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Autonomous Parvovirus LuIII Encapsidates Equal Amounts of Plus and Minus DNA Strands

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Autonomous parvoviruses are thought to uniquely encapsidate single-stranded DNA of minus polarity. In contrast, the defective adeno-associated viruses separately encapsidate equal amounts of plus and minus DNA strands. We reexamined the uniqueness of minus strand encapsidation for the autonomous parvoviruses. Although we found that Kilham rat virus and H-1 virus encapsidate varying but small amounts of complementary-strand DNA, it was unexpected to find that LuIII virus encapsidated equal amounts of plus and minus DNA. The extracted LuIII DNA possessed properties of double-stranded replicative-form DNA, including insensitivity to S1 endonuclease, cleavage by restriction enzymes, and conversion to unit-length, single-stranded DNA when electrophoresed under denaturing conditions. However, the inability of this DNA to form single-stranded DNA circles when denatured and then renatured in the presence of formamide and the lack of double-stranded DNA circle formation after treatment with exonuclease III and reannealing shows a lack of sequence homology of the 3′ and 5′ termini of LuIII DNA, in contrast to adeno-associated virus DNA. Digestion of LuIII double-stranded DNA with *Eco*RI and *Hin*cII and separation of plus and minus DNA strands on composite agarose-acrylamide gels identified a heterogeneity present only in the plus DNA strand. These results suggest that strand specificity of viral DNA encapsidation is not a useful property for differentiation between the autonomous and defective parvoviruses. Furthermore, encapsidation by LuIII of equal amounts of complementary DNA strands in contrast to encapsidation of minus strands by H-1 virus, when propagated in the same host cell type, suggests that selection of strands for encapsidation is a virus-coded rather than host-controlled event.

The genus *Parvovirus* in the family Parvoviridae is represented by the autonomous or nondefective viruses which do not require a helper virus for their replication. Kilham rat virus (KRV), H-1 virus, and minute virus of mice are rodent viruses representative of this genus, and these contain a unique (i.e., minus) DNA strand complementary to the mRNA with nonidentical 5′ and 3′ terminal hairpin sequences (4; C. E. Snyder, C. R. Wobbe, C. C. Morse, H. B. Scott, and S. Mitra, manuscript in preparation). The genus *Dependovirus* contains the defective adeno-associated viruses (AAV) that require a helper virus. In contrast to the automonous parvoviruses, AAV encapsidates equal amounts of plus and minus DNA strands which possess identical 5′ and 3′ terminal hairpin sequences. In addition to the helper virus requirement and terminal sequence characteristics, the strand specificity of viral DNA encapsidation has been the basis for classification of parvoviruses as defective or autonomous (2).

We reexamined the uniqueness of minus strand encapsidation of the autonomous parvoviruses in light of the finding (A. K. Saemundsen, M.S. thesis, Virginia Polytechnic Institute and State University, Blacksburg, 1980) that a significant fraction (up to 20 to 30%) of the DNA molecules isolated from bovine parvovirus virions exhibited double-stranded characteristics. In the present study, two parvoviruses of rodent origin, KRV and H-1 (23), were chosen for comparison to LuIII parvovirus (22, 23). Although LuIII was isolated from and can grow only in human cells, its origin is unknown, but our recent studies on sequence homology (3) strongly suggest that it is closely related to the rodent parvoviruses, including H-1 virus. However, H-1 can be propagated in either rodent or human cells (23). Electrophoresis of the DNA from the three viruses in nondenaturing gels revealed that the majority of the DNA of the rodent viruses, KRV and H-1, was present in a band corresponding to single-stranded DNA. However, for LuIII, most of the DNA after a reannealing treatment was in the position of replicative-form (RF) DNA. This observation for LuIII DNA taken with other results demonstrates that plus and minus strands are encapsidated separately in equal amounts in a manner analogous to that of AAV (4, 17). These results indicate that the uniqueness of strand specificity of the virion DNA may not be related to the nondefectiveness of the virus or to the identical 5′ and 3′ terminal hairpin sequences present in AAV DNA, but not present in LuIII DNA.

