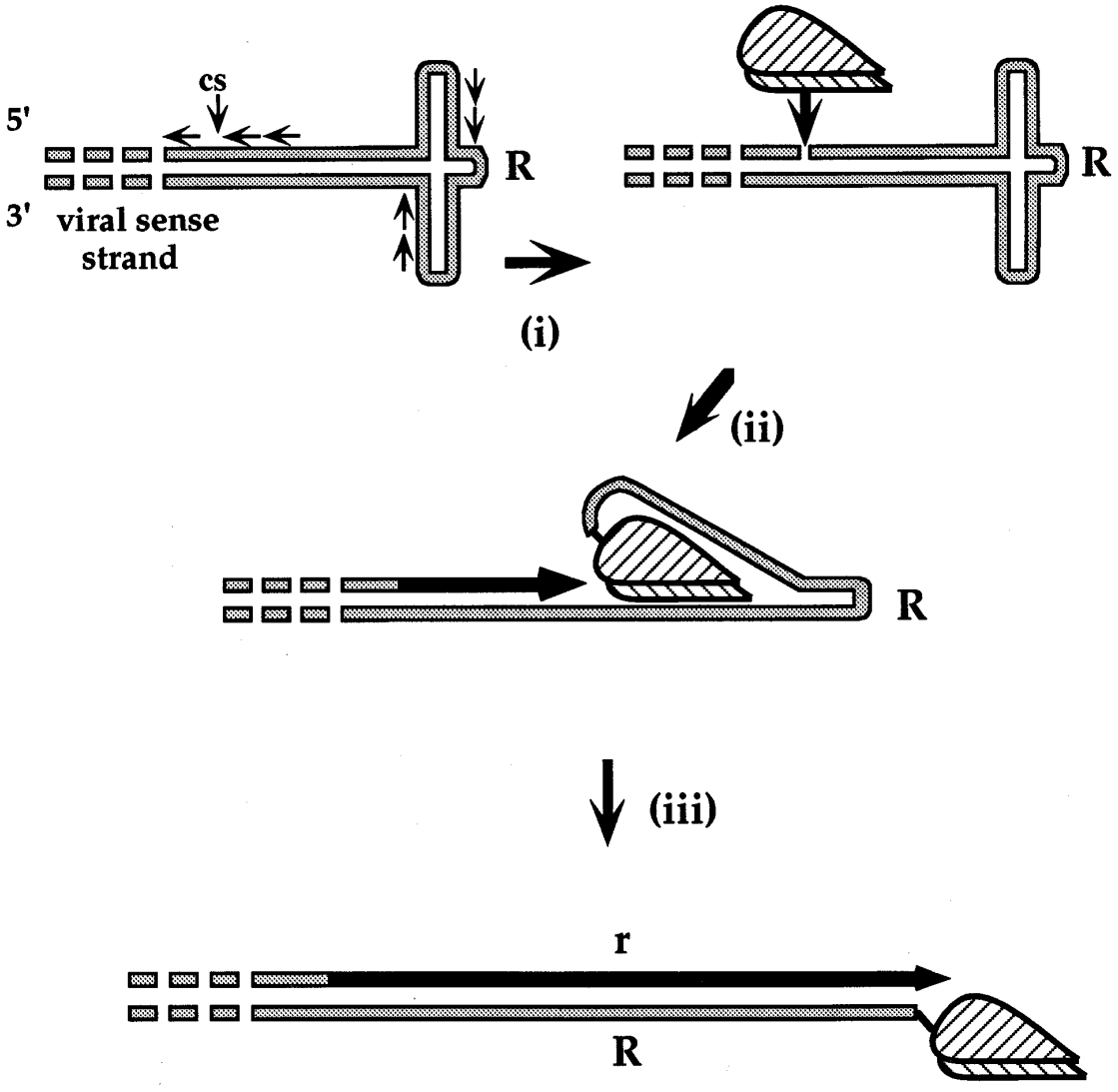

Figure 3. Terminal resolution at the MVM right-hand end. The diagram is structured as in Figure 2. **R** and **r** denote sequences which are the inverted complements of one another. The double image of the NS1 protein indicates that the active form is believed to be a dimer. Small arrows indicate (ACCA) DNA sequences recognized by NSI.

