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Borrelia burgdorferi EbfC, a Novel, Chromosomally Encoded Protein, Binds Specific DNA Sequences Adjacent to *erp* Loci on the Spirochete's Resident cp32 Prophages

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All examined isolates of the Lyme disease spirochete, *Borrelia burgdorferi*, naturally maintain numerous variants of a prophage family as circular cp32 episomes. Each cp32 carries a locus encoding one or two different Erp outer membrane, surface-exposed lipoproteins. Many of the Erp proteins bind a host complement regulator, factor H, which is hypothesized to protect the spirochete from complement-mediated killing. We now describe the isolation and characterization of a novel, chromosomally encoded protein, EbfC, that binds specific DNA sequences located immediately 5′ of all *erp* loci. This is one of the first site-specific DNA-binding proteins to be identified in any spirochete. The location of the *ebfC* gene on the *B. burgdorferi* chromosome suggests that the cp32 prophages have evolved to use this bacterial host protein for their own benefit and that EbfC probably plays additional roles in the bacterium. A wide range of other bacteria encode homologs of EbfC, none of which have been well characterized, so demonstration that *B. burgdorferi* EbfC is a site-specific DNA-binding protein has broad implications across the eubacterial kingdom.

Every Lyme disease spirochete isolated from nature has been found to contain multiple members of a DNA element family that replicate as circular plasmids (57). Those plasmids are designated "cp32s" in light of their circular plasmid nature and approximate size of 32 kb (11, 12, 56, 57). All cp32s are essentially identical to each other except in three loci: one that is involved in plasmid replication and segregation and two loci that encode surface-exposed lipoproteins (18, 48, 52, 57, 63). Intriguingly, cp32s appear to be the genomes of lysogenic bacteriophages, which evidently infect all Lyme spirochetes (12, 15, 19, 20, 57, 62).

Each cp32 element carries a mono- or bicistronic *erp* locus, which often varies in sequence between plasmids (51). The Erp lipoproteins are among the specific repertoire of proteins synthesized by *Borrelia burgdorferi* during mammalian infection (3, 16, 21, 26, 28, 43–46, 50, 51, 57). Synthesis of Erp proteins during mammalian infection is consistent with a known function of some Erp proteins: the binding of host serum protein factor H, an important fluid phase regulator of the alternative pathway of complement activation (4, 29, 32, 33, 42, 53). Factor H is normally bound by receptors on surfaces of host cells, where it protects those cells by inhibiting C3 cleavage and promoting C3b degradation. Binding of factor H via Erp and other *B. burgdorferi* outer-surface proteins is hypothesized to likewise protect the pathogen from complement-mediated destruction (34, 35, 37).

To date, the cp32 family members and *erp* loci of four different *B. burgdorferi* isolates have been characterized. The *B. burgdorferi* species type strain, B31, contains 10 different cp32 family members and 17 *erp* genes. Since one bicistronic locus is present in three identical copies and another locus, *erpH*, is naturally defective, individual bacteria of strain B31 can simultaneously produce up to 13 distinct Erp lipoproteins (11, 12). Strains 297 and Sh-2-82 proved to be nearly clones of each other, holding 9 cp32s and 13 *erp* genes in common, including 3 copies of a bicistronic locus (2, 54). As evidence of naturally occurring transduction of cp32s among *B. burgdorferi* strains, strain Sh-2-82 also contains an additional cp32 that appears to be identical to a cp32 of strain B31 (54). Strain N40 carries six cp32s and nine unique *erp* genes (54). Considerable diversity occurs between *erp* gene sequences, both within an individual bacterium and between strains, giving each Erp protein unique functional and antigenic characteristics (2, 51, 54, 57). However, a unifying feature of all *erp* genes is that each locus is preceded by a highly conserved DNA sequence. Within that region are the transcriptional promoter and two separate sites that specifically bind different bacterial cytoplasmic proteins (Fig. 1) (7, 22, 41, 51, 56, 57). Our laboratory previously demonstrated that the protein-binding region closest to the *erp* promoter, designated operator 2, is required for proper regulation of *erp* transcription (7). Continuing those studies, we used DNA affinity chromatography to purify a *B. burgdorferi* protein that binds specific DNA sequences within the boundaries of *erp* operator 2, which we identified as the novel protein EbfC. Characterization of the DNA sequences specifically recognized by that chromosomally encoded protein are also presented.

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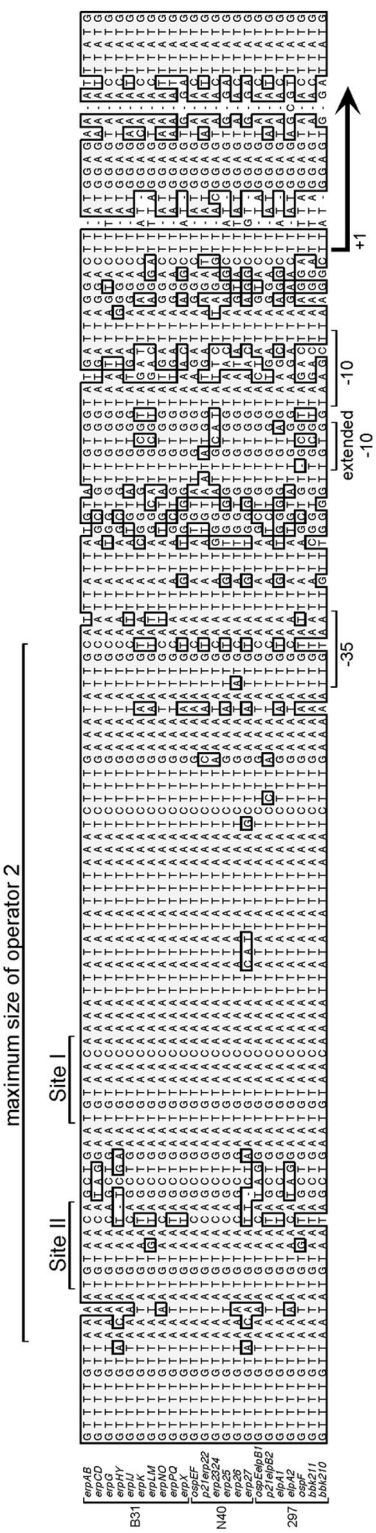


