

# Linear Plasmids of *Borrelia burgdorferi* Have a Telomeric Structure and Sequence Similar to Those of a Eukaryotic Virus

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Spirochetes of the genus *Borrelia* have double-stranded linear plasmids with covalently closed ends. The physical nature of the terminal connections was determined for the 16-kb linear plasmid of the B31 strain of the Lyme disease agent *Borrelia burgdorferi*. Native telomeric fragments representing the left and right ends of this plasmid were isolated and subjected to Maxam-Gilbert sequence analysis. At the plasmid ends the two DNA strands formed an uninterrupted, perfectly palindromic, AT-rich sequence. This *Borrelia* linear plasmid consisted of a continuous polynucleotide chain that is fully base paired except for short single-stranded hairpin loops at each end. The left and right telomeres of the 16-kb plasmid were identical for 16 of the first 19 nucleotide positions and constituted an inverted terminal repeat with respect to each other. The left telomere of the 49-kb plasmid of strain B31 was identical to the corresponding telomere of the 16-kb plasmid. Different-sized plasmids of other strains of *B. burgdorferi* also contained sequences homologous to the left end of the 16-kb plasmid. When the borrelia telomeres were compared with telomeric sequences of other linear double-stranded DNA replicons, sequence similarities were noted with poxviruses and particularly with the iridovirus agent of African swine fever. The latter virus and a *Borrelia* sp. share the same tick vector. These findings suggest that the novel linear plasmids of *Borrelia* originated through a horizontal genetic transfer across kingdoms.

Bacteria of the genus *Borrelia* are host-associated spirochetes that shuttle between arthropods and vertebrates. The genus includes *Borrelia burgdorferi*, the agent of Lyme disease, as well as *B. hermsii* and other species that cause relapsing fever (3). These organisms, perhaps uniquely among prokaryotes, possess linear double-stranded DNA plasmids with covalently closed ends. These unusual plasmids have been found in all species examined and range in size from 15 to 200 kb (2, 13, 29). The genes for major surface proteins of these organisms, namely, *ospA* and *ospB* of *B. burgdorferi* and the *vmp* gene family of *B. hermsii* involved in antigenic variation, are located on linear plasmids (2, 29). *Borreliae* also possess plasmids with the circular supercoiled form typical of other bacteria (2). These observations, and the finding that the *Borrelia* chromosome migrates in pulsed-field electrophoresis gels as a linear molecule of 950 kb (12), raise the possibility that the linear plasmids are actually minichromosomes.

The telomeres of linear replicons have special features that stabilize the ends and allow for their complete replication. The latter is a long-recognized problem for linear DNA molecules (34). Previous studies of the linear plasmids of *B. burgdorferi* indicated that the two strands of the duplex DNA are covalently connected at each end (2). These linear plasmids rapidly reannealed upon removal of denaturing conditions, and electron microscopy of samples of a denatured 49-kb plasmid revealed single-stranded DNA circles of approximately 100 kb. Moreover, the connections between the two DNA strands were susceptible to single-strand-specific endonucleases but not to protease treatment.

Telomeric DNAs from the 49- and 16-kb linear plasmids of *B. burgdorferi* B31 and from two 28-kb linear plasmids of *B.*

*hermsii* have been cloned and sequenced (18, 21). The cloning procedure, however, required the removal of the terminal cross-links. In the present study, we determined the complete sequence of uncloned, native telomeres of the *B. burgdorferi* plasmids. The results demonstrate that hairpin loops, formed by perfectly palindromic AT-rich sequences, link the two strands at the ends of *Borrelia* linear plasmids. Additional characteristics of the telomeres were identified by analysis of isolated plasmid end fragments. We also compared the *Borrelia* linear plasmid telomeres with those of structurally similar eukaryotic viruses.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** Telomeric DNA was isolated and sequenced from the 16-kb (lpB31.16) and 49-kb (lpB31.49) linear plasmids of *B. burgdorferi* B31 (ATCC 35210). Other strains of *B. burgdorferi* used are listed in Table 1; the sources of these strains are given in references 4 and 5. Strain IP90 was provided by E. Korenberg and V. Kryuchnikov, and strain G1 was provided by B. Wilske. *B. hermsii* HS1 (ATCC 35209) serotype 14 was also used. Plasmid pTL16 contains 570 bp and pTL49 contains 600 bp of the left ends of the 16- and 49-kb linear plasmids, respectively (18). Plasmid pTR16 contains 1.7 kb of the right end of the 16-kb linear plasmid (18).

