Asymmetric Resolution of a Parvovirus Palindrome In Vitro

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Cell extracts from murine A9 or human HeLa cells containing wild-type copies the NS1 polypeptide of minute virus of mice (MVM), produced from a recombinant vaccinia virus, can support the resolution of viral 3' termini from palindromic junction fragments of dimeric, replicative-form MVM DNA. Resolution resulted in the generation of two new viral termini, one associated with each arm of the junction palindrome. Telomeres were created in two configurations, "extended" forms, which were covalently associated with NS1 molecules, and smaller "turn-around" forms in which a single arm of the palindrome terminated at the axis of dyad symmetry in a covalent bond which cross-linked the two strands. The *in vitro* resolution reaction was asymmetric, generating predominantly extended-form termini from one arm of the palindrome but predominantly turn-around forms from the other. This asymmetry was independent of the type of cell used to prepare the *in vitro* extract or the orientation of the palindrome in the plasmid and was obtained for all cloned junction sequences of 156 bp or more. Two modified forms of the duplex junction fragment, which appeared to be intermediates in the resolution process since they were nicked, covalently linked to NS1, and associated with newly synthesized DNA, were identified. The structures of these intermediates suggest that resolution is initiated by preferential nicking at one of the two candidate resolution sites. The asymmetric nature of this resolution reaction is discussed in terms of current models of MVM DNA replication.

Parvoviruses have linear, single-stranded DNA genomes, which are replicated by cellular polymerases through a series of monomeric and concatemeric duplex intermediates (30, 33, 35). The 3' hydroxyl group at the end of the viral genome can be used directly to prime DNA synthesis because the termini contain complex palindromic sequences which allow them to fold into hairpin-like structures in which the terminal nucleotide is paired to a complementary internal base. Synthesis of a complementary DNA strand thus results in the formation of a duplex molecule with a covalently closed (viral 3') terminus, which must be cleaved and copied to duplicate the original telomere. It was suggested initially (6, 34) that these structures could be resolved by a hairpin transfer mechanism, such as that proposed by Cavalier-Smith (8), in which a specific endonuclease cuts the parental DNA strand at the inboard junction of the terminal palindrome and in so doing inverts the original palindrome onto the 5' end of the daughter strand. This nick provides a base-paired 3' hydroxyl group at the end of the parental strand to prime synthesis of a new telomere, by using the original palindrome as template. Since parvoviral termini are imperfect palindromes, the original parental telomere is replaced by a sequence which is its inverted complement. This type of hairpin transfer with concomitant sequence inversion has been demonstrated *in vitro*, using the covalently joined terminal sequences of a defective parvovirus, adeno-associated virus type 2 (AAV2) as substrate (21, 22, 31). However, this mechanism cannot satisfactorily explain the regeneration of the 3' telomeres of some of the autonomous parvoviruses, since these imperfect palindromes are conserved in a single orientation both in packaged viral DNA and in duplex intracellular replicative-form (RF) DNA (1–3, 16).

Minute virus of mice (MVM) is one such virus. In order to accommodate a self-priming replication mechanism with

sequence conservation at the 3' terminus of the negative-sense MVM genome, Astell and colleagues (1, 3) suggested that this telomere might be resolved asymmetrically from a different type of duplex substrate. During viral replication, synthesis of dimeric and tetrameric duplex forms of the genome in which unit-length molecules are fused in left-end to left-end (viral 3'-to-3') and right-end to right-end (viral 5'-to-5') orientations, respectively (35), occurs. Astell and colleagues suggested that instead of nicking the covalently closed, left-end terminus of monomer RF, the initiating endonuclease might only be able to cleave after recognizing a complex DNA sequence present on one side of the imperfect palindrome which makes up the junction region of dimeric replication intermediates. Introduction of a single nick into this structure would permit the use of a single DNA strand as the template for DNA synthesis, (1, 3). These investigators suggested that subsequent steps in the resolution process might mimic those employed by the gene A protein of phage ϕ X174 to replicate single-stranded, circular phage DNA (17). This step in their model, which has been dubbed "the modified rolling hairpin model," is diagrammed in Fig. 1A. Briefly, the initiator endonuclease introduces a site-specific, single-strand nick on one side of the junction sequence, and the endonuclease itself becomes covalently attached to the 5' end of the DNA at this site. Replication initiates at the new 3' hydroxyl group, and displacement synthesis continues across the apex of the palindrome until a second, latent, cut site is encountered in the opposite strand. The initiator protein is then able to nick the new site and exchange itself across the analogous phosphodiester bond. This ligates the duplex on one side of the junction, giving rise to an unreplicated, covalently closed, viral terminus, while the other side of the junction is released as an extended-form telomere containing one newly synthesized DNA strand.

We have shown previously that junction fragments from dimeric and tetrameric replication intermediates of MVM, cloned into *Escherichia coli* plasmids, can act as replication origins when transfected into murine cells superinfected with

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FIG. 1. (A) Diagram of the resolution step in the modified rolling hairpin model of Astell et al. (1, 3), showing how the parental sequence orientation at the left end can be transferred to progeny genomes (as described in the Introduction). Thick horizontal arrows indicate newly synthesized DNA; vertical arrows indicate the axes of symmetry; V and C denote viral and complementary sense strands, respectively; par and prog denote parental and progeny strands, respectively. The so-called bubble sequences, in which the triplets 5'-GAA-3' and 5'-TTC-3' in one arm of the palindrome are paired, in the hairpin configuration, to the doublets 5'-GA-3' and 5'-TC-3', respectively, in the other arm, are indicated. These sequences are located 27 nucleotides inboard of the potential endonuclease cut sites and appear to be critical in determining the selection of the single cut site. (B) Plasmid map of pLEB 711, showing the MVM 3' concatemer junction sequence (crosshatched), and the positions of various restriction endonuclease sites. The two arms of the MVM palindrome have slightly different sequences and are designated A and B (in crosshatched sections) in the figure. Those asymmetries in the so-called bubble sequence, are indicated, in order to allow orientation of the inserts with the viral genome. In their respective hairpin configurations, 5'-GAA-3' in the A arm would be paired to 5'-GA-3' in the B arm, and 5'-TTC-3' in the A arm would be paired to 5'-TC-3' in the B arm. The terminal residue at the 3' end of the viral genome is probably at position -3 with regard to the numbering in the published sequence (4, 14, 16). This nucleotide is, therefore, adjacent to the presumed resolution sites (marked with large arrows) on either side of the palindrome. These resolution sites have identical sequences. Small arrows indicate the positions of MVM nucleotide 415 on each strand, located at the virus-vector junction. (C) Predicted structure of the duplex, Y-shaped hairpin which forms the 3' terminus of single-stranded virion DNA. The bubble sequence 5'-GAA-3' paired to 5'-GA-3' is indicated. This structure is in the flip orientation, which is found at the 3' terminus of all virion DNA (1-3). (D) Diagram of the predicted structure of the template DNA and the resolution products following digestion with *ScaI*, giving their calculated lengths. MVM sequences are shown as crosshatched sections, and newly synthesized DNA is indicated by a bold line with an arrowhead indicating the direction of synthesis. The filled circle at the end of the DNA strand represents the covalently associated NS1 polypeptide. A and B in the crosshatched sections indicate the two arms of the palindrome as shown in panel B; ext indicates the extended-form configuration, and trn indicates the turn-around configuration of the resolved termini.

