JOURNAL OF VIROLOGY, July 1989, p. 3180–3184
0022-538X/89/073180-05$02.00/0

Vol. 63, No. 7

Identical Ends Are Not Required for the Equal Encapsidation of Plus- and Minus-Strand Parvovirus LuIII DNA

NANETTE DIFFOOT,* BRUCE C. SHULL, KATHERINE C. CHEN, ERNEST R. STOUT, MURIEL LEDERMAN, AND ROBERT C. BATES

Department of Biology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

Received 16 November 1988/Accepted 14 March 1989

Sequence analyses of the left and right termini of LuIII virus show they are nonidentical imperfect palindromes of 122 and 211 nucleotides, respectively. The left terminus of the minus strand of LuIII DNA, uniquely in the flip conformation, can assume a T-shaped structure. The right terminus of the minus strand of LuIII DNA can assume a U-shaped structure, and it exists in either the flip or flop conformation. The termini of LuIII shared a high degree of sequence homology and showed conserved secondary structure with those of the rodent parvoviruses MVMp and H-1. LuIII, like adeno-associated virus, encapsidates equal amounts of plus- and minus-strand DNA. However, the sequence data for LuIII virus demonstrate that identical termini are not required for this encapsidation pattern.

Parvoviruses are small, icosahedral, single-stranded DNA viruses with characteristic palindromes at the left and right termini. Studies of defective interfering particles (13, 14), genomes with terminal deletions (16, 18, 25, 29), and site-specific mutants (17) suggest that the *cis* signals necessary for DNA replication and encapsidation reside in the genomic termini, possibly in the 200 nucleotides (nt) at each end. The helper-dependent adeno-associated virus (AAV) has identical terminal repeats and separately encapsidates equal amounts of plus and minus strands (6, 8). All autonomous parvoviruses of known sequences have nonidentical ends (with the possible exception of B19) (27), and their encapsidation patterns allow separation into three distinct subgroups (for reviews, see references 11 and 31). The rodent parvoviruses minute virus of mice (MVM) (4), rat virus (3, 24), and H-1 virus (21, 22) have closely related terminal sequences and encapsidate 99% minus strand. Bovine parvovirus (10) and lapine parvovirus (B. Metcalf, unpublished results) both encapsidate about 90% minus strand. Parvoviruses B19 and LuIII have encapsidation patterns similar to that of the defective parvovirus AAV. Like AAV, both viruses encapsidate both plus and minus strands with equal frequency (5, 32). The equal production of both strands of AAV is thought to result from the symmetry of the terminal palindromes (7). This may be the case for B19 virus (27) but not for LuIII virus. Bates et al. (5) showed that unlike AAV, LuIII, given appropriate reannealing conditions, did not form panhandles, suggesting that it has nonidentical ends. We have sequenced the left and right termini and found them to be nonidentical; therefore, identical termini are not required for equal encapsidation of plus and minus strands.

LuIII virus was propagated in newborn human kidney cells (NBE) transformed by simian virus 40 (28). The virion DNA was purified as described previously for bovine parvovirus (10), resulting in fully double-stranded DNA due to reannealing of plus and minus strands. This DNA was end repaired with the Klenow fragment of *Escherichia coli* DNA polymerase I (Amersham Corp., Arlington Heights, Ill.) and digested with *Eco*RI or *Hin*dIII. LuIII has one recognition site for each of these restriction enzymes (20) at about map units 20 and 50, respectively. The vectors were prepared by digesting plasmid pUC18 DNA with *Sma*I and *Eco*RI and plasmid pUC19 DNA with *Sma*I and *Hin*dIII, followed by treatment with calf intestinal phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The two *Hin*dIII fragments of LuIII were cloned into pUC19 (Fig. 1A), and the two *Eco*RI fragments were cloned into pUC18 (Fig. 1B). Ligations were carried out at 15°C for approximately 18 h. Restriction enzymes were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.).