FIG. 1. Alignment of noncoding DNA sequences located immediately 5′ of the determined *erp* loci of *B. burgdorferi* strains B31, N40, and 297 (the *erp* loci of strain Sh-2-82 are identical to those of strain 297 plus the *erpNO* locus of strain B31). Identical nucleotides found in the majority of loci are boxed and shaded. Initiation methionine codons of the first gene in each locus are to the far right. The maximum size of *erp* operator 2, as determined previously using competitive EMSA and transcriptional fusions to reporter genes (7), is indicated. The two EbfC-binding sites (TGT[A/T]ACA) determined by the present work are indicated as Site I and Site II above the alignment.

TABLE 1. Oligonucleotides used in this work (all sequences are shown 5′ to 3′)

Function or name	Sequence
DNA affinity purification and EMSA probe production	
Bio-E43	Biotin-AAAATTTTAGTCAAATTTGGAGTG
R8	GCAATATTTCAAAGATTTAAA
Bio-G14A	Biotin-TTGTAATGAGTAGAGCATTTG
VLSF-27B	Biotin-AATAGTTTGCCTAAGGAAAAAAGCG
VLSR-8	CAACTTCTCCAATTGCAGCAGTAC
Cloning of *ebfC*	
BATA-1	GCTTCAAAATTATAAAGG
BATA-2	AATTATAAAGCACTTAAC
462-M	CACCATGGCAGTAAATCCGTTAG
462-L	CACCTTGGAGCAAGTGAAGTTCTG
462-R	CTACATTCCAAAAGGAAGAACTCC
EMSA competitor production	
R8	GCAATATTTCAAAGATTTAAA
G14A	TTGTAATGAGTAGAGCATTTG
104F	TGTTAAAATGTAACAGCTGAATGTAACAAA
104R	TTTGTTACATTCAGCTGTTACATTTTAACA
100F	TAAAATGTAACAGCTGAATGTAACAAAATT ATAT
100R	ATATATAATTTTGTTACATTCAGCTGTTACA TTTTA
100NCF	AAATGTAACAGCTGAATGTAACAAAAT
100NCR	ATTTTGTTACATTCAGCTGTTACATTT
100NC-2F	GAATGTAACAAAAT
100NC-2R	ATTTTGTTACATTC
100-1F	ATGTAACAGCTGAATGTA
100-1R	TACATTCAGCTGTTACAT
100-2F	GAATGTAACAAAATTATAT
100-2R	ATATAATTTTGTTACATTC
100-3F	AAATGTAACAATCAGGTGTAACAAAAT
100-3R	ATTTTGTTACACCTGATTGTTACATTT
100-4F	AAACACAACAGCTGAATGTAACAAAAT
100-4R	ATTTTGTTACATTCAGCTGTTGTGTTT
100-5F	AAATGTAACAGCTGAACACAACAAAAT
100-5R	ATTTTGTTGTGTTCAGCTGTTACATTT
100-6F	AAATGTAACAAAATAATGTAACAAAAT
100-6R	ATTTTGTTACATTATTTTGTTACATTT
100-7F	AAATGTAACAGCTGAATGTAACAGCTG
100-7R	CAGCTGTTACATTCAGCTGTTACATTT
100-8F	GAATGTAACAGCTG
100-8R	CAGCTGTTACATTC
100-9F	GCCTGTAACAAAAT
100-9R	ATTTTGTTACAGGC
100-10F	GAATGTAACAGCTGTATA
100-10R	TATACAGCTGTTACATTC
64F	ACAAAATTATATATTTAAATCTTTGAAATA
64R	TATTTCAAAGATTTAAATATATAATTTTGT
FLA-6	TTCAGGGTCTCAAGCGTCTTG
FLA-7	GCATTTTCAATTTTAGCAAGTG

MATERIALS AND METHODS

Bacteria. All studies used a virulent, clonal subculture of the *B. burgdorferi* species type strain, B31-MI-16 (46). Spirochetes were cultured at 34°C in Barbour-Stoenner-Kelly II (BSK-II) medium (8). Soluble protein extract was prepared from *B. burgdorferi* as previously described (6, 7).

DNA affinity purification. A segment of 5′ noncoding DNA from the strain B31 *erpG* locus, containing the promoter and both identified operator sites, was amplified by PCR using oligonucleotide primers R8 and biotinylated E43 (Table 1) (6, 7, 56). Due to the modification of oligonucleotide E43, the resulting amplicon contained a biotin moiety at the end distal to the *erp* promoter. Reaction mixtures were separated by agarose gel electrophoresis followed by extraction and purification of the amplicon. The biotin-conjugated amplicon was then affixed to streptavidin magnetic beads (Dynal, Brown Deer, WI) as follows. Magnetic beads were washed twice in equal volumes of 2× binding and wash buffer (2× B&W; 10 mM Tris [pH 7.5], 1 mM EDTA, 2 M NaCl) using a magnetic stand (Dynal) to adhere the beads to the side of the tube during removal of the wash buffer. Beads were then resuspended in 550 μl 1× B&W containing the biotin-