MVM (15), giving rise to linear plasmid molecules with viral telomeres which are structurally indistinguishable from those generated at the viral termini during infection. Moreover, we have shown that junction fragments from the right end of the viral genome, which does show sequence inversion *in vivo*, are resolved symmetrically *in vitro*, giving rise to similar structures from both arms of the palindrome (11). In this paper, we examine the resolution of junction clones from the left end of the viral genome *in vitro* and show that these clones are resolved by an asymmetric reaction, leaving one arm of the junction in the extended configuration and one in the turn-around form as predicted by Astell and colleagues. Analysis of two partially replicated structures which are generated *in vitro*, however, suggests that the initiating single-strand nick is not introduced at the site predicted by the model, indicating that either the resolution assay or the model needs to be refined.

MATERIALS AND METHODS

Templates. The pUC 18-based plasmid, pLEB 711, containing the palindromic, 711-bp *Pst*I fragment (MVM nucleotide 415) derived from the left-end to left-end (viral 3'-to-3') junction of dimeric RF DNA has been described previously (15) and is diagrammed in Fig. 1B. Other plasmids were obtained by excising the bridge region of this construct with the appropriate enzymes and recloning them back into pUC vectors. Thus, pLEB 157 contains the *Pme*I (MVM nucleotide 136) bridge fragment of pLEB 711 cloned into the *Sma*I site of pUC 19, and pLEB 407 contains the *Nco*I (MVM nucleotide 259) bridge fragment from pLEB 711 cloned into an *Nco*I linker in the *Sma*I site of pUC 19. Plasmid pLEB 407-REV was derived from pLEB 407 by excising the viral insert at adjacent polylinker sites and reversing it with respect to the vector sequences by recloning it into equivalent sites in pUC 18.

Recombinant vaccinia viruses. Recombinant vaccinia viruses expressing wild-type copies of the MVM NS1 polypeptide from a phage T7 promoter have been described elsewhere (27). This recombinant, vv-NS1_{wt}, provides efficient expression of full-length, nuclear NS1 in both HeLa and A9ouab⁺11 cells when they are coinfecting with a second recombinant vaccinia virus, vTF7-3, which expresses T7 RNA polymerase (18).

Cell extracts and resolution assays. Cytoplasmic extracts were prepared as described by Stillman and Gluzman (32) from HeLa cells or from the murine L-cell derivative A9ouab⁺11 grown in suspension culture (5×10^5 cells per ml) in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Control nuclear extracts containing T7 polymerase alone and extracts containing the polymerase together with wild-type copies of NS1 were prepared from both cell types grown in monolayer culture approximately 14 h after infection with 15 PFU per cell of vTF7-3 alone or 15 PFU each of vTF7-3 and vv-NS1_{wt} per cell, as previously described (11). Resolution assays contained cytoplasmic extracts from uninfected cells supplemented with control nuclear extracts or nuclear extracts containing NS1, deoxynucleotides, ATP and an ATP-regenerating system, supercoiled substrate DNA (2 μ g/ml), and [³²P]dGTP as previously described (11). Reaction products were processed for analysis as described previously (11).

RESULTS

Structure of the substrate and predicted resolution products. The substrates used in these assays, pLEB 711, pLEB 407, pLEB 407-REV, and pLEB 157, are pUC 18- or pUC 19-based plasmids containing the viral 3'-to-3' (left-end to left-end) palindromic junction of dimeric MVM RF DNA. The structure of pLEB 711 is shown in Fig. 1B, and the positions of the *Nco*I and *Pme*I sites, which bracket the viral inserts in clones pLEB 407 (and pLEB 407-REV) and pLEB 157, respectively, are indicated. The long arrows indicate the positions of the two candidate resolution sites which are situated on either side of the axis of symmetry, in the complementary strand opposite the future 3' end of progeny single-stranded virion DNA. Sequence comparison with that of the closely related parvovirus LuIII (16) and the position of the MVM 5' cut site (14) suggest that the 3' nick is introduced 3 nucleotides upstream of the published MVM 3' end sequence, at the position indicated in Fig. 1C.

The region of the junction fragment located between the two putative resolution sites is an imperfect palindrome in which 108 of the 121 nucleotides can be base paired in the hairpin form (4). The two arms of the palindrome bearing these sequence disparities are indicated by hatched segments labeled "A" and "B" in Fig. 1B and D. If the resolved plasmid DNA is digested with a restriction enzyme such as *Sca*I, which cuts once within the vector sequences, resolution products of different lengths are obtained from the two arms of the palindrome. This allows us to track the fate of viral sequence asymmetries. The 3' end of progeny virion DNA carries a hairpin form of one of the DNA strands from the apex region, in the so-called "flip" orientation (1-3). As shown in Fig. 1C, this sequence is most stably folded into a Y-shaped configuration in which the nick site is positioned on the stem of the Y, 45 nucleotides from the base of the apical fork structure. Within this stem, the sequences are predominantly base paired, the major exception being a "bubble" located 27 bp inboard of the nick site in which the sequence 5'-GAA-3' in the inboard arm of the telomere is aligned opposite the sequence 5'-GA-3' in the outboard arm. These asymmetric bubble sequences are also included in the plasmid map to indicate the orientation of the viral sequences in the construct.

To initiate resolution of substrates such as pLEB 711, we hypothesize that the viral insert must first be nicked at one or both of the candidate resolution sites, thus making available 3' hydroxyl groups which can prime DNA synthesis by host polymerase(s), through the axis of symmetry, to the position of the resolution site on the complementary strand and thereby replicate the sequence of the terminal palindrome. The 3' termini of MVM duplex RF DNA are known to occur *in vivo* in both extended and turn-around configurations (9, 35). Extended-form termini are covalently associated with NS1 molecules via their 5' ends (13) and contain a duplex copy of the 121-bp palindrome which is located at the apex of the junction fragment and which eventually gives rise to the hairpin structure at the 3' end of progeny single-strand DNA (4, 16). Termini in the turn-around configuration contain a single copy of the palindrome, terminating at the axis of dyad symmetry in a covalent bond which cross-links the two strands. If pLEB 711 were to be resolved into similar structures, it would give the *Sca*I digestion products indicated in Fig. 1D.