**DNA preparation and techniques.** DNA was prepared from cells grown in BSK II medium (3). Total DNA of *B. burgdorferi* B31 was extracted as described previously (4). A plasmid-enriched DNA fraction of the *B. burgdorferi* and *B. hermsii* strains was obtained by the method of Barbour (1). Isolated linear plasmids were excised and purified from 0.2% agarose gels of fractionated total DNA, using a UEA electroelutor (International Biotechnologies, Inc., New Haven, Conn.). Restriction endonucleases, calf intestinal alkaline phosphatase, and T4 polynucleotide kinase were purchased

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TABLE 1. *B. burgdorferi* strains

Strain	Geographic location	Source
B31	New York	<i>Ixodes dammini</i>
HB19	Connecticut	Human blood
297	Connecticut	Human spinal fluid
Veery	Connecticut	Veery bird
6A	California	<i>Ixodes pacificus</i>
ACA II	Sweden	Human skin
G25	Sweden	<i>Ixodes ricinus</i>
G1	Germany	<i>Ixodes ricinus</i>
Munich	Germany	Human spinal fluid
IP90	Eastern Soviet Union	<i>Ixodes persulcatus</i>

from Boehringer-Mannheim Biochemicals, Indianapolis, Ind., and used as recommended by the manufacturer.

**Gel electrophoresis analysis.** Total *B. burgdorferi* DNA was digested with restriction enzymes and fractionated on nondenaturing 4% polyacrylamide gels in TBE buffer (90 mM Tris [pH 8.0], 90 mM borate, 2 mM EDTA). The linear plasmid end fragments were eluted from the gels (27) and were digested with other enzymes to produce shorter end fragments. The resultant fragments were dephosphorylated with calf intestinal alkaline phosphatase, extracted with phenol and chloroform, and precipitated in ethanol. The fragments were then 5'-end-labelled with [ $\gamma$ -<sup>32</sup>P]ATP and T4 polynucleotide kinase. A portion of the end-labelled digests was subjected to electrophoresis in 3% agarose, 8% polyacrylamide, and a denaturing 8% polyacrylamide gel containing 98% (vol/vol) formamide (24). TBE buffer was used for agarose and nondenaturing acrylamide gel electrophoresis, and phosphate buffer (16 mM Na<sub>2</sub>HPO<sub>4</sub>, 4 mM NaH<sub>2</sub>PO<sub>4</sub>) was used for the denaturing gel. Denaturing gel samples contained 30% formamide and were heated at 95°C for 3 min prior to loading. The agarose gel was dried for autoradiography.

**DNA sequencing and analysis.** Approximately 0.5  $\mu$ g of each of the three end-labelled digests was fractionated on a 4% nondenaturing acrylamide gel. Telomeric fragments were recovered from the gels, and their nucleotide sequences were determined by the Maxam-Gilbert method (27). Products of the chemical reactions were separated on a 6% acrylamide sequencing gel containing 50% (wt/vol) urea, using 100 mM Tris (pH 8.0)–100 mM borate–2 mM EDTA as buffer. Telomeric sequences were compared with bacterial and viral nucleic acid sequences in the GenBank data base release 59.0 and EMBL data base release 18.0, using programs of the University of Wisconsin Genetics Computer Group (GCG; Sequence Analysis Software Version 6.0) on a VAX computer (Digital Equipment Corp.).