ylated PCR amplicon, incubated at room temperature for 30 min, and then washed three times with equal volumes of 2× B&W buffer. The beads were next washed twice in BS/THES buffer (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 50 mM KCl, 20% glycerol, 20 mM Tris, 5 mM EDTA, 90 mM NaCl, and protease and phosphatase inhibitor cocktails [both from Sigma, St. Louis, MO] at concentrations of 6.7 μg and 1.7 μg per ml, respectively) and then once in BS/THES buffer containing 30 μg/ml poly(dI-dC). Following these treatments, beads were incubated for 15 min on ice with 7.5 mg *B. burgdorferi* soluble protein extract in BS/THES buffer plus 200 μg poly(dI-dC), the supernatant was removed, and the beads were then incubated again with a second, similar aliquot of protein extract plus poly(dI-dC). Next, the beads were washed twice with BS/THES buffer containing 30 μg/ml poly(dI-dC). Bound proteins were sequentially eluted by washing with 50 mM Tris [pH 7.5] and 10 mM EDTA plus either 500 mM, 750 mM, or 1 M NaCl. Aliquots of eluted proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by staining with SYPRO-Ruby (Molecular Probes, Eugene, OR). Protein bands were extracted and analyzed by matrix-assisted laser desorption ionization–time of flight (mass spectrometry) (University of Louisville, Louisville, KY). Spectrometry results were compared with the known sequence of *B. burgdorferi* strain B31 using Mascot (Matrix Science, Boston, MA).

Recombinant EbfC. As a first step, a region of the *B. burgdorferi* strain B31-MI-16 chromosome extending from approximately 100 bp 5′ of *ebfC* through approximately 60 bp 3′ of the gene was PCR amplified using oligonucleotide primers BATA-1 and BATA-2 (Table 1). The resulting amplicon was cloned into pCR2.1 (Invitrogen) and the insert completely sequenced. Using this plasmid clone as a template, the *ebfC* open reading frame (ORF) was amplified using either oligonucleotide primer 462-L (for the presumed lysine start site) or 462-M (for the presumed methionine start site) in conjunction with 462-R (reverse primer). Each of the two PCR products was cloned into the pET200 Champion TOPO expression vector (Invitrogen). The insert of one clone of each was completely sequenced to confirm that errors had not been introduced during the PCR or cloning processes. Recombinant proteins were produced in *Escherichia coli* BL-21 Star (pLysS) (Novagen, San Diego, CA) and overnight dual media (Zymo Research, Orange, CA). Bacteria were lysed by lysozyme treatment and sonication, debris was cleared by centrifugation, and recombinant EbfC was purified using Ni-nitrilotriacetic acid spin kits (QIAGEN). Recombinant protein quality and purity were assessed by SDS-PAGE followed by either staining with Coomassie brilliant blue or immunoblotting with horseradish peroxidase-conjugated anti-His-tag antibodies (QIAGEN) and chemiluminescence.

Electrophoretic mobility shift assays. Electrophoretic mobility shift assays (EMSAs) were performed using a biotin-labeled probe and light shift chemiluminescence (Pierce), as previously described (7). The probe used for specific binding studies was a 124-bp fragment of the *B. burgdorferi* strain B31 *erpG* 5′ noncoding DNA, PCR amplified from a recombinant plasmid template (56) using oligonucleotide primers Bio-G14A and R8 (Table 1). With the exception of competitor 1 (a nonbiotinylated version of the 124-bp *erp* probe DNA) and the P*flaB* competitor, double-stranded competitor DNAs were produced from complementary single-stranded DNAs by heating the two DNAs together and then slowly cooling them to room temperature (7). Oligonucleotides used to produce competitor DNAs are listed in Table 1, with each competitor being produced from the two oligonucleotides bearing the same number-letter designation and either "F" or "R" (e.g., c100 from 100F plus 100R, c104 from 104F plus 104R, etc.). Competitor 1 was produced in the same manner as the biotinylated *erp* probe, except with the use of nonbiotinylated oligonucleotide G14A. The P*flaB* competitor was produced by PCR from a cloned *flaB* gene using oligonucleotides FLA-6 plus FLA-7 and purified as previously described (6). All competitor DNAs were used in 100-fold excess over the labeled probe.

An internal fragment of the *B. burgdorferi* strain B31 *vlsE* gene was amplified from a plasmid clone of that gene, using oligonucleotides VLSF27-B and VLSR8, and used as a labeled EMSA probe for examination of nonspecific DNA binding by EbfC.

Size fractionation chromatography. The ability of the polyhistidine-tagged recombinant EbfC protein to form multimers was determined by gel filtration chromatography, using a Waters 600 pump and controller equipped with a Waters 996 photodiode array UV/Vis detector (Waters, Milford, MA). A Superdex 75 10/300 GL column (GE Healthcare) was prepared with a mobile phase consisting of 200 mM NaCl, 50 mM Tris-HCl (pH 7.5), and 1% (vol/vol) glycerol. The column was run with a flow rate of 0.20 ml per min. The elution of each standard was determined by monitoring the A_{280}. A calibration curve was created using an MW-GF-70 low-molecular-weight calibration kit (Sigma-Aldrich), and the void volume, V_0, was determined by injection of 200 μl of 1 mg/ml blue dextran in elution buffer with 5% glycerol. The remaining protein standards, bovine lung aprotinin (6.5 kDa), horse heart cytochrome *c* (12.4 kDa), bovine carbonic anhydrase (29 kDa), and bovine serum albumin (66 kDa), were individually prepared in elution buffer with 5% glycerol to a total concentration of 0.3 mg/ml each. The molecular-mass calibration curve was generated by plotting the log (molecular mass) versus V_0/V_e (5). A 200-μl sample of recombinant EbfC (approximately 0.2 mg/ml) was then injected and its elution compared to the established curve.