In vitro resolution requires NS1. Resolution assays contained cytoplasmic extracts from murine A9 cells or HeLa cells supplemented with either (i) control nuclear extracts

FIG. 2. Autoradiograph of a 1.1% agarose gel showing deproteinized *ScaI* digests of [32 P]dGTP-labeled in vitro reaction products generated when pUC 18 (lanes 2 and 3) or pLEB 711 (lanes 4 to 8) are incubated in cytoplasmic extracts from A9 cells (lanes 2 to 5) or from HeLa cells (lanes 6 to 8) with the addition of control nuclear extracts from A9 cells infected with vTF7-3 alone (lanes 2, 4, and 6) or nuclear extracts from A9 cells containing the MVM NS1 polypeptide expressed from a recombinant vaccinia virus (lanes 3, 5, 7, and 8). Lanes 2 to 6 and 8 contain 2.5% of the total products from a single reaction, and lane 7 contains 0.625% of the total products. Lin denotes monomeric, linear forms of the plasmids; A and B denote the two arms of the palindrome, and ext and trn denote extended and turn-around forms of the resolution products, respectively. Molecular weight standards (indicated on the left in thousands) are shown in lane 1.

obtained from cells infected with the recombinant vaccinia virus vTF7-3 which expresses the T7 polymerase gene or (ii) nuclear extracts from cells dually infected with vTF7-3 and vv-NS1_{wt}, the recombinant vaccinia virus carrying a wild-

type NS1 gene expressed under the control of a T7 promoter (27). When the MVM concatemer junction plasmid pLEB 711 or its pUC 18 parent was incubated in control reaction mixtures without NS1, relatively little [32 P]dGTP was incorporated into the DNA (Fig. 2, lanes 2, 4, and 6). It seems likely that this limited incorporation reflects nick translation, or repair synthesis, which requires only cellular enzymes that are present in the extract. In contrast, when the extracts contained NS1, incorporation of [32 P]dGTP into the MVM junction plasmid increased dramatically (Fig. 2, lanes 5, 7, and 8), while incorporation into its pUC 18 parent was barely affected (Fig. 2, lane 3).

Plasmids containing palindromic MVM sequences migrate on neutral agarose gels as a complex mixture of slowly migrating forms (15) (Fig. 3A, lane 2). To determine whether the input plasmid was specifically resolved within the viral sequences and to discriminate between the products of the two arms of the palindrome, we cut the DNA with *ScaI* (Fig. 1B). DNA synthesis therefore would generate 32 P-labeled linear 3,397-bp molecules from unresolved plasmids and a number of smaller 32 P-labeled fragments (diagrammed in Fig. 1D) from those molecules which had been resolved. *ScaI* would be expected to give extended-form fragments that were 1,346 and 2,171 bp from the A and B arms of the pLEB 711 palindrome, respectively, and fragments that were 1,284 and 2,112 bp, respectively, if these termini were also present in the turn-around configuration. Digestion products approximately corresponding in size to all four types of termini were generated from the reaction products of the pLEB 711 substrate incubated in the presence of NS1 (Fig. 2, lanes 5, 7, and 8), although those corresponding to extended forms of the A arm and turn-around forms of the B arm clearly predominate.

The in vitro system used here relies upon DNA synthesis to label the reaction products. Although this system offers the major advantage of identifying the relatively small proportion of substrate molecules processed in the reaction (5 to 10%, as estimated by Southern transfer), the results require careful interpretation. Incorporation of label by nick trans-

FIG. 3. Autoradiographs of 1.1% agarose gels showing the deproteinized [32 P]dGTP-labeled products obtained when pLEB 711 is incubated in reaction mixtures containing A9 (A) or HeLa (B) cytoplasm. Reaction products are shown before immunoprecipitation (lane 2) and after immunoprecipitation with control hyperimmune rabbit serum directed against the viral capsid (lane 3) or with anti-NS1 serum (lane 4). Similar anti-NS1 immunoprecipitates were digested with *ScaI* (lane 5) or were digested with *ScaI* and recentrifuged to give an NS1-associated pellet fraction (lane 6) and an NS1-free supernatant (lane 7). lin, ext, and trn are the same as described in the legend to Fig. 2. A 4.2-kb *ScaI* restriction fragment thought to be derived from the tetrameric product generated by rolling hairpin replication initiating at the extended form of the A arm following resolution is designated rh1. Molecular weight standards (indicated on the left in thousands) are shown in lane 1.

lation or repair synthesis would be expected to be random throughout the plasmid. This type of labeling should, thus, be proportional to the size of the fragment, emphasizing larger fragments, such as those derived from the A arm of pLEB 711, and underrepresenting smaller ones, such as those derived from the B arm. Current models of MVM DNA replication predict that extended forms of the viral termini would contain a new 119-base DNA strand, while turn-around forms would not contain any newly synthesized terminal sequences. Thus, we would expect turn-around forms to be underrepresented in the autoradiographs. A further complication results from labeling because of a type of rolling-circle DNA synthesis, which we discuss in the next section. This labels DNA to high specific activity and is markedly directional since it initiates at one resolution site in preference to the other. This complex pattern of [32 P]dGTP incorporation results in the effective labeling of all reaction products but means that labeling is not uniform, so that, for example, turn-around forms of the termini are always underrepresented relative to extended forms.

Synthesis of high-molecular-weight DNA in HeLa-cell extracts. The results of syntheses shown in Fig. 2 were all obtained by using NS1-containing nuclear extracts derived from vaccinia virus-infected A9 cells, while the cytoplasmic extracts were prepared from either murine A9 cells (lanes 2 to 5) or human HeLa cells (lanes 6 to 8). When reaction mixtures contained A9 cytoplasm, NS1-mediated DNA synthesis was largely confined to unit-length plasmid molecules and their resolution products (Fig. 2, lane 5). When HeLa cell cytoplasm was used, there was a substantial increase in the extent of DNA synthesis, as seen by comparing samples in lanes 5 and 8 of Fig. 2, which contain equal aliquots of the reaction products obtained in otherwise matched reactions with A9 and HeLa cytoplasm, respectively. The sample shown in lane 7 contains one quarter of the HeLa cell products shown in lane 8, for qualitative comparison with the A9 products. As seen for the A9 cytoplasm, there is substantial NS1-mediated DNA synthesis associated with unit-length pLEB 711 molecules and their resolution products, but there is also pronounced synthesis of high-molecular-weight DNA, which is refractory to digestion with *ScaI*.

The origin and structure of this high-molecular-weight material are not fully understood and will require further analysis, but it appears to reflect several different modes of synthesis. Some minor forms which correspond to specific structures predicted by current models of MVM replication will be detailed in a later section. However, much of the high-molecular-weight material generated in HeLa extracts appears to be the product of a type of rolling-circle synthesis, initiated following the introduction of a single NS1-mediated nick at one of the two resolution sites in the plasmid. The replication fork established at this site then proceeds past the second resolution site without completing resolution. Such a fork could continue strand displacement synthesis around the plasmid, leading to the production of a 32 P-labeled duplex circle attached to a displaced, NS1-associated single-strand tail or, if lagging strand synthesis occurred, to a 32 P-labeled NS1-associated duplex. The exact nature of this synthesis and whether it is in any way physiological with respect to MVM replication remain to be determined. Southern transfer analysis (not shown) indicates that these molecules, although highly labeled, are rare.

