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Juxtaposition of expressed variable antigen genes with a conserved telomere in the bacterium *Borrelia hermsii*

(relapsing fever/spirochete/antigenic variation/plasmid/outer membrane)

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ABSTRACT **_Borrelia hermsii_, an agent of relapsing fever, survives in mammals through antigenic variation. Change in serotype-specific variable outer membrane proteins (Vmps) occurs when a Vmp gene at an expression site is replaced with a previously silent gene for another Vmp. Silent and active genes are on separate linear plasmids. The upstream site for a nonreciprocal recombination between two linear plasmids is near the 5′ ends of the expressed and silent genes. In the present study we sought the downstream recombination sites in two serotypes, 7 and 21. Restriction fragments containing plasmid telomeres were identified by susceptibility to digestion with BAL-31 and rapid reannealment following denaturation. Whereas both silent genes and a minority population of both expression-linked genes were several kilobases from the telomeres, the predominant population of both expressed genes had 3′ ends near plasmid telomeres. Sequence analysis of the predominant expression plasmids revealed that the telomeric sequences were the same in serotypes 7 and 21. Identical sequence was also downstream of silent Vmp genes. Switching of Vmp genes appears to occur by recombination that involves both upstream and downstream sites. The expression plasmid's telomere is preserved in the recombination event.**

Borrelia hermsii, a spirochete, causes relapsing fever (1, 2). This infection is characterized by episodes of fever and bacteremia separated by periods of well-being. Borreliae evade the host's antibody response through multiphasic antigenic variation; 25 antigenically distinct serotypes have been identified in the progeny of a single cell of *B. hermsii* (2, 3). Antigenic variation is the consequence of sequential expression of genes for variable outer membrane proteins known as Vmps (4). Activation of a different Vmp gene (*vmp*) follows a nonreciprocal, unidirectional recombination event in which a copy of an untranscribed *vmp* is fused to a common expression site and the formerly active *vmp* is lost (5, 6). In *B. hermsii*, silent and expressed copies of *vmp* genes are located separately on linear plasmids of 28–32 kilobases (6). In the related species *Borrelia burgdorferi* the natural ends, or telomeres, of similar replicons are covalently closed (7, 8).

Vmp7 and Vmp21 are similar in protein sequence at their amino termini (9). The presumed similarity between the *vmp7* and *vmp21* genes at their 5′ ends suggested that recombination could occur at this region, and, in fact, the upstream recombination site for a switch from expression of *vmp7* to *vmp21* was mapped to a 700-base-pair (bp) *Hin*dIII fragment containing the 5′ end of the genes (6). As no comparable downstream recombination site was found, it was suggested that translocation between linear plasmids was the result of a single crossover (6). To better characterize *vmp* gene switches, regions downstream of expressed and silent *vmp* genes of serotypes 7 and 21 were physically mapped and sequenced.

MATERIALS AND METHODS

Strains. The isogenic serotypes 7 and 21 of *B. hermsii* strain HS1 (ATCC 35209) were studied (2, 4). A different clonal population of serotype 21, which represented the progeny of a cell that switched from serotype 7, was also examined (3). Serotypic identities were confirmed with monoclonal antibodies (4). Borreliae were grown in BSK II medium (10).

DNA Isolation and Analysis. Plasmid-enriched DNA was isolated from borreliae as described (7). Plasmid DNA from *Escherichia coli* was extracted by the alkaline lysis method (11). Restriction fragments and intact linear plasmids were examined by constant field electrophoresis and field inversion gel electrophoresis, respectively, as described (11).

BAL-31 Digestion. DNA was incubated at 30°C with 0.3 unit of BAL-31 (Boehringer Mannheim) per μg of DNA for various periods. EGTA was then added to a concentration of 15 mM, the samples were heated for 15 min at 65°C, and DNA was recovered by ethanol precipitation.

DNA Denaturation. After digestion of DNA with *Pst* I, EDTA was added to a final concentration of 10 mM. One-half of the sample was heated to 65°C and the other half was heated to 95°C for 10 min in a heating block. Both halves were placed on ice for 2 min before electrophoresis.