**Southern blot analysis.** Linear plasmid DNA was separated in a 1.0% agarose gel by field-inversion gel electrophoresis, using the PPI-100 power inverter (MJ Devices, Waltham, Mass.). Electrophoresis was run at 7 V/cm, with a forward time of 0.5 s and an initial reverse time of 0.25 s, which was incremented by 0.1 s at each step of a three-step cycle repeated throughout the 24-h run. Following ethidium bromide staining, the DNA in the gel was nicked by acid depurination prior to transfer to a nylon membrane (25). Probe DNA was radiolabelled with [ $\alpha$ -<sup>32</sup>P]dATP by nick translation, using a commercial kit (Bethesda Research Laboratories, Gaithersburg, Md.). Southern hybridization was performed at 37°C as described previously (4). Prior to autoradiography, final washes of the Southern blots were at 42°C in 0.1 $\times$  SSC (1 $\times$  SSC is 150 mM NaCl plus 15 mM

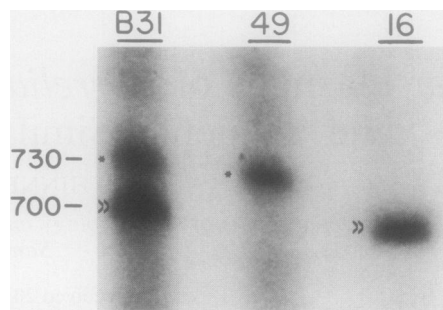


FIG. 1. Left terminal *SalI* fragments of the 16-kb (lpB31.16) and 49-kb (lpB31.49) linear plasmids of *B. burgdorferi* B31. End-labelled *SalI* digests of total *B. burgdorferi* (B31), 49-kb linear plasmid (49), and 16-kb linear plasmid (16) DNAs were fractionated on a 4% acrylamide gel. The portion of the autoradiograph containing the 700-bp (indicated by double arrows) and 730-bp (indicated by asterisks) linear plasmid end fragments is shown.

sodium citrate)–0.1% sodium dodecyl sulfate (SDS)–1 mM EDTA. Probe DNA was stripped from the blot by incubation in 0.2 M NaOH at 42°C for 30 min. The blot was then washed in 0.1 $\times$  SSC–20 mM Tris (pH 7.5)–0.5% SDS at 42°C for 30 min before prehybridization and hybridization with a different probe.

## RESULTS

**Isolation of telomeric fragments.** To determine the physical nature of the terminal connections of *Borrelia* linear plasmids, native telomeric restriction fragments were collected and sequenced directly. Physical mapping of the 16- and 49-kb plasmids revealed a *SalI* site 700 and 730 bp, respectively, from their designated left ends (17, 18). As *Borrelia* DNA contains few *SalI* sites (17), this enzyme was used to generate left telomeric fragments of the two plasmids. Figure 1 shows that *SalI* digestion of *B. burgdorferi* genomic DNA produced the two expected fragments of 700 and 730 bp which were well separated from other fragments on a polyacrylamide gel. Both of these fragments hybridized on Southern blot (not shown) with recombinant plasmids pTL16 and pTL49, which contain sequences from the left ends of the 16- and 49-kb linear plasmids, respectively (18). Sequence analysis of these two clones had revealed that the left ends of the 16- and 49-kb linear plasmids were identical except for a 30-bp segment that was present in pTL49 but absent from pTL16 (18). Figure 1 demonstrates that this 30-bp difference is also seen in the uncloned telomeric fragments: the terminal *SalI* fragment from the 16-kb plasmid is 700 bp, and the terminal *SalI* fragment from the 49-kb plasmid is 730 bp.

The analogous telomeric fragment from the right end of the 16-kb plasmid was produced with *ClaI*. Sequence analysis of pTR16, a clone containing DNA from the right end of the 16-kb linear plasmid (18), predicted that this enzyme would generate a 715-bp fragment containing the right telomere. Gel electrophoresis of *ClaI*-digested *Borrelia* DNA yielded a 715-bp fragment which hybridized on Southern blot with plasmid pTR16 (not shown).