Covalent attachment of NS1 at the nick site. Many of the DNA forms labeled in the *in vitro* reactions could be immunoprecipitated with antisera directed against the NS1 polypeptide (Fig. 3, compare lane 2 with lane 4 in each panel)

but not by control, antcapsid rabbit serum (Fig. 3, lanes 3). Since the reaction products were incubated at 60°C for 30 min in 2% sodium dodecyl sulfate to denature and dissociate the protein component of the extract prior to precipitation, this suggests that much of the labeled DNA is covalently associated with NS1. When these immunoprecipitates were digested with *ScaI* (Fig. 3, lanes 5) the species generated were similar to those observed previously when the total reaction mixture was digested (Fig. 2). This indicates that all of the observed digestion products were derived from DNA molecules which bore a direct association with NS1.

Resolution is asymmetric. Following digestion with *ScaI*, anti-NS1 immunoprecipitates could be fractionated by centrifugation into those fragments which remained directly attached to NS1 after restriction, and which thus pelleted with the immunoabsorbent (Fig. 3, lanes 6), and those which lacked a direct association with NS1 and which were consequently released into the supernatant by restriction endonuclease cleavage (Fig. 3, lanes 7). In both the A9-based reactions (Fig. 3A) and those employing HeLa cell cytoplasm (Fig. 3B), fragments from both arms of the palindrome which appeared to be extended-form termini on the basis of their molecular weights were seen to be quantitatively associated with NS1. In contrast, fragments from both arms which had the mobility expected of turn-around forms were quantitatively released from the immunoabsorbent following digestion, as would be expected for structures in this "endless" configuration. In both cell systems, most of the [32 P]dGTP label present in the resolved fragments was associated with extended forms of the A arm of the junction palindrome and with turn-around forms of the B arm, leading to the conclusion that resolution of the left-end bridge is asymmetric.

This result was confirmed by two alternate approaches which we do not present in detail here. First, we used prelabeled, linearized substrate molecules rather than relying upon direct [32 P]dGTP incorporation into supercoiled plasmids during the course of the reaction. For this, pLEB 711 molecules were linearized with *ScaI* and labeled at their 3' ends with [32 P]dGTP by using T4 polymerase (data not shown). Second, we analyzed the reaction products of a standard reaction, using supercoiled substrate, by Southern transfer analysis after allowing the incorporated [32 P]dGTP to decay (data not shown). In both of these systems, relatively few substrate molecules were processed, but the results unequivocally support the asymmetry reported here. Thus, although there are inequalities in the extent to which different fragments are labeled in the *in vitro* system employed here, they do not obscure the underlying observation that resolution products from the B arm of pLEB 711 were generated predominantly in the turn-around configuration, while those from the A arm were predominantly in the extended form.

Asymmetry is controlled by sequences within the terminal palindrome. We resected the pLEB 711 insert to the *NcoI* sites diagrammed in Fig. 1B and recloned it into pUC vectors in both sequence orientations. In this way we obtained pLEB 407, which has a 407-base viral palindrome inserted into the plasmid in the same orientation as the pLEB 711 insert, and pLEB 407-REV, which has the insert inverted with respect to the vector. Figure 4A shows the *ScaI* fragments generated from the resolution products of these two constructs in a HeLa extract. Digestion products of both total DNA (lanes 2 and 3) and the immunoprecipitated DNA (lanes 4 and 5) suggest that resolution of these two substrates is asymmetric and that the direction of the asymmetry is

FIG. 4. (A) Autoradiograph of a 1.1% agarose gel showing the deproteinized [32 P]dGTP-labeled products obtained when pLEB 407 (lanes 2, 4, 6, and 7) or pLEB 407-REV (lanes 3, 5, 8, and 9) is incubated in reaction mixtures containing HeLa cytoplasm. Reaction products digested with *ScaI* before (lanes 2 and 3) and after immunoprecipitation with anti-NS1 serum (lanes 4 and 5) are shown. Similar *ScaI*-digested anti-NS1 immunoprecipitates were recentrifuged to give an NS1-associated pellet fraction (lanes 6 and 8) and an NS1-free supernatant (lanes 7 and 9). lin, ext, and trn are the same as described in the legend to Fig. 2. Fragments rh2 and rh3, identified by dots, are consistent with being the products of rolling hairpin replication initiating at the extended terminus generated during resolution and are equivalent to fragment rh1 described in the legend to Fig. 3. (B) Autoradiograph of a 1.1% agarose gel showing the deproteinized [32 P]dGTP-labeled products obtained when pLEB 157 is incubated in reactions containing HeLa cytoplasm. Reaction products are shown digested with *ScaI* before (lane 1) and after (lane 2) immunoprecipitation with anti-NS1 serum. Similar *ScaI*-digested anti-NS1 immunoprecipitates were recentrifuged to give an NS1-associated pellet fraction (lane 3) and an NS1-free supernatant (lane 4). Molecular weight standards (indicated in thousands) are shown in lanes 1 (panel A) and 5 (panel B).

governed by the sequence of the insert rather than by the orientation of the insert within the plasmid backbone. This conclusion is confirmed by fractionation of the *ScaI*-digested immunoprecipitates (Fig. 4A, lanes 6 through 9).

We then digested the insert from pLEB 711 with *PmeI* and recloned the 157-bp bridge fragment into pUC vectors. Figure 4B shows the *ScaI* digestion products obtained when one of these plasmids, pLEB 157, with the viral sequence in the same orientation as pLEB 711, was resolved in a HeLa extract. Once again, the construct was resolved asymmetrically and in the predicted orientation. Thus, all the information required for asymmetric resolution is contained within a fragment which spans the presumed resolution sites and extends for only 18 bp into the inboard sequence on each side.

Is there evidence for a rolling-hairpin mode of replication in HeLa extracts? As mentioned before, the origin and structure of the high-molecular-weight material generated in HeLa extracts are complex and will require further analysis. Although much of it appears to be the product of directional rolling circle synthesis initiated following the introduction of a single NS1-mediated nick at one of the resolution sites in the circular plasmid, some of the minor high-molecular-weight species do coincide with intermediates predicted by current models of MVM replication (1, 3, 12). Thus, a faint band at 4.2 kb, labeled "rh1" in Fig. 3B, lane 7, could be the *ScaI* fragment derived from a tetrameric intermediate generated by repeated rounds of rolling hairpin synthesis following resolution of the cloned junction. This assignment has been strengthened by partially purifying the band and monitoring its digestion with various restriction enzymes (not shown). In the experiment shown in Fig. 4A, inversion of the viral insert with respect to the vector gives two fragments of different sizes consistent with predicted equiv-

alent rolling hairpin products (Fig. 4A, lanes 7 and 9; marked rh2 and rh3, respectively). Thus, it seems likely that enzymes and accessory factors present in the HeLa extract may eventually allow in vitro reconstruction and analysis of further steps in the rolling hairpin replication scheme proposed for MVM.