Southern Blot Analysis. Probes I–III were prepared from recombinant plasmids p7.16 and p21.4 (6). Probe IV was taken from a recombinant plasmid (p21.20) that was created for this study by ligating a 3.0-kilobase (kb) *Pst* I fragment containing the 3′ sequences of *vmp21* into pBR322. Restriction fragments as probes were radiolabeled with [α-^{32}P]dATP using a random primed labeling kit (Boehringer Mannheim) or nick-translation kit (BRL). Southern blot analysis was carried out as described (8); the final wash was at 67°C in 15 mM NaCl/1.5 mM sodium citrate/0.1% SDS/1 mM EDTA. Densitometry of autoradiographs was performed with a GS 300 scanning densitometer (Hoefer).

Cloning of Telomeric Fragments. Terminal restriction fragments were cloned as follows: DNA (15 μg) from serotype 7 or 21 cells was digested with *Kpn* I, precipitated, and resuspended in 30 mM NaOAc, pH 5.0/50 mM NaCl/10 mM $MgCl_2$/1 mM $ZnCl_2$/5% glycerol, in a total volume of 120 μl. Mung bean nuclease (60 units; Pharmacia) was added, and the solution was incubated at 30°C for 30 min. The reaction was stopped by adding SDS to 0.2%, Tris·HCl (pH 9.4) to 40 mM, and EDTA to 1 mM. After phenol/$CHCl_3$ extraction, the DNA was treated with Klenow fragment in the presence of dNTPs and separated by electrophoresis. Isolated fragments were ligated into *Sma* I-digested pUC13 and transformed into *E. coli* DH5α. Transformants were screened by colony blot hybridization (6).

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DNA Sequencing. Sequence was determined directly from recombinant plasmids by double-stranded sequencing (12) using Sequenase (United States Biochemical). Custom synthetic oligonucleotides for primer-directed sequencing were obtained from Midland Certified Reagent (Midland, TX). Sequences were analyzed using GENEUS software (13) and University of Wisconsin Genetics Computer Group sequence analysis software, version 6.0 (14).

RESULTS

Telomere Linkage of Expressed *vmp* Genes. Expressed and silent versions of *vmp7* and *vmp21* were cloned previously (5, 6). When initial efforts to clone restriction fragments downstream of expressed *vmp* genes failed, we wondered whether the expressed form of the gene was near the sealed telomere of its plasmid. To test this hypothesis, DNA samples from populations of serotype 7 and 21 cells were treated with BAL-31, which digests the linear plasmids from their ends (6, 8).

Fig. 1 *Upper* shows the results of an experiment in which DNA from serotype 7 or 21 cells was treated with BAL-31 for different durations, digested with *Pst* I, and probed with a *vmp21*-specific probe (III in Fig. 2). In untreated (0 min) samples four *Pst* I fragments (3.0, 2.8, 1.9, and 1.5 kb) of serotype 21 DNA and two fragments (3.0 and 1.5 kb) of serotype 7 DNA hybridized with this probe. The 2.8- and 1.9-kb fragments thus encompassed the expression-linked