of mRF molecules have extended forms at both ends, whereas most of the remaining molecules are covalently closed at their left ends and extended at their right ends, and only a few copies of the right-hand turn-around form are observed, suggesting that this form must be quickly converted to the extended form. Moreover, most dRF and higher order concatemers contain single-stranded nicks in the left-end;left-end junction sequences, indicating that while resolution of dRF into two mRFs does occur by a site-specific endonucleolytic process, nicking is followed by a rate-limiting step which is not seen for the right-hand telomere.[30] These observed differences in the way in which the two termini are processed are reflected in the different resolution strategies, terminal resolution and junction resolution, described below.

Terminal resolution at the MVM right-end origin

Duplex replication intermediates which are covalently cross-linked at the right end by a single copy of the terminal palindrome, as produced by step (iii) in Figure 2, are presumed to be resolved to an extended form configuration containing two copies of the palindrome, by a process described as terminal resolution. First suggested by Cavalier-Smith[31] as a theoretical solution to the problem of maintaining the sequence of linear chromosome ends during cellular DNA replication, to date this type of resolution has only been recapitulated *in vitro* using substrates derived from the genome of the helper-dependent parvovirus AAV2,[27,32,33] although a similar process is thought to occur at the 5', but not the 3', terminus of MVM.[34]

The right-end of MVM RF DNA is depicted in Figure 3 in the turn-around configuration with the new viral-sense DNA strand at the bottom of the diagram. Although shown in its cruciform configuration, this sequence can also be arranged into a relatively simple linear configuration with a single mismatch region involving sequences at the tips of the cruciform arms. Since differences in the energy states of these two configurations are low, either or both forms may exist *in vivo*. NS1 is presumed to bind to the ACCA sequences [marked by arrows] and introduce a nick at a specific site (**cs**) within the nick consensus sequence CTWWTCA. Cleavage results in the formation of a phosphodiester bond between the phosphoryl group of the 5' nucleotide and an aromatic hydroxyl group from tyrosine 210 in the NS1 amino acid sequence,[16] and effectively transfers the original copy of the complex palindrome onto the 5' end of the viral-sense strand, while providing a base-paired 3' hydroxyl to prime synthesis of its complement. Terminal resolution thus replaces the original sequence of the hairpin, '**R**', with its inverted complement '**r**'. Since this inversion is repeated with every round of terminal resolution, progeny genomes contain equal numbers of right-end termini in each of the two sequence orientations. The terminal palindromes of all parvoviruses are imperfect, so that these alternate sequence orientations, dubbed 'flip' and 'flop', can be readily identified. The absence of such heterogeneity at the left-end terminus of MVM first alerted Astell and her colleagues[6] to the likely existence of an alternate mechanisms for telomere regeneration, described below as 'junction resolution'.

Structure of the MVM left-end origin

In MVM the 121 nucleotide left-end telomere (Figure 4, top left) exists as a single flip sense sequence containing internal palindromes designated the 'ears', and an asymmetric, mismatch 'bubble' in the stem where the triplet GAA on the inboard arm is opposed to the doublet GA on the outboard strand.[4-6] During rolling hairpin replication this sequence is extended and copied to form the palindromic (left-end:left-end) duplex bridge structure which links unit length genomes in the dimer intermediate [Figure 2, step (iv)], and it is this duplex structure, rather than a single hairpinned terminus, which contains the replication origin.[15] Figure 4, step (i) shows how the asymmetries in the terminus are disposed and copied in the bridge sequence. Since it contains two copies of the terminal hairpin, one in each sequence orientation, it also contains two candidate nick sites (**cs**) located on opposite DNA strands and one on each arm of the palindrome. However, only the nick site on the arm carrying the GA/TC doublet functions as a replication origin.[15] Since the replication fork initiated at this site is undirectional and supports the synthesis of a single, continuous DNA strand, use of a single nick site inevitably results in the synthesis of DNA in a single sequence orientation.