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MATERIALS AND METHODS

Purification of viral DNA. KRV 171 originally isolated by Kilham and Moloney (14) and obtained from R. Tennant was grown in normal rat kidney cells (18). Plaque-purified H-1 was a gift from S. Rhode, and LuIII virus was a gift from K. Soike originally obtained from G. Siegl. H-1 and LuIII were grown in newborn human kidney cells transformed by simian virus 40 (21, 24). KRV and LuIII were plaque purified twice in the cells described above after diluting the virus-containing lysate in 0.5% sodium dodecyl sulfate thus preventing infection with aggregates of virus particles (R. C. Bates, unpublished data). The serological identity of each virus after plaque purification was reconfirmed by hemagglutination-inhibition assay with specific antisera. The general procedure for growth and purification of the viruses labeled with [^{3}H]thymidine has been described previously (16). Viral DNAs obtained by band sedimentation in alkaline sucrose were dialyzed against 1 M NaCl containing 10 mM Tris-

hydrochloride (pH 8.0) and 1 mM EDTA at room temperature for 3 to 6 h to facilitate reannealing, extracted with a 1:1 mixture of phenol-chloroform, and ethanol precipitated. Ratios of absorbance of the DNAs at 260 and 280 nm were 1.7 to 1.85 for different preparations.

Enzymes. Digestion of DNA with S1 endonuclease was carried out as described previously (8), except that 0.3 M NaCl and pH 5.0 were used. The conditions for digestion of LuIII DNA with *Eco*RI and *Hin*cII were as specified by the manufacturer (Bethesda Research Laboratories).

Gel electrophoresis and strand separation. Electrophoresis of viral DNA was carried out in slab gels containing 1 or 1.4% agarose with Tris-acetate buffer (pH 7.8). For separation of LuIII DNA strands, a modification of the procedure described by Hauswirth et al. (12, 13) was used. Composite 1% agarose–1% acrylamide gels were prepared in 45 mM Tris borate (pH 8.3) and 2.5 mM EDTA buffer. ^{3}H-labeled LuIII DNA was denatured in 15 mM methylmercuric hydroxide as described previously (25) and loaded directly onto the gel. Nondenatured LuIII DNA was loaded in the absence of methylmercuric hydroxide. Electrophoresis was carried out at 90 V for 18 h, and the DNA was visualized by ethidium bromide staining. The bands containing ^{3}H-labeled DNA were excised, and the DNA was electroeluted from the gel slices.

Electron microscopy. AAV and LuIII DNAs were denatured in 0.1 N NaOH and reannealed for 2 h at room temperature in the presence of 50% formamide at a DNA concentration of 0.3 μg/ml (9). The samples were quick chilled and immediately spread for electron microscopy as described previously (1, 18).

RESULTS

Strandedness of autonomous parvoviruses under nondenaturing and denaturing conditions. We examined the strandedness of the reannealed DNAs of KRV, H-1, and LuIII by electrophoresis in 1% agarose (Fig. 1A). Under the nondenaturing conditions used, single-stranded DNA has a greater mobility than RF DNA. After staining the gels containing comparable amounts of each DNA with ethidium bromide, several bands were seen for KRV and H-1. The majority of KRV and H-1 DNA (80 to 90% based on radioactivity of isolated bands) was present in the fastest band corresponding to single-stranded DNA. A smaller amount of DNA was distributed in three slower moving bands (A, B, and C [Fig. 1A]). Bands A and C, seen in preparations of KRV and H-1 DNAs, contained less than 1% of the DNA based on radioactivity. Band B had a mobility similar to that of KRV RF DNA synthesized in vitro from KRV DNA with T5 phage DNA polymerase. More suprisingly, in the case of reannealed LuIII DNA, no band corresponding to single-stranded DNA was seen as expected (22) after staining with ethidium bromide (Fig. 1A). The LuIII DNA was almost totally present in a single band (Fig. 1A, band B) with a mobility similar to that of KRV RF DNA, but a detectable amount of DNA did not enter the gel. The apparent mobility of such molecules (Fig. 1A, band B) in agarose gels suggested that they were like RF DNA produced in vivo during viral replication or that they can be synthesized in vitro by replication of virion DNA with purified bacterial or other DNA polymerases (6). This is reminiscent of AAV DNA where the equal amounts of plus and minus strands of DNA reannealed during DNA purification. The size of the LuIII DNA (Fig. 1A, band B) when electrophoresed under nondenaturing conditions was approximately 5,000 base pairs which is equivalent to that of KRV RF DNA. This would exclude the possible encapsidation of truncated duplex forms of LuIII DNA which would be smaller.

