

## Genetic Variation of the *Borrelia burgdorferi* Gene *vlsE* Involves Cassette-Specific, Segmental Gene Conversion

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**The Lyme disease spirochete *Borrelia burgdorferi* possesses 15 silent *vls* cassettes and a *vls* expression site (*vlsE*) encoding a surface-exposed lipoprotein. Segments of the silent *vls* cassettes have been shown to recombine with the *vlsE* cassette region in the mammalian host, resulting in combinatorial antigenic variation. Despite promiscuous recombination within the *vlsE* cassette region, the 5' and 3' coding sequences of *vlsE* that flank the cassette region are not subject to sequence variation during these recombination events. The segments of the silent *vls* cassettes recombine in the *vlsE* cassette region through a unidirectional process such that the sequence and organization of the silent *vls* loci are not affected. As a result of recombination, the previously expressed segments are replaced by incoming segments and apparently degraded. These results provide evidence for a gene conversion mechanism in *VlsE* antigenic variation.**

*Borrelia burgdorferi*, the agent of Lyme disease, possesses an elaborate genetic system, designated *vmp*-like sequence (*vls*), on a 28-kb linear plasmid (lp28-1) (41). The presence of lp28-1 correlates with the high-infectivity phenotype in strains of *B. burgdorferi*, and homologous plasmids are present in low-passage, infectious strains of the Lyme disease spirochetes *Borrelia afzelii* and *Borrelia garinii* (41). The *vls* system has been characterized in *B. burgdorferi* B31 clone 5A3 (B31-5A3) (41), and in this strain consists of a *vls* expression site (*vlsE*) located near the right telomere of lp28-1 and 15 silent *vls* cassettes upstream. *vlsE* encodes a surface-exposed lipoprotein with a predicted molecular mass of 34 kDa. The coding sequence of *vlsE* in B31-5A3 contains a *vls* cassette region in the middle and two stretches of 5' and 3' flanking sequences which will be called noncassette regions hereafter. The central *vlsE* cassette region is separated from the noncassette regions by a 17-bp direct repeat sequence, which also separates the 15 silent *vls* cassettes (41).

The *vlsE* cassette region of B31-5A3 has up to 92% DNA sequence identity with the silent *vls* cassettes. The 15 silent cassettes begin ~200 bp upstream of *vlsE* and are oriented in the opposite direction, away from the telomere. They form a nearly contiguous, 8-kb open reading frame interrupted by only one stop codon and two frameshifts, but they lack promoter sequences and apparently are not expressed. The silent cassettes are 474 to 594 bp in length, and each is delimited by the same 17-bp direct repeat found in the *vlsE* expression site (with the exception of the 5' and 3' ends of the entire silent cassette locus). In this manner, the *vls* silent cassettes closely resemble the middle portion of *vlsE*.

Most sequence differences among the *vls* cassettes are confined within six highly variable regions (41). DNA segments of the silent cassettes are able to recombine in an apparently random manner into the *vlsE* cassette region in C3H/HeN mice throughout the course of infection (41, 42). Sequence

results are consistent, with roughly 6 to 11 recombination events with multiple silent *vls* cassettes during the first 28 days of infection (41). The promiscuous recombination events at the *vlsE* site lead to extensive genetic and antigenic variation in *VlsE* variants.

A recent publication by Kawabata et al. (16) shows that a similar, yet divergent, *vls* system exists in *B. burgdorferi* 297. Comparisons of patient and tick isolates from New York also indicate that the *vls* sequences of some strains closely resemble those of *B. burgdorferi* B31-5A3, whereas others are quite different (14). The sequence information available for comparison is incomplete at this point, and further analysis will be needed to provide a more complete picture of the heterogeneity of the *vls* system among Lyme disease isolates.

The *vls* system resembles a previously characterized genetic system encoding surface-exposed variable major proteins (VMP) in the relapsing fever agent *Borrelia hermsii* (2). *B. hermsii* has at least 26 *vmp* genes on multiple linear plasmids; only 1 *vmp* gene, located at a *vmp* expression site near one end of a linear plasmid, can be expressed by a single organism at a given time (2). Recombination events between the expressed and silent *vmp* genes lead to antigenic variation and thus evasion of the host immune response during the course of mammalian infection (3, 25, 28).

Antigenic variation of surface-exposed proteins has been identified as an important immune evasion mechanism in a number of additional pathogenic bacteria and parasites. In most cases, antigenic variation results from gene conversion events between the expressed genes and silent or nonexpressed genes or copies (5, 35). For example, nonreciprocal replacement of the expressed *vmp* sequence by silent *vmp* genes is the primary mechanism for *vmp* antigenic variation in *B. hermsii* (2), although other mutations and recombination events can also occur (29, 30). A similar mechanism appears to be responsible for recombination between the expressed pilin gene (*pilE*) and silent copies (*pilS*) in *Neisseria gonorrhoeae* (8, 10, 43). Antigenic variation of variable surface glycoprotein (VSG) in African trypanosomes also results in part from gene conversion events in which the silent *vsg* genes recombine into the telomeric expression sites (5).