The active MVM left-end origin (Figure 4, bottom) is a multi-domain structure of approximately 50 base pairs, extending from an Activated Transcription Factor (ATF) consensus-binding site at one end to a position some seven base pairs beyond the cut site. An

$(ACCA)_2$ NS1 binding sequence is positioned 17 nucleotides from the cut site, separated from the ATF consensus by the TC bubble dinucleotide. The actual sequence of the bubble dinucleotide appears unimportant, suggesting that it is not an essential part of the binding site for a cellular replication factor, but insertion of any third residue here totally inactivates the origin. This suggests that the bubble represents a critical spacer element, presumed to control the interaction, with $(ACCA)_2$-bound NS1, of a cellular protein which binds at the ATF consensus site. This precisely controlled interaction appears to activate the endonuclease function of NS1, since purified NS1 alone is unable to nick the origin sequence with appreciable efficiency or detectable specificity. Mutations in the ATF-consensus impair origin function, suggesting that a cellular site-specific DNA-binding protein may well interact with this sequence to allow initiation, but while purified recombinant ATF 1, 2 or 3, or the related CREB-1 homodimer, bind avidly to the site, they suppress rather than stimulate, origin-specific replication *in vitro* (Christensen, Cotmore & Tattersall, in preparation). In contrast, a substantially enriched cellular protein fraction from 293 cells contains a factor(s) which is able to cooperate with NS1 to mediate left-end origin-specific nicking and replication *in vitro*, but which is not required for activation of the MVM right-end or the SV40 origin (Christensen, J., Cotmore, S.F. and Tattersall, P., in preparation). This fraction, dubbed **pif** for 'parvovirus initiation factor', contains a 120kd protein which shows sequence-specific cross-linking to a double-stranded oligonucleotide containing the MVM ATF-binding site sequence. Although this protein is the subject of much current interest, its role in the biology of the host cell has yet to be determined.

The replication fork initiated on the left-end origin is aphidicolin-sensitive, unidirectional and supports the synthesis of a single, continuous DNA strand, suggesting that polymerase δ and its accessory