The resulting recombinant plasmids were sequenced directly by the dideoxy method (26). Two primers which anneal to plasmid sequences just outside the multiple-cloning site were used, one to obtain plus-strand sequence of the left end and the other to obtain minus-strand sequence of the right end. On the basis of these sequences, two additional primers were synthesized with sequences complementary to the viral DNA just inside the terminal palindromes to obtain the sequences of the opposite strands. At the left palindrome, the primer annealed to bases 157 to 171 of the plus strand and provided minus-strand sequence. At the right palindrome, the primer annealed to bases 279 to 291 of the minus strand (see Fig. 3 for numbering convention) and provided plus-strand sequence. Sequencing reactions were carried out by using Klenow fragment or avian myeloblastosis reverse transcriptase (Boehringer Mannheim) and [^{35}S]dATP (500 Ci/mmol) or [^{35}S]dCTP (1290 Ci/mmol; New England Nuclear Corp., Boston, Mass.) as the label. The nucleotide analog deazaguanosine triphosphate (deaza GTP; American Bionetics, Hayward, Calif.) was used in some reactions with Klenow fragment to resolve regions high in G+C content (19).

The left palindrome of LuIII consists of 122 bases which can assume a T-shaped intrastrand base-paired structure. The sequence of this terminus was obtained by analyzing 15 independent left-end clones. Nine of these clones began 5′-ATC (Fig. 2A). The other eight each had a different terminal nucleotide and therefore appeared to represent terminal deletions ranging in size from 11 to 37 nt.

The sequence of the left terminus of LuIII was virtually identical to that of the left terminus of MVMp (4) and of H-1 (22) (Fig. 2B). All 15 clones had the sequence conformation designated flip by Astell et al. (4) in studies of MVMp. One of the arms of the LuIII hairpin differs from the published

* Corresponding author.