In the present study, we analyzed the *vlsE* and silent *vls* cassette loci of *B. burgdorferi* M1e4A and M1e4C (41) obtained

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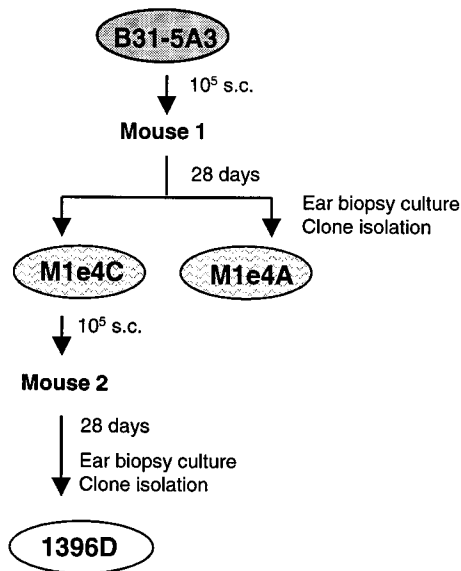


FIG. 1. Derivation of *B. burgdorferi* B31 clones in C3H/HeN mice. Clone B31-5A3 was the parental strain used in this study. Clones M1e4C and M1e4A were cultured from a single ear biopsy from a C3H/HeN mouse at 28 days postinfection with B31-5A3 (41). Similarly, clone 1396D was derived from a skin biopsy of a mouse inoculated with  $10^5$  M1e4C 28 days previously.

from a C3H/HeN mouse infected 28 days previously with the parental strain B31-5A3. The results indicate that the silent *vls* cassette locus and the *vlsE* noncassette regions are preserved during the course of *vlsE* variation, findings consistent with a gene conversion mechanism in which segments of the silent *vls* cassettes replace corresponding regions in the *vlsE* expression site.

#### MATERIALS AND METHODS

**Spirochete strains.** *B. burgdorferi* B31 clone 5A3 (B31-5A3) was isolated from the infectious low-passage strain B31 and identified as a high-infectivity strain by Norris et al. (27). B31-5A3 was previously used to characterize the *vls* system and *vlsE* antigenic variation (41). *B. burgdorferi* clones M1e4A and M1e4C were isolated from a single ear biopsy specimen from a C3H/HeN mouse infected 28 days previously with B31-5A3 (Fig. 1) (41). The spirochetes were cultured in BSK II medium as described previously (26). Ear biopsy isolate 1396 was obtained

from a C3H/HeN mouse 28 days postinfection with M1e4C; clone 1396D was cultured from isolate 1396 by subsurface colony plating (27).

**PCR techniques.** All PCR amplifications were carried out in a Minicycler thermal cycler (MJ Research, Watertown, Mass.) with the Thermalase PCR kit (Amresco, Solon, Ohio). All primers were obtained from GenoSys Biotechnologies, Inc. (Woodlands, Tex.) and dissolved to a final concentration of 100  $\mu$ M in  $H_2O$  as stocks. The entire coding regions of *vlsE* alleles were amplified by PCR by using forward (+ strand) primer F4224 and reverse (- strand) primer R4225 at a final concentration of 20  $\mu$ M (Table 1). Primers F4224 and R4225 are located at the 5' and 3' noncoding regions of *vlsE*, respectively (41).

The silent *vls* cassette sequences in *B. burgdorferi* M1e4C and 1396D were PCR amplified with *vls* cassette-specific primers at a final concentration of 20  $\mu$ M. The sequences and combinations of these primers used are described in Table 1 and in the Results section, respectively. *B. burgdorferi* plasmid DNA was prepared according to the method of Hinnebusch et al. (12) and used as a template. The PCR parameters utilized included 35 cycles of denaturation at 95°C for 40 s, with annealing at 64°C for 40 s followed by extension at 72°C for 2 min in a reaction volume of 100  $\mu$ l. The final cycle was followed by an additional extension at 72°C for 5 min.

**Southern hybridization.** *B. burgdorferi* plasmid DNA was prepared with stationary-phase organisms as previously described (12). *B. burgdorferi* total DNA was prepared with stationary-phase organisms by proteinase K digestion and phenol-chloroform extraction according to the method of Walker et al. (40). *B. burgdorferi* DNA was digested with restriction enzymes, separated by agarose gel electrophoresis, and transferred to nylon filters as described previously (41).