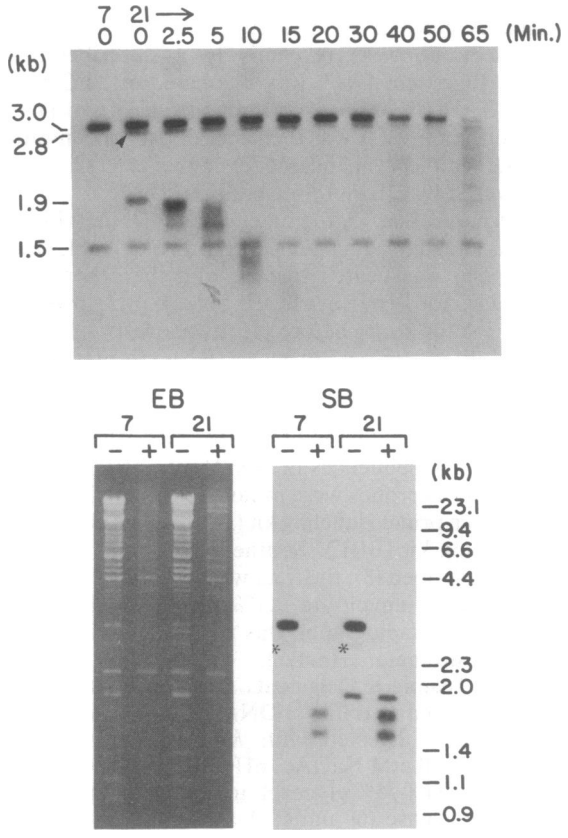

FIG. 1. Telomere linkage of expressed *vmp21*. (*Upper*) DNA from *B. hermsii* serotype 7 cells (leftmost lane) or 21 cells (remaining lanes) was treated with BAL-31 for various times (min) and then digested with *Pst* I. The blot was hybridized with *vmp21*-specific probe III (see Fig. 2). The arrowhead indicates a band of 2.8 kb in serotype 21 DNA. (*Lower*) Serotype 7 or 21 DNA was digested with *Pst* I and heated to 65°C (−) or to 95°C (+). The EtdBr-stained gel (EB) and Southern blot (SB) are shown. The blot was hybridized with probe IV (see Fig. 2), which is specific for *vmp21* and its downstream sequences. Asterisks indicate minor bands present in both serotypes.

form of *vmp21*, and the 3.0- and 1.5-kb fragments contained silent *vmp21*. The 1.9-kb fragment was the first to be affected, being shortened by BAL-31 within 2.5 min. The next fragment detectably degraded was the 2.8-kb *Pst* I fragment, which contains the 5′ end of expressed *vmp21* (5, 6). The third fragment to be shortened was the 3.0-kb fragment of the silent *vmp21* locus. The 1.5-kb *Pst* I fragment, which contains the 5′ end of silent *vmp21* (6), was not affected through 65 min of BAL-31 digestion. These findings indicated that the expressed *vmp21* gene is near a plasmid terminus and the silent *vmp21* is not. In addition, the experiment suggested that active and silent *vmp21* genes were oriented similarly with respect to the nearest telomere and that *vmp21* transcription is directed toward that telomere.

To confirm that expressed genes were telomere linked we used a second approach to identify plasmid ends. If telomeric fragments with covalently closed ends are heated, they should rapidly reanneal when cooled. The left panel of Fig. 1 *Lower* shows an EtdBr-stained gel containing serotype 7 and 21 DNA digested with *Pst* I and heated to 65°C (−) or 95°C (+) before electrophoresis. The right panel shows a blot of this gel probed with a *vmp21*-specific probe (IV in Fig. 2). Heat treatment followed by rapid cooling did not alter the migration of serotype 21's 1.9-kb *Pst* I fragment, which bears the 3′ end of expressed *vmp21*. In contrast, the 3.0-kb *Pst* I fragment, which contains the end of a silent *vmp21*, was converted by the same treatment into its two strands. (The 2.8-kb fragment marked with an asterisk is discussed later.) The stained gel shows that some restriction fragments of 4.4 kb and larger reannealed, indicating that renaturation is not dependent on fragment size. This experiment confirmed that the expressed *vmp21* is telomere linked and that silent *vmp21* is internally sited.

Experiments to locate *vmp7* on its expression and silent plasmids were also performed. Expressed *vmp7* was assigned to a 3.2-kb *Kpn* I fragment that was shortened within 2.5 min by BAL-31; silent *vmp7* was in a 16-kb *Kpn* I fragment not detectably affected for at least 30 min (not shown). When *Kpn* I-digested serotype 7 DNA was heated, quickly cooled, and probed with a *vmp7*-specific sequence, the 16-kb fragment containing silent *vmp7* remained in two single strands and the 3.2-kb *Kpn* I fragment bearing expressed *vmp7* reannealed (not shown). These findings indicated that the expressed *vmp7* is terminally located on its expression plasmid and that silent *vmp7* occupies an internal position on its plasmid.