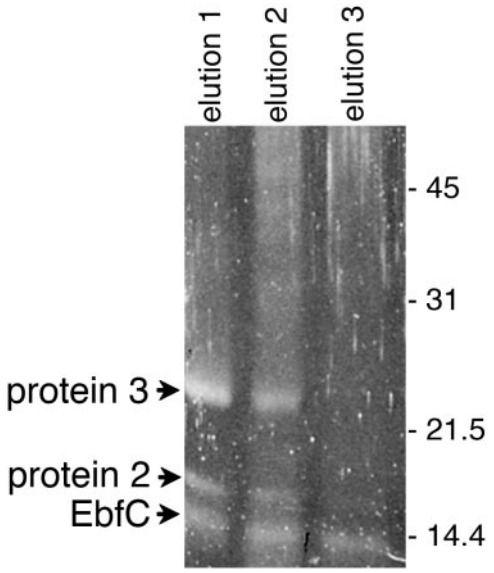


FIG. 2. *B. burgdorferi* cytoplasmic proteins purified by affinity chromatography using *erpG* 5′ noncoding DNA as bait. Proteins in elutions 1, 2, and 3 were eluted with NaCl at concentrations of 500, 750, and 1,000 mM, respectively. Proteins were separated by SDS-PAGE and visualized with SYPRO-Ruby. Numbers on the right indicate positions of molecular mass standards.

Protein cross-linking. Aliquots (10 μg) of purified recombinant EbfC in band shift buffer (10 mM HEPES [pH 8.0], 50 mM KCl, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride) (6) were incubated at room temperature for 20 min, and then formaldehyde was added to a final concentration of 2% (vol/vol) and the aliquots were incubated for an additional 20 min at room temperature. As a control, protein was also incubated without added formaldehyde. Proteins were then separated by SDS-PAGE, transferred to nitrocellulose membranes, and detected by immunoblotting using horseradish peroxidase-conjugated anti-His-tag antibodies (QIAGEN) and chemiluminescence.

RESULTS

Identification of EbfC. We previously used a fragment of the strain B31 *erpG* 5′ noncoding region in EMSAs to identify DNA sequences that specifically bound cytoplasmic proteins. A similar DNA fragment, containing all identified protein-binding regions, was therefore used as bait to fish for those proteins by DNA affinity chromatography. Biotinylated DNA was affixed to avidin-linked magnetic beads, incubated with *B. burgdorferi* cytoplasmic extract, and washed extensively, and then bound proteins were eluted. This protocol yielded three proteins which eluted under high-salt-concentration wash conditions, having molecular masses of approximately 11, 16, and 25 kDa (Fig. 2). Eluted proteins were separated by SDS-PAGE, extracted from gels, and analyzed by matrix-assisted laser desorption ionization–time of flight (mass spectrometry). Results were compared with the determined genome sequence of strain B31 to provide potential identities to the *erp* operator-binding proteins. The 11-kDa protein was further characterized and designated as "EbfC" (*erp-b*inding *f*actor, *c*hromo-

FIG. 3. Alignment of the predicted amino acid sequences of *B. burgdorferi* EbfC and a homologous protein encoded by *H. influenzae*. Identical amino acids found in both proteins are boxed in black and similar residues in gray. The three-dimensional structure of the *H. influenzae* protein has been determined, but it has not been otherwise characterized (38).

somal), as described below. The other two purified proteins have yet to be fully characterized.

Four peptide fragments of the 11-kDa protein matched the predicted amino acid sequence of a chromosomal ORF of strain B31, providing 30% coverage (data not shown). This ORF is annotated as ORF BB0462 in the strain B31 genome database and ORF BG0475 in the sequence database of the related Lyme disease spirochete *Borrelia garinii* strain PBi (25, 27). Intriguingly, many other bacteria encode orthologs of this protein, which have been grouped as "domain of unknown function" (DUF)-149, Pfam 2575, COG-0718, "YbaB"-like, and "YaaK"-like proteins (40). None of those related proteins appear to have been functionally defined prior to the current work. Serendipitously, the *Haemophilus influenzae* ortholog (Fig. 3) was crystallized and its three-dimensional structure solved by the University of Maryland Center for Advanced Research in Biotechnology as part of their "Structure to Function" project to solve structures of otherwise uncharacterized proteins (38) (http://s2f.umbi.umd.edu). The *H. influenzae* DUF-149 family member forms a homodimer containing a pair of protruding, parallel alpha helices that form a "pincerlike" shape. The gap between the two "pincer" arms is approximately the same width as the diameter of a DNA double helix, although the recombinant *H. influenzae* protein did not bind DNA nonspecifically (38). To the best of our knowledge, no further information on that protein or any other EbfC homolog has been published.

