Linear Plasmids of the Bacterium *Borrelia burgdorferi* Have Covalently Closed Ends

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The genetics of spirochetes, a division of eubacteria, has been little studied. Double-stranded linear plasmids were found in *Borrelia burgdorferi*, the agent of Lyme disease. A 49-kilobase linear plasmid contained the *ospA* and *ospB* genes, which encode the major outer membrane proteins of strain B31. Molecules of the 49-kilobase plasmid rapidly reannealed after alkaline denaturation; rapid renaturation was prevented if the 49-kilobase plasmids were first treated with S1 nuclease. When denatured plasmid molecules were examined directly, single-stranded circles of approximately 100-kilobase circumference were seen. These studies provide direct visual evidence that the linear plasmids have covalently closed ends. This form of DNA occurs in some animal viruses, but it has not heretofore been described in prokaryotic organisms.

SPIROCHETES CONSTITUTE A DISTINCT and ancient division of eubacteria (*1*). In comparison to several other groups of bacteria, spirochetes have not been well studied at the level of molecular biology; their genetics remain uncharted (*2*). In the course of an investigation of *Borrelia hermsii*, a spirochete that is an agent of relapsing fever, we found that this species' DNA organization was novel: the gene for an outer membrane protein was located on an extrachromosomal element that was linear (*3*). *Borrelia burgdorferi*, the cause of Lyme disease (*4*), has two major outer membrane proteins, OspA and OspB (*5*), the genes for which are cotranscribed (*6*). In the present study we aimed to ascertain whether the *osp* genes of *B. burgdorferi* were located on a plasmid, and, if so, to determine the structure of the plasmid.

Borrelia burgdorferi B31 (ATCC 35210) was grown in BSK II medium (*7*). A DNA extraction procedure was used to partition a cell lysate into plasmid-rich and chromosome-rich fractions. The plasmid fraction was then centrifuged in an ethidium bromide and cesium chloride density gradient (*8*). Two bands were observed in the centrifuge tubes: one, of higher density, contained supercoiled DNA as seen by electron microscopy (*9, 10*); the other, of lower density, consisted of linear duplexes. Chromosomal DNA was separated from a small amount of plasmid material contaminating the chromosome-rich fraction by electroeluting, from agarose gels, DNA with an apparent size greater than 60 kb (*11*).

The three resultant DNA preparations were designated "chromosome," "supercoiled plasmid," and "linear plasmid." Samples of the three fractions were examined by electrophoresis in a low concentration of agarose (Fig. 1A) and by direct gel hybridization (Fig. 1B). The hybridization probe was a 0.4-kb fragment of *ospB*, the gene that encodes OspB (*5, 6*). A 49-kb band in the linear plasmid lane of the gel hybridized

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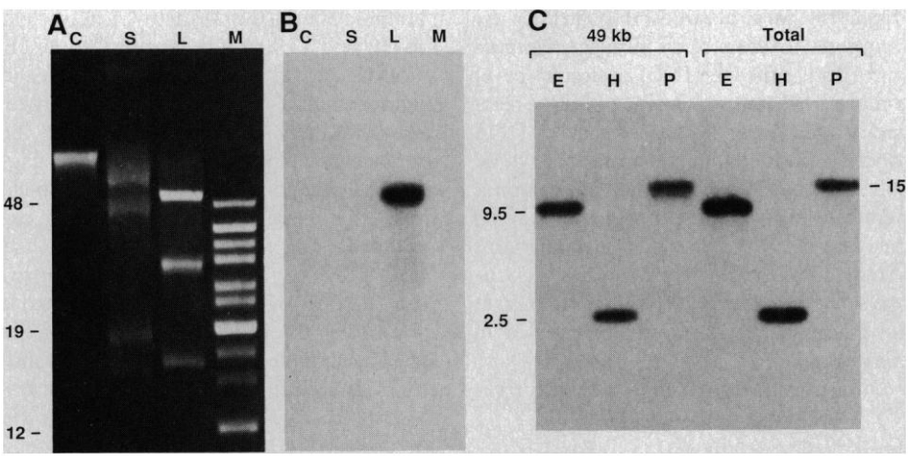