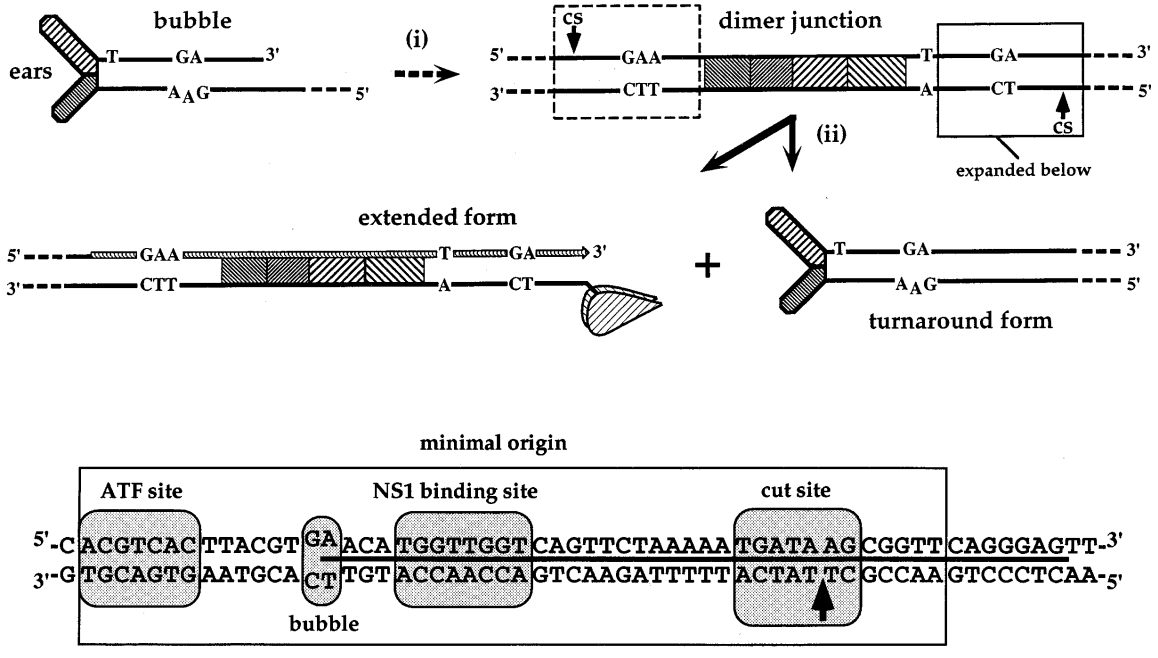

Figure 4. Junction resolution reaction for the left-end of MVM. Step (i) condenses the in-vivo steps (i) through (iv) of Figure 2, showing the organization of the left-end hairpin sequences within the dimer junction in greater detail. Cross-hatched boxes represent the palindromic sequences which fold to give the internal 'ears' in the hairpin form of the 3' end of the genome. Step (ii) represents the in-vitro resolution reaction described in the text. The potential cut sites are denoted **cs**, and newly synthesized DNA is depicted as a hatched bar with an arrow head at its 3' end. The domains within the minimal origin described in the text are boxed, and the underline indicates the sequences protected by NS1 from DNAseI digestion.[11]

proteins are required for its synthesis. This possibility is supported by the finding that neutralizing monoclonal antibodies directed against polymerase α:primase fail to inhibit MVM replication *in vitro*, while biochemical studies clearly indicate an absolute requirement for PCNA and RPA (Christensen, J., Cotmore, S.F. and Tattersall, P., in preparation). Since MVM gene expression coincides with a rapid reduction in the rate of cellular DNA replication,[21] it also seems likely that the virus inactivates a critical cellular replication protein which it does not itself require.

Since insertion of any third nucleotide into the bubble dinucleotide TC inactivates the origin, the analogous sequence derived from the inboard arm of the viral telomere, which is identical to the origin except for the presence of a triplet bubble sequence GAA, is completely inactive.[15] It seems likely that the use of such a replication strategy is an adaptation which, by restricting NS1-mediated nicking to the outboard arm of the telomere, allows similar, but subtly different, configurations of these same sequences on the inboard arm and in the mismatched, hairpinned configuration of the terminus to be dedicated to transcription.[35,36]

Junction resolution at the left-end origin

At the 5' end of the MVM genome a terminal resolution process results in the generation of equal numbers of termini in flip and flop configurations, while at the left-end telomere a more restricted, 'junction resolution' mechanism generates termini in a single orientation. While junction resolution mechanisms probably operate on all parvoviral termini when these are presented as the junction fragments of concatemeric replication intermediates, in MVM and many of the other autonomous viruses, a critical asymmetry in the stem of the left-end hairpin, the bubble, restricts this mechanism to the synthesis of a single sense DNA strand.[15]