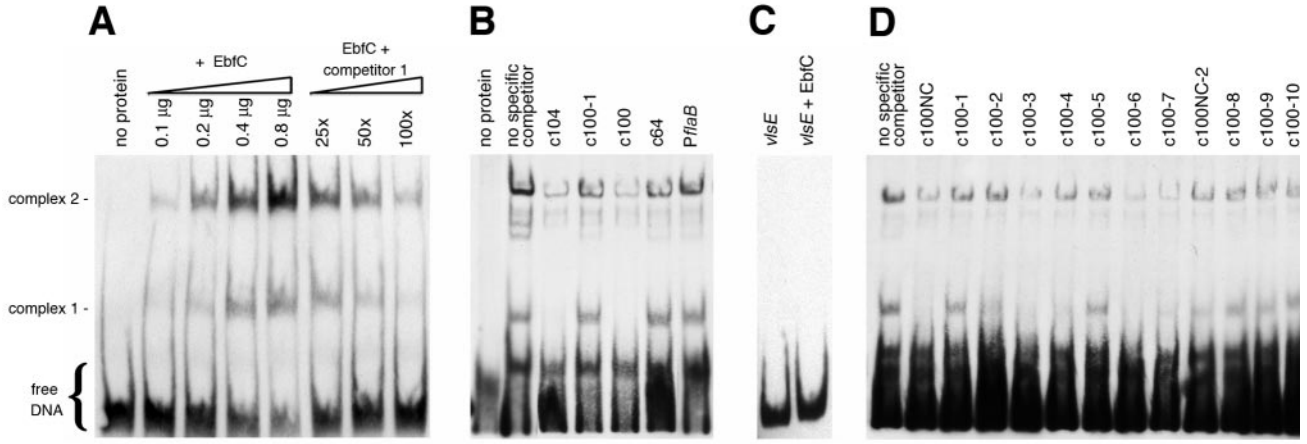
We next assessed whether the *B. burgdorferi* EbfC protein could specifically bind *erp* operator DNA. Our examination of the *B. burgdorferi* strain B31 chromosome sequence suggested a 99-codon *ebfC* gene that could encode an 11.0-kDa protein, initiating with an AUG methionine codon and preceded by a near-consensus GGAGGGA Shine-Delgarno sequence. Use of that translational initiation site yields an amino-terminal protein sequence comparable to those of EbfC homologs of other bacterial species (Fig. 3 and data not shown). However, annotation of strain B31 by the Institute for Genomic Research proposed a sequence with a leucine initiation codon 11 residues further 5′, with a very weak AGA Shine-Delgarno sequence, encoding a 12.5-kDa protein (25). The Institute for Genomic Research annotation did not provide a rationale for suggesting use of that weak translation initiation site. Two recombinant, N-terminal polyhistidine-tagged proteins were produced from strain B31 DNA, one beginning at the methionine codon and a second, longer protein extending from the leucine codon. Both purified proteins were analyzed by EMSA, and both bound comparably well to *erpG* operator/promoter DNA (Fig. 4 and data not shown), suggesting that the residues between the postulated leucine and the first methionine do not contribute to DNA binding. The three-dimensional structure of the *H. influenzae* EbfC homolog places the protein's amino terminus at the end of an alpha helix, apart from the remainder of the protein and distal to the proposed DNA-binding site (38). These data, together with the DNA sequence comparisons described above, suggest that the methionine is the most likely initiation amino acid of native EbfC. The shorter recombinant protein was used for all further studies of EbfC function.

EMSA using purified EbfC yielded two specific DNA-protein complexes (Fig. 4). EMSA signals from both complexes increased in intensity with the addition of increasing concentrations of EbfC (Fig. 4A). Similarly, EMSA signals of both complexes diminished as increasing levels of unlabeled, specific competitor were added (Fig. 4A). This may have been due to the presence of two identical, adjacent EbfC-binding sites in the *erp* operator DNA (see below). Alternatively, DNA-EbfC complexes may form higher-ordered structures, as suggested by the ability of EbfC to form homotetramers in solution (see below). The more slowly migrating complex 2 appeared to represent a somewhat higher affinity DNA-protein complex than did complex 1.

Competition analyses using 100-fold excesses of unrelated DNAs, including the 5′ noncoding region of the constitutively expressed *B. burgdorferi flaB* gene, indicated that EbfC binding to the *erp* operator DNA was sequence specific (Fig. 4B). EMSA using a labeled 184-bp fragment of the *B. burgdorferi vlsE* gene as the probe did not detect any binding by EbfC, further indicating the specificity of that DNA-binding protein (Fig. 4C). As additional controls, EMSAs were performed using cellular extracts from *E. coli*, but an electrophoretic mobility shift was not observed in those studies (data not shown), demonstrating that it was the recombinant proteins and not potential contaminating *E. coli* proteins that were responsible for the mobility shifts.

EbfC forms dimers and higher-ordered multimers. Since the EbfC homolog produced by *H. influenzae* formed a dimer when crystallized (38), we examined the *B. burgdorferi* EbfC for its ability to multimerize. To that end, purified recombinant EbfC protein was applied to a size fractionation column, and apparent molecular mass was determined. While a proportion of EbfC eluted with an apparent molecular mass of 13.8 kDa (corresponding with the calculated mass of EbfC plus the fusion partner polypeptide), additional peaks were obtained having apparent masses of 26.9 and 64.1 kDa, corresponding with the calculated masses of EbfC dimers and tetramers (Fig. 5A).

Multimerization of EbfC was also examined by incubation of purified recombinant protein with formaldehyde, which causes cross-linking between closely associated proteins (36), followed by separation by SDS-PAGE. In addition to the EbfC monomer, a band having the size of a dimer was detected (Fig. 5B). Both those protein bands were of comparable intensity, indicating a strong propensity of EbfC to dimerize in solution.



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competitor name	sequence (5′ to 3′)	competition?
c-104	TGTTAAAA**TGTAACA**GCTGAA**TGTAACA**AAA	+
c-100	TAAAA**TGTAACA**GCTGAA**TGTAACA**AAATTATAT	+
c-100NC	AAA**TGTAACA**GCTGAA**TGTAACA**AAAT	+
c-100-1	A**TGTAACA**GCTGAA**TGTA**	-
c-100-2	GAA**TGTAACA**AAATTATAT	-
c-100-3	AAAcac**AACA**GCTGAA**TGTAACA**AAAT	+
c-100-4	AAA**TGTAACA**atcagg**TGTAACA**AAAT	+
c-100-5	AAA**TGTAACA**GCTGAAcac**AACA**AAAT	-
c-100-6	AAA**TGTAACA**aaatAA**TGTAACA**AAAT	+
c-100-7	AAA**TGTAACA**GCTGAA**TGTAACA**gctg	+
c-100NC-2	GAA**TGTAACA**AAAT	-
c-100-8	GAA**TGTAACA**gctg	-
c-100-9	Gcc**TGTAACA**AAAT	-
c-100-10	GAA**TGTAACA**gctgTATA	-

