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Analysis of the Termini of the DNA of Bovine Parvovirus: Demonstration of Sequence Inversion at the Left Terminus and Its Implication for the Replication Model

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The distribution of terminal-sequence orientations in the viral DNA of bovine parvovirus (BPV), an autonomous parvovirus, was studied by end labeling and restriction enzyme digestion and also by cloning. The left (3′) end of the minus strand of BPV was found in two alternative sequence orientations (designated as flip and flop, which are reverse complements of each other), with a 10-fold excess of flip. This is in contrast to the autonomous rodent parvoviruses which encapsidate minus-strand DNA with only the flip orientation at this end. The right (5′) end of the minus strand of BPV contained both sequence orientations with equal frequencies, as in the rodent parvoviruses. Sequence inversions were also detected at both ends of the plus strand, which makes up about 10% of the encapsidated BPV DNA. Each terminus of BPV DNA had a characteristic ratio of flip to flop forms, and this ratio was restored in the progeny DNA resulting from transfection with genomic clones of different defined terminal conformations. Replicative-form DNA showed the same distribution of terminal-sequence orientations as the reannealed plus and minus virion DNAs, suggesting that the distribution of flip and flop forms observed in virion DNA is not due to selective encapsidation, but rather to the specific distribution of replicative forms. The current replication model for autonomous parvoviruses, which was based on the available data for the rodent parvoviruses, cannot account for the observed distribution of BPV DNA. An alternative model is suggested.

Parvoviruses are small icosahedral viruses with single-stranded DNA genomes containing terminal palindromic sequences. These terminal regions have *cis* signals important in various steps of replication (6, 8, 9, 14). A number of related models have been proposed for the replication of parvoviruses (1, 3, 7, 8, 14, 16, 21, 30, 31). An essential feature included in all these models is a hairpin transfer mechanism originally proposed by Cavalier-Smith (11) for the replication of linear molecules containing terminal palindromes. According to this mechanism, if a terminal palindrome is imperfect, two terminal sequences will result from the replication process and each will be the reverse complement of the other, arbitrarily assigned as flip or flop (3, 25). If a restriction enzyme recognition sequence lies within such an unpaired region in the single-stranded DNA, that restriction enzyme will be diagnostic for the flip and flop orientations, since it will cut double-stranded DNA at two alternative sites depending on the orientation (21).

The dependent parvovirus, adeno-associated virus (AAV), encapsidates equal amounts of plus- and minus-strand DNA. The left and right termini are identical, and the two alternative sequence orientations are found with equal frequencies at both ends of the plus- and minus-strand DNA, consistent with the hairpin transfer mechanism (8, 17). The autonomous parvoviruses encapsidate various amounts of minus-strand DNA, from 99% for the rodent parvoviruses, which include minute virus of mice (MVM), H-1, and rat virus, to 90% for bovine parvovirus (BPV), to 50% for LuIII (14, 28). The left and right termini of the autonomous parvoviruses are not identical. The terminal-sequence orientations have so far been studied in detail only for the rodent parvoviruses (2–4, 24). The right (5′) termini of the minus strands of these viral genomes have been shown to exist in either orientation with equal frequencies, but the left (3′) termini are unique. A modified rolling hairpin model (1, 14) was developed to account for the absence of sequence inversion at the left end. However, the generality of this model for the other autonomous parvoviruses remains to be determined.

In this study, the distribution of terminal-sequence orientations in the viral DNA population of BPV, a nonrodent parvovirus, was investigated by end labeling and restriction enzyme analyses and also by cloning. The results demonstrated the presence of both flip and flop conformations at the left end of the minus strand of BPV, in contrast to the unique conformation at this end for the rodent parvoviruses. The relation of this finding to the models of parvovirus replication is discussed.

MATERIALS AND METHODS

Clone construction. The construction and characterization of BPV infectious genomic clones pVT501, pVT502, and pGCSma20 have been described previously (27). Left and right terminal fragments of BPV were cloned into pUC8, and terminal *Sma*I fragments were cloned into M13mp18 by standard procedures (18).