Fig. 2 summarizes results of these and other mapping studies. The location and orientation of expression-linked *vmp* genes are indicated by arrows. Plasmids with telomeric copies of expression-linked *vmp*s were designated "bp7E" or "bp21E". The 3′ ends of *vmp7* and *vmp21* were found to be 1.2 and 1.4 kb, respectively, from what were arbitrarily assigned the right termini of the expression plasmids. Telomeric fragments from silent plasmids were identified, as described above, with BAL-31 and heat denaturation treatments. In these plasmids, named "bp7S" and "bp21S", the 3′ ends of *vmp7* and *vmp21* were found to be 4.3 and 6.1 kb, respectively, from the right telomeres. Black boxes upstream of *vmp* genes depict upstream recombination sites identified previously (6). The plasmids "bp7e" and "bp21e" and the downstream homology block in Fig. 2 are discussed below.

Two Forms of Expression Plasmids. During mapping studies, we found infrequent, longer forms of expression plasmids. In one experiment serotype 7 and 21 DNA was treated for different durations with BAL-31, digested with *Bgl* II, and probed with the *vmp21*-specific probe III (Fig. 3 *Upper*). The probe bound to *Bgl* II fragments of 3.4 kb (at the end of bp21E), 14 kb (at the end of bp21S), and 9.4 kb. The 9.4-kb fragment was unique to serotype 21. To determine whether this fragment was expression linked, the blot was stripped of the first probe and rehybridized with an expression site-

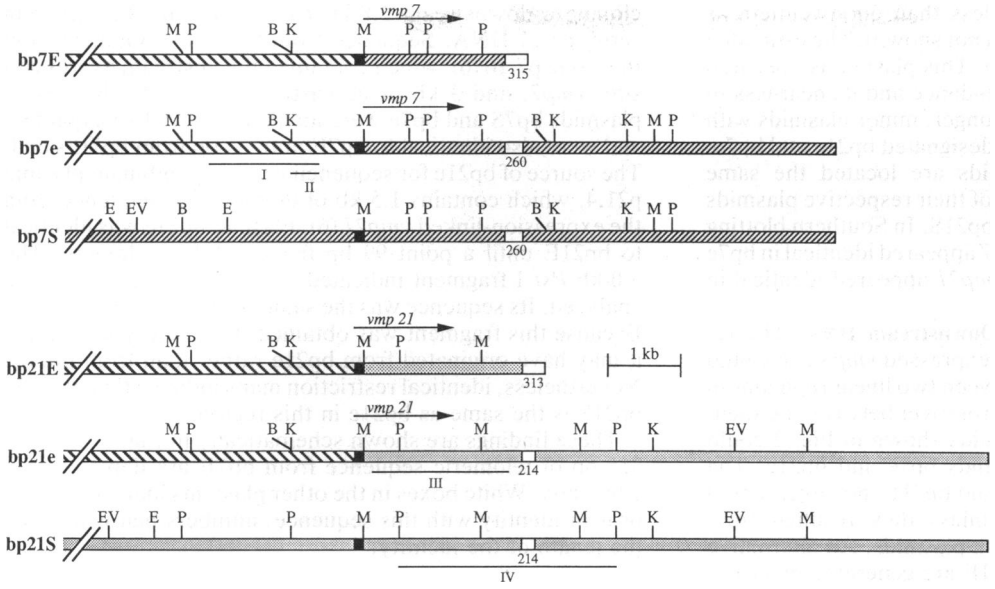

FIG. 2. Restriction maps of right arms of *B. hermsii* linear plasmids bearing expressed and silent *vmp7* and *vmp21*. Identity in pattern indicates highly similar or identical sequences. The region with the bold hatched pattern is the common upstream expression site; expressed *vmp* genes are indicated by arrows. Black and white boxes represent upstream and downstream recombination sites, respectively; numbers beneath indicate their lengths in base pairs. Horizontal lines with roman numerals (I–IV) denote DNA probes. Restriction enzymes are *Msp* I (M), *Pst* I (P), *Bgl* II (B), *Kpn* I (K), *Eco*RI (E), and *Eco*RV (EV).