Fig. 1. The *ospB* gene of *B. burgdorferi* is located on a 49-kb linear plasmid. (**A** and **B**) Direct gel hybridization: The three forms of extracted DNA, chromosome (lanes C), supercoiled plasmid (lanes S), and linear plasmid (lanes L), were applied to a 0.2% agarose gel (Seakem GTG), which was run with recirculated 10 m*M* sodium phosphate buffer (*p*H 6.5) at 1.4 V/cm. Molecular size standards (Bethesda Research Laboratories) were in the M lanes; the numbers at the left give the sizes (in kilobases) of selected standards. The DNA in the gel was stained with ethidium bromide (A) and then denatured with a mixture of 0.5*N* NaOH and 0.15*M* NaCl for 30 minutes. After neutralization (1*M* tris, *p*H 7.6, for 30 minutes), the gel was dried under vacuum at 65°C onto a 1.2-μm nylon membrane. After prehybridization, the DNA in the dried mounted gel was directly hybridized with radiolabeled probe (B). In this experiment the probe was a 0.7-kb Hind III–Hind III fragment of recombinant plasmid pTRH45; the fragment contained 0.4 kb of the *ospB* gene (*6*). Conditions for hybridization were 50% formamide, 6× SSC (standard saline citrate), 5× Denhardt's, 0.5% SDS, 0.1 mg/ml of denatured herring sperm DNA, and 0.1% sodium pyrophosphate; incubation was at 37°C for 18 hours. After hybridization the blots were washed in a Turbo-Blot apparatus (American BioNuclear) with 0.1× SSC containing 0.1% SDS and 1 m*M* EDTA at 65°C. (**C**) Southern blot analysis: The 49-kb plasmid (49 kb) was isolated by electroelution and along with total linear plasmid DNA (Total) digested with Eco RI (lanes E), Hind III (lanes H), or Pst I (lanes P). The restriction fragments were separated in a 1.0% agarose gel, transferred to a nylon membrane, and hybridized with the *ospB* probe as described above.

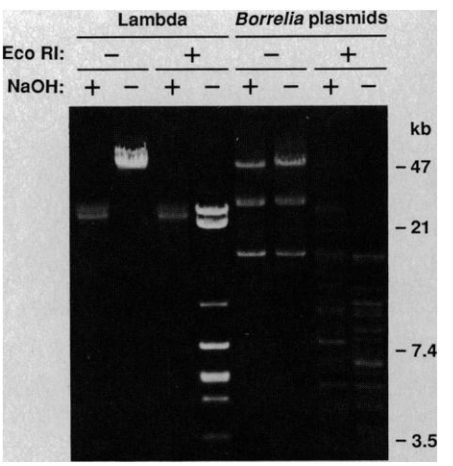

Fig. 2. *Borrelia* linear plasmids rapidly reanneal after denaturation. To either bacteriophage λ DNA (Sigma) or total *B. burgdorferi* linear plasmids was added one-tenth volume of 1*N* NaOH (+). Ten minutes later one-tenth volumes of 2*M* tris-HCl and then 0.2*M* $MgCl_2$ were added. For control tubes (−), the tris-HCl solution was added before the NaOH incubation and addition of $MgCl_2$. Some samples (+) were digested with Eco RI for 1 hour at 37°C; control tubes (−) were incubated under similar conditions but without added restriction enzyme. The DNA samples so treated were examined in 0.2% agarose gels run with tris (90 m*M*), borate (90 m*M*), and EDTA (2 m*M*) buffer at 1.4 V/cm. The electrophoretic migrations of duplex λ DNA and of selected Eco RI restriction fragments of lambda (in kilobase pairs) are shown on the right.

under high stringency conditions to the probe. There was no detectable hybridization of the probe to the other, smaller linear plasmids, to a supercoiled plasmid, or to the chromosome fraction with autoradiograph exposures 20 times as long as those of the originals. A probe specific for *ospA*, a gene cotranscribed with *ospB* (*6*), also hybridized to the 49-kb plasmid under the same conditions. Furthermore, the presence of *ospA* sequences was confirmed directly by heteroduplex analysis in which a 1.3-kb region of homology could be identified near the middle of the 49-kb molecule.