Oligonucleotides R4232, F4234, F4289, and R4290 were used as probes for Southern hybridization. The locations and sequences of these oligonucleotides are shown in Fig. 2 and Table 1, respectively. Portions (30 pmol) of oligonucleotides were radiolabeled by using T4 polynucleotide kinase (Promega, Madison, Wis.) and [ $\gamma$ - $^{32}P$ ]ATP (Amersham Life Sciences, Arlington Heights, Ill.) in a total volume of 25  $\mu$ l by standard methods (1). The radiolabeled probes were then purified through STE Select-D G-25 columns (5 Prime $\rightarrow$ 3 Prime, Boulder, Colo.) and hybridized with the *B. burgdorferi* DNA blots at 65°C for 16 h as described previously (41). The blots were washed sequentially with 0.5% sodium dodecyl sulfate in 2 $\times$  SSC (1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C for 30 min, 1 $\times$  SSC at 65°C for 30 min, and 0.1 $\times$  SSC at room temperature for 20 min (34). Autoradiography was carried out by using X-Omat film (Kodak, Rochester, N.Y.) with enhancing screens.

**DNA sequence analysis.** PCR products were purified by using Wizard columns (Promega, Madison, Wis.), and the remaining salts were removed in Microcon-100 columns (Millipore, Bedford, Mass.). DNA sequences were determined with an ABI 377 DNA sequencer (Perkin-Elmer/ABI, Foster City, Calif.) at the University of Texas—Houston Microbiology and Molecular Genetics DNA Core Facility. Sequences were analyzed with Genetics Computer Group (Madison, Wis.) programs as previously described (41). PILEUP and BOXSHADE programs were used to produce graphic output of sequence alignments.

**Nucleotide sequence accession numbers.** DNA sequences of the *vlsE* allele (*vlsE1*) and silent *vls* cassettes in the parental strain B31-5A3 are available under the GenBank entries U76405 and U76406, respectively. The complete coding sequences of *vlsE* alleles *m1e4A* and *m1e4C* are contained in the updated versions of U84554 and U84556, respectively. The *vlsE* cassette sequence of clone 1396D was deposited in GenBank under accession entry AF030082.

TABLE 1. Sequences and locations of oligonucleotides

| Oligonucleotide <sup>a</sup> | DNA sequence                              | GenBank accession no. | Location <sup>b</sup>           |
|------------------------------|---|-----------------------|---------------------------------|
| R4084                        | 5'-ACGGCAGTTCACAGAACCTGTACTATCT-3'        | U76405                | 389-418                         |
| F4219                        | 5'-TGCTGAGTCTGCAGTTCGCAAAG-3'             | U76405                | 1010-1032                       |
| F4224                        | 5'-AGATGATAAAGTTATACTTTCATTATAAGGAGACG-3' | U76405                | 34-68                           |
| R4225                        | 5'-CAATCATGAGGGCATAGTCGTGCCATACA-3'       | U76405                | 1156-1185                       |
| R4232                        | 5'-TCAGCCTCACTAGCAGCCGACC-3'              | U76406, U84556        | 5437-5459, 645-674 <sup>c</sup> |
| F4234                        | 5'-GGGAAAGTTGATGATGCTCATGCTGGGGAC-3'      | U76406, U84554        | 4741-4770, 747-769 <sup>c</sup> |
| F4265                        | 5'-CTGATGCAATTGGAGAAGTTGTGGATA-3'         | U76406                | 5437-5459                       |
| R4277                        | 5'-AACTCCGATCCGCATTACCCTT-3'              | U76406                | 4741-4770                       |
| R4279                        | 5'-GGTTAGTGTGGGGAGCAGATAT-3'              | U76406                | 5392-5413                       |
| F4280                        | 5'-CTGGTGTGGTGCTAATGCTGG-3'               | U76406                | 5165-5175 <sup>d</sup>          |
| F4289                        | 5'-ACCAATCTGTCATACAATTAGGTAACGGAT-3'      | U76405                | 175-204                         |
| R4290                        | 5'-AAAACCTTTCGCAACTGCAGACTCAGC-3'         | U76405                | 1011-1036                       |

<sup>a</sup> R and F represent reverse (- strand) and forward (+ strand) sequences, respectively.

<sup>b</sup> Coordinates in the GenBank source.

<sup>c</sup> Due to deletions and insertions relative to the sequence of the parental clone B31-5A3, the *vlsE* coordinates in M1e4C and M1e4A (GenBank entries U84556 and U84554, respectively) do not match those in B31-5A3 (GenBank entry U76405 and Fig. 2).

<sup>d</sup> Only the 11-bp 5' end of primer R4280 is identical to the corresponding sequence of silent cassette *vls11*.