End-label analysis. Procedures for isolating DNAs (single- and double-stranded virion DNAs, as well as replicative-form [RF] DNA) were described previously (12). DNA, 1 to 2 μg per 50-μl reaction, was labeled at the 3′-hydroxyl ends with 50 μCi of [^{35}S]ddATP (Dupont, NEN Research Products, Boston, Mass.) and 20 U of terminal deoxynucleotidyltransferase (Dupont) in a solution containing 100 mM potassium cacodylate, 25 mM Tris hydrochloride, 0.2 mM dithiothreitol, and 1 mM $CoCl_2$ (pH 7.6) (23). After incubation at 37°C for 2 h, DNA was precipitated and washed three

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times with 70% ethanol. The labeled DNA was digested with the restriction enzymes diagnostic for the flip and flop orientations as indicated in Results. The digestion products were analyzed on a sequencing gel (10) by using M13mp18 sequencing reactions as size markers. End-labeled restriction fragments would be one base longer than the sizes predicted from the DNA sequence as a result of the extra base (ddA) added by the terminal transferase.

RESULTS

BPV encapsidates approximately 90% minus-strand DNA and 10% plus-strand DNA, and virion DNAs of opposite polarity readily reanneal under the conditions used for isolation (12). Virion DNA was recovered in two fractions: double-stranded DNA, consisting of reannealed plus and minus strands, and single-stranded DNA of minus polarity only. We assume that the minus strands in reannealed virion DNA are a representative sample of the total minus-strand population. By convention, parvoviral genomes are presented with the coding sequence (plus strand) reading from left to right; thus, the 5′ end of the plus strand or the 3′ end of the minus strand is referred to as the left end. This convention will be followed here to discuss the distribution (the proportion of various components in a population) of terminal-sequence orientations of BPV DNA.

Sequence heterogenity of the left end of BPV demonstrated by cloning of terminal fragments. To obtain a population of cloned terminal fragments, reannealed double-stranded virion DNA of BPV was used to generate clones of the left-terminal (map units 0 through 17) and right-terminal (map units 92 through 100) *Eco*RI fragments of BPV. For the right end, clones with either flip or flop orientations were readily obtained. For the left end, most of the clones obtained were in the flip conformation. Surprisingly, clone p3′V8 containing a 34-base-pair (bp) deletion was found to be in the flop conformation at this terminus. This conformation had not been observed from other autonomous parvovirus genomes.

Since a sequence inversion within the left terminus of BPV could occur during cloning as an artifact (27), it was necessary to confirm the presence of the flop conformation in the native DNA by other means. The unique *Sma*I recognition sequence (CCC/GGG) of BPV lies within an unpaired region of the left hairpin (Fig. 1A). Hence, the enzyme will cut double-stranded DNA at either 44 or 106 nucleotides (nt), depending on whether the DNA is in the flip or flop form. The ability to clone a left terminus of 106 nt from the double-stranded virion DNA after digestion with *Sma*I would provide direct evidence for the existence of the flop conformation at the left end of both strands of DNA. When a series of terminal *Sma*I clones were sequenced, one with a 96-bp insert was found. This clone, M13Sma199, had the expected sequence for the flop conformation but with a 10-bp deletion at the terminus. This finding provided direct proof, by cloning, of the existence of the flop conformation at the left end of both the plus and minus strands. Sequence inversion during cloning could not give rise to a 96-bp fragment from a 44-bp fragment.

Demonstration of both flip and flop conformations at the left (3′) end of the minus-strand DNA by end-label analysis. The occurrence of the flip or flop forms can also be visualized and the relative amounts of each can be assessed by end-label analysis as described in Materials and Methods. On the basis of the sequence data (12), the left end of the minus strand of BPV DNA can exist in a most stable (maximum base pairing) Y-shaped configuration (Fig. 1A). There is a unique *Hha*I (GCG/C) site in one of its arms. End labeling (which added a ddA base to the 3′-hydroxyl end and made the fragment one base longer) of the minus strand, followed by digestion with *Hha*I, should yield bands of 77 and 66 nt, corresponding to the flip and flop conformations, respectively.

When the single-stranded BPV DNA (minus polarity only) was analyzed in this way, bands at 79 and 64 nt, which corresponded well with the expected lengths, were observed, along with a number of secondary bands (Fig. 2). The band corresponding to the flip conformation at the 3′ terminus was about 10-fold more intense than that corresponding to the flop conformation at the 3′ terminus. Therefore, roughly 90% of the minus strands had the flip orientation, and only 10% had the flop orientation at the left end. *Hha*I tends to make single-strand cuts within the duplex region containing the recognition site (3). Weaker bands in the end-label analysis (Fig. 2), at nt 71 and 83, agreed well with the expected values of 73 and 84 predicted for cleavage at the distal sides of the *Hha*I sites (Fig. 1A). Bands at 77, 78, and 80 nt (Fig. 2) were the result of variations in the position of the terminal nucleotide at the left end, as reported for other parvoviruses (3, 15). The discrepancies between the expected terminal-fragment lengths and the observed ones are due, at least in part, to the effect of secondary structure on the migration of these fragments in a sequencing gel.