The 49-kb plasmid was electroeluted from the gel (*11*), and contour-length measurements of the linear duplex molecules were made (*9, 10*); the mean size of 50 molecules was 48.8 ± 1.6 kb (SD). Southern blot analysis of restriction digests of isolated 49-kb plasmid and the total linear plasmid fraction (lane L in Fig. 1A) showed that the 49-kb plasmid itself accounted for the *ospB* probe hybridization pattern seen with the mixture of linear plasmids (Fig. 1C). The electrophoretic migrations of neither the 49-kb plasmid itself nor constituent restriction fragments were altered by prior treatment with proteinase K (*12*).

Electron microscopy studies revealed that the linear plasmids of *B. burgdorferi* rapidly reannealed after formamide-mediated denaturation. Other experiments had shown that linear plasmids of *Borrelia* were not susceptible to exonuclease III or to terminal digestion with bacteriophage λ exonuclease (*13*) but were shortened by Bal 31 (*3*). This last enzyme progressively shortens linear chromosomal molecules from their ends (*14*). These studies suggested that the linear plasmids might have covalently closed ends. To better define the terminal structure of these plasmids we carried out additional denaturation experiments.

In one study we compared the electrophoretic migrations of the linear plasmid and bacteriophage λ DNA before and after alkali denaturation (Fig. 2). The DNA of phage λ has free termini, and it separated into single strands upon exposure to alkali, as expected. In this denatured state λ DNA was not susceptible to the actions of restriction endonucleases such as Eco RI. The *B. burgdorferi* linear plasmids, in contrast, were seemingly unaffected by similar treatment with alkali: either the DNA molecules never completely denatured or they quickly reannealed after neutralization. At low DNA concentrations, rapid renaturation would only be possible if the separated strands were held in apposition at some point along their lengths. The differences in the patterns of restriction enzyme fragments between the two samples of *Borrelia* DNA indicated that the plasmids may not have reannealed in every location.

During exposure to high alkalinity (*p*H 12), λ DNA denatured (Fig. 3). When aqueous mounts of the treated DNA were made and examined by electron microscopy, the single strands collapsed into "bushes" as a consequence of intrastrand bonding. Purified 49-kb plasmids were only found as linear duplexes whether or not they had been treated with alkali. However, when samples of the 49-kb plasmid were first treated with concentrations of S1 nuclease that cleave only single-stranded regions of DNA (*15*), bushes characteristic of denatured DNA were observed.

These findings are consistent with a linear duplex structure containing a single-stranded loop at each end. The ability of both Bal 31 exonuclease and S1 nuclease to "unseal" the ends were indications that the cross-linking was not mediated by a protein. Moreover, treatment of the 49-kb plasmid with first proteinase K and then phenol (*12*) prior to alkaline denaturation did not increase the number of single-stranded molecules seen in electron microscopic examinations.

Inherent in a structural model specifying covalently closed termini is a prediction that single-stranded circles having a circumference twice the length of the linear duplex will be found, if viewed in a completely denatured state. Complete denaturation was achieved by dialyzing a solution of the 49-kb plasmid against 10 m*M* methylmercuric hydroxide and 1*M* glyoxal, heating the sample to 60°C, and then mounting the DNA in high formamide concentrations; this procedure had been used to demonstrate the terminal cross-links of a poxvirus (*16*). Figure 4 shows one of the single-stranded circles that were produced; the mean circumference of ten measured circles was 100.1 ± 3.6 kb (SD). The predominant circular plasmid of *B. burgdorferi* B31 had a contour length of 28.0 ± 1.9 kb (20 circles measured). Thus, the 100-kb single-stranded circles seen in the preparation were un-