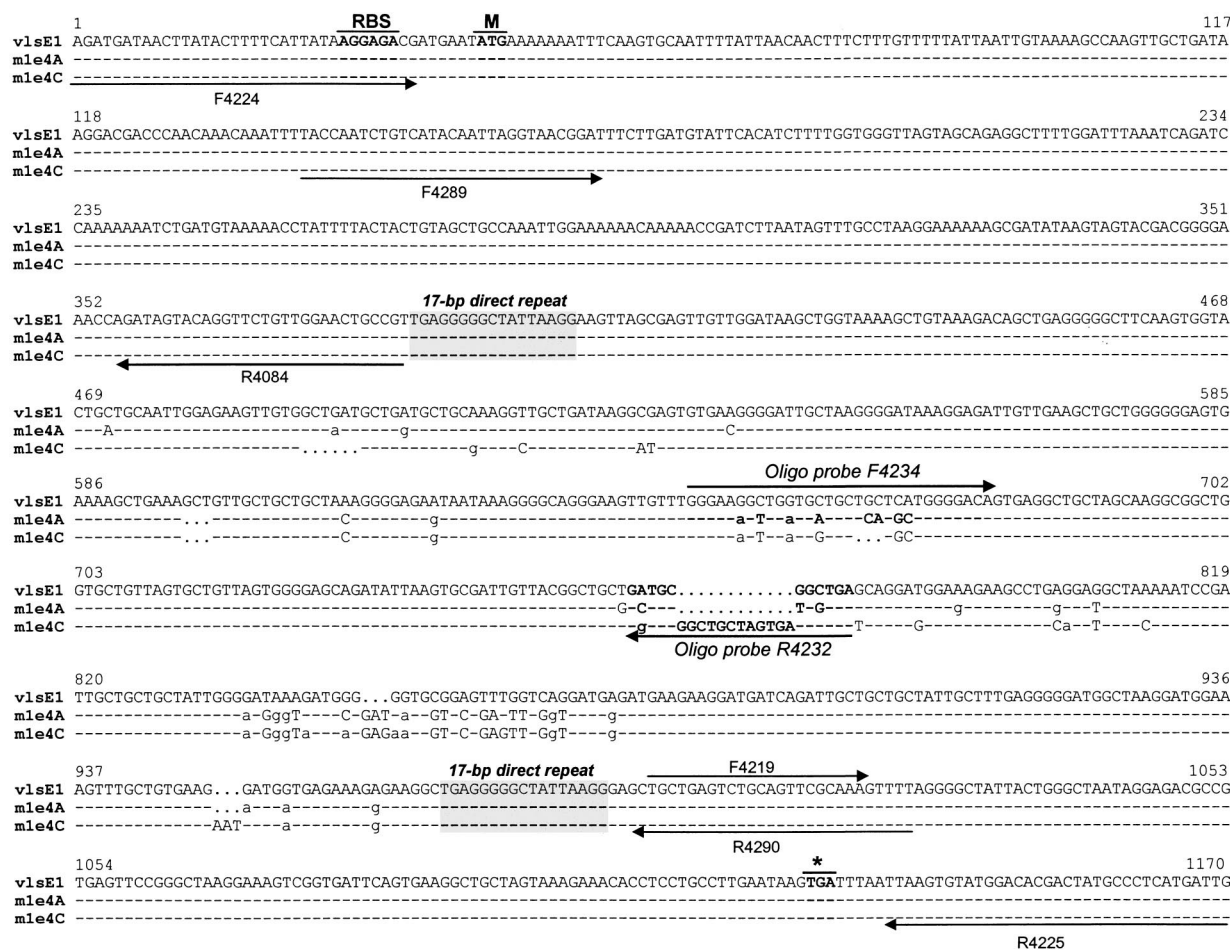


FIG. 2. Nucleotide sequence comparison between the parental *vlsE* allele (*vlsE1*) and two derivative alleles, *m1e4A* and *m1e4C*. Identical nucleotides and gaps are marked as dashes and dots, respectively; nonidentical nucleotides are shown as letters. The 17-bp direct repeats flanking the *vlsE* cassette region are shaded. The predicted ribosome binding site (RBS), putative start codon (M), and putative stop codon (\*) are indicated. Locations and directions of oligonucleotides used in this study are marked with arrows.

## RESULTS

### The 5' and 3' noncassette regions of *vlsE* remain unchanged.

It has been shown previously that considerable sequence variation occurred within the *vlsE* cassette region of *B. burgdorferi* during infection of C3H/HeN mice (41). However, the sequences outside the cassette region were not determined in variant clones, so it was not known whether the 5' and 3' noncassette regions of *vlsE* are affected during recombination events. To determine the sequences of the noncassette regions following infection in mice, we chose two *B. burgdorferi* clones, M1e4C and M1e4A, both of which were isolated from the same C3H/HeN mouse 28 days postinfection with the parental clone B31-5A3 (Fig. 1). The cassette sequences of *vlsE* alleles *m1e4A* and *m1e4C* have been previously determined (41).

The entire coding regions of *vlsE* for both strains were PCR amplified by using flanking primers F4224 and R4225 localized in the 5' and 3' noncoding regions (Table 1), and the PCR products were sequenced directly. Sequence analysis revealed that the 5' and 3' noncassette *vlsE* sequences for progeny clones M1e4A and M1e4C were identical to those of the parental strain B31-5A3 (Fig. 2). In contrast, both *vlsE* alleles exhibited numerous nucleotide sequence changes within the *vlsE* cassette region (Fig. 2), resulting in extensive changes in the predicted amino acid sequences (41). Thus, neither the 5'

nor the 3' noncassette region is altered during *vlsE* cassette region recombination in the mammalian host.