specific probe (II in Fig. 2). Fig. 3 *Lower* shows that the 3.4- and 9.4-kb fragments of serotype 21 hybridized with the probe, indicating that both fragments are expression linked. The *Bgl* II fragment to the 5′ side of the 3.4-kb fragment on plasmid bp21E was found to be 10.7 kb in length (not shown). The 9.4-kb band is not, therefore, a partial digestion product of the 3.4-kb and neighboring 10.7-kb *Bgl* II fragments. We concluded that the 9.4-kb *Bgl* II fragment was part of a second expression plasmid that was larger but less common than bp21E.

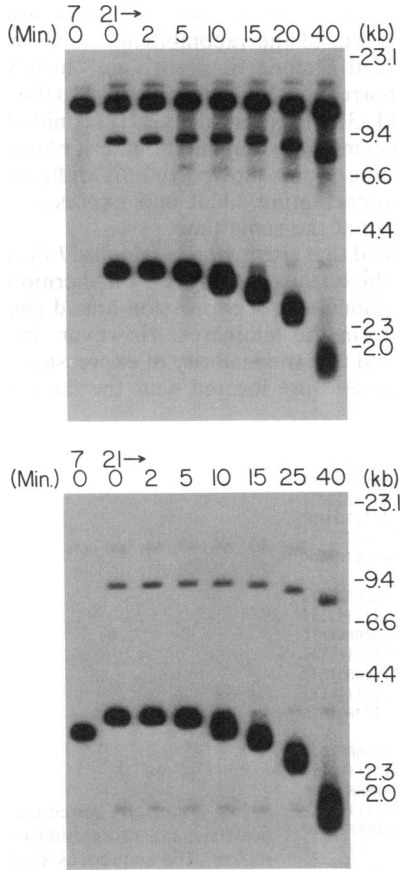

FIG. 3. Southern blot analysis with *vmp21*- and expression site-specific probes of *B. hermsii* serotypes 7 and 21. (*Upper*) Plasmid DNA was treated with BAL-31, digested with *Bgl* II, transferred to a membrane, and hybridized with probe III (Fig. 2). (*Lower*) The same blot stripped of bound probe and hybridized with probe II (Fig. 2), which is specific for the expression site.

To confirm this assessment, another expression site probe (I in Fig. 2) was hybridized with intact linear plasmids of serotypes 7 and 21 (Fig. 4). The left panel shows a 3-hr exposure of the blot. The predominant expression plasmids had apparent sizes of 28 kb, as previously found (6). With further exposure (right panel) additional hybridizing bands were detected. These included 50-kb plasmids in both serotypes and a 34-kb plasmid unique to serotype 21. The 34-kb plasmid was estimated with densitometry to be no more than one-fourteenth as abundant as bp21E. The size and relative abundance of this plasmid are consistent with it being the minor expression plasmid postulated above.

We also examined DNA from a population of serotype 21 cells derived directly from serotype 7. This line of serotype 21 also had infrequent, long expression-linked plasmids that were indistinguishable in size and restriction sites from the forms described above. Neither the long nor short form of *vmp21*-bearing expression plasmid was detectable in the parent serotype 7 culture (data not shown).

We next determined whether two forms of a *vmp7*-bearing expression plasmid were present in a population of serotype 7 cells. Southern blots using probes specific for *vmp7* and for the upstream expression site indicated that a longer form of expression plasmid exists in serotype 7 cells. Densitometry

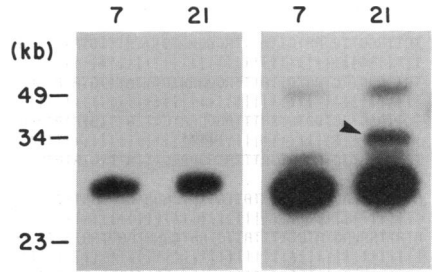

FIG. 4. Two forms of expression plasmid in serotype 21 cells. Linear plasmids of serotypes 7 and 21 were separated by field inversion gel electrophoresis and subjected to Southern blot analysis with an expression site probe (I in Fig. 2). The relative migrations of selected high molecular weight markers are shown at the left. (*Left*) Three-hour exposure showing the predominant (28 kb) expression plasmids in serotype 7 and 21 cells. (*Right*) Eighteen-hour exposure of the same blot. The arrowhead indicates a 34-kb plasmid.