**Demonstration of the hairpin structure of the telomeric fragments.** A map of the 16-kb linear plasmid (lpB31.16), the left telomeric *SalI* fragment, and right terminal *ClaI* fragment is shown in Fig. 2. The left and right telomeric fragments of the 16-kb plasmid and the left telomeric *SalI*

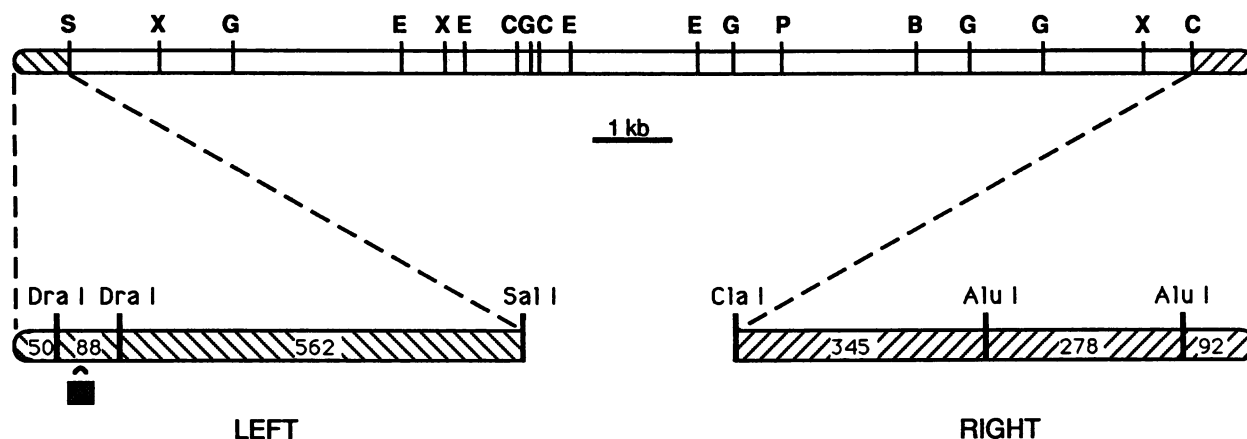


FIG. 2. Partial restriction enzyme map of the 16-kb linear plasmid (lpB31.16) of *B. burgdorferi* B31 (18). The left terminal *Sall* fragment and the right terminal *ClaI* fragment are shown in greater detail below. Numbers refer to sizes of the fragments in base pairs, determined from their nucleotide sequences (18). The shaded box indicates the 30-bp segment that is present in the left end fragment of the 49-kb plasmid but absent from the 16-kb plasmid. The recognition sites for *Bam*HI (B), *Bgl*III (G), *Cla*I (C), *Eco*RI (E), *Sall* (S), and *Xba*I (X) are indicated.

fragment of the 49-kb linear plasmid (lpB31.49) were isolated from preparative gels and digested next with *DraI* or *AluI*, as indicated in Fig. 2. These smaller subfragments were 5'-end-labelled with [ $\gamma$ - $^{32}$ P]ATP and separated on a 3% agarose gel (Fig. 3). To verify that the 50-bp *DraI* fragments and the 92-bp *AluI* fragment that were observed were, in fact, the expected plasmid ends, the digests were also subjected to 8% polyacrylamide gel electrophoresis under nondenaturing and denaturing conditions. Inasmuch as telomeric fragments are effectively cross-linked at one end, we predicted that they would unfold to twice their double-stranded length under denaturing conditions. In this analysis, the 50- and 92-bp left and right telomeric fragments (arrows) migrated at their expected sizes in the nondenaturing gels, but their apparent sizes were doubled in the denaturing gel, as expected.

This analysis also revealed that the subterminal fragments from both ends of the 16-kb plasmid migrated anomalously. The 562-bp left subterminal *DraI-Sall* fragment and the 278-bp *AluI* and 345-bp *AluI-ClaI* right subterminal fragments migrated more slowly in the polyacrylamide gels than in the agarose gel. These fragments migrated at their expected sizes in the agarose gel, but at approximately twice their expected sizes in the nondenaturing acrylamide gel. Analysis of the same DNA in recombinant form and prepared from *Escherichia coli* yielded identical results (not shown), an indication that the unusual migration was due to sequence-directed curvature (26) or other distinctive secondary structure of the DNA, and not to DNA modification that occurs in borrelia cells.