As seen in Figure 4 (middle), MVM left-end bridge sequences are resolved and replicated asymmetrically *in vitro*.[14,37] Each junction is resolved into two viral telomeres, one in the extended configuration containing a duplex copy of the entire palindrome and one containing a single copy of the palindrome in the covalently-continuous, hairpinned configuration. As shown, one of the strands of the extended-form terminus is newly-synthesized, whereas its unreplicated complement is now covalently attached through its 5' end to an NS1 molecule. How such resolution occurs is uncertain. It was originally suggested that NS1 might carry out a sequence of nicking and joining reactions analogous to those performed by the gene A protein of phage ØX174.[6] According to this model the initiating nick would be introduced at the GAA-proximal site, thus liberating a 3' hydroxyl capable of priming synthesis of a single, flip-sense strand across the axis of the palindrome. The NS1 molecule attached to the 5' end of the displaced strand, remaining in the replication fork as it traversed the bridge, would then encounter the 'latent' nick site on the opposite, TC, strand, and terminate the reaction at this second site by exchanging itself across analogous phosphodiester bonds.[5,6] This is an attractive model since it would recapitulate processes employed in prokaryotic rolling circle replicons, but it is unfortunately incompatible with initiation in the TC arm of the palindrome, as this would lead to the synthesis of DNA strands with the flop (TC/TCC) sequence, rather than allowing persistence of the flip (GAA/GA) sequence, as observed *in vivo*.

In order to synthesize a DNA strand in the flip configuration from a fork initiating in the TC arm, the bridge could be re-configured into a cruciform structure at a point soon after establishment of the replication fork, as shown in Figure 5, steps (i–ii). This would allow the fork to copy sequences in the flop orientation through the axial asymmetries of the palindrome, producing a new flip sense strand via a series of discrete linear [step (i)] and cruciformed [steps (ii-iv)] intermediates. The limited experimental data available tends to support such a mechanism;[14,15] replication forks are established on linear sequences derived from a single-arm of the bridge but resolution of dimer bridge substrates in murine cell extracts leads to the accumulation of nicked and partially-replicated bridge intermediates having structures compatible with those illustrated in Figure 5, steps (i) through (iv). Moreover, resolution *in vitro* only occurs if the two arms of the bridge are sufficiently palindromic to favor formation of a cruciform structure (Cotmore and Tattersall, unpublished). While it thus seems probable that resolution proceeds through a cruciformed intermediate, how this structure is then cleaved is entirely a matter of speculation. Since we are unable to reconcile a NS1-mediated termination reaction with the available data, we suggest the recombinase-mediated strategy shown in Figure 5, step (v). While this is currently entirely speculative, it points out that there are mechanisms available in the cell by which the final products could easily be explained.