NS1 remains associated with the junction sequence in A9 extracts. Total extracts and anti-NS1 immunoprecipitates of the pLEB 711 resolution products generated in A9-based assays were digested with *PvuII*, which cleaves the vector DNA twice, once on either side of the MVM insert (Fig. 1B). This leaves short vector sequences with sizes of around 115 and 206 bp attached to the A and B arms of the bridge, respectively. When digested with *PvuII* the unmodified substrate gives two fragments: a 2,364-bp fragment, which is derived from vector sequences only, and a 1,032-bp fragment, which contains the viral insert. Resolution at the predicted sites would cleave the 1,032-bp *PvuII* fragment into extended-form fragments with sizes of 531 and 622 bp from the A and B arms of the bridge, respectively, and turn-around fragments of 470 and 561 bp. Figure 5 shows digests of this type. Immunoprecipitates digested with *PvuII* (Fig. 5, lane 2) were fractionated by centrifugation into those fragments which were directly attached to NS1 molecules (lane 4) and those which lacked such a direct association and so were released into the supernatant (lane 3). Both fully resolved (A and B) and unresolved (J) forms of the junction fragment are apparent, but this analysis also reveals partially resolved or modified forms of the junction, designated MJ1 and MJ2, which, unlike fragment J, are associated with NS1 (Fig. 5, lane 4).

Pellet fractions (Fig. 5, lane 4) contained extended-form resolution products from both sides of the junction sequence, although, as expected, the 531-bp fragment gener-

FIG. 5. Autoradiograph of a 1.6% agarose gel showing the deproteinized [32 P]dGTP-labeled products obtained when pLEB 711 is incubated in reaction mixtures containing A9 cytoplasm. Reaction products digested with *Pvu*II before (lane 1) and after (lane 2) immunoprecipitation with anti-NS1 serum are shown. *Pvu*II-digested anti-NS1 immunoprecipitates were recentrifuged to give an NS1-free supernatant (lane 3) and an NS1-associated pellet fraction (lane 4). Additional anti-NS1 immunoprecipitates were doubly digested with *Pvu*II and either *Hind*III (lane 5) or *Xba*I (lane 6) prior to electrophoresis. A and B denote the two arms of the MVM palindrome, and ext and trn denote extended and turn-around forms, respectively, of the resolution products. A vector-derived 2,364-bp *Pvu*II fragment is labeled vect, and the unresolved junction fragment is labeled J. MJ1 and MJ2 denote the modified junction fragments discussed in the text. Molecular weight standards (indicated on the right in thousands) are shown in lane 7.

ated from the A arm was substantially more intense than the 622-bp fragment generated from the B arm. We were also able to detect the 561-bp turn-around fragment predicted from the B arm in the supernatant fraction, although a turn-around form from the A arm (470 bp) was barely detectable at this exposure. Double digestion of the *Pvu*II-cut immunoprecipitated DNA with enzymes which cut on one side of the insert in the vector polylinker (as shown in Fig. 1B) confirms the origin of these fragments (Fig. 5, lanes 5 and 6). Thus, the *Pvu*II digests confirm the asymmetric nature of the resolution products observed previously.

Supernatant fractions contained copies of the unresolved junction fragment (1,032 bp). Two modified, NS1-associated forms of this fragment, designated MJ1 and MJ2 in Fig. 5, were also found in the pellet fraction (lane 4), but there were remarkably few NS1-associated molecules larger than these modified bridge structures. This suggests that in A9 extracts, replication forks established following the nicking and attachment of NS1 at a resolution site frequently pause, or terminate, within the junction fragment.

The 2,364-bp *Pvu*II fragment derived exclusively from vector sequences is significantly labeled with [32 P]dGTP in A9 extracts, but it is not NS1 associated (Fig. 5, lanes 2 to 4). Since A9 extracts supplemented with NS1 do not markedly

FIG. 6. Two-dimensional gel analysis showing deproteinized [32 P]dGTP-labeled products obtained when pLEB 711 is incubated in reaction mixtures containing A9 cytoplasm and the reaction products are immunoprecipitated with anti-NS1 serum and digested with *Pvu*II. Two equivalent samples were first electrophoresed through a 1.4% neutral agarose gel (lane A), and then one of these tracks was excised and turned through 90° before being reelectrophoresed in a denaturing 1.4% alkaline agarose gel (lanes B). Abbreviations are the same as those in the legend Fig. 5.

promote DNA synthesis in plasmids lacking MVM resolution sites (Fig. 2, lane 3), this could be explained most easily if NS1-associated replication forks which failed to resolve at the first resolution site that they encountered simply circled around the plasmid until they encountered the site for the second time.

Turn-around forms generated in vitro are covalently cross-linked. *Pvu*II digests of anti-NS1-immunoprecipitated DNA from an A9 assay, similar to those seen in Fig. 5, lane 2, were electrophoresed first through a neutral agarose gel (Fig. 6, lane A). This track was then turned through 90°, and the DNA was reelectrophoresed through an alkaline denaturing gel (Fig. 6B). Extended-form resolution products from the A and B arms of the palindrome (530 and 621 bp, respectively) exhibited the same mobility under native and denaturing conditions. In contrast, the putative turn-around telomere generated from the B arm was about twice its native length, migrating slightly more slowly than the unresolved junction fragment in the alkaline dimension. This mobility was expected since the fragment should be about 90 bases larger than the unresolved junction fragment, having two copies of the 206-base vector sequence flanking the B arm of the junction, rather than the 206-base fragment from the B arm plus the 115-base fragment from the A arm present in the unresolved fragment. At this exposure, the turn-around form of the A arm was also just visible as a faint spot, with a mobility slightly greater than that of the unresolved junction fragment. In fact, it should be approximately 90 nucleotides smaller, for the converse of the reasons outlined above. These results strongly suggest that the resolution products which we have described as being in the turn-around configuration are indeed duplex fragments which are covalently continuous, or cross-linked, at one end. Thus, the structures of the viral telomeres generated in vitro appear to conform well with those generated in vivo during the course of a viral infection (9, 35).