**Nucleotide sequence of the telomeres.** For direct sequencing of the telomeres, the 5'-end-labelled 50-bp left telomeric *DraI* fragments of the 16- and 49-kb plasmids, as well as the 92-bp right telomeric *AluI* fragment of the 16-kb plasmid, were isolated from polyacrylamide gels. As these were hairpin fragments, they were labelled at only one end and were sequenced by the Maxam-Gilbert method without further treatment. The perfectly palindromic sequences read on the denaturing gel down the labelled 5' strand around the hairpin loop region and back along the 3' strand. The results for the left and right telomeres of the 16-kb plasmid are presented in Fig. 4. The sequence of the left telomere of the 49-kb plasmid was identical to that of the left telomere of the

16-kb plasmid (not shown). A predicted secondary structure of the telomeres, with a 4-nucleotide single-stranded hairpin loop, is shown in Fig. 5. The sequences are very AT rich, and 16 of the first 19 bp at each end of the 16-kb plasmid were

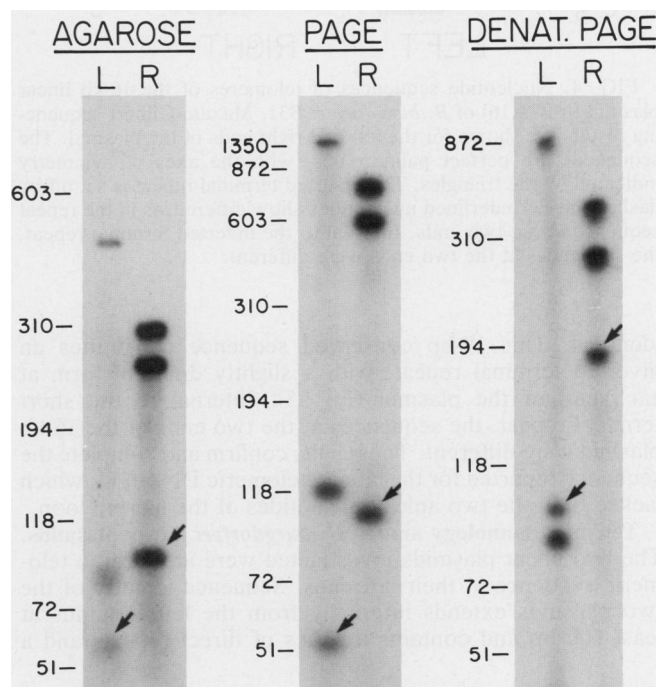


FIG. 3. Terminal cross-links of telomeric fragments of the 16-kb linear plasmid (lpB31.16) of *B. burgdorferi* B31: agarose and polyacrylamide gel electrophoresis (PAGE) of end-labelled *DraI* digest of the left terminal fragment and *AluI* digest of the right terminal fragment under nondenaturing and denaturing conditions. Arrows indicate the 50-bp left telomeric *DraI* fragment and the 92-bp right telomeric *AluI* fragment, whose apparent sizes double on the denaturing gel. The other (subterminal) fragments migrated more slowly in the PAGE gels than predicted from their sizes. The sizes in base pairs of *Hae*III-digested  $\phi$ X170 DNA and the 51-bp *KpnI-DraI* fragment of plasmid pTL16 (18), which were used as molecular weight standards, are indicated.