site II site I

FIG. 4. EMSA using a labeled 124-bp fragment of *erpG* 5′ noncoding DNA, recombinant EbfC protein, and various double-stranded DNA competitors. DNA alone is labeled "free." Addition of EbfC resulted in two major protein-DNA complexes. Additional complexes are also visible, possibly due to EbfC-mediated aggregation of DNA (see the text). (A) Effects of addition of increasing concentrations of EbfC or unlabeled competitor on formation of EbfC-DNA complexes. Second through fifth lanes, addition of 0.1, 0.2, 0.4, or 0.8 μg EbfC to labeled *erpG* promoter/operator DNA; sixth through eighth lanes, labeled *erpG* DNA plus 0.8 μg EbfC plus 25-fold, 50-fold, or 100-fold excesses, respectively, of unlabeled DNA competitor 1 (the same 124 bp of *erpG* promoter/operator DNA). (B) Competition studies using large DNAs spanning EbfC-binding sites I and II (c104 and c100) or DNAs lacking those two sites (c100-1 and c64). The promoter region of the constitutively expressed *B. burgdorferi flaB* gene was included as a competitor for nonspecific protein binding. (C) Analysis of a 184-bp labeled fragment of *B. burgdorferi vlsE*, without and with added EbfC (0.8 μg), further demonstrating the DNA specificity of EbfC binding. (D) Competition studies using smaller DNAs containing either wild-type EbfC-binding sites or mutants thereof. (E) Sequences of DNA competitors used in the EMSAs shown in panels B and D. Consensus EbfC-binding sites I and II are illustrated in boldface type and are underlined. Nucleotides within competitors that differ from those of the *erpG* promoter/operator are indicated by lowercase letters. The ability of a competitor to prevent EbfC binding to the biotinylated probe was scored as plus (inhibition of complex 1 and >50% inhibition of complex 2) or minus (no detectable inhibition of either complex).

Furthermore, a significant protein band of a size corresponding with 4 EbfC subunits was also observed.

Identification of EbfC-binding DNA sequences. A variety of unlabeled, double-stranded DNAs were next used as EMSA competitors to identify DNA sequences capable of binding EbfC. For each competitor, two complementary oligonucleotides were synthesized and then annealed together, allowing us to efficiently test various fragments of *erp* 5′ DNA and mutants thereof. A 100-fold excess of each competitor DNA was used for each analysis.

Both the 31-bp c104 and the 34-bp c100 DNAs effectively competed away EbfC from the labeled *erp* operator DNA probe (Fig. 4B and D). A shorter, 27-bp DNA fragment, c100NC, consisting of a sequence contained within c100, also competed away EbfC binding (Fig. 4D). All three competitor DNAs completely inhibited formation of complex 1 and reduced complex 2 formation by greater than 50%. These data indicate that a specific EbfC-binding site(s) is located within the sequences shared by c100, c100NC, and c104. This DNA is within the maximum boundaries of *erp* operator 2 that we previously mapped through use of EMSA with larger DNA competitors and transcriptional fusions to a reporter gene (7). Deletion of *erp* operator 2 resulted in constitutive expression, indicating that a DNA sequence(s) within that region is necessary for regulation of transcription (7).

The ability of EbfC to form dimers and the known structure of the homologous *H. influenzae* protein provided important clues

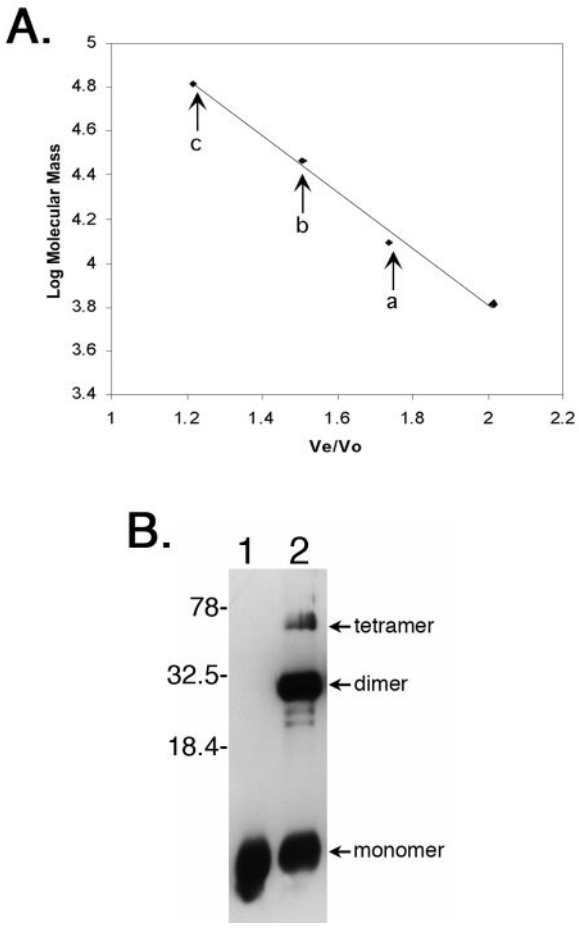

FIG. 5. EbfC forms dimers and higher-ordered multimers in solution. (A) Size fractionation analysis of recombinant EbfC, with arrows denoting V_e/V_0 values of three 280-nm-absorbing peaks corresponding to monomer (a), dimer (b), and tetramer (c) forms of the protein, having apparent molecular masses of 13,800, 26,900, and 64,100 Da, respectively. Diamonds indicate elution positions of molecular mass standards, left to right: bovine serum albumin (66,000 Da), bovine carbonic anhydrase (29,000 Da), horse heart cytochrome *c* (12,400 Da), and bovine lung aprotinin (6,500 Da). (B) Cross-linking of purified recombinant EbfC in solution. Lane 1, no formaldehyde cross-linking agent added; lane 2, protein incubated with formaldehyde. Protein bands with molecular masses corresponding to monomeric, dimeric, and tetrameric EbfC are indicated. Note that the recombinant protein is larger than wild-type EbfC due to the inclusion of the N-linked polyhistidine tag and linker residues (approximately 14 kDa versus 11 kDa for native EbfC). Numbers on the right indicate positions of molecular mass standards.