Structure of the modified junction fragments, MJ1 and MJ2. Figure 6B also illustrates the nature of the labeled DNA fragments present in the NS1-associated, modified junction fragments, designated MJ1 and MJ2. These two forms presumably represent specific steps in the resolution process at which the mechanism pauses. Most of the label in the more intensely labeled, higher-molecular-weight MJ1 form comigrates under denaturing conditions with extended-form fragments from the B arm of the palindrome, while most of the label in the lower (and less intensely labeled) MJ2 form appears to comigrate with extended-form fragments from the A arm. The simplest way to interpret these data would be to suggest that most of the molecules in the upper, more intensely labeled form have been nicked in the B arm of the viral palindrome and the 3' hydroxyl group exposed at this site extended to the position of the resolution site in the A arm. This would give a labeled fragment that is the size of the extended form of the B arm. The lower mobility of this form under native conditions would be explained by the presence of a displaced single-strand loop 119 bases long. Similarly, it is possible that the less intensely labeled MJ2 form could be generated by nicking the resolution site in the A arm and extending the 3' end created at this site through to the resolution site in the B arm. Although this would produce a labeled fragment of the observed size (equal to that of extended forms of the A arm) under denaturing conditions, it would not explain why this structure fails to comigrate with the more abundant (MJ1) form under native conditions, since it would have essentially the same structure. Alternatively, the majority of molecules in both the MJ1 and MJ2 forms may have been nicked at the same resolution site (in the B arm of the palindrome), but the exposed 3' hydroxyl groups in the more slowly migrating MJ1 form fully extended across the junction to the resolution site in the opposite arm, while in the MJ2 forms relatively few, if any, nucleotides have been added. This would explain why MJ2 forms almost comigrate with the unmodified junction fragment in native gels. In light of a number of other lines of experimentation currently in progress (10), the latter explanation seems most probable. If this explanation is correct, then it would imply that the asymmetry of the system is established by the position of the first nick and that this nick is introduced predominantly into the B arm of the substrate. According to this interpretation, MJ2 forms would result from a pause in the resolution process occurring immediately after introduction of the first nick, perhaps allowing assembly of the replication fork or rearrangement of the substrate, while MJ1 forms would accumulate as the replication fork pauses in the vicinity of the second candidate resolution site prior to the final nicking and religation process.

Structure of the resolved plasmids. In this paper we have detailed the structure of [^{32}P]dGTP-labeled termini in both the extended and turn-around configurations generated from both arms of the junction palindrome and have drawn the conclusion that resolution is asymmetric and generally leads to the production of one extended-form telomere associated with the A arm of the palindrome and one turn-around form associated with the B arm (Fig. 7D). However, we know from Southern analysis that only a relatively small percentage (5 to 10%) of the input plasmid molecules is resolved in the present system (data not shown), so that the individual termini which we have characterized could be derived from separate resolution events occurring on different plasmid subsets. Because the two termini resolved from an individual junction remain physically attached by the vector se-

quences, this possibility could be addressed directly. We took advantage of our finding that all extended-form termini are covalently associated with NS1. Plasmids carrying such structures were immunoprecipitated with anti-NS1 serum, and the precipitates were analyzed on native and denaturing gels. The samples shown in Fig. 7A and B are from a time course of pLEB 711 resolution in A9 extracts. Under native conditions (Fig. 7A), several NS1-associated forms are apparent even at the first time point (15 min, lane 2). In particular, there are major bands which migrate at approximately 3,397 bp, which is the size of the linear input plasmid (designated 1X), and forms of twice and three times this size which are probably multimers (designated 2X and 3X). With time, labeling of all forms intensifies, but at different rates, so that by 2 to 4 h molecules migrating at approximately the position of monomer length duplexes (3,397 bp, designated 1X) greatly predominate (lanes 5 and 6). It is probable that some dimer forms of the plasmid preexist in the bacterially derived input DNA. Since such forms would have four resolution sites, they are likely to be labeled rather efficiently in the subsequent reactions.

A form migrating at around 4 kb, designated MJ for modified junction, was also observed. When similar precipitates from 15-min reactions were digested with *Pvu*II, we could not detect any extended- or turn-around form resolution products, but the predominant NS1-associated fragment was the modified junction MJ1 described earlier (data not shown). This leads us to propose that the form we have designated MJ in Fig. 7A is probably a circular duplex which has been nicked and extended at one of the resolution sites but has not yet been fully resolved. In addition, a high-molecular-weight form of over 12 kb, as yet uncharacterized, is gradually lost from the reaction products as the time course proceeds.

Under denaturing conditions (Fig. 7B), the major form precipitated in all samples migrated at twice the unit length of the input plasmid, at around 7 kb (designated 2X). Minor 1X and 3X forms were also observed, in addition to an uncharacterized high-molecular-weight form which might represent four genome lengths. These observations were extended by subjecting a similar immunoprecipitate, from a 2-h reaction, to electrophoresis in two dimensions on native (Fig. 7C, lane 2) and then denaturing gels (Fig. 7C, lanes 3). As expected, molecules which migrated at two times the unit length under denaturing conditions were seen to arise predominantly from 1X unit-length duplexes. Thus, as diagrammed in Fig. 7D, the major products of the resolution reaction in A9 extracts are linear, duplex molecules slightly larger than the input plasmid, which are covalently associated with NS1 and in which the two strands are covalently cross-linked at the end derived from the B arm.

DISCUSSION

We have shown that the left-end to left-end (viral 3'-to-3') palindromic junction region from dimeric RF MVM DNA can be resolved *in vitro* in cell extracts supplemented with wild-type copies of the NS1 polypeptide. This resolution reaction is remarkable because it is not symmetric, generating telomeres from one arm of the junction palindrome which are predominantly in the extended configuration while those from the other arm are predominantly in the turn-around form. The asymmetry is sequence dependent and can be monitored at the level of individual junction fragments, since the products of a single resolution reaction remain covalently associated with each other via plasmid sequences.

FIG. 7. Autoradiograph of a 1.1% native agarose gel (A) and a 1.1% alkaline agarose gel (B) showing the deproteinized [^{32}P]dGTP-labeled products obtained when pLEB 711 was incubated in reaction mixtures containing A9 cytoplasm and the reaction products were immunoprecipitated with anti-NS1 serum. Reactions were stopped after 15 min (lane 2), 30 min (lane 3), 1 h (lane 4), 2 h (lane 5), 4 h (lane 6), or 20 h (lane 7). Molecular weight standards (in thousands indicated on the left) are shown in lane 1. (C) Two-dimensional analysis showing deproteinized [^{32}P]dGTP-labeled products obtained when anti-NS1 immunoprecipitates from a 2-h A9-based resolution reaction were first electrophoresed through a 1.1% neutral agarose gel (lane 2), then turned through 90°, and finally reelectrophoresed in a denaturing 1.1% alkaline gel (lanes 3). (D) Diagram of the predicted structure of the major resolved form of pLEB 711 generated in reaction mixtures containing A9 cytoplasm. Viral sequences are crosshatched. The circle at the end of one DNA strand represents the covalently associated NS-1 polypeptide. A and B in the crosshatched sections refer to the two arms of the junction palindrome as described in the legend to Fig. 1B.

In contrast, junction fragments from concatemeric MVM RF DNA containing right-end to right-end (viral 5'-to-5') fusions are resolved symmetrically in comparable assays, as shown previously (11), and give rise to similar, predominantly extended-form structures from both arms of the palindrome. This disparity between the products obtained from the two substrates is striking because resolution of both junctions appears to be initiated by a site-specific, NS1-dependent endonuclease reaction.