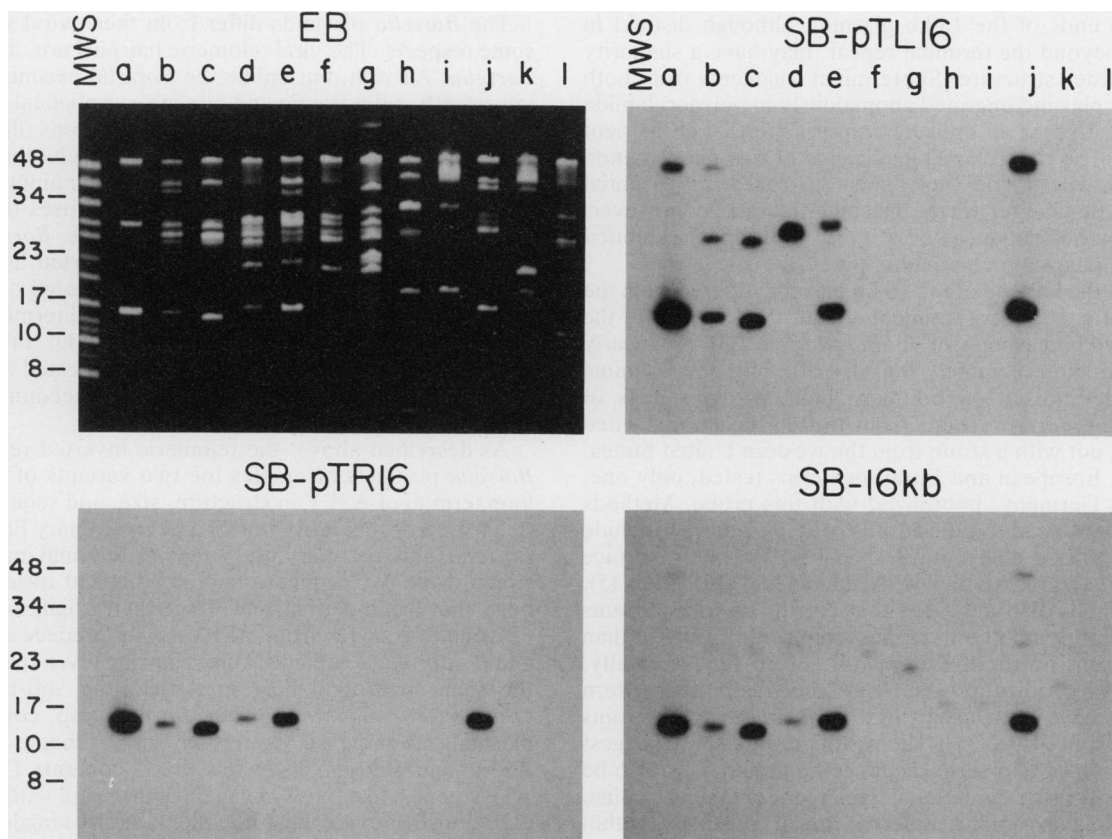


FIG. 6. Homology of telomeric sequences of the 16-kb linear plasmid (lpB31.16) of *B. burgdorferi* B31 with linear plasmids from other strains. EB, ethidium bromide-stained gel of linear plasmid DNA from different strains of *B. burgdorferi* separated by field-inversion gel electrophoresis. The Southern blot of the gel was sequentially hybridized with radiolabelled pTL16(SB-pTL16), pTR16(SB-pTR16), and 16-kb linear plasmid DNA (SB-16kb). pTL16 and pTR16 contain the left and right terminal *Hind*III fragments, respectively, of the 16-kb linear plasmid (18). Lanes a to f contain linear plasmids from the following North American strains of *B. burgdorferi*: a, B31 (high passage); b, B31 (low passage); c, HB19; d, 297; e, Veery; f, 6A. Lanes g to k contain linear plasmids from the following European strains of *B. burgdorferi*: g, ACA II; h, G25; i, G1; j, Munich; k, IP90. Lane l, linear plasmids of *B. hermsii*. MWS, 8.3- to 48.5-kb DNA size standards (Bethesda Research Laboratories).

of symmetry of the palindromes, i.e., the nucleotides forming the hairpin loops, are in the middle. The 37-nucleotide ASFV sequence is 67% identical to the 38-nucleotide terminal repeat sequence of *Borrelia* linear plasmids. The similarity includes an 8-nucleotide region of identity (5'-TATA ATTT-3') adjacent to the hairpin loop termini. A 19-bp palindromic sequence beginning 6 bp from the vaccinia virus terminus is also similar to the hairpin loop sequence of

*Borrelia* telomeres (Fig. 7). A corresponding similarity was not noted, however, between the *Borrelia* terminal repeat and Shope fibroma virus, another poxvirus whose hairpin loop termini have been sequenced (33). No similarities were found between the *Borrelia* telomeric sequences and other viral or prokaryotic sequences in the GenBank data base.