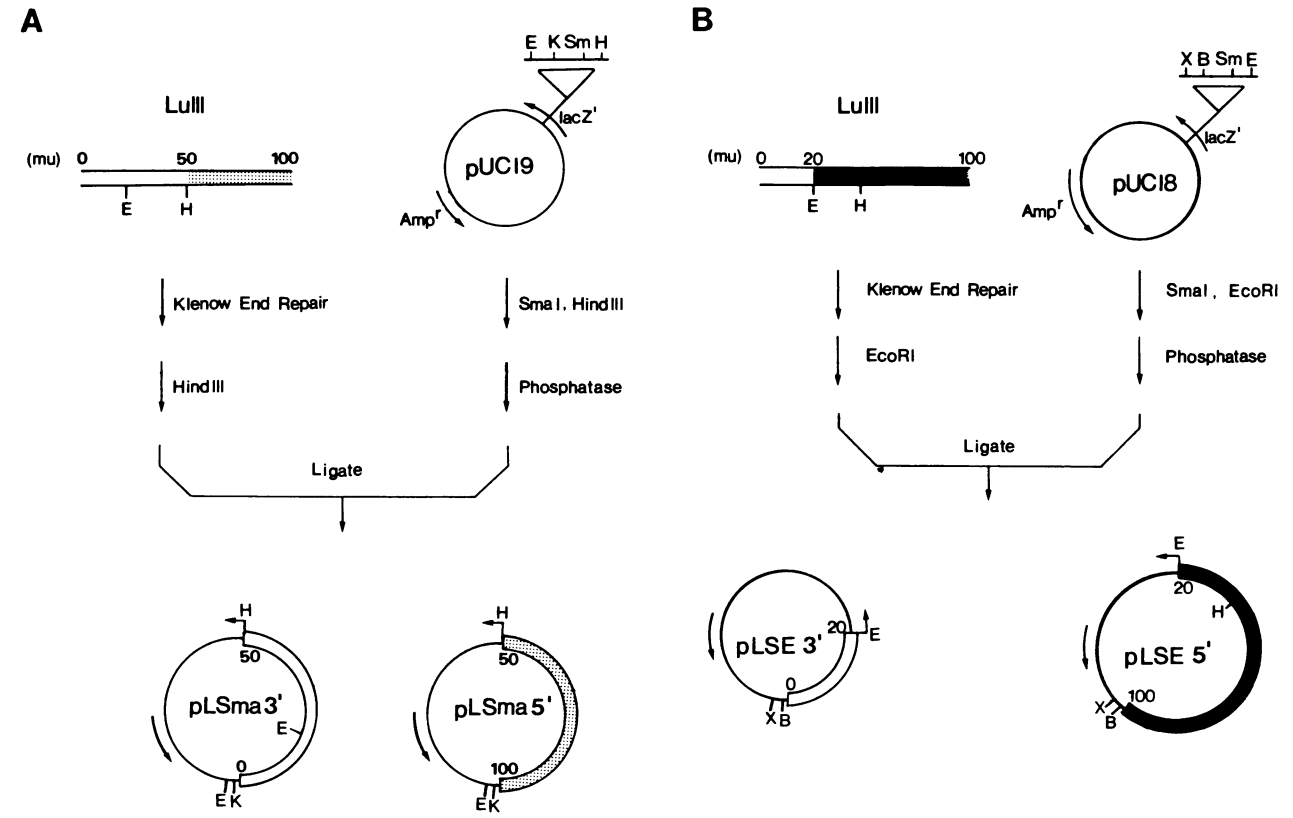

FIG. 1. Strategy for cloning LuIII genome fragments into pUC18 and pUC19. Restriction sites of the vector point outward, and restriction sites of the LuIII insert point inward. (A) Clones of the pLSma series were constructed by ligation of the *Hin*dIII fragments of LuIII into the *Hin*dIII-*Sma*I sites of pUC19. (B) Clones of the pLSE series were constructed by ligation of the two *Eco*RI fragments of LuIII into the *Eco*RI-*Sma*I sites of pUC18. Abbreviations for the restriction enzyme sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; Sm, *Sma*I; X, *Xba*I. mu, Map unit.

sequence of MVMp in having an A-T base pair at nt 64 and 74, whereas MVMp has an unpaired T at nt 69 (4). Astell et al. (2) recently suggested that this T residue may in fact occur at nt 70, the equivalent of the T residue at nt 74 in LuIII. However, the sequence of MVMi shows the A-T pair at the same location as in LuIII (23). As these differences are unlikely to affect the stability of the hairpin, the secondary structure of the left palindrome appears to be fully conserved between the viruses.

The right palindrome of LuIII can assume a 211-nt U-shaped intrastrand base-paired hairpin. A portion of this terminal sequence was obtained from the analyses of four independent clones, while the sequence of nt 131 to 229 was obtained from a clone with a deletion at the right terminus. The structure of the right hairpin in the two possible orientations is shown in Fig. 3A. When the sequence of the right palindrome of LuIII was compared with that of parvoviruses MVMp and H-1 (Fig. 3B), only minor differences were observed. Nucleotides in LuIII which are not in common with MVMp and H-1 have their complements altered as well to maintain base pairing within the right palindrome. The differences seen are unlikely to affect the overall secondary structure of the right hairpin.

The terminal sequence inversions of LuIII were analyzed by digestion with the restriction enzyme *Hha*I (3). The *Hha*I recognition sequence (GCG/C) occurs within the arms of the left hairpin (Fig. 2A) and in an unpaired region of the right hairpin (Fig. 3A) of LuIII. The *Hha*I site of the left end may be digested in either single-stranded or double-stranded (reannealed plus and minus strands) virion DNA, while the right end site can be digested only in double-stranded virion DNA.

Reannealed double-stranded LuIII virion DNA was end labeled at the 3' hydroxyl with [^{35}S]ddATP (1,372 Ci/mmol; New England Nuclear) followed by digestion with *Hha*I. This results in labeling of the minus strand at the left end and of the plus strand at the right end. The labeled fragments were run on a sequencing gel by using M13mp18 sequencing reactions as size markers (Fig. 4). Four possible fragments resulting from *Hha*I digestion were expected from the left end of LuIII as a consequence of the two overlapping restriction sites (Fig. 2A). Digestion of DNA in the flip conformation at the left end would result in fragments of 50, 52, 59, and 61 nt, while digestion of flop-conformation DNA would result in fragments of 61, 63, 70, and 72 nt. Strong bands of 47, 49, and 56 nt were observed which corresponded well with the expected fragment sizes for DNA, whose terminus of the minus strand was in the flip orientation. The secondary structure assumed by the fragments during migration in the sequencing gel could be responsible in part for the observed deviation (9). The extra bands of lesser intensity probably reflect the heterogeneity in the position of the terminal nucleotide of LuIII, as seen in the cloning studies described above and for other parvovirus genomes (3, 15, 29). No bands of sufficient length to correspond to the flop orientation of the left terminus of the minus strand were observed. Because of the inability to label the 5' end of the plus strand (unpublished result), no conclusions