showed that the longer form is less than one-twentieth as abundant as the shorter form (data not shown). The estimated size of the longer form was 32 kb. This plasmid is obscured in Fig. 4 because of its lesser abundance and its nearness to the 28-kb plasmid. In Fig. 2, the longer, minor plasmids with expression-linked *vmp* genes are designated bp21e and bp7e. The *vmp* genes in these plasmids are located the same distance from the right telomeres of their respective plasmids as are the *vmp* genes in bp7S and bp21S. In Southern blotting experiments, sequences 3′ to *vmp7* appeared identical in bp7e and bp7S, and sequences 3′ to *vmp21* appeared identical in bp21e and bp21S (not shown).

Sequencing of Telomeric and Downstream DNA. The recombination site upstream of the expressed *vmp* gene would be sufficient for translocation between two linear replicons in a single crossover (6, 15). Such a crossover between the silent and expressed *vmp*-bearing plasmids shown in Fig. 2 could yield the minor expression plasmids bp7e and bp21e. The major expression plasmids bp7E and bp21E, however, could not be generated in this way unless they resulted from shortening of the minor expression plasmids. An alternative explanation is that bp7E and bp21E are generated by crossovers 3′ as well as 5′ to the *vmp* genes. The second model predicts sequence identity between bp7E and bp21E at their termini. To distinguish between these models, the ends of bp7E and bp21E were examined. Our cloning strategy was based on the finding that plasmid telomeres are unsealed by mung bean nuclease (11). A *Kpn* I digest of serotype 7 and serotype 21 DNA was treated with mung bean nuclease and Klenow fragment to produce fragments with blunt ends. The 3.2-kb fragment from plasmid bp7E and the 3.4-kb *Kpn* I fragment from plasmid bp21E containing the *vmp* genes and their downstream sequences (see Fig. 2) were cloned for sequence analysis.

Fig. 5 shows the sequence of the terminal 340 bp of bp7E and bp21E and corresponding regions in bp7S, bp7e, and bp21e. For 315 bp, bp7E and bp21E termini were identical with exception of 4 nucleotides. DNA further upstream in these plasmids was not similar. The underlined terminal nucleotides in Fig. 5 are identical to terminal sequences from linear plasmids of *B. burgdorferi* (11). This suggests that this sequence is telomere specific. To assess whether other sequences shared by bp7E and bp21E were unique to these plasmids, sequences downstream from the *vmp* genes on minor expression plasmids or silent plasmids were needed. Sequences downstream from silent *vmp7* were obtained by cloning a downstream 2.8-kb *Pst* I fragment (Fig. 2) from serotype 21 DNA. Sequence from bp7e was obtained from the clone p7.16 (6), which contained the upstream expression site, *vmp7*, and 3 kb of downstream DNA. In this region plasmids bp7S and bp7e were identical, as we had expected, and were also identical to bp7E until 55 bp from bp7E's end. The source of bp21e for sequencing was recombinant plasmid p21.4, which contains 1.5 kb of downstream sequence from the expression-linked *vmp21* (6). Plasmid bp21e was identical to bp21E until a point 99 bp from bp21E's telomere. The 3.0-kb *Pst* I fragment indicated as "IV" in Fig. 2 was also analyzed. Its sequence was the same as that shown for bp21e. Because this fragment was obtained from serotype 21 cells, it may have originated from bp21e rather than from bp21S. Nevertheless, identical restriction maps indicate that plasmid bp21S is the same as bp21e in this region.

These findings are shown schematically in Fig. 2. The last 315 bp of telomeric sequence from bp7E are depicted as a white box. White boxes in the other plasmids indicate perfect or near identity with this sequence; numbers below indicate the length of the identity.