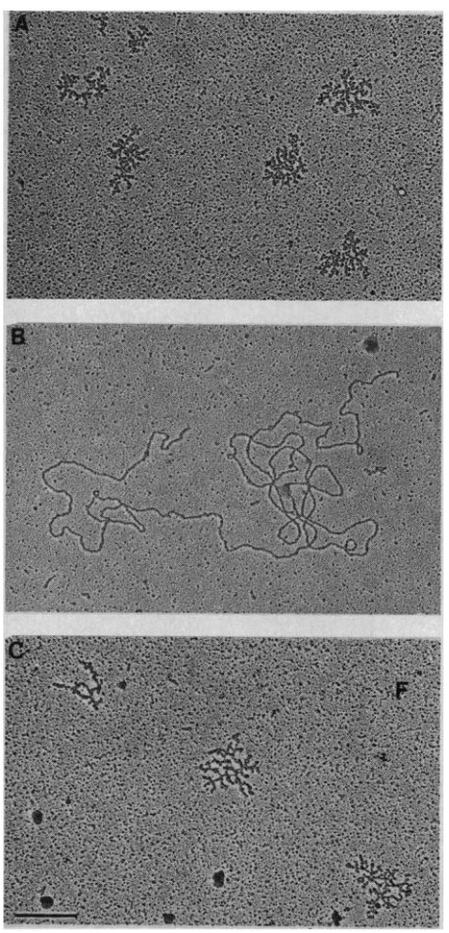

Fig. 3. Prior S1 nuclease treatment prevents rapid renaturation of the 49-kb plasmid DNA after alkaline denaturation. Two volumes of 0.2*N* NaOH were added to either λ DNA (**A**) or electroeluted 49-kb plasmid DNA (**B**) at 10 μg/ml of TE. After a 10-minute incubation, one-fifth volume of 2*M* tris HCl was added, and aqueous grids were immediately prepared (*11*). The 49-kb plasmid DNA was also treated with S1 nuclease (*15*) before denaturation (**C**). Scale bar, 1.0 μm.

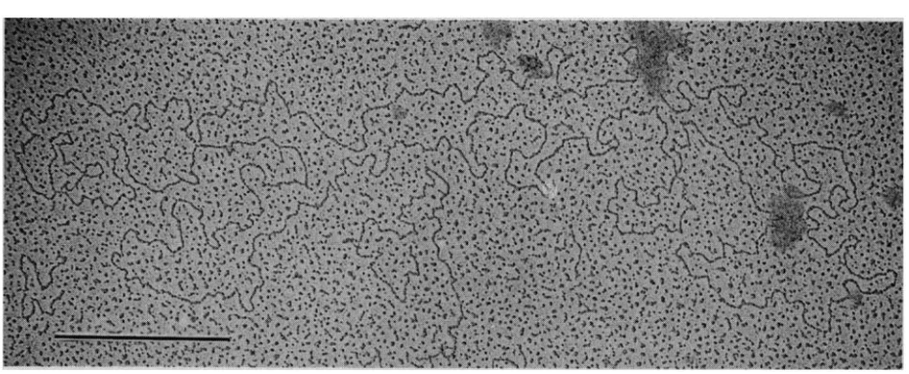

Fig. 4. The 49-kb plasmid forms a single-stranded circle when completely denatured. Electroeluted plasmid in TE was dialyzed against 10 m*M* methylmercuric hydroxide (Alfa), 1*M* deionized glyoxal (Aldrich), and 50 m*M* sodium phosphate, *p*H 6.5, for 3 hours (*16*). The DNA was heated briefly to 60°C and then mounted in 80% formamide for electron microscopy. Scale bar, 1.0 μm.

likely to have been derived from circular plasmids contaminating the preparation.

From these experiments we conclude that the *ospA* and *ospB* genes of *B. burgdorferi* are arrayed on linear plasmids and that the linear plasmids have covalently closed termini. The first finding, while not unexpected [genes encoding outer membrane proteins of *B. hermsii* had been found on plasmids of that species (*3*)], further demonstrates the novel arrangement of DNA in this group of bacteria. The second finding is perhaps of greater significance: only among eukaryotic organisms and their viruses have covalently closed ends of DNA been found. Vaccinia virus has been most extensively studied (*17*). Bacteriophages can have linear DNA, too, but known double-stranded forms with free ends, as found in phage λ, or protein-bound 5′ ends, as found in Phi29, are separable along their entire lengths (*18*). It is conceivable, though, that linear plasmids of *Borrelia* species do derive from bacteriophages since viruses have been observed in both *B. burgdorferi* and *B. hermsii* (*2, 19*).