To exclude the possibility that the slower-moving bands in the different DNAs arose from longer than unit-length DNA molecules packaged into virions, the DNAs were electrophoresed under denaturing conditions in agarose containing 10 mM methyl mercury. The electrophoretic pattern of the DNAs under this denaturing condition showed that all of the DNAs were present in single bands (25). The size of the DNAs in these bands was determined to be just over 5,000 nucleotides for the rodent parvoviruses and for LuIII (25). The complete denaturation of the slower-moving bands into a single DNA band for LuIII supports the explanation of the RF-like DNA consisting of annealed unit-length plus and minus strands.

S1 endonuclease analysis of LuIII RF-like DNA. To show that LuIII DNA was indeed like RF and double stranded, KRV single-stranded DNA and LuIII DNA were treated with S1 endonuclease, which was specific for single-stranded DNA, and electrophoresed as described above (Fig. 1B). As expected, KRV DNA was degraded into small pieces and ran out of the gel, whereas LuIII DNA remained unchanged, except for the formation of a slightly faster minor band. Extensive digestion with S1 endonuclease made 85% of the KRV DNA and only 9.5% of the LuIII DNA acid soluble. A fraction which varied from preparation to preparation of LuIII DNA but not the others did not enter the gel (Fig. 1, lane 2). However, the possibility that this DNA contains tightly bound protein, as in the case of H-1 RF DNA (19), is unlikely because extensive deproteinization of LuIII DNA with a mixture of phenol and $CHCl_3$ or prior digestion with

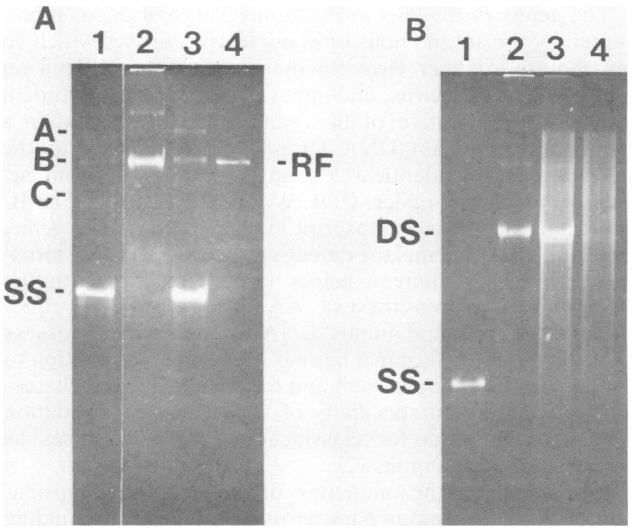

FIG. 1. Electrophoresis of DNA in nondenaturing agarose gels. (A) Strandedness of parvovirus DNA. Lane 1, KRV DNA; lane 2, LuIII DNA; lane 3, H-1 DNA; and lane 4, in vitro KRV RF DNA. Electrophoresis was as described in the text. Single-stranded DNA (SS) (0.75 μg) and 0.4 μg of KRV RF DNA (RF) were loaded on the gel. Slower moving bands (A, B, and C) are described in the text. (B) Effect of treatment with S1 endonuclease on electrophoresis of LuIII DNA. Lanes 1 and 2, untreated KRV DNA (SS) and LuIII DNA (DS), respectively; lanes 3 and 4, S1 endonuclease-treated LuIII DNA and KRV DNA, respectively, in the absence of carrier DNA added to the S1 reaction mixture. The background in lane 3 results from digestion of small amounts of single-stranded DNA and single-stranded regions of network structures present in LuIII DNA preparations (see text).