Equal frequencies of flip and flop forms at the right (5′) end of the minus strand. The conformation of the right (5′) end of the minus strand can be demonstrated by end-label analysis of in vitro-replicated minus strand. In vitro replication would yield a double-stranded DNA whose conformation at the right end is defined by the conformation of the minus strand. *Nde*I (CA/TATG) digestion would cut the in vitro-replicated BPV DNA at nt 5437 in the flip orientation and at nt 5422 in the flop orientation (12). Replicated BPV virion DNA was thus analyzed, and two bands of 55 and 73 nt, with approximately equal intensities, were observed (12).

The right terminus of BPV was previously assigned to nt 5491 on the basis of the data mentioned above and the sequence analysis of cloned S1 nuclease-resistant fragments of single-stranded virion DNA (12). However, in vitro replication of palindromic DNA by *Escherichia coli* DNA polymerase I Klenow fragment is often incomplete and arrests at specific sites that are capable of forming hairpin structures, as previously reported by Cotmore and Tattersall (13) and as described below. During cloning both of the right terminus of BPV and of infectious genomic clones from double-stranded virion DNA (27), several of the clones obtained had the right terminus 26 bp beyond the previously assigned terminus of the genome. Also, one clone of the S1 nuclease-resistant right terminus containing the same 26 additional bp was found (K. C. Chen, unpublished results). In addition, the fragment lengths of end-labeled, *Nde*I-digested double-stranded virion DNA were found to be 20 to 22 bp longer than those obtained from the in vitro-replicated DNA (see below). On the basis of this evidence, the revised terminus of BPV is placed at nt 5517 (Fig. 1B).

Even though the terminal fragments of *Nde*I-digested in vitro-replicated minus-strand DNA were truncated because of incomplete replication, the band intensities indicated that both the flip and flop forms occur with equal frequencies at the right end of the minus-strand virion DNA, as demonstrated for other parvoviruses (1, 17, 21, 24). This result is expected if the hairpin transfer mechanism were involved in the synthesis of minus strand with the plus strand as the template and its right-end palindrome as the self-primer.

(A) Left (3') terminus

(1) Flip conformation

```
      A
 80—A G
   |G C|←
   |C G| HhaI
  →|G C|     100          120                   140                  160
   |C G|      |            |                     |                    |
    G CGCGTAA^C A^TT CCGG^ATTA GCCCGTCGGTTATACCACCTACGTCCTTTACTCATTATTTTTATATCTCGGACAC--5'
    C
    A TCGCATT_A T_T -GGCC----CGGGCAGCCAATATGGTGGATGCAGGAAATGAGTAATAAAAATAT  3'
    G C—\             ‾‾‾‾‾‾‾‾‾  |                      |                 |
    C G 60               SmaI  40                     20                 1
    G C
    G C
    C G
    G A
     C
```

(2) Flop conformation

```
     G
    C T
    G C
    C G
    C G
 80—G C   100         SmaI          120                  140                  160
    C G    |       _________         |                    |                    |
    T AGCGTAA^T A-^A CCGG----GCCCGTCGGTTATACCACCTACGTCCTTTACTCATTATTTTTATATCTCGGACAC--5'
    G
    C GCGCATT_G T_AA GGCC_TAAT CGGGCAGCCAATATGGTGGATGCAGGAAATGAGTAATAAAAATAT  3'
   |G C| |                 |                      |                 |
   |C G|←60               40                     20                 1
  →|G C| HhaI
   |C G|
    T C
     T
```

(B) Right (5') terminus

(1) Flip conformation

```
5341                5360                 5380                 5400                 5420
 |                   |                    |                    |                    |
3'--GAAATATAAAAATATGTGACTAAGTGAGAAAGGTGGTAGAAAATCAACATAAGAATCAATAGTTATTTCCGCGCTTCGCGGTAT^TTTTTA
                                                                                                  A
    5' ATATTTTTATACACTGATTCACTCTTTCCACCATCTTTTAGTTGTATTCTTAGTTATCAATAAAGGCGCGAAGCGCCATA_TGACAT
       |                |        *         |                    |                    |
     5517              5500               5480                 5460                 5440
                                                                                    NdeI
```