for the further characterization of the *B. burgdorferi* EbfC-binding site. Homodimers generally interact with palindromic DNA sequences, with each subunit binding one side of the palindrome. Two different palindromes are contained within the sequence of competitor c-100NC: CAGCTG and two copies of TGT(A/T)ACA (Fig. 4E). The CAGCTG sequence within c-100NC was mutated to either CAATCA (c100-4) or CAAAAT (c100-6), but both still effectively competed for EbfC binding, indicating that the CAGCTG palindrome is not directly involved in EbfC interactions (Fig. 4).

Mutation of the 3′ TGTaACA sequence to CACAACA in c100-5 prevented competition (Fig. 4). In contrast, that same mutation of the identical 5′ TGTAACA palindrome in c100-3 did not detectably influence EbfC competition. These results indicate that the TGT(A/T)ACA palindrome is involved in the binding of EbfC, although flanking DNA is also important. We designated the 3′ TGTAACA sequence of the *erp* operator as site I and the 5′ sequence as site II (Fig. 1 and 4). Alterations of the sequences immediately 5′ or 3′ of site I on competitors c100-4 and c100-7, respectively, did not perceptibly affect EbfC binding, indicating that specific flanking DNA sequences are not required for interactions with EbfC. DNAs that contained 4 or more base pairs on either side of the palindrome effectively competed for EbfC binding, while those palindromes with only 3 flanking base pairs did not compete (Fig. 4). Together, these data suggest that efficient EbfC binding requires a TGTaACA sequence bordered by at least 4 nonspecific bases. Those additional bases may be important for correct formation of the DNA double helix in the competitor or may nonspecifically interact with the EbfC protein.

All *erp* loci from every examined strain of *B. burgdorferi* contain an absolutely conserved EbfC site I (Fig. 1), suggesting that this sequence is critical to the bacterium and/or the cp32 prophage. On the other hand, considerable variation was observed among site II sequences, with several loci containing sequences in that location that our data predict to be unable to efficiently bind EbfC.

Searches of the *B. burgdorferi* strain B31 genome indicated that the palindrome TGT(A/T)ACA occurs only 4 to 7 times in each cp32 element and 92 times on the chromosome, a frequency of approximately 1 site every 5 to 10 kb. Other than the two palindromes described above, there are no further copies of the sequence within several kilobases of *erp* loci. *erp* operator 2 is the only locus in the *B. burgdorferi* strain B31 genome in which two EbfC-binding palindromes were found in close proximity.

Additional borrelial proteins bind *erp* operator 2. In a previous study, we demonstrated that one or more proteins in *B. burgdorferi* cell extracts bind to *erp* operator 2 DNA (7). Moreover, EMSAs revealed a strong DNA-protein complex whose intensity was inversely proportional to the levels of *erp* transcription (7), suggesting that the observed complex represented a transcriptional repression mechanism. Comparing EMSAs of labeled *erp* operator DNA incubated with either *B. burgdorferi* cell lysates or purified recombinant EbfC revealed different band shift patterns for each (Fig. 6). Noting that the *B. burgdorferi* cell extract includes EbfC (Fig. 2), we conclude that at least one other borrelial protein binds *erp* operator 2 DNA to repress *erp* transcription levels.

DISCUSSION

All natural isolates of the Lyme disease spirochete contain multiple cp32 elements, each of which carries an *erp* locus. Two DNA regions immediately 5′ of *erp* promoters specifically bind *B. burgdorferi* cytoplasmic proteins, with operator 2 being involved in transcriptional regulation. We have now identified a novel DNA-binding protein, EbfC, as binding to operator 2, interacting with the interrupted palindromic sequence TGT(A/T)ACA. EbfC forms dimers in solution, consistent with its recognition of an inverted-repeat DNA sequence. Additional studies of EbfC, including creation of *B. burgdorferi ebfC* mu-

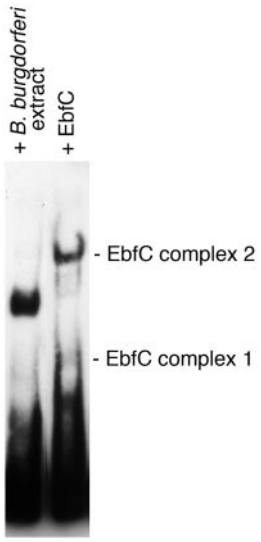

FIG. 6. EMSAs of *B. burgdorferi* cell extract and purified recombinant EbfC binding to labeled *erp* operator DNA. Although the bacterial extract contains EbfC (Fig. 2), it appears that at least one additional protein contributes to the DNA-protein complex observed in the left lane.

tants and bacteria carrying *erp* loci with specifically mutated EbfC-binding sites, are currently under way to precisely elucidate the function(s) of this chromosomally encoded protein.