**The 5' and 3' noncassette regions of *vlsE* are present only at a single *vlsE* site.** In a previous study, we identified a *vlsE* site in *B. burgdorferi* clone B31-5A3 by cloning and sequencing analysis (41). Multiple *vsg* expression sites have been shown in African trypanosomes, although only one of them appears to be expressed at a given time (5). To determine whether *B. burgdorferi* possesses multiple *vlsE* sites for *vls* recombination, the oligonucleotides F4289 and R4290 were used as markers of *vlsE* to probe the plasmid DNA blots of *B. burgdorferi* B31-5A3, M1e4A, and M1e4C by Southern hybridization. Oligonucleotides F4289 and R4290 (Table 1) are located at the 5' and 3' noncassette regions of *vlsE*, respectively (Fig. 2).

In the parental strain and the two variants, only one major DNA band corresponding to *vlsE* was detected by both the 3' probe R4290 (Fig. 3) and 5' probe F4289 (data not shown). The sizes of the hybridizing fragments and were identical for the two probes except for the *RsaI* and *Sau3AI* digests, for which the probes are predicted to hybridize with fragments of different sizes. The presence of weakly hybridizing bands is most likely due to cross-hybridization with other unrelated sequences. These results indicated that the 5' and 3' noncassette

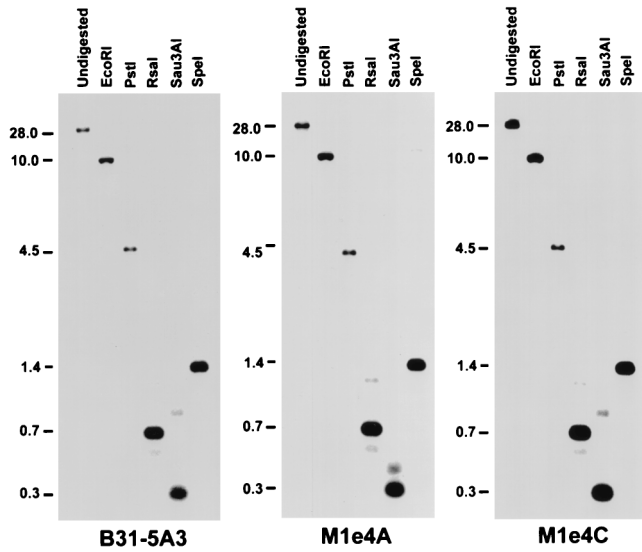


FIG. 3. Detection of the *vlsE* noncassette sequences. Southern blots of restriction enzyme-treated plasmid DNA from the parental strain B31-5A3 and two progeny strains M1e4A and M1e4C were hybridized with oligonucleotide probe R4290, representing the 3' noncassette region of *vlsE*. The molecular sizes of DNA bands are indicated in kilobases on the left side.

regions of *vlsE* are not present in other *B. burgdorferi* plasmids. Similar results were observed when using *B. burgdorferi* total DNA blots and the entire noncassette regions as probes (data not shown). In these experiments, the 5' and 3' noncassette regions were amplified by using primer sets of F4289-R4084 and F4219-R4225 and then used as probes (Fig. 2; Table 1). The extreme 5' end of *vlsE* was not included in the probe because it is partially homologous to the 5' end of the silent cassette *vls2* (41). These results provide evidence that only one *vlsE* locus on linear plasmid lp28-1 is present in the parental strain B31-5A3 and the two progeny variants M1e4A and M1e4C. They also show that the region surrounding *vlsE* does not undergo extensive rearrangement during recombination, as demonstrated by the nearly identical patterns obtained with the parent strain and the two variants.

**Segments of silent *vls* cassettes are duplicated into the *vlsE* site.** Our previous study (41) indicated that segments of the silent *vls* cassette sequences replaced portions of the *vlsE* cassette region. However, the mechanism by which the segments recombined into the *vlsE* cassette region was not known. There are at least two possibilities involving reciprocal and nonreciprocal recombination mechanisms (35). Reciprocal recombination would result in exchange of sequences between the silent *vls* cassettes and *vlsE*. In this case, the sequences of the silent *vls* cassettes would be altered. Conversely, nonreciprocal recombination would allow duplication of the silent *vls* cassette sequences into *vlsE*, preserving the sequence and structure of the silent *vls* cassette locus. To test these possibilities, several oligonucleotide probes representing variable *vls* segments were hybridized with the blots of the plasmid DNA from the parental strain B31-5A3 and its derivative clones digested with restriction enzymes.