(2) Flop conformation

```
5341                5360                 5380                 5400                 5420  NdeI
 |                   |                    |                    |                    |  ______
3'--GAAATATAAAAATATGTGACTAAGTGAGAAAGGTGGTAGAAAATCAACATAAGAATCAATAGTTATTTCCGCGCTTCGCGGTAT^ACTGTA
                                                                                                  T
    5' ATATTTTTATACACGGATTCACTCTTTCCACCATCTTTTAGTTGTATTCTTAGTTATCAATAAAGGCGCGAAGCGCCATA_AAAAAT
       |                |        *         |                    |                    |
     5517              5500               5480                 5460                 5440
```

FIG. 1. Nucleotide sequences of the flip and flop conformations at the left (3') terminus (A) and the right (5') terminus (B) of the minus strand of BPV. Restriction enzyme recognition sequences for *Hha*I, *Nde*I, and *Sma*I are indicated. The position of the previously assigned right (5') terminus (*) is shown.

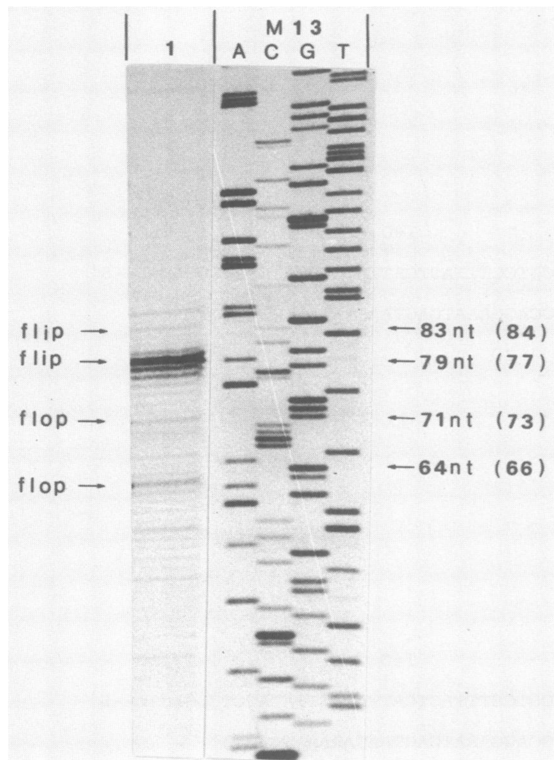

FIG. 2. End-label analysis of the left terminus of the minus strand of BPV. Single-stranded virion DNA was end labeled, digested with *HhaI*, and analyzed as described in Materials and Methods. Lane 1 contains wild-type virion DNA. Bands corresponding to the flip and flop orientations are indicated on the left. The observed and the expected (shown in parentheses) sizes are indicated on the right.

Demonstration of sequence inversions at both ends of the plus strand by end-label analysis of double-stranded virion DNA. The distribution of terminal-sequence orientations in the plus-strand virion DNA can be deduced from the analysis of double-stranded (reannealed plus and minus strands) virion DNA, provided that the distribution within the minus strand is known, as illustrated below.

Reannealing of BPV virion DNA plus and minus strands would result in double-stranded molecules which are either fully base paired (in the flip or flop conformations) or partly unpaired in terminal regions because of various terminal-sequence orientations. The former would be cut by the diagnostic restriction enzyme and produce fragments characteristic of the sequence orientation, but the latter would not. For a restriction enzyme to generate a fragment with a given conformation, either flip (i) or flop (o), both plus (c) and minus (v) strands in the double-stranded (d) DNA have to be in the same orientation. The probability (P) of cutting at such a conformation is the product of the probabilities that each strand is in that conformation, as given by $P_d(i) = P_c(i) \times P_v(i)$ and $P_d(o) = P_c(o) \times P_v(o)$, where $P_d(i)$ denotes the probability of double-stranded DNA being in the flip orientation. The ratio of flip to flop observed in the double-stranded DNA is then the product of the ratios for the individual strands,