DISCUSSION

These experiments provide further information about the organization of genes for the serotype-specific Vmp proteins. The results lead us to propose a model for antigenic variation in *B. hermsii*.

In the study that demonstrated the siting of *vmp* genes on linear plasmids, the expressed and silent *vmp* genes for serotypes 7 and 21 appeared to be several kilobases from plasmid ends (6). Evidence for this supposition was the following. (*i*) One of the recombinant clones of expressed *vmp7* resembled a clone of silent *vmp7* in having several kilobases of borrelia DNA sequence distal to the 3′ end of the gene. (*ii*) BAL-31 digestion of linear plasmids followed by restriction enzyme digestion and Southern blot analysis with a *vmp21*-specific probe showed two hybridizing fragments, presumably representing silent and expressed *vmp21*, decreasing in size at the same time.

We confirmed that silent *vmp7* and *vmp21* occupy internal locations on the linear plasmids (6). Furthermore, we found that a minor population of expression-linked *vmp* genes was also distant from the telomeres. However, in the present study, we found that the majority of expression-linked *vmp7* and *vmp21* genes were located near the telomeres of their

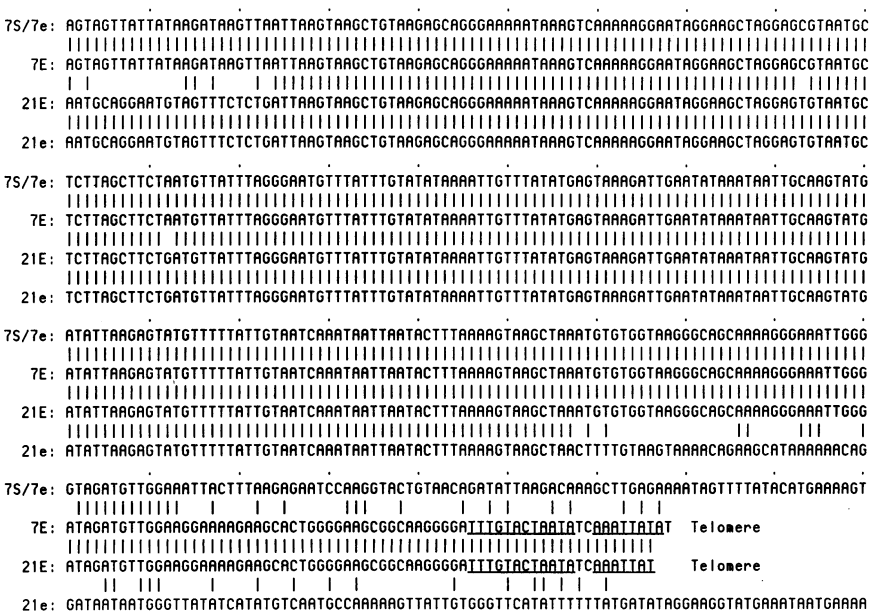

FIG. 5. DNA sequence of the downstream recombination site region. The sequences shown are from the plasmids indicated at the left of each line. (Sequences from plasmids bp7S and bp7e are identical in this region.) The underlined sequences are identical to terminal sequences of linear plasmids in *B. burgdorferi* (11).

plasmids. The previously obtained recombinant clones with expressed *vmp21* or *vmp7* and 1.5–3 kb of downstream sequence were obtained from bp21e and bp7e, the minority forms of expression-linked plasmids. Full-length *vmp7* sequences from the majority plasmid bp7E are difficult to clone by standard methods because of the proximity of *vmp7* gene's 3′ end to the unligatable telomere. In the previous study with BAL-31, only bp21e and bp21S *vmp21* genes were detected (6). However, the probe used in that study would not have revealed the telltale 1.9-kb *Pst* I fragment that bears the terminus of bp21E.

The detection of a conserved homology block downstream from *vmp7* and *vmp21* in both active and silent versions was also surprising; Southern blots had not suggested its presence. This discrepancy is probably explained by the small size and terminal location of the homology block. Fig. 1 *Lower* shows a blot that was probed with the downstream homology block from bp21S (IV in Fig. 2). The 2.8-kb *Pst* I fragment (asterisk) containing the downstream homology block from bp7S and bp7e hybridizes faintly and could have been missed.