Sequence analysis revealed two sequences specific for the silent *vls* cassettes. The sequence corresponding to the 23-mer R4232 (Table 1) was present in the *vlsE* expression site of M1e4C but was found only in the silent cassette *vls11* of the parental strain B31-5A3 (Fig. 2 and 4A), indicating that this *vls11* sequence had recombined into the *vlsE* gene of M1e4C.

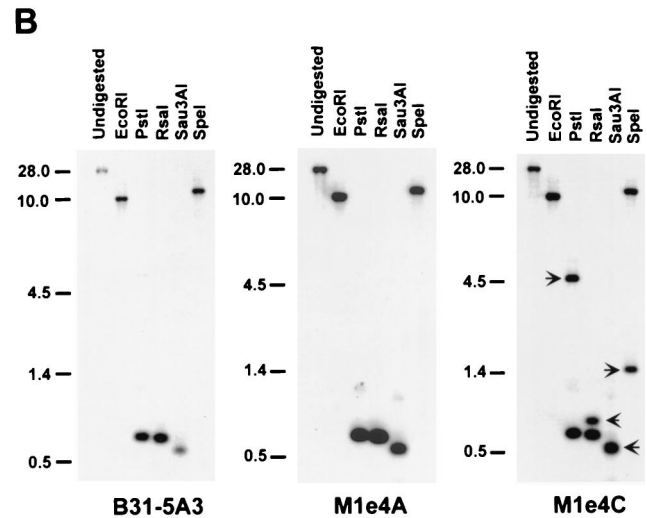
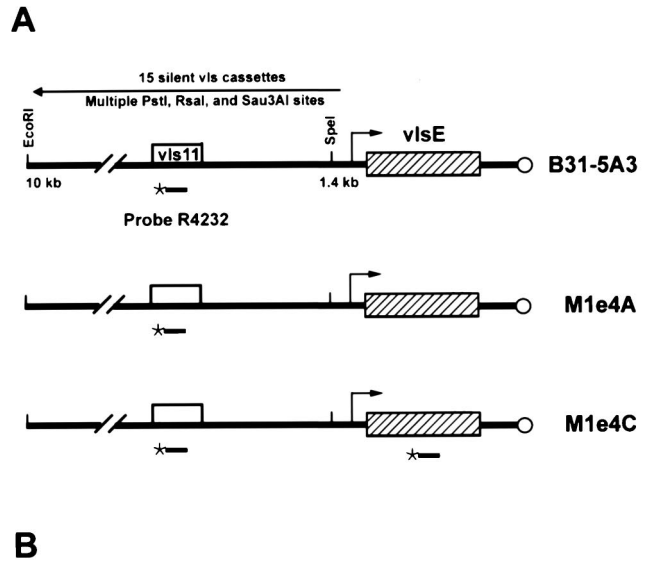


FIG. 4. Evidence for unidirectional recombination of silent *vls* cassette segments into *vlsE*. (A) Diagram of locations of oligonucleotide probes in *vlsE* and the silent *vls* cassettes in the parental strain B31-5A3 and its derivative strains M1e4A and M1e4C. (B) Hybridization of oligonucleotide probe R4232 with the blots of restriction enzyme-digested plasmid DNA preparations from B31-5A3, M1e4A, and M1e4C. In all lanes, the probe hybridized to a single band corresponding to a region containing the silent cassette *vls11* in both strains B31-5A3 and M1e4A. However, for strain M1e4C, two bands were detected in each digest that corresponded to the *vlsE* region with the second band (arrows). The molecular sizes of DNA bands are indicated in kilobases on the left side.

Similarly, the sequence represented by the 30-mer oligonucleotide F4234 (Table 1) is present only in the variable region 3 (VR-III) of the silent cassette *vls10* (41). The specific region of *vls10* represented by oligonucleotide F4234 had recombined into the *vlsE* cassette region of mouse isolate M1e4A (41). These probes thus served as specific markers of the *vls11* and *vls10* sequences located either in the silent cassette loci or in both the silent cassette region and *vlsE*.

Based on the restriction sites present in *vlsE* and the silent *vls* cassettes, *PstI*, *RsaI*, *Sau3AI*, and *SpeI* would produce restriction fragments for *vlsE* that are distinct from those of the silent *vls* cassettes. These enzymes were chosen to digest plasmid DNA from B31-5A3, M1e4C, and M1e4A for Southern blots. The blots were hybridized with radiolabeled oligonucleotides F4234 and R4232.

Only the silent cassette *vls11* contains sequence of oligonucleotide R4232 in clones B31-5A3 and M1e4A (Fig. 2 and 4A); therefore, only a single band was detected for all of the lanes (Fig. 4B). In contrast, in strain M1e4C, *Pst*I, *Rsa*I, and *Spe*I digestions yielded two hybridizing bands with the same probe (Fig. 4B). These results showed that strain M1e4C contains two copies of the probe sequence, one in *vlsE* and the other in the silent cassette *vls11*. *Sau*3AI digestion of the *vlsE* allele *m1e4C* was predicted to produce a 429-bp DNA fragment, while the *Sau*3AI fragment of the silent cassette *vls11* is 489 bp in length. Therefore, the hybridization band in the *Sau*3AI lane in strain M1e4C presumably represented two comigrating DNA fragments (Fig. 4B). Similarly, the *vls10*-specific oligonucleotide F4234 hybridized with both *vls10* and *vlsE* bands in the restriction pattern of strain M1e4A and with only the silent *vls10* copy in B31-5A3 and M1e4C Southern blots (data not shown).