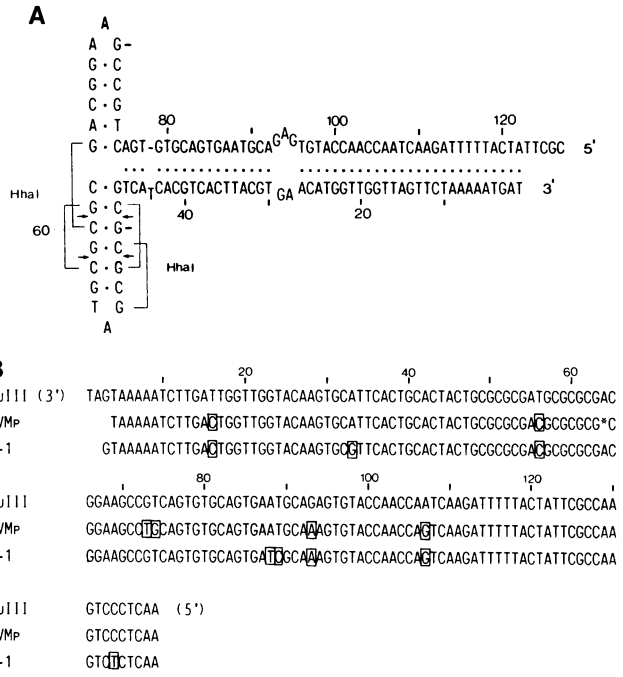

FIG. 2. (A) DNA sequence at the left terminus of the minus strand of LuIII. The cleavage sites for *Hha*I are indicated by arrows. (B) Comparison of the DNA sequence at the left termini of rodent parvoviruses MVMp (4), H-1 (22), and LuIII. Boxes indicate nucleotides nonhomologous to the LuIII sequence. A space required for maximal alignment of the sequences is indicated by an asterisk.

can be made about the conformation of this strand at the left terminus.

From the sequence of the cloned right terminus of LuIII (Fig. 3A), two bands of 82 and 129 nt, corresponding to the flip and flop conformations, are expected after end labeling and *Hha*I digestion. Strong bands were observed at 99 nt, 17 nt longer than expected, and at 144 nt, 15 nt longer than expected, for the flip and flop conformations, respectively (Fig. 4). The difference between the observed and expected sizes should be the same for the flip and flop fragments, and the small deviation seen could be due to the secondary structure assumed by the fragments during migration or to the heterogeneity at the ends of virion DNA molecules as mentioned above. Irrespective, the data suggest that the mature right terminus is longer than the cloned terminus. Since only the 3′ end of the plus strand was labeled, no information is available on the length of the minus strand. The sequence predicted for the ultimate nucleotides suggests that they could form a small hairpin, making molecules with this hairpin inaccessible to cloning. The presence of a covalently linked terminal protein at the mature terminus, similar to the one described for MVM (12), might also interfere with the efficient cloning of full-length DNA molecules. Attempts to label the 5′ end of LuIII virion DNA were unsuccessful (unpublished results), suggesting that the 5′ end of both plus- and minus-strand LuIII DNA is also blocked by a terminal protein. These extra nucleotides, if actually present at the right terminus, are not crucial for viral replication. An LuIII genomic clone with a right terminus identical to that shown in Fig. 3A was highly infectious (N. Diffoot, unpublished results).