proteinase K (200 μg/ml for 2 h at 37°C in the presence of 0.5% sodium dodecyl sulfate, 1 mM EDTA, and 10 mM Tris-hydrochloride [pH 8.0]) followed by extraction with phenol did not alter the electrophoretic pattern (data not shown). It is more likely that molecules not entering the gel have a large size from intermolecular interactions forming network structures (Fig. 2B) as explained below.

Electron microscopic visualization of LuIII DNA. The fact that no significant amount of longer or shorter than unit-length viral DNA was seen in denaturing gels indicates that the slower-moving bands in native gels contained DNA molecules that were produced as a result of noncovalent or hydrogen bonding between unit-length single strands of DNA. By electron microscopy, it was seen that about 5% of the DNA was determined to be single stranded. If the reannealing step was omitted during viral DNA purification, greater amounts of single-stranded DNA were observed.

Some DNA in the LuIII DNA preparations appeared too large to enter nondenaturing gels (Fig. 1A). Also, 10 to 20% of the viral DNA collected in a 66% sucrose cushion when it was centrifuged in a 5 to 20% netural sucrose gradient (data not shown). Electron microscopic examination of such DNA oligomers shows that they consist of DNA molecules which are partially single stranded with intervening double-stranded regions involving several single strands (Fig. 2B). Under denaturing conditions, these DNA oligomers, formed by intermolecular interactions between complementary sequences of plus and minus strands, are converted to uniform single strands (25). Similar network-like structures were previously described for AAV DNA (7). This type of structure was not observed for KRV and H-1 DNA where strands isolated from virions are almost entirely of one polarity. Since LuIII possesses plus and minus strands, it is expected that network-like structures would occur as was observed for AAV DNA.

Examination of LuIII for complementarity of termini. It is well documented that AAV DNA plus and minus strands can self-anneal as a result of the self-complementary inverted repeat at their termini (4) forming single-stranded circular molecules (5, 15). Because of our observation that LuIII encapsidates equal amounts of plus and minus single strands, we investigated the ability of denatured LuIII DNA strands to reanneal in the presence of 50% formamide to form single-stranded circular DNA molecules as a probe of their terminal sequence structure. When LuIII and AAV DNAs were prepared under identical conditions, no single-stranded circles were observed for LuIII (Fig. 2D), whereas 25% (50 of 200 unit-length molecules) of the AAV DNA