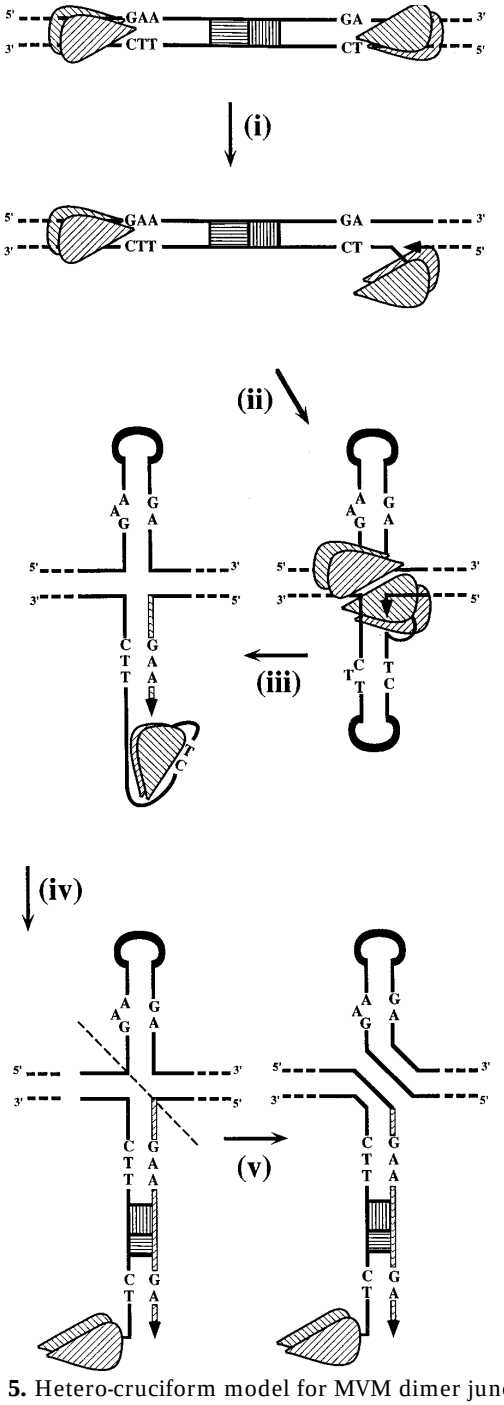

Figure 5. Hetero-cruciform model for MVM dimer junction resolution. Duplex forms of the internal 'ear' palindromes are represented by hatched boxes, while hairpinned forms of these sequences are shown as thick black lines. NS1 proteins are shown bound to each arm of the junction as dimers, and from step (iii) on, the complex on the 'inactive' arm is omitted for clarity. The dashed diagonal line indicates the preferred resolution axis of the cellular recombinase hypothesized to recognize the hetero-cruciform formed in step (iv) as a 'Holliday structure' substrate, and to resolve it in step (v).

Synthesis of progeny single-stranded virion DNA

Although low levels of progeny single-strand DNA synthesis can be detected within 5 hours of the start of S-phase, late in infection this type of replication predominates.[21] Such synthesis is entirely dependent upon the availability of preformed capsids and all single-stranded DNA appears to be encapsidated. However, since packaging requires on-going viral DNA synthesis,[29] it is not a simply a process of melting out and sequestering preformed DNA strands.

All the *cis*-acting information required for packaging is carried within the viral termini,[38,39] but little is known about the mechanism. The virus LuIII shares 80% identity with MVM[40] and has highly homologous unique termini,[41] but it encapsidates approximately equal amounts of both strands.[42] Both strands of a transgene construct flanked by the LuIII termini were encapsidated equally well,[39] suggesting that the minor sequence differences in the termini of LuIII and MVM may be critical. However, the reciprocal experiment with MVM termini has yet to be reported, and it has been suggested[39] that packaging both strands may be the default pathway. According to this explanation, there would have to be specific sequences within the body of MVM, but not LuIII, which suppress packaging of the positive sense strand. LuIII does contain a unique A/T-rich region near the 5' end of its genome, but the function of this region remains speculative. Alignment of the published LuIII and MVM sequences indicates that this A/T-rich region exists as an insertion into an element that, in MVM, binds cellular proteins.[39,43]

Since packaged, defective genomes invariably contain sequences from the 5' end of the genome,[38] and because displacement synthesis would release single-strands from the duplex in a 5' to 3' direction, we presume packaging initiates at the 5' end of the genome. NS1 molecules are present on the 5' ends of all newly synthesized single-strand DNA, and during packaging these molecules remain on the outside of the particle, tethered by a 24 nucleotide sequence.[17] This suggests that NS1 may mediate encapsidation, either by establishing the primary interaction with the empty particle and/or by oligomerizing with NS1 molecules bound to their cognate sites throughout replicating duplex DNA. Subsequent ATP hydrolysis would lead NS1 to release the DNA and could provide the energy required to drive sequestration of the single-strand as it was displaced.

An alternative 3'-to-5' packaging scheme has been

proposed[44] based on the observation that viral capsids bind strongly to the left (3' end) of the Aleutian disease virus (ADV) genome when these sequences are present in the covalently-closed 'turn-around' configuration, but not when they are re-arranged into linear extended forms. A similar interaction was later demonstrated between MVM capsids and turn-around forms of the MVM left-end palindrome, where it was shown to be mediated by sequences at the branch point between stem and fork regions of the hairpin.[45] These interactions are undoubtedly very strong and are likely to mediate genome–virion association at some point in the viral life cycle, although exactly when and where remains unclear.

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