Recognizing the similarity between pox viral termini and the ends of chromosomes, some investigators have referred to the hairpins of poxviruses as telomeres (*20*). This term cannot be applied to ends of the linear molecules found in *Borrelia* species; palindromic sequences conducive to hairpin formation would have to be demonstrated. Nevertheless, the present study reveals in a bacterium a form of DNA that was thought to be unique to eukaryotes.

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9. The DNA was mounted for electron microscopy by means of the Kleinschmidt aqueous technique (*10*). Adenovirus 2 DNA (Bethesda Research Laboratories) was the standard used to calibrate contour-length measurements. Grids were examined in a JEOL 100B electron microscope at 40-kV accelerating voltage. Electron micrographs were taken on Kodak Electron Image plates at a magnification of ×7000. The magnification was calibrated for each set of plates with a grating replica (E. F. Fullam), and contour lengths were measured with a Numonics Graphics calculator interfaced with a Tektronik 4052A computer. The mean plus standard deviation of the measured lengths are given.
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11. DNA molecules of greater than 40-kb apparent size were recovered from agarose gels by electroelution into a salt sink of 3*M* sodium acetate in an IBI model UEA electroeluter. The buffer was 20 m*M* tris (*p*H 8.0), 5 m*M* NaCl, and 0.2 m*M* EDTA, and the elution was carried out for 2 hours at 100 V. DNA to be used in electron microscopy and denaturation studies was not stained with ethidium bromide prior to electroelution. Pipette tips with large bores at their ends were used in all DNA manipulations.
12. Electroeluted 49-kb plasmid DNA at 0.1 mg/ml was incubated with proteinase K (Boehringer Mannheim) at 0.5 mg/ml in TE containing 1% SDS for 30 minutes at 65°C. The mixtures were extracted with a mixture of phenol and chloroform before being precipitated with ethanol. Control samples were treated in the same way except for the absence of protease.
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Neuronal pp60$^{c\text{-}src}$ Contains a Six–Amino Acid Insertion Relative to Its Non-Neuronal Counterpart

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Neuronal cells express a pp60$^{c\text{-}src}$ variant that displays an altered electrophoretic mobility and a different V8 peptide pattern relative to pp60$^{c\text{-}src}$ expressed in tissues of non-neuronal origin. To determine whether the neuronal form of pp60$^{c\text{-}src}$ is encoded by a brain-specific messenger RNA, a mouse brain complementary DNA (cDNA) library was screened with a chicken c-*src* probe and a 3.8-kilobase c-*src* cDNA clone was isolated. This clone encodes a 60-kilodalton protein that differs from chicken or human pp60$^{c\text{-}src}$ primarily in having six extra amino acids (Arg-Lys-Val-Asp-Val-Arg) within the NH_2-terminal 16 kilodaltons of the molecule. S1 nuclease protection analysis confirmed that brain c-*src* RNA contains an 18-nucleotide insertion at the position of the extra six amino acids. This insertion occurs at a position that corresponds to a splice junction in the chicken and human c-*src* genes. The isolated c-*src* cDNA clone encodes a protein that displays an identical V8 peptide pattern to that observed in pp60$^{c\text{-}src}$ isolated from tissues of neuronal origin.

THE PROTO-ONCOGENE C-*src* ENcodes a 60-kD tyrosine-specific protein kinase (pp60$^{c\text{-}src}$). Recently, Brugge *et al.* (*1, 2*) showed that neuronal cells express an altered form of pp60$^{c\text{-}src}$ which exhibits a slightly slower electrophoretic migration on sodium dodecyl sulfate (SDS)–polyacrylamide gels relative to pp60$^{c\text{-}src}$ isolated from non-neuronal tissues. They mapped this alteration in a 16-kD NH_2-terminal peptide obtained after V8 protease digestion and postulated that this structural alteration resides in the primary amino acid sequence of pp60$^{c\text{-}src}$ (*2*). To examine whether the neuronal form of pp60$^{c\text{-}src}$ [pp60$^{c\text{-}src(+)}$] is encoded by a brain-specific messenger RNA (mRNA), a size-selected

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