The sequence homology between the termini of LuIII, MVM, and H-1 parvoviruses, the conservation of secondary

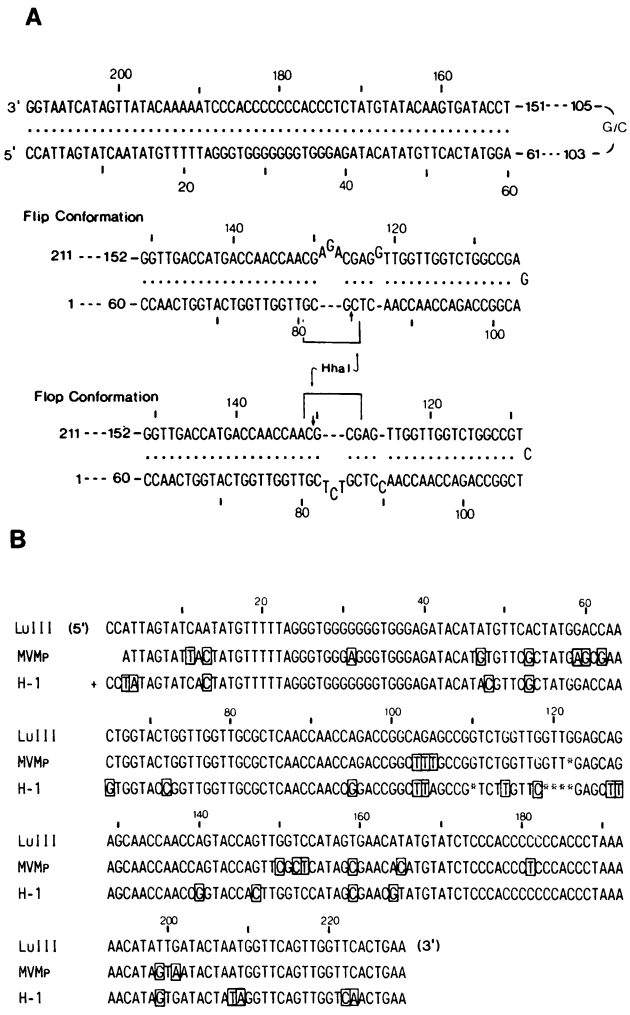

FIG. 3. (A) DNA sequence at the right terminus of the minus strand of LuIII. The cleavage sites for *Hha*I are indicated by arrows. (B) Comparison of the DNA sequence at the right termini of rodent parvoviruses MVMp (4), H-1 (22), and LuIII. Boxes indicate nucleotides nonhomologous to the LuIII sequence. Spaces required for maximal alignment of the sequences are indicated by asterisks.

structure in spite of minor sequence differences, and the unique flip conformation at the left terminus of the minus strand of LuIII suggest that this virus is closely related to the rodent parvoviruses, even though it was originally isolated from a human cell line (30). The finding of equal amounts of plus- and minus-strand LuIII progeny DNA with a unique conformation at the left terminus of the minus strand means that neither the replication model for AAV (7) nor the replication model for MVM (1) can explain the replication of LuIII. The replication model for AAV predicts hairpin transfer at both (left and right) ends with equal efficiency. This would result in equal amounts of plus- and minus-strand DNA, concomitant with equal proportions of flip-flop at both ends. Conversely, the replication model for MVM explains the formation of progeny DNA with only one conformation at the left terminus but simultaneously limits the nature of the progeny DNA to strands of only minus polarity, in contrast to LuIII.

The available data (3, 9) suggest that the virion DNA is not positively selected at the encapsidation step. A kinetic