The initial rolling hairpin model of MVM DNA replication (34) predicted that any terminus which was not a perfect palindrome would be replicated with equal frequency in two sequence orientations, related by being the inverted complements of one another, which were dubbed flip and flop. Subsequent analyses showed that such asymmetries did exist at both ends of the viral genome (2, 4) and that flip and

flop sequences were generated in equal numbers from cloned right-end (viral 5') termini *in vivo* (19, 26). Parallel studies of sequences present in the AAV2 terminal repeat (24) showed that a similar interconversion of flip and flop orientations also occurred during replication of this virus, and subsequent *in vitro* analyses (31) have confirmed that these termini can be replicated by a simple hairpin-transfer mechanism. However, the left (viral 3') end of the genome of MVM is present only in the single flip orientation in both packaged virus and intracellular viral RF DNA (1-3), and asymmetric resolution of a duplex junction structure, as observed here, was suggested as a possible mechanism by which this conservation could be achieved. The investigators who described the modified rolling hairpin model (1, 3) demonstrated that a protein, now known to be NS1 (13), was covalently associated with the 5' termini of viral DNA and

that this linkage was probably mediated by a tyrosine residue. This suggested an analogy with the gene A protein encoded by phage ϕ X174, which initiates rolling circle replication of single-strand phage DNA by introducing a site-specific nick and becomes attached to the 5' end of the DNA at this site via a tyrosine linkage (17). The A protein then functions as part of the replication fork established at the nick site and is translocated around the circular viral genome until it encounters the reconstructed nick site once again. A second active-site tyrosine residue, located 4 amino acids away from the first, attacks the new site, and the A protein molecule is transferred onto the new 5' end, ligating its previous DNA attachment site to the newly exposed 3' nucleotide. Adapted to the MVM 3' junction sequence, this type of mechanism would ligate the two strands of the duplex on one side of the junction and generate an extended form at the other side, as shown in Fig. 1A. Such a reaction would explain why turn-around forms of the left-end terminus are always far more abundant than similar forms of the right-end terminus and how they might progressively accumulate during infection (9). It would also explain how input single-strand viral genomes could replicate to give a form of duplex RF molecule (termed M1) in which both termini are covalently cross-linked (9).

Attractive though this model would seem, direct evidence for it has remained elusive. The *in vitro* results presented here support three of its specific predictions: (i) dimer junction fragments can be resolved to give mature telomeres; (ii) the A arm of the bridge, containing the bubble sequence 5'-GAA-3', is resolved into an extended-form telomere, while the B arm, containing the bubble sequence 5'-TC-3', is resolved into a structure in the turn-around configuration; and (iii) despite the fact that the sequences in the immediate vicinity of the candidate resolution sites in the two arms of the junction are identical, one site appears to be nicked much more efficiently than the other. However, to generate new terminal sequences exclusively in the flip orientation, the model requires the initial nick to be introduced into the viral strand on the inboard side of a flip-sense palindrome, corresponding to the A arm of the pLEB 711 palindrome, while the data generated *in vitro* suggest that the primary nick site is in the B arm. Thus, the *in vitro* results support the proposition that sequence conservation at the MVM 3' terminus is achieved by restricting the initial endonuclease cleavage site to one arm of the duplex junction region of a dimer intermediate but suggest that subsequent steps in the resolution of this structure may differ from those outlined in the current model.

Whether the model or the *in vitro* assay requires modification is not clear at present. In the experiments presented here, the resolution substrate was a circular, supercoiled plasmid, while the *in vivo* substrate is presumably linear. However, linearized, 3' end-labeled, plasmid sequences can be used in the *in vitro* assay (data not shown), and the resolution reaction shows the same asymmetry and site specificity as described for the supercoiled substrates. In theory, site specificity could also be influenced by the presence of 5' termini in the same substrate molecule as the 3' junction or by the presence of other components of the viral replication apparatus, such as the virally coded NS2 molecules or the capsid polypeptides. Further experiments will be required to explore these possibilities.

The first duplex forms of the 3' resolution site to be generated during infection *in vivo* are in the turn-around configuration (35), and these forms progressively accumulate at later times (9). Thus, an asymmetric dimer resolution

mechanism could only predominate *in vivo* if 3' termini in the turn-around configuration were refractory to nicking by the site-specific endonuclease. The direct interactions of NS1 with structures in this configuration have yet to be explored in detail. However, in an AAV2-based *in vitro* resolution system, substrates in this configuration do serve as resolution targets for the initiator endonucleases Rep68 and Rep78, which are virally coded proteins analogous to the MVM NS1 polypeptide (21, 22, 28, 31). The complex hairpin structure of AAV2 termini is known to be essential for efficient DNA replication *in vivo* (7, 23), and multiple copies of Rep68 and Rep78 bind efficiently *in vitro* to turn-around forms of the terminus but only poorly to extended forms (20–22, 29). These observations have led to speculation that the secondary structure of the turn-around form is critically required for the endonuclease reaction *per se* (21). This appears not to be the case for MVM 3' termini, although it can be argued that the resolution substrates used in the present study could adopt a cruciform structure, comprising two turn-around forms, prior to nicking *in vitro*. In order to address this possibility, we have recently constructed plasmid clones containing only the B arm of the junction fragment, extending from MVM residue 50 through MVM residue 1 and on through the coding region, which lack the ability to adopt such cruciform structures. These substrates are nicked efficiently and become covalently complexed with NS1 in this *in vitro* system (10), indicating that the ability to form a hairpin structure is not necessary for the nicking reaction at MVM left-end-left-end junctions.

Even if the endonuclease initiating resolution does not recognize the secondary structure of the MVM 3' terminus, it must still be able to scan sequences at a distance from the nick site, because the site itself is identical in the two arms of the junction fragment and in the covalently closed end of monomer RF. The modified rolling hairpin model proposes that site recognition is critically influenced by local asymmetries, in particular the asymmetric bubble sequence, shown in Fig. 1, which is located around 27 nucleotides from the cut site. Moreover, the 3' and 5' termini of MVM have very different sequences, sizes, and predicted secondary structures, and yet both are resolved by NS1-mediated reactions. This suggests that the initiating endonuclease, most probably NS1 itself, is also able to recognize two different resolution sites in substantially different sequence contexts.

The 3' telomeric structure of the rodent parvoviruses is highly conserved (2), and where examined, this structure has been shown to be associated with the conservation of one particular sequence orientation (1–3, 16). Thus, conservation of one sequence orientation (designated flip) over the other at the left end, but not at the right end, of these genomes appears to have conferred some evolutionary advantage. One potentially significant difference between these conserved left-end termini and the left terminus of AAV2, which is expressed in both flip and flop orientations, is their relative distance from the transcriptional promoter which initiates viral gene expression. In the case of MVM, the earliest mRNAs initiate at nucleotide 201 (5), some 83 nucleotides downstream of the putative NS1 nick site, whereas for AAV2 the equivalent mRNAs initiate at nucleotide 287, 162 nucleotides downstream from the Rep cut site (21, 25). Thus, it is more likely that critically spaced upstream transcriptional promoter elements might reside in the MVM terminal palindrome than within the equivalent sequences of AAV2. However, whether it is the sequence orientation itself or the mechanism and intermediates employed in replicating this

sequence which provide the adaptive advantage remains to be determined.