Except for the extra *vlsE* bands for recombinant strains M1e4A and M1e4C, the hybridization patterns for all three strains were identical, indicating that *vls* recombination does not result in gross DNA rearrangement in the silent *vls* cassette locus. Thus, the overall structure of the silent *vls* cassettes appears to be conserved during *vls* recombination. In terms of the recombination mode, segments of the silent *vls* cassettes appear to be simply duplicated into the *vlsE* cassette region. Therefore, a unidirectional recombination mechanism seems to be operative in *vls* recombination.

#### The sequence of the silent *vls* cassette locus is preserved.

The oligonucleotide hybridization results described above suggested that the overall structure of the silent *vls* cassette locus is not altered during *vlsE* recombination (Fig. 4B). However, these hybridization experiments only showed the presence of these *vls* segments and did not provide further information about possible sequence changes in other regions of the silent *vls* cassette locus. Due to considerable sequence redundancy in the silent *vls* cassette locus, it is difficult to identify unique primer sets to amplify and verify the sequences of the silent cassettes by standard PCR methods.

We were able to PCR amplify and sequence portions of several silent *vls* cassettes in strain M1e4C and its variant 1396D, derived as shown in Fig. 1. A 697-bp region of *vls5* was amplified by using primers F4265 and R4277 (Table 1). Similarly, primers F4234 and R4232 allowed us to amplify a 720-bp fragment covering the 3' region of *vls10* and the 5' region of *vls11*. Finally, a 249-bp region of *vls11* was amplified with primers F4280 and R4279. These silent *vls* cassettes in strains M1e4C and 1396D were identical to those in the parental strain B31-5A3 in terms of DNA sequence (data not shown), although sequences from these regions had recombined into the *vlsE* expression site in these variant progeny. These results provide further evidence that the silent *vls* cassette locus does not undergo sequence variation during *vlsE* recombination.

**The previously expressed *vls* segments are lost.** The regions of the *vlsE* cassette that are replaced during recombination could be conserved by progeny. This could occur either by retention of lp28-1 plasmid copies within the same cell that did not undergo recombination or by transfer of those sequences to another, undefined site. In *B. hermsii*, an expressed *vmp* gene is degraded and lost when it is replaced by a silent *vmp* gene (17, 25, 28). To test whether the expressed *vls* segments are removed or retained in the *B. burgdorferi* genome after being replaced by other *vls* segments, oligonucleotide R4232 was radiolabeled and used to probe the restriction enzyme-digested total DNA of *B. burgdorferi* clone 1396D. Clone 1396D was derived from strain M1e4C during infection of a

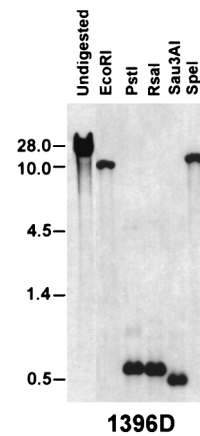


FIG. 5. Loss of the previously expressed *vls* cassette segments. The total DNA blot of M1e4C-derived clone 1396D was hybridized with oligonucleotide probe R4232. In all lanes, the probe hybridized to a single band containing the silent cassette *vls11*. The molecular sizes of DNA bands are indicated in kilobases on the left side.

C3H/HeN mouse (42) (Fig. 1) and had lost part of the oligonucleotide R4232 sequence from the *vlsE* site.

In contrast to the presence of two copies of probe R4232 in the parental strain M1e4C (Fig. 4B), the same probe detected only a single band in each restriction digest in the progeny strain 1396D, representing the silent *vls11* cassette in all lanes (Fig. 5). These results indicated that the displaced *vls* segments from the *vlsE* locus are not preserved elsewhere in the *B. burgdorferi* genome, a finding consistent with a gene conversion and loss of the replaced sequences.

## DISCUSSION

The results presented in this study have provided evidence for a gene conversion mechanism in *vlsE* genetic and antigenic variation. First, we showed that genetic duplication of *vls* silent cassette segments into the *vlsE* expression site appears to be responsible for the extensive sequence variation within the *vlsE* cassette region (41) (Fig. 4B). Second, sequence and Southern blot hybridization analyses indicated that the sequence and organization of the silent *vls* cassettes were conserved during recombination in the three isogenic *B. burgdorferi* B31 strains examined. Finally, direct comparison of restriction patterns between the parental and progeny strains revealed that the *vlsE* cassette sequences are degraded and are not retained elsewhere in the genome following recombination events.