$$P_d(i)/P_d(o) = [P_c(i)/P_c(o)] \times [P_v(i)/P_v(o)] \quad (1)$$

End labeling and *SmaI* digestion of double-stranded, reannealed virion DNA from wild-type virus gave a major band at 42 nt and a minor band at 103 nt (Fig. 3A, lane 1), which corresponded well with the expected 45 and 107 nt for the flip and flop conformations at the left end, respectively. Bands at 40, 41, and 43 nt were the result of terminal variations of the left end, as observed with *HhaI* digestion. The existence of a band corresponding to the flop conformation of the left end from double-stranded DNA provided additional evidence for its presence in the minus strand. The relative intensities indicated that the flip conformation was predominant at the left end. The flip-to-flop ratio in double-stranded, reannealed virion DNA was about the same as that observed in the minus strand after digestion with *HhaI* (Fig. 2). Since the two ratios were about the same, this suggested, from equation 1, that the ratio for the plus strand must be close to 1. That is, for the virion DNA that was encapsidated as plus strand, half of it was in the flip orientation at its left end, and the other half was in the flop orientation. This would be the result expected if plus strands were synthesized by the hairpin transfer mechanism with minus strand as the template and its 3′ hairpin as the self-primer.

Similar analysis of the right end of double-stranded virion DNA with *NdeI* as the diagnostic enzyme showed two bands of 77 and 93 nt (Fig. 3B, lane 1). With the reassigned right end of BPV, the expected sizes of the flip and flop fragments would be 81 and 96 nt (Fig. 1B), which were in reasonable agreement with the observed values. The flop band was about twice as intense as the flip band. Since the minus strand had equal proportions of flip to flop at this end, the ratio observed in the double-stranded DNA must result from the flip-to-flop ratio in the plus strands (see equation 1). Hence, for the plus strand, roughly one-third of it was flip and two-thirds of it was flop at the right end.

Terminal structures of BPV RF DNA. In vivo RF DNA (mostly of monomer length) (22) was isolated from BPV-infected cells 20 to 24 h postinfection by a modification of the Hirt procedure (29). This DNA was subjected to end-label analysis by using *SmaI* for the left end and *NdeI* for the right end. The restriction fragments from these reactions were compared to those obtained by digesting reannealed, double-stranded virion DNA with the same enzymes (Fig. 3A and B). The ratio of flip to flop at each terminus was the same for both RF and double-stranded virion DNA. Selective encapsidation could not be responsible for the observed ratios of terminal-sequence orientations in virion DNA, because the same ratios of flip to flop forms were observed in the RF population. Furthermore, the sizes of terminal *NdeI* fragments obtained from the two DNAs were the same. There was no extension of the right end of BPV RF DNA compared with the encapsidated virion DNA (1), in contrast to MVM.

Fate of flip and flop forms of transfecting genomic clones. Full-length genomic clones pVT501 (flop at left end) and pVT502 (flip at left end) have the same right end (in the flip form) and differ only in the orientation at the left end (27). When the left ends of the progeny DNA of amplified transfections initiated with either pVT501 or pVT502 were analyzed by end labeling and *SmaI* digestion, both flip and flop conformations were observed and the ratio of the two forms was identical to that found in a normal infection (Fig. 4). This suggested that, at the left end, one conformation could generate the other and that the flip form always accumulated preferentially in the viral DNA, regardless of the conformation present in the clone used for transfection. Similar analyses were performed on a different infectious genomic clone, pGCSma20 (flip at both ends [27]), which is deleted by 3 bases at the left end and 35 bases at the right end. Both ends were repaired to wild-type length and again the ratio of