Our previous studies that mapped the maximum boundaries of *erp* operator 2 utilized large (>100-bp) EMSA competitor DNAs and deletion mutations spanning 100 or more bp (7). For this reason, the actual operator 2 region may be much smaller than illustrated in Fig. 1. The two EbfC-binding sites are located within the determined maximum boundaries of *erp* operator 2. In the present studies, binding of purified EbfC to *erp* operator 2 was effectively blocked with the 31-bp competitor c104, while our earlier experiments with this competitor found that it did not effectively compete for binding of proteins from total *B. burgdorferi* cytoplasmic extracts (7). This may mean that the wild-type protein has a greater affinity for long stretches of DNA than does the recombinant fusion protein and cannot be as effectively competed away with short linear DNA fragments. Alternatively, additional cytoplasmic factors may interact with EbfC to facilitate binding of a larger DNA region which cannot be competed with small DNAs such as c104. Comparisons of EMSA results using purified EbfC and *B. burgdorferi* cell lysates support the hypothesis that additional borrelial proteins also bind *erp* operator 2. Our DNA affinity purification techniques identified two other proteins that bind *erp* 5′ noncoding DNA, one or both of which are likely to be the hypothesized additional protein. We are continuing to characterize these as yet undefined proteins to determine the roles they and EbfC play in control of *erp* gene expression.

As mentioned above, there is a significant body of evidence demonstrating that the cp32 elements are in fact bacteriophage genomes (12, 15, 19, 20, 57, 62). While relapsing-fever spirochetes such as *Borrelia hermsii* also contain cp32 elements similar to those of *B. burgdorferi*, the *B. hermsii* cp32s lack *erp* loci, yet every single identified cp32 from a Lyme disease spirochete contains such a locus (51, 54, 55, 57). This suggests that *B. burgdorferi* cp32 bacteriophages have acquired and maintain *erp* genes for reasons specific to that host bacterium. The role of some Erp proteins in the binding of vertebrate factor H and subsequent protection from complement-mediated killing illustrates one reason for the phages to carry these genes, since the Erp proteins' contribution to survival of the bacterium also enhances the probability of phage survival. Bacteriophages of many other bacterial species have also acquired such "moron" DNAs, including the serum-resistance-encoding *bor* gene of *E. coli* phage lambda and the toxin-producing genes of *Vibrio cholerae* (9, 10, 30, 31, 59, 60). It is intriguing to note that the *B. burgdorferi* cp32 elements have evolved to use a DNA-binding protein encoded by the bacterial host chromosome, possibly tapping into a bacterial regulatory network for their own benefit. Some phages of other bacterial species use host regulatory machinery to control viral gene expression (13, 59). Alternatively, since bacterial DNAs are constrained into highly organized structures by DNA-binding proteins (58), it is possible that EbfC is used to mold both *B. burgdorferi* genetic material and the cp32 prophage genomes. The ability of EbfC to form homomultimers supports such a hypothetical role in DNA topological modulation. Further exploration of the interrelationships between *B. burgdorferi*, cp32s, *erp* genes, and EbfC will undoubtedly shed additional light not only on *B. burgdorferi* pathogenesis but on the interplay between bacteria and phages in general.

A broad spectrum of other bacterial species contain genes homologous to *B. burgdorferi ebfC*, but surprisingly little is known about either those genes or their encoded proteins. Most information has stemmed from characterization of neighboring genes. A physical linkage of *dnaX* immediately 5′ and *recR* immediately 3′ of the *ebfC* homolog is frequently found, leading to speculation that EbfC homologs may be involved in either DNA replication or repair, although those possibilities appear never to have been tested. Arguing against that circumstantial evidence, *ebfC* homologs are not always found adjacent to either *dnaX* or *recR*: *B. burgdorferi ebfC* is bordered to the 3′ by a probable *ndk* gene, and this bacterium does not even contain a homolog of *recR* (25), while the *H. influenzae* homolog is flanked on the 5′ side by genes involved with DNA uptake (17). Studied organisms both cotranscribe *dnaX* and the *ebfC* homolog and transcribe the second gene alone from promoters located within the *dnaX* open reading frame, thereby allowing independent expression of the EbfC homolog. There are at least two separate promoters within the *E. coli dnaX* gene that independently drive transcription of its *ebfC* homolog, one of which is controlled by the stress response sigma factor σ^E, while *Corynebacterium glutamicum* regulates a promoter within *dnaX* as part of the ClgR regulon (14, 23, 24, 39, 49, 61). Of relevance to those observations, *B. burgdorferi* lacks homologs of both σ^E and ClgR (25). Mutants of *H. influenzae* and *Streptomyces coelicolor* with deleted *ebfC* homologs are viable (1, 17, 47), raising our hopes that Δ*ebfC* mutants of *B. burgdorferi* can also be obtained and the effects of that lesion can be studied. Our studies were the first to determine a function for an EbfC family member, that of a site-specific DNA-binding protein, placing the spirochete *B. burgdorferi* in the unusual position of being the model organism for studying this widely dispersed but barely characterized protein.

In conclusion, we purified and characterized a novel, chro-

mosomally encoded DNA-binding protein of *B. burgdorferi*. The location of *ebfC* on the *B. burgdorferi* main chromosome suggests that the EbfC protein performs bacterial host functions in addition to those postulated for the cp32 prophages. Competition binding analyses demonstrated that EbfC binds the broken palindrome TGT(A/T)ACA. The EbfC-binding sites of *erp* loci are within the previously mapped maximum boundaries of operator 2, which is required for regulation of *erp* transcription (7). Two closely linked TGTAACA sequences are found within the operator, one of which is absolutely conserved in every *erp* locus yet examined. This localization suggests that EbfC may play a role in the regulation of *erp* transcription. Alternatively, EbfC may affect DNA conformation of the cp32 prophages and the bacterial host's genome. Homologs of EbfC are encoded by a wide range of other bacterial species, where they presumably also function as site-specific DNA-binding proteins. Continued characterization of EbfC and its effects on cp32 elements and the *erp* and other *B. burgdorferi* genes will continue to provide insight into the biology of the Lyme disease spirochete as well as many other medically and environmentally important bacteria.

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