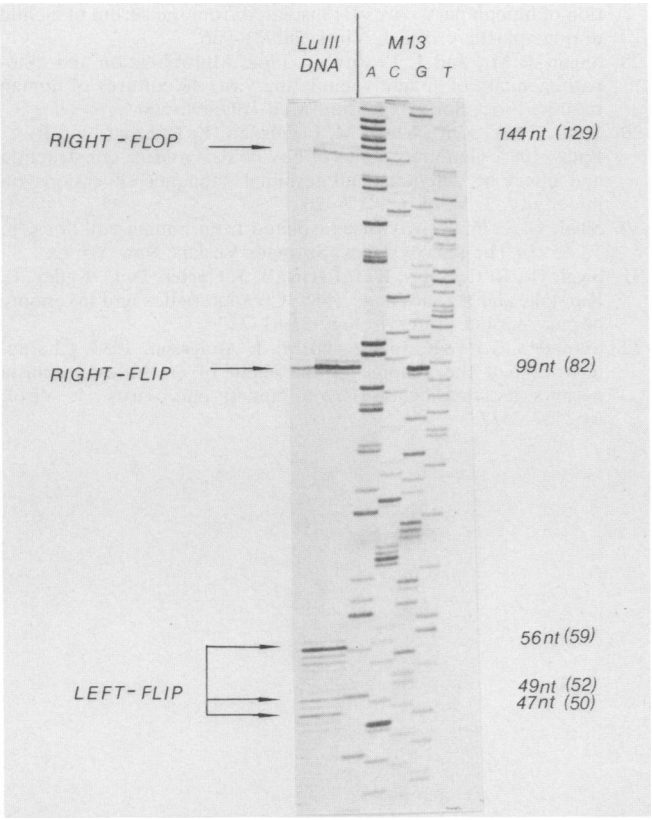

FIG. 4. End-label analysis of double-stranded LuIII virion DNA. Reannealed plus- and minus-strand virion DNA was end labeled and digested with *Hha*I for analysis of sequence inversions at the termini. The labeled fragments were run on a sequencing gel with M13mp18 sequencing reactions as size markers. The positions of the observed and expected (shown in parentheses) sizes of fragments in the flip and flop conformations are indicated.

analysis (K. C. Chen, J. J. Tyson, M. Lederman, E. R. Stout, and R. C. Bates, J. Mol. Biol., in press) proposes that the characteristic distribution of virion DNA is defined by the differential rates of hairpin transfer at various 3′ hydroxyl terminal palindromes. These rates determine the distribution of amplified replicative intermediates during the infectious cycle and the efficiency with which replicative forms generate single-stranded DNAs of characteristic polarity and terminal orientation, which are then packaged with equal efficiencies. The differential rates of hairpin transfer are determined by the flip-flop conformation of the terminal palindrome as well as by cellular and viral factors.

It is unlikely that the signals necessary for strand selection during replication are defined solely by the termini; otherwise, this would predict encapsidation of only minus-strand LuIII DNA on the basis of its virtual sequence identity with MVM DNA, unless the minor nucleotide differences between the terminal palindromes of LuIII and MVMp are sufficient to account for the different encapsidation patterns observed for the two viruses. Further studies of the mechanism of LuIII replication will be of great importance in understanding the biology of parvoviruses, since LuIII shares characteristics with two highly studied extremes of the parvovirus spectrum, AAV and the rodent parvoviruses MVM and H-1 virus.

This work was supported by American Cancer Society grant MV220.