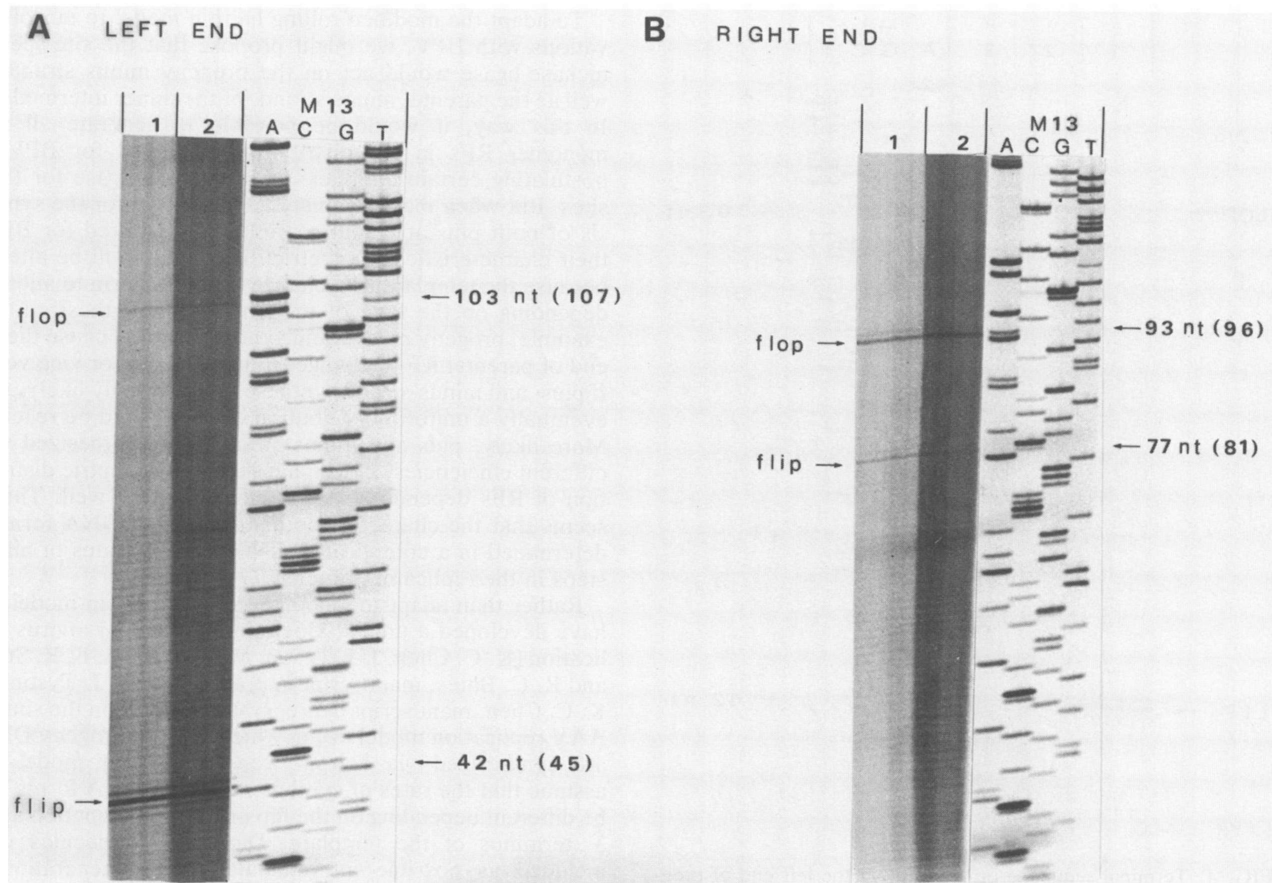

FIG. 3. End-label analyses of double-stranded virion DNA and in vivo RF DNA. Reannealed plus- and minus-strand virion DNA (lane 1) and RF DNA (lane 2) were end labeled and digested either with *Sma*I for analysis of sequence inversions at the left end (A) or with *Nde*I for analysis of sequence inversions at the right end (B). The positions and observed and expected (shown in parentheses) sizes of the flip and flop conformations are indicated.

flip to flop at each terminus was indistinguishable from that for wild-type virion DNA (data not shown). The same phenomenon has been reported for AAV (26) and the right terminus of MVM (cited in reference 14).

DISCUSSION

The distribution of terminal-sequence orientations of BPV virion DNA was analyzed by end labeling and restriction enzyme digestion. The results demonstrated specific ratios of flip and flop conformations at both ends for DNA of either plus or minus polarity. In contrast, sequence inversion at the left end of the minus strand was absent in all the rodent autonomous parvoviruses studied to date (14).

The presence of approximately equal amounts of flip and flop forms at the right ends of the minus strands, as well as at the left ends of the plus strands, suggests that hairpin transfer processes occur for synthesis of BPV virion DNA of either polarity. In either case, the complement to the plus or minus strand would serve as the template and self-primer from its 3′-terminal palindrome.