## DISCUSSION

We describe in this study several features of the natural ends, or telomeres, of *Borrelia* linear plasmids. The structure of the telomeres was determined by direct chemical sequence analysis. The nucleotide sequences of the plasmid ends were perfectly palindromic "hairpins." Thus, the terminal cross-links between the two strands of a *Borrelia* linear plasmid are in the form of short single-stranded hairpin loops of a continuous polynucleotide chain. The 16-kb linear plasmid contained an AT-rich 19-bp inverted terminal repetition, with a slightly different form at each end. The end of the 49-kb plasmid that was examined was identical to one end of the 16-kb plasmid. A nearly identical sequence is found at the ends of *B. hermsii* plasmids (21). The hairpin loop structure and short inverted terminal repeat sequence thus appear to be conserved features of *Borrelia* linear plasmid telomeres.

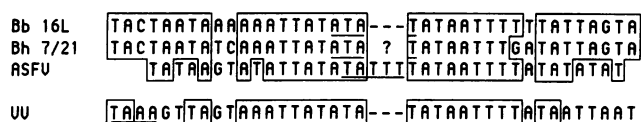


FIG. 7. Comparison of telomeric sequences of *Borrelia* spp. linear plasmids, ASFV, and vaccinia virus (VV). The left terminal repeat of the *B. burgdorferi* 16-kb linear plasmid (Bb 16L), the terminal sequence of two linear plasmids of *B. hermsii* (Bh 7/21) (21), and one conformation of the hairpin loop of ASFV (16) are written 5' to 3' as single-stranded palindromes. One strand of the vaccinia virus telomere (6) is also shown, written 5' to 3' from the apical nucleotide of the hairpin loop. Nucleotides which form the terminal hairpin loops are underlined. Dashes indicate a gap introduced to facilitate alignment between the sequences. The hairpin loops of the *B. hermsii* plasmids have not been sequenced directly.

The two ends of the 16-kb plasmid, although distinct in sequence beyond the terminal repeat, may have a similarity in higher order structure. Subterminal fragments from both ends of the plasmid migrated anomalously in polyacrylamide gels, suggestive of an unusual conformation, such as bent DNA (26). The nature and significance of this phenomenon are not known. It does not appear to reflect an essential structural element of linear plasmid telomeres, however, since telomeric sequences of *B. hermsii* plasmids exhibited normal gel migration characteristics (17).

Whereas the left end of the 16-kb plasmid differed from the right end after the short terminal repeat, the left ends of the 16- and 49-kb plasmids of *B. burgdorferi* B31 are nearly identical for approximately 0.6 kb (18). This left terminal sequence hybridized on Southern blots with plasmids of other *B. burgdorferi* strains from the northeastern United States, but not with a strain from the western United States. Of the five European and Eurasian strains tested, only one, from West Germany, hybridized with this probe. Methods that have been used to type strains of *B. burgdorferi* include electrophoretic profiles of plasmids (1) or outer surface proteins (4) and reactivities with monoclonal antibodies (5). These methods also indicated that North American strains are more closely related to strains from central Europe than to those from northern Europe (4). More fundamentally, DNA-DNA hybridization and rRNA gene restriction pattern differences have been used to group *B. burgdorferi* strains into two distinct taxa (31). The results of this study suggest that detection of telomeric sequence homology may also be useful in indicating evolutionary relatedness among strains.

A relationship among different linear plasmids within individual strains was also evident from this analysis. The left terminal sequence was present on as many as three different linear plasmids of a single strain. This may be the result of telomeric translocations or rearrangements among the different linear plasmids within a cell. In *B. hermsii*, a relapsing fever species, frequent telomeric translocations occur between linear plasmids (21, 29).