LITERATURE CITED

1. **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.
2. **Astell, C. R., E. M. Gardiner, and P. Tattersall.** 1986. DNA sequence of the lymphotropic variant of minute virus of mice, MVM(i), and comparison with the DNA sequence of the fibrotropic prototype strain. J. Virol. **57:**656–669.
3. **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 the origins of DNA replication. Cell **17:**691–703.
4. **Astell, C. R., M. Thompson, 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.
5. **Bates, R. C., C. E. Snyder, P. T. Bannerjee, and S. Mitra.** 1984. Autonomous parvovirus LuIII encapsidates equal amounts of plus and minus DNA strands. J. Virol. **49:**319–324.
6. **Berns, K. I., and R. A. Bohensky.** 1987. Adeno-associated viruses: an update. Adv. Virus Res. **32:**243–306.
7. **Berns, K. I., and W. W. Hauswirth.** 1984. Adeno-associated virus DNA structure and replication, p. 1–31. *In* K. I. Berns (ed.), The parvoviruses. Plenum Publishing Corp., New York.
8. **Berns, K. I., and M. Labow.** 1987. Parvovirus gene regulation. J. Gen. Virol. **68:**601–614.
9. **Chen, K. C., B. C. Shull, M. Lederman, E. R. Stout, and R. C. Bates.** 1988. Analysis of the termini of the DNA of bovine parvovirus: demonstration of sequence inversion at the left terminus and its implication on the replication model. J. Virol. **62:**3807–3813.
10. **Chen, K. C., B. C. Shull, E. A. Moses, M. Lederman, E. R. Stout, and R. C. Bates.** 1986. Complete nucleotide sequence and genome organization of bovine parvovirus. J. Virol. **60:**1085–1097.
11. **Cotmore, S. F., and P. Tattersall.** 1987. The autonomously replicating parvovirus of vertebrates. Adv. Virus Res. **33:** 91–174.
12. **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.
13. **de la Maza, L. M., and B. J. Carter.** 1980. Molecular structure of adeno-associated virus variant DNA. **255:**3194–3203.
14. **Faust, E. A., and D. C. Ward.** 1979. Incomplete genomes of the parvovirus minute virus of mice: selective conservation of genome termini, including the origin for DNA replication. J. Virol. **32:**276–292.
15. **Fife, K. H., K. I. Berns, and K. Murray.** 1977. Structure and nucleotide sequence of the terminal regions of adeno-associated virus DNA. Virology **78:**475–487.
16. **Labow, M. A., L. H. Graf, Jr., and K. I. Berns.** 1987. Adeno-associated virus gene expression inhibits cellular transformation by heterologous genes. Mol. Cell. Biol. **7:**1320–1325.
17. **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.
18. **Merchlinsky, M. J., P. J. Tattersall, J. J. Leary, S. F. Cotmore, E. M. Gardiner, and D. C. Ward.** 1983. Construction of an infectious molecular clone of the autonomous parvovirus minute virus of mice. J. Virol. **47:**227–232.
19. **Mizusawa, S., S. Nishimura, and F. Seela.** Improvement of the dideoxy chain termination method of DNA sequencing by use of deoxy-7-deazaguanosine triphosphate in place of dGTP. Nucleic Acids Res. **14:**1319–1324.
20. **Rhode, S. L.** 1978. Replication process of parvovirus H-1. X. Isolation of a mutant defective in replicative-form DNA replication. J. Virol. **25:**215–223.
21. **Rhode, S. L., and B. Klaassen.** 1982. DNA sequence of the 5′ terminus containing the replication origin of parvovirus replicative form DNA. J. Virol. **41:**990–999.
22. **Rhode, S. L., and P. R. Paradiso.** 1983. Nucleotide sequence of H-1 and mapping of its genes by hybrid-arrested translation. J.

Virol. **45**:173–184.
23. **Sahli, R., G. K. McMaster, and B. Hirt.** 1985. DNA sequence comparison between two tissue-specific variants of the autonomous parvovirus, minute virus of mice. Nucleic Acids Res. **13**:3617–3633.
24. **Salzman, L. A., and P. Fabisch.** 1979. Nucleotide sequence of the self-priming 3′ terminus of the single-stranded DNA extracted from the parvovirus Kilham rat virus. J. Virol. **30**: 946–950.
25. **Samulski, R. J., A. Srivastava, K. I. Berns, and N. Muzyczka.** 1983. Rescue of adeno-associated virus from recombinant plasmids: gene correction within the terminal repeats of AAV. Cell **33**:135–143.
26. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
27. **Shade, R. O., B. C. Blundell, S. F. Cotmore, P. Tattersall, and C. R. Astell.** 1986. Nucleotide sequence and genome organization of human parvovirus B19 isolated from the serum of a child during aplastic crisis. J. Virol. **58**:921–936.
28. **Shein, J. M., and J. F. Enders.** 1962. Multiplication and cytopathogenicity of simian vacuolating virus 40 cultures of human tissues. Proc. Soc. Exp. Biol. Med. **109**:495–500.
29. **Shull, B. C., K. C. Chen, M. Lederman, E. R. Stout, and R. C. Bates.** 1988. Genomic clones of bovine parvovirus: construction and effect of deletions and terminal sequence inversions on infectivity. J. Virol. **62**:417–426.
30. **Siegl, G.** 1976. Parvoviruses isolated from human cell lines, p. 72–85. *In* The parvoviruses. Springer-Verlag, New York.
31. **Siegl, G., R. C. Bates, K. I. Berns, B. J. Carter, D. C. Kelley, E. Kurstak, and P. Tattersall.** 1985. Characteristics and taxonomy of parvoviridae. Intervirology **23**:61–73.
32. **Summers, J., S. E. Jones, and M. J. Anderson.** 1983. Characterization of the genome of the agent of erythrocyte aplasia permits its classification as a human parvovirus. J. Virol. **64**:2527–2532.