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REFERENCES

- Astell, C. R., M. B. Chow, and D. C. Ward. 1985. Sequence analysis of the termini of virion and replicative forms of minute virus of mice DNA suggests a modified rolling hairpin model for autonomous parvovirus DNA replication. *J. Virol.* **54**:171-177.
- Astell, C. R., M. Smith, M. B. Chow, and D. C. Ward. 1979. Structure of the 3' hairpin termini of four rodent parvovirus genomes: nucleotide sequence homology at origins of DNA replication. *Cell* **17**:691-703.
- Astell, C. R., M. Thomson, M. B. Chow, and D. C. Ward. 1983. Structure and replication of minute virus of mice DNA. *Cold Spring Harbor Symp. Quant. Biol.* **47**:751-762.
- Astell, C. R., M. Thomson, M. Merchlinsky, and D. C. Ward. 1983. The complete DNA sequence of minute virus of mice, an autonomous parvovirus. *Nucleic Acids Res.* **11**:999-1018.
- Ben-Asher, E., and Y. Aloni. 1984. Transcription of minute virus of mice, an autonomous parvovirus, may be regulated by attenuation. *J. Virol.* **52**:266-276.
- Berns, K. I., and W. W. Hauswirth. 1979. Adeno-associated viruses. *Adv. Virus Res.* **25**:407-432.
- Bohenzky, R. A., R. B. LeFebvre, and K. I. Berns. 1988. Sequence and symmetry requirements within the internal palindromic sequences of the adeno-associated virus terminal repeat. *Virology* **166**:316-327.
- Cavalier-Smith, T. 1974. Palindromic base sequences and replication of eukaryotic chromosome ends. *Nature (London)* **250**:467-470.
- Cotmore, S. F., M. Gunther, and P. Tattersall. 1989. Evidence for a ligation step in the DNA replication of the autonomous parvovirus minute virus of mice. *J. Virol.* **63**:1002-1006.
- Cotmore, S. F., J. P. F. Nüesch, and P. Tattersall. Unpublished observations.
- Cotmore, S. F., J. P. F. Nüesch, and P. Tattersall. 1992. In vitro excision and replication of 5' telomeres of minute virus of mice DNA from cloned palindromic concatemer junctions. *Virology* **190**:365-377.
- Cotmore, S. F., and P. Tattersall. 1987. The autonomously replicating parvoviruses of vertebrates. *Adv. Virus Res.* **33**:91-174.
- Cotmore, S. F., and P. Tattersall. 1988. The NS-1 polypeptide of minute virus of mice is covalently attached to the 5' termini of duplex replicative-form DNA and progeny single strands. *J. Virol.* **62**:851-860.
- Cotmore, S. F., and P. Tattersall. 1989. A genome-linked copy of the NS-1 polypeptide is located on the outside of infectious parvovirus particles. *J. Virol.* **63**:3902-3911.
- Cotmore, S. F., and P. Tattersall. 1992. In vivo resolution of circular plasmids containing concatemer junction fragments from minute virus of mice DNA and their subsequent replication as linear molecules. *J. Virol.* **66**:420-431.
- Diffoot, N., B. C. Shull, K. C. Chen, E. R. Stout, M. Lederman, and R. C. Bates. 1989. Identical ends are not required for the equal encapsidation of plus- and minus-strand parvovirus LuIII DNA. *J. Virol.* **63**:3180-3184.
- Eisenberg, S., and A. Kornberg. 1979. Purification and characterization of ϕ X174 gene A protein. A multifunctional enzyme of duplex DNA replication. *J. Biol. Chem.* **254**:5328-5332.
- Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss. 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA* **83**:8122-8126.
- Gardiner, E. M., and P. Tattersall. 1988. Evidence that developmentally regulated control of gene expression by a parvoviral allotropic determinant is particle mediated. *J. Virol.* **62**:1713-1722.
- Im, D.-S., and N. Muzyczka. 1989. Factors that bind to adeno-associated virus terminal repeats. *J. Virol.* **63**:3095-3104.
- Im, D.-S., and N. Muzyczka. 1990. The AAV origin binding protein Rep68 is an ATP-dependent site-specific endonuclease with DNA helicase activity. *Cell* **61**:447-457.
- Im, D.-S., and N. Muzyczka. 1992. Partial purification of adeno-associated virus Rep78, Rep52, and Rep40 and their biochemical characterization. *J. Virol.* **66**:1119-1128.
- Lefebvre, R. B., S. Riva, and K. I. Berns. 1984. Conformation takes precedence over sequence in adeno-associated virus DNA replication. *Mol. Cell. Biol.* **4**:1416-1419.
- Lusby, E., K. H. Fife, and K. I. Berns. 1980. Nucleotide sequence of the inverted terminal repetition in adeno-associated virus DNA. *J. Virol.* **34**:402-409.
- Lusby, E. W., and K. I. Berns. 1982. Mapping of 5'-termini of two adeno-associated virus RNAs in the left half of the genome. *J. Virol.* **41**:518-526.
- Merchlinsky, M. J. 1984. Studies on MVM DNA replication using an infectious molecular clone. Ph.D. thesis. Yale University, New Haven, Conn.
- Nüesch, J. P. F., S. F. Cotmore, and P. Tattersall. 1992. Expression of functional parvoviral NS1 from recombinant vaccinia virus: effects of mutations in the nucleotide-binding motif. *Virology* **191**:406-416.
- Owens, R. A., and B. J. Carter. 1992. In vitro resolution of adeno-associated virus DNA hairpin termini by wild-type Rep protein is inhibited by a dominant-negative mutant of Rep. *J. Virol.* **66**:1236-1240.
- Owens, R. A., J. P. Trempe, N. Chejanovsky, and B. J. Carter. 1991. Adeno-associated virus Rep proteins produced in insect and mammalian expression systems: wild-type and dominant-negative mutant proteins bind to the viral replication origin. *Virology* **184**:14-22.
- Siegl, G., R. C. Bates, K. I. Berns, B. J. Carter, D. C. Kelly, E. Kurstak, and P. Tattersall. 1985. Characteristics and taxonomy of Parvoviridae. *Intervirology* **23**:61-73.
- Snyder, R. O., R. J. Samulski, and N. Muzyczka. 1990. In vitro resolution of covalently joined AAV chromosome ends. *Cell* **60**:105-113.
- Stillman, B. W., and Y. Gluzman. 1985. Replication and supercoiling of simian virus 40 DNA in cell extracts from human cells. *Mol. Cell. Biol.* **5**:2051-2060.
- Straus, S. E., E. Sebring, and J. Rose. 1976. Concatamers of alternating plus and minus strands are intermediates in adeno-virus-associated virus DNA synthesis. *Proc. Natl. Acad. Sci. USA* **73**:742-746.
- Tattersall, P., and D. C. Ward. 1976. Rolling hairpin model for replication of parvovirus and linear chromosomal DNA. *Nature (London)* **263**:106-109.
- Ward, D. C., and D. Dadachanji. 1978. Replication of minute virus of mice DNA, p. 297-313. In D. C. Ward and P. Tattersall (ed.), *Replication of mammalian parvoviruses*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.