Transfection with genomic clones of BPV containing defined conformations at the ends resulted in progeny virion DNAs with distributions identical to that found in a normal infection. The same ratio of plus to minus strands (27), as well as the same ratios of flip to flop forms at the ends of reannealed virion DNAs (Fig. 4), were observed. This indicated that there must be inherent constraints on DNA replication for the establishment of such a specific distribution. Evidence exists that both cellular and viral factors are involved in these processes. Both H-1 and LuIII can infect NBE cells; however, H-1 encapsidates predominantly minus strands, whereas LuIII encapsidates equal amounts of plus and minus strands (5, 19, 20). The cellular influence is indicated by the observation that H-1 encapsidates various amounts of plus strand when propagated in different cell types (S. Rhode, personal communication).

The specificity of the distribution of terminal-sequence orientations in virion DNA is determined at the RF stage and not at the encapsidation stage (Fig. 3). The presence of the same ratios of flip to flop forms in RF DNA and in double-stranded virion DNA for BPV argues against encapsidation of a subpopulation of progeny DNAs. If this were the mechanism for selection, the ratios in reannealed virion DNA would be expected to differ significantly from those in RFs. The data indicated instead that the distribution of RFs with different terminal-sequence orientations is responsible for the observed distribution of terminal-sequence orientations in virion DNA. The same conclusion was reached for MVM (1) from the observation that a unique sequence orientation was detected at the left end of both RF and virion DNA.

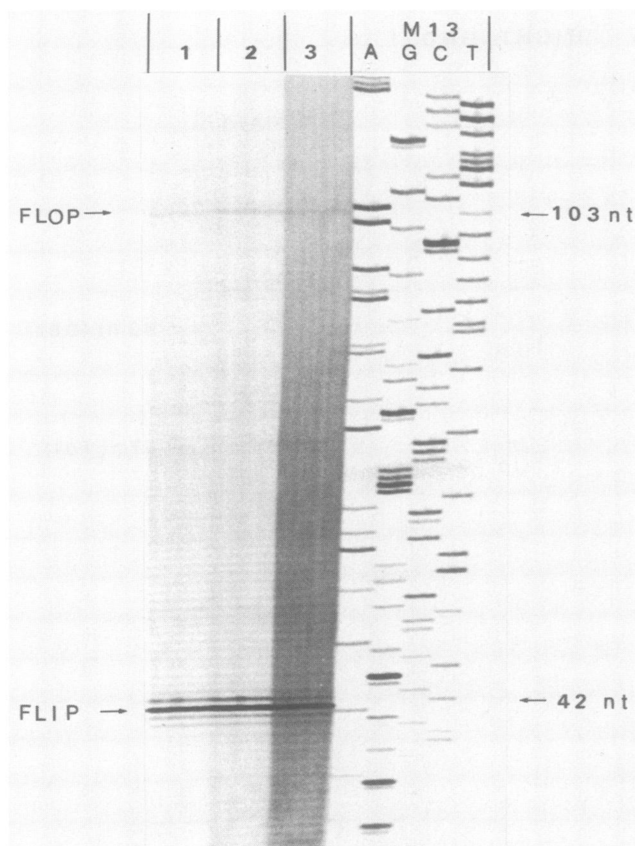

FIG. 4. Terminal-sequence orientations at the left end of progeny DNA resulting from amplified transfections with pVT501 and pVT502. Double-stranded virion DNA was purified from cell cultures infected with progeny virions of cells transfected with pVT501 (lane 1), pVT502 (lane 2), or wild-type BPV (lane 3). The DNA was end labeled and digested with *Sma*I.

However, the mechanisms for establishing specific RF and progeny DNA distributions currently proposed for AAV (8) and MVM replication (1, 14) are not adequate to explain the data for BPV. The replication mechanism for AAV, which allows hairpin transfer with equal efficiency at both termini, would predict equal distribution in both RFs and virion DNAs.

The modified rolling hairpin model (1, 14), postulated as a general model for autonomous parvovirus replication, proposed a mechanism for selective generation of certain RFs to account for the distribution of virion DNA in MVM. In that model, two obligatory dimer intermediates and site-specific nicks and ligations generate, in equal proportions, two monomer RFs whose left ends are in the same orientation as that of the input virion DNA. The two RFs interconvert from one to the other while generating minus strands by repetitive displacement synthesis on plus-strand templates. However, the constraints that restrict these RFs to minus-strand synthesis were not indicated.