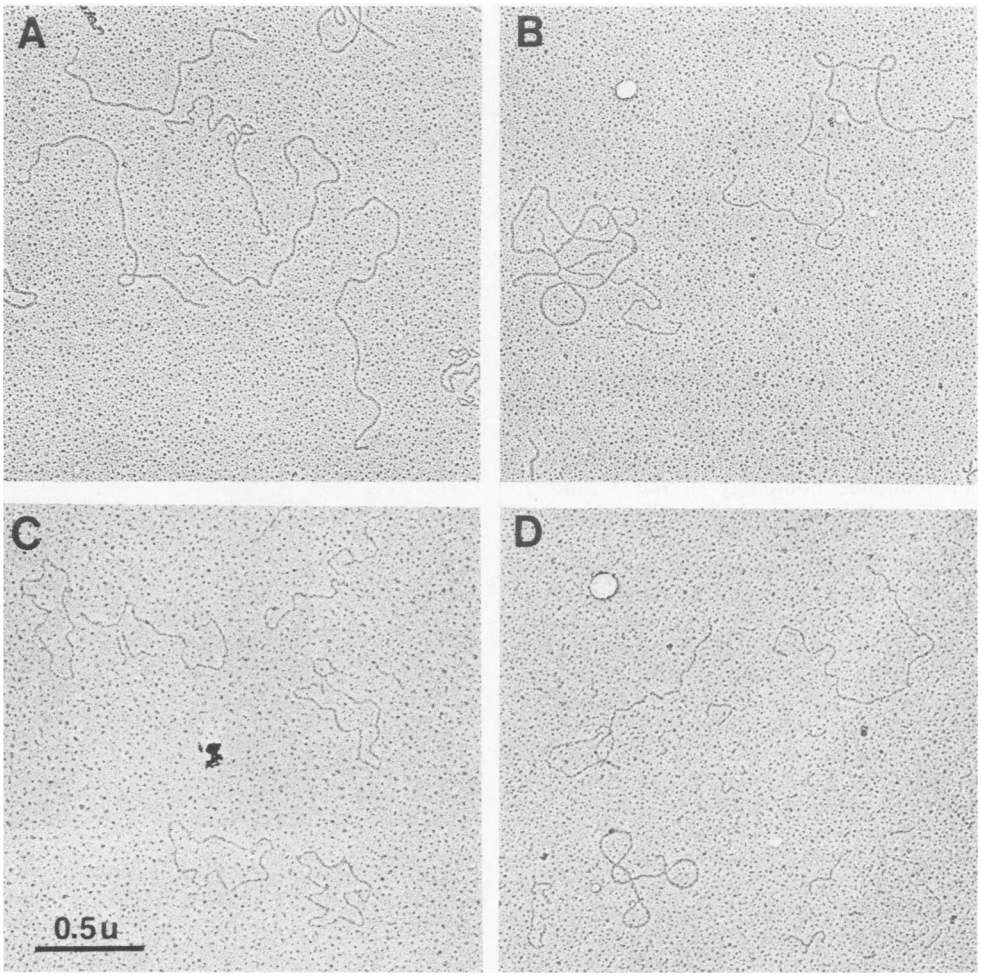

FIG. 2. Electron microscopic examination of native and reannealed LuIII and AAV DNA. (A) LuIII DNA; (B) LuIII DNA network formation through intermolecular interaction; (C) AAV DNA reannealed in the presence of formamide demonstrating circle formation through self-complementary terminal repeats; (D) LuIII DNA reannealed in the presence of formamide.

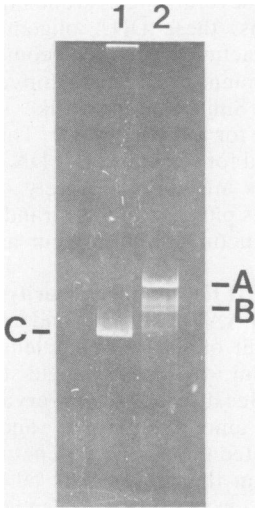

FIG. 3. Separation of ^{3}H-labeled LuIII DNA strands by electrophoresis on composite agarose-acrylamide gels. Lane 1, nondenatured LuIII DNA; lane 2, LuIII DNA denatured with 15 mM methylmercuric hydroxide. The slow migrating band (A) corresponds to the minus viral DNA strand, and the faster migrating doublet band (B) corresponds to the plus strand DNA (lane 2). The amount of ^{3}H in DNA eluted from band A was equal to that present in band B. In this gel system, double-stranded DNA (C) migrates faster than single-stranded DNA (13).

strands reannealed to form circles (Fig. 2C). It is possible that single-stranded DNA circles were not observed for LuIII because the plus and minus strands were not separated before the reannealing step. Therefore, we separated the LuIII DNA strands (Fig. 3) but still did not observe any circle formation (data not shown). In the same manner as was shown for AAV DNA (11), we attempted to demonstrate the formation of double-stranded circles after treatment of LuIII duplex DNA with *Escherichia coli* exonuclease III and reannealing, but none were observed. If it is assumed that single-stranded LuIII DNA has sequences capable of forming hairpins at the termini like other parvovirus DNAs (4), the lack of single-stranded circle formation shows the lack of sequence homology of the 3′ and 5′ termini. The absence of duplex circle formation also supports this lack of sequence homology.