Programmed gene rearrangements (4) or genetic variation (31) have been described in both prokaryotic and eukaryotic organisms. The classic example of programmed gene rearrangement in eukaryotes is the V(D)J rearrangement and isotype switching that occur in immunoglobulin and T-cell receptor expression (7, 19). This process involves site-specific recombinases that recognize inverted nonamer and heptamer sequences at either side of the gene segment "joints" in the case of V(D)J rearrangement or switch-site specific sequences upstream of each constant region locus in isotype switching (19). Although the *vls* recombination process involves replacement rather than deletion, it is possible that the 17-bp direct repeats at either end of the expressed and silent *vls* cassette regions or other conserved sequences are involved in site-specific recognition by the putative recombinase(s) responsible for this activity.

Our studies thus far have suggested that *B. burgdorferi* has an

elaborate system to ensure this unidirectional recombination. The unidirectional and segmental recombination features of *vlsE* antigenic variation in *B. burgdorferi* resemble those of the pilin antigenic variation in *N. gonorrhoeae*. *N. gonorrhoeae* pilin encoded by *pilE* is the main subunit of the surface-exposed pili, which have been shown to promote gonococcal infection (36). Segments of several silent pilin gene copies (*pilS*) scattered on the gonococcal chromosome can replace the existing sequences in *pilE* through unidirectional recombination events (8, 10, 43). The resulting changes in the amino acid sequence of the pilin protein can lead to antigenic variation (37) and variation in human tissue tropism (15). The molecular mechanisms of gonococcal pilin antigenic variation are still unclear, although the RecA protein has been shown to be essential (18). Homologous sequences in pilin genes have been shown to be important, including the *SmaI-ClaI* region (38, 39) and a conserved *cys2* region (13).

A site-specific invertase responsible for inversion of a DNA segment and pilin phase variation in *Moraxella lacunata* has been characterized (22, 23). This invertase apparently recognizes specific 19-bp repeat sequences (32) at the inversion sites. A putative *N. gonorrhoeae* recombinase (*gcr*) was able to recognize the same *M. lacunata* sequences and to catalyze the pilin gene inversion in a surrogate *Escherichia coli* system, but it is still unclear whether *gcr* plays a role in gonococcal pilin variation or virulence (33). Gonococcal mutants deficient in pilin antigenic variation have been generated recently by transposon mutagenesis (24). These mutants had undetectable pilin gene recombination at the *pilE* expression site. Based on these observations, we believe that conserved sequences of *vlsE* and silent *vls* cassettes are necessary for *vlsE* antigenic variation and that one or more site-specific proteins are involved in the recombination process. The required sequences may include the 17-bp direct repeats that flank the cassette regions of *vlsE* and the silent *vls* cassettes. The genome sequence of *B. burgdorferi* (6) contains several plasmid-associated genes encoding putative "transposase-like proteins," but these genes most closely resemble transposases associated with insertion sequences and also contain frameshifts. Therefore, these genes are unlikely to encode the *vls* recombinase protein(s), and other candidate genes have not as yet been identified.

There are also notable differences between *vlsE* and gonococcal pilin antigenic variation systems. Unlike the silent pilin genes which are scattered across the gonococcal chromosome (9), the silent *vls* cassettes are organized in a compact, head-to-tail array in lp28-1 (41). In addition, *N. gonorrhoeae* is capable of taking up extracellular DNA, which has been implicated as a source for pilin antigenic variation (11). However, natural competence in DNA transformation has not been reported in *B. burgdorferi*, although lateral transfer of genetic information has been suggested based on the sequence heterogeneity of several *B. burgdorferi* genes (20, 21). These distinctions may reflect other differences in the molecular mechanisms of the two antigenic variation systems.

The 5' and 3' noncassette regions of three *vlsE* alleles examined did not vary in the mouse isolates examined, despite extensive sequence variation with the *vlsE* cassette region (Fig. 2). Site specificity of a proposed recombinase may be a factor in this respect. It is possible that both noncassette regions are required for an as-yet-unknown function of the VlsE protein, so that there is a selection pressure against sequence variation in these regions. Finally, lack of sequence variation in the noncassette regions may simply be due to lack of corresponding silent copies in the genome.

Like "germline" immunoglobulin and T-cell receptor loci of vertebrates, the silent *vls* cassette locus appears to serve as a

stable reservoir for *vlsE* antigenic variation. By multiplying the number of possible amino acids at each variable position in the silent *vls* cassettes, the number of possible amino acid combinations has been estimated to be over  $10^{30}$  (unpublished data). This estimate assumes that recombination can occur at any location within the *vlsE* cassette and that all possible combinations are permissible for the survival and growth of *B. burgdorferi*. Even with these potential limitations, *B. burgdorferi* may have a nearly inexhaustible capacity for *vlsE* sequence variation.

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