*Borrelia* linear plasmids are unusual bacterial replicons. Among prokaryotes, only an uncharacterized *E. coli* prophage has been reported to have ends with a similar structure (32). Other linear replicons in bacteria, including linear plasmids of *Streptomyces rochei* (19) and certain bacteriophages (11), have a protein covalently attached to each 5' end; their ends are not cross-linked. Some phages of *E. coli*, such as T7, exist in linear form but have non-cross-linked ends without terminal proteins (34). The *Borrelia* plasmids can also be compared with replicons of higher organisms. Eukaryotic replicons with hairpin termini include *Paramecium* mitochondrial DNA (15), linear plasmids of yeasts (20), and plastids of barley chloroplasts (9). In these replicons, however, only one of the two ends is covalently closed. Parvoviruses contain single-stranded DNA with terminal T-shaped hairpins at each end (23).

The genomes of poxviruses, including vaccinia virus, and the iridovirus that causes African swine fever are architecturally most like the *Borrelia* plasmids (6, 16). Comparison of telomeric sequences revealed that the strongest resemblance was between the *Borrelia* linear plasmids and ASFV. Although the identity between these AT-rich sequences might be attributable to chance, their telomeric location suggests a functional equivalence in the two replicons. The short terminal repeat is the only conserved sequence element of different linear plasmid telomeres of two species of *Borrelia*. This is another indication that this sequence is essential for telomeric function in that genus.

The *Borrelia* plasmids differ from these viral genomes in some respects. The viral telomeric hairpins are, like those of *Borrelia*, AT rich, but unlike the *Borrelia* plasmids they are imperfectly palindromic and contain extrahelical bases. The viral telomeres can exist in two conformations (flip and flop) that are inverted and complementary to each other. Each of these alternate forms is present in equimolar amounts at both ends of the genome in a population of viruses (6, 16). The two forms of the terminal repeat of the *Borrelia* 16-kb plasmid, on the other hand, are not inverted and complementary to each other and are specific to the left or right end. These viruses contain much longer inverted terminal repeats than *Borrelia* plasmids. Approximately 10 kb, or about 6%, of the 180-kb vaccinia virus genome is repeated at each end (14), whereas the 19-bp terminal repeat accounts for only 0.1% of the 16-kb *Borrelia* plasmid.

As described above, the telomeric inverted repeat of the *Borrelia* plasmids resembles the two variants of the hairpin loop termini of ASFV in structure, size, and sequence. Does this telomeric similarity reflect an evolutionary link between *Borrelia* and a eukaryotic virus? Additional parallels between these two replicons support this and raise the possibility that the linear plasmids trace their origin to a horizontal genetic transfer from ASFV or its predecessor. ASFV and *B. duttoni*, a noncultivable relapsing fever species, share the same arthropod host in Africa, the soft-bodied tick *Ornithodoros moubata* (3, 30). *Borrelia* spp. contain linear plasmids as large as the 170-kb viral genome (13). Both ASFV and *Borrelia* have low G+C contents (3, 10), and ASFV genes, like those of vaccinia virus, are without introns (22). Further evidence for either a trans-kingdom genetic transfer or common ancestry would come from detecting other similarities, especially in the replication systems. Insight into the manner in which *Borrelia* linear plasmids replicate awaits the analysis of replicative forms of the plasmids, which have not been detected to date. An evolutionary connection among a group of phylogenetically diverse linear bacteriophages and eukaryotic viruses has also been discerned by comparison of their DNA polymerases (7).

The same telomeres could also have arisen independently in *Borrelia* spp. and ASFV, in an example of analogous structures and convergent evolution. In any case, this terminal configuration may contribute to genetic rearrangements with important biological consequences for these replicons and their hosts. The ends of poxviruses are known to be highly recombinogenic, a characteristic which may play a role in determining host range (28). ASFV has telomere-associated multigene families which likely arose by recombination and which vary among strains (8). Recombination among members of a family of surface protein genes located near the telomeres of linear plasmids also underlies antigenic variation in the relapsing fever agent *B. hermsii* (21, 29).

#### ACKNOWLEDGMENTS

We thank T. Kitten and S. Hardies for discussions and D. LeBlanc and S. Deb for comments on the manuscript.

This research was supported by Public Health Service grants AI 29731 and AI 24424 from the National Institutes of Health.

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