We have observed a complex distribution of single-stranded BPV DNA. All eight possible configurations (flip or flop at each end of plus or minus strands) occur in unequal amounts. Consequently, all four monomer RFs (flip or flop at either end) must also be present, in unequal amounts, to serve as templates for progeny DNA synthesis. This distribution of RFs must be established and maintained to generate the particular distribution of virion DNA observed.

To adapt the modified rolling hairpin model to our observations with BPV, we might propose that the site-specific nickase-ligase would act on the progeny minus strand, as well as the parental minus strand, of the dimer intermediate. In this way, it would be possible to generate all four monomer RFs in the distribution observed for BPV by postulating certain affinities of the nickase-ligase for these sites. But when these template RFs are used for the synthesis of both plus and minus strands (as required for BPV), their characteristic asymmetric distribution will be altered, because the template RFs change from one form to another, depending on the type of progeny DNA synthesized. For example, progeny plus-strand synthesis would cause the left end of parental RF to change from flip to flop or vice versa. If plus- and minus-strand syntheses were equally likely, then eventually a uniform distribution of RFs would be reached. More likely, plus and minus strands are synthesized with different efficiencies, in which case the asymmetric distribution of RFs depends on these efficiencies as well. Thus it seems that the characteristic distribution of DNA forms is determined in a complicated fashion by the rates of all the steps in the replication process.

Rather than adapt the modified rolling hairpin model, we have developed a unified kinetic model for parvovirus replication (K. C. Chen, J. J. Tyson, M. Lederman, E. R. Stout, and R. C. Bates, manuscript in preparation; J. J. Tyson and K. C. Chen, manuscript in preparation) based on the simpler AAV replication model (8), in which RF and progeny DNAs are synthesized via hairpin transfer. In our model, we assume that the rates of synthesis of these DNA forms may be different depending on the flip or flop conformation at the 3′ terminus of the template. Dimer RF molecules were included as possible intermediates in the generation of monomer RFs. At some point in the infectious cycle, the various forms of RF and progeny DNAs would reach steady-state distributions, based on their rates of synthesis and rates of conversion to other forms by hairpin transfer. These rate constants would be characteristic for each parvovirus and could be modulated by cellular or viral factors or both.

For example, suppose that the rates of RF and progeny DNA synthesis were the same regardless of the conformation at the ends of the template. Then a uniform distribution of RF and virion DNA would be established, as observed for AAV. To account for BPV distribution, suppose that the rates of synthesis with the flip form at the left terminus were very slow compared with the rates with the flop form at the left terminus or either form at the right terminus. Then most of the RF would be in the flip form at the left terminus mainly because these RFs with the flip conformation at the left end would be used less frequently as templates and remain in the flip form. Since the left terminus would be used as the primer and template for plus-strand synthesis and the right terminus would be used for minus-strand synthesis, mostly minus strands with the flip form at the left end would be produced from these RFs as a result of the different rates of synthesis from the ends. This would be the case for BPV, and if the rate were even slower for utilization of the flip form of the left terminus, the distribution seen with MVM could be obtained. With this kinetic model, the observed virion DNA and RF DNA distributions of all parvoviruses studied to date, including LuIII (N. Diffoot and B. Shull, unpublished results), can be accounted for by assuming different sets of rate constants for synthesis of RF and progeny DNA with different primers-templates.

In the rolling hairpin model, dimer intermediates are obligatory and must be processed by a sequence of nicking

and ligation steps in order to generate two monomer RFs (of MVM) with a unique conformation at the left end. However, dimer intermediates may not be obligatory for BPV replication. The four monomer RFs in BPV theoretically can be generated from any given form of input virion DNA, with or without a dimer intermediate, by the hairpin transfer processes. In vivo RF isolated from BPV-infected cells was found to be predominantly of monomer length (22), unlike the large proportion of dimers observed for MVM (32). In our kinetic model, dimer RFs are possible intermediates but are not necessary in order to generate a specific distribution of RFs and virion DNA forms. If dimer intermediates are formed, they can be processed to two monomers by staggered nickings and repair of both strands.

The observation of two alternative orientations at the left terminus of the minus-strand DNA of the autonomous parvovirus BPV shows greater diversity in the replication strategy of these viruses than had previously been suspected. To account for this diversity, we suggest that the relative abundance of the various viral DNA species are determined by the rate constants for synthesis of RF and progeny DNA from various templates. A more detailed description of this kinetic model for parvovirus DNA replication is in preparation.

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