Identification of LuIII DNA strand polarity and terminal heterogeneity. In some nondenaturing gels for different preparations of LuIII DNA, we observed two very closely spaced bands at the double-stranded DNA position. The presence of two bands could be accounted for by differences in secondary structure or by differences in size. Electrophoresis of the DNA under denaturing conditions (25) did not reveal a difference in size of the plus and minus DNA strands. However, when the DNA was electrophoresed to separate strands in a composite agarose-acrylamide gel under nondenaturing conditions three single-stranded DNA species were observed (Fig. 3). Based on our previous observation that LuIII DNA and the DNAs of several rodent parvoviruses share significant sequence homology (3), it was possible to identify the polarity of the separated LuIII DNA strands. The extent of hybridization of ^{3}H-labeled LuIII DNA to KRV minus single-stranded DNA was determined. The faster-migrating doublet band hybridized to the KRV minus DNA strand 15 times better than did the slower-migrating band (Table 1). Therefore, the slower-migrating band was a minus DNA strand, and the faster-migrating doublet band was a plus DNA strand (Fig. 3). The relative migration rates and polarity of the strands is the same as that previously determined for AAV (12, 13).

TABLE 1. Identification of LuIII DNA strand polarity by hybridization

Input DNA[a]	Immobilized DNA[b]	Bound cpm (%)[c]
LuIII DNA, 3,000 cpm/filter (band A, Fig. 3)	KRV minus strand	57 (1.9)
LuIII DNA, 3,000 cpm/filter (band B, Fig. 3)	KRV minus strand	844 (28.1)

[a] Labeled with [^{3}H]thymidine and electroeluted from composite 1% agarose–1% acrylamide gels (see Fig. 3).
[b] Two micrograms of unlabeled KRV minus strand DNA per Schleicher & Schuell B-6 nitrocellulose filter.
[c] Hybridization was in the presence of formamide (10), and the counts per minute hybridized were corrected for background and nonspecific binding to blank filters.

Since the apparent difference in migration of the double-stranded species and the plus strands can be accounted for by secondary structure, it was of interest to determine the location of this heterogeneity. By use of a restriction enzyme which cleaves the genome into a large and small fragment, it was possible to make this identification. *Eco*RI is predicted to cleave LuIII DNA at about map unit 21 from the 3′ end of the minus strand on the basis of sequence homology (3), map alignment (20), and our mapping experiments (S. Mitra and R. Bates, unpublished data). Therefore, if the difference in secondary structure of the two populations of LuIII plus strands is at the 5′ end of these strands (complementary to the 3′ end of the minus strand), cleavage with *Eco*RI followed by nondenaturing agarose gel electrophoresis should yield two rather than one small fragment. Figure 4A shows that cleavage with *Eco*RI yields one large fragment

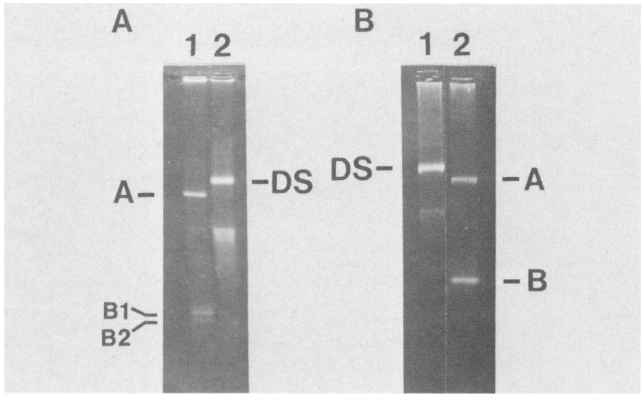

FIG. 4. Demonstration of heterogeneity at the 5′ end of the plus strand of LuIII DNA. LuIII DNA was digested with *Eco*RI or *Hin*cII and electrophoresed on 1.0% agarose. (A) For DNA digested with *Eco*RI, one large fragment (A) and two small fragments (B1 and B2) were observed (lane 1). Uncleaved LuIII DNA (DS) is shown in lane 2. (B) For DNA digested with *Hin*cII, one large fragment (A) and one small fragment (B) were observed (lane 2). Lane 1 is uncleaved LuIII DNA (DS).

and two small fragments differing slightly in mobility. However, if LuIII DNA was cleaved with *Hin*cII, which cleaves near the right end of the genome (S. Mitra and R. Bates, unpublished data), only one large and one small fragment were observed (Fig. 4B). The *Hin*cII large fragment did not show the heterogeneity observed for the *Eco*RI small fragments since it was too large. This experiment demonstrates that a heterogeneity is located near the 5′ end of the plus strands of LuIII DNA, but not at the 3′ end.