For a hypothetical switch from expression of Vmp7 to Vmp21 protein in a single borrelia, our model specifies the following events involving the plasmids shown in Fig. 2. Pairing between bp21S and bp7E plasmids is promoted by sequence similarities in the upstream and downstream regions of silent *vmp21* and active *vmp7*. In the process of this recombination between the two plasmids, a formerly silent *vmp21* and about 1 kb of distal 3′ sequence replace the *vmp7* gene and corresponding downstream sequences. The recombination event is consistent in these features with gene conversion. A gap in the expression plasmid may be repaired with the silent gene as template, and the gap may be widened until the downstream homology block is reached. In a variation of this mechanism, bp21E is created by separate upstream and downstream crossovers between bp21S and bp7E with loss of the reciprocal product of the recombination. At this time, these two types of recombination cannot be distinguished as they are applied to *B. hermsii*.

In an alternative, but less likely, model, bp21E and bp7E are products of bp21e and bp7e editing. In this model, the downstream homology block is not a locus for recombination but instead constitutes a recognition site for a specific endonuclease. Though this mechanism cannot presently be ruled out, neither can it readily account for (*i*) the cleavage of bp21e and bp7e without cleavage of identical sequences in silent plasmids and (*ii*) the 99 bp at the terminus of bp21E and 55 bp at the end of bp7E that are absent in the corresponding minor expression plasmids.

The larger expression plasmids themselves may arise in different ways. For instance, bp21e and bp7e may be the result of a single crossover involving the *vmp* gene itself and its distal sequences, including the downstream telomere. It would follow then that short expression plasmids result from either independent double crossovers, as discussed above, or from secondary single crossovers at the downstream homology blocks of bp21e or bp7e. The first prediction is difficult to reconcile with the fact that clonal populations were examined here and that only one type of *vmp* gene was expression linked in the populations. The second prediction assumes high recombination rates for long expression plasmids. If secondary recombinations do occur, it is more likely, in our view, that long expression plasmids derive from short ones than vice versa. For example, bp21e may be the product of recombination between plasmid bp21E and bp21S in the postswitch serotype 21 population. This order of events would explain the higher numbers of short plasmids.

Further evaluation of these mechanisms will likely require examination of the genomes of individual postswitch cells. Of additional importance for definition of the mechanism is determination of plasmid copy numbers.

The switching of *vmp* genes in the bacterium *B. hermsii* resembles antigenic variation in certain protozoans. Like relapsing fever borreliae, African trypanosomes are blood-borne, are arthropod-transmitted, and sequentially display surface antigens from an extensive repertoire (reviewed in ref. 16). Active variable antigen genes of trypanosomes are found at one of a few expression sites, all of which are near the telomere of a chromosome (16, 17). The telomere linkage of expressed Vmp genes in borreliae is remarkably similar. Activation of variable antigen genes by trypanosomes occurs by more than one mechanism, but the most common is indistinguishable from that observed in *B. hermsii*—namely, replacement of the resident gene at a telomeric expression site with a silent gene (16, 18).

Possible reasons for the preferred placement of variable antigen genes at the ends of linear replicons in a prokaryote and eukaryote include the following. (*i*) If silent *vmp* genes, like silent variant surface glycoprotein (VSG) genes (19), occur in clusters on a plasmid or chromosome, the presence downstream of other *vmp* genes in an expression plasmid could result in the inappropriate expression of more than one *vmp* by the cell. A telomeric siting of the *vmp* or VSG obviates this possibility. (*ii*) Also foreclosed by a terminal location of *vmp* and VSG genes would be the chance of oppositely directed transcription from genes closer to the telomere. The discovery that the majority of expressed Vmp7 and Vmp21 genes of *B. hermsii* are telomere linked, together with previous findings that expressed surface antigen genes in trypanosomes are also terminally located, indicates an importance of the telomeric environment for gene regulation in linear replicons.

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