DISCUSSION

A distinctive feature of the autonomous parvoviruses, in contrast to the defective parvoviruses, was believed to be the uniqueness of the encapsidation of the single-stranded DNA, namely the minus strand. It was unexpected to find that virtually all of the DNA extracted from LuIII virions exhibited a mobility under nondenaturing conditions equal to that of parvovirus RF DNA (Fig. 1A). The double-stranded nature of this LuIII DNA was confirmed by its insensitivity to S1 endonuclease, its cleavage by restriction enzymes, and its denaturation to unit-length, single-stranded DNA. Also, there was no detectable difference in the behavior of the double-stranded DNA after treatment with proteinase K, eliminating the possibility that protein was responsible for the duplex nature of the DNA. The ratio of plus to minus strands was equal as demonstrated by the radioactivity associated with separated strands (Fig. 3) and by the observation that virtually all of the DNA was present as duplex DNA on nondenaturing gels. Since all of the parvoviruses characterized to date have nearly identical particles, capsid proteins, and DNA sizes, it was unlikely that LuIII DNA was double stranded within the virion. Therefore, we concluded that LuIII encapsidated equal amounts of plus and minus strands in separate virions, as does the defective parvovirus, AAV (4).

The similarity between LuIII and AAV with respect to strand encapsidation raised the possibility that LuIII DNA might also have identical hairpin sequences at the 5′ and 3′ termini. To investigate this possibility, separated and reannealed LuIII DNA single strands were examined by electron microscopy for single-stranded circle formation and compared with parallel preparations of AAV DNA (Fig. 2). The absence of circles demonstrated that the 5′ and 3′ terminal sequences for each LuIII DNA strand lacked sequence homology and, thus, that LuIII DNA was like that of other autonomous parvoviruses. Further supporting the lack of sequence homology was the inability of LuIII duplex DNA to form double-stranded circles after exonuclease III treatment and reannealing. Although LuIII plus and minus DNA strands were of the same size, as demonstrated on denaturing gels (25), it was apparent from strand separation gels (Fig. 3) and from analysis with *Eco*RI (Fig. 4) that a terminal heterogeneity existed at the 5′ end of the plus strand. This apparent structural heterogeneity accounting for differences in mobility on nondenaturing gels might be due to a flip-flop sequence or to folding of the hairpin region in different ways.

LuIII virus is serologically distinct from any other known parvovirus, and it is well documented that this virus possesses physicochemical and biological properties consistent with its classification as an autonomous parvovirus (23). The production in newborn human kidney cells of LuIII virions containing equal amounts of plus and minus strands in separate virions is in contrast to another apparently closely related (3) autonomous parvovirus, H-1, which can also be propagated in newborn human kidney cells, but it predominantly encapsidates minus DNA strands. This suggests that strand selection for encapsidation may be a virus-coded rather than host-controlled event. Recently, however, others have observed increased amounts of complementary strand encapsidated in H-1 virions produced in newborn human kidney cells (S. L. Rhode, and M. Goulian, personal communications). Encapsidation of complementary strands is apparently not unique to LuIII, as we observed variable amounts of RF-like double-stranded DNA in DNA purified from bovine parvovirus. The viral or cellular factors, or both, regulating the strand selection and encapsidation steps of parvovirus replication remain to be elucidated.

(While this manuscript was in preparation, D. E. Muller and G. Siegl, J. Gen. Virol. **64**:1043–1054, 1983, also reported the encapsidation of LuIII plus and minus DNA strands.)

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