

## Regulation of OspE-Related, OspF-Related, and Elp Lipoproteins of *Borrelia burgdorferi* Strain 297 by Mammalian Host-Specific Signals

P. SCOTT HEFTY,<sup>1</sup> SARAH E. JOLLIFF,<sup>1</sup> MELISSA J. CAIMANO,<sup>2</sup> STEPHEN K. WIKEL,<sup>3</sup>  
JUSTIN D. RADOLF,<sup>4,5</sup> AND DARRIN R. AKINS<sup>1\*</sup>

*Department of Microbiology and Immunology, The University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104,<sup>1</sup> and Departments of Pathology,<sup>2</sup> Physiology,<sup>3</sup> Medicine,<sup>4</sup> and Genetics and Developmental Biology,<sup>5</sup> Center for Microbial Pathogenesis, University of Connecticut Health Center, Farmington, Connecticut 06030*

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In previous studies we have characterized the cp32/18 loci in *Borrelia burgdorferi* 297 which encode OspE and OspF orthologs and a third group of lipoproteins which possess OspE/F-like leader peptides (Elps). To further these studies, we have comprehensively analyzed their patterns of expression throughout the borrelial enzootic cycle. Serial dilution reverse transcription-PCR analysis indicated that although a shift in temperature from 23 to 37°C induced transcription for all nine genes analyzed, this effect was often markedly enhanced in mammalian host-adapted organisms cultivated within dialysis membrane chambers (DMCs) implanted within the peritoneal cavities of rats. Indirect immunofluorescence assays performed on temperature-shifted, in vitro-cultivated spirochetes and organisms in the midguts of unfed and fed ticks revealed distinct expression profiles for many of the OspE-related, OspF-related, and Elp proteins. Other than BbK2.10 and ElpA1, all were expressed by temperature-shifted organisms, while only OspE, ElpB1, OspF, and BbK2.11 were expressed in the midguts of fed ticks. Additionally, although mRNA was detected for all nine lipoprotein-encoding genes, two of these proteins (BbK2.10 and ElpA1) were not expressed by spirochetes cultivated in vitro, within DMCs, or by spirochetes within tick midguts. However, the observation that *B. burgdorferi*-infected mice generated specific antibodies against BbK2.10 and ElpA1 indicated that these antigens are expressed only in the mammalian host and that a form of posttranscriptional regulation is involved. Analysis of the upstream regions of these genes revealed several differences between their promoter regions, the majority of which were found in the –10 and –35 hexamers and the spacer regions between them. Also, rather than undergoing simultaneous upregulation during tick feeding, these genes and the corresponding lipoproteins appear to be subject to progressive recruitment or enhancement of expression as *B. burgdorferi* is transmitted from its tick vector to the mammalian host. These findings underscore the potential relevance of these molecules to the pathogenic events of early Lyme disease.

Lyme disease, the most common arthropod-borne infection in the United States, is a multisystem disorder caused by spirochetes of the *Borrelia burgdorferi* sensu lato complex (7, 30). *B. burgdorferi* is maintained in nature through a complex enzootic cycle involving *Ixodes* ticks and small mammalian hosts, typically rodents (26). An interesting feature of this pathogen, which distinguishes it from most other prokaryotes, is its complex genome. The borrelial genome is comprised of a small, linear chromosome of approximately 1 Mb and a variable number of linear and circular extrachromosomal DNA elements (plasmids), as many as 21 in some instances (9, 18). Several lines of investigation have indicated that the numerous plasmids harbored by this organism are essential for *B. burgdorferi* virulence (6, 31, 35, 49, 52). Consequently, identification of plasmid-encoded virulence determinants has become a major objective in the Lyme disease field.

Among the borrelial plasmids are the well-characterized 32- and 18-kb circular plasmids (designated cp32/18) (2–4, 8, 10, 42–44). Prior studies have revealed that single strains can har-

bor as many as nine different cp32/18 plasmids and that they are largely homologous (reviewed in reference 44). However, despite this sequence conservation, three regions of hypervariability have been identified. These are comprised of the putative plasmid maintenance locus and two loci which encode differentially expressed lipoproteins (44, 46, 51). We have focused our studies on the cp32/18 hypervariable regions which encode orthologs of outer surface protein E (OspE) and OspF (2–4, 8). In the B31 strain these orthologs have been designated Erps (OspE/F-related proteins) (10, 43). Our analysis of *B. burgdorferi* strain 297, however, has demonstrated that these polypeptides fall into three evolutionarily distinct groups: (i) OspE-related orthologs, (ii) OspF-related orthologs, and (iii) Elps, which contain OspE/F-like leader peptides but are otherwise unrelated to both OspE and OspF (3).

Prior studies with strain B31 have shown that the Erps are all regulated by temperature (41). However, we and others have shown in different strains that other factors also appear to be important in the overall regulation of these proteins (2, 4, 13, 45). For example, Fikrig and coworkers have characterized the OspE homolog p21 from strain N40 and found that it is expressed only during mammalian infection (13, 45), while the OspF homolog designated pG from strain ZS7 also is expressed exclusively in the mammalian host environment (47).

\* Corresponding author. Mailing address: Department of Microbiology and Immunology, The University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104. Phone: (405) 271-8668. Fax: (405) 271-3117. E-mail: darrin-akins@ouhsc.edu.

TABLE 1. Oligonucleotide primers used in this study

Designation	Sequence <sup>a</sup>	Description <sup>b</sup>
<i>ospA</i> -1	5'-ATGAAAAAATATTATTGGGAATAG-3'	PCR; bases 1-25 (+strand)
<i>ospA</i> -819	5'-TTTAAAGCGTTTTAATTTTCATCA-3'	PCR; bases 795-819 (-strand)
<i>ospC</i> -1	5'-TGTAATAATTCAGGGAAAGATGGGA-3'	PCR; bases 1-25 (+strand)
<i>ospC</i> -576	5'-AGGTTTTTTTGGACTTTCTGCCACA-3'	PCR; bases 552-576 (-strand)
<i>flaB</i> -453	5'-AGAGCTTGGAAATGCAGCCT-3'	PCR; bases 453-462 (+strand)
<i>flaB</i> -993	5'-GGGAACCTTGATTAGCCTCGC-3'	PCR; bases 973-993 (-strand)
<i>ospE</i> -78	5'-TGATGGGCAAAGTAATGGAGAGG-3'	PCR; bases 78-100 (+strand)
<i>ospE</i> -282	5'-AAAGAATGTAGCGGTGTATCCTGC-3'	PCR; bases 259-282 (-strand)
<i>elpB1</i> -630	5'-AGAGGCAGAAAAAAGGCAAGTTG-3'	PCR; bases 630-652 (+strand)
<i>elpB1</i> -832	5'-CATCTTCCAAATCCCCCAAG-3'	PCR; bases 812-832 (-strand)
<i>p21</i> -81	5'-TGAGCAAAGTAGTGGTGAGAT-3'	PCR; bases 81-101 (+strand)
<i>p21</i> -300	5'-TGAAAAGAATGTAGCCGAATGTCC-3'	PCR; bases 277-300 (-strand)
<i>elpB2</i> -848	5'-TTAGCGTAGGAGCAGAAGAAGTTAGAG-3'	PCR; bases 848-874 (+strand)
<i>elpB2</i> -948	5'-ATCTCCACCACCCCAAGTTTGGC-3'	PCR; bases 924-948 (-strand)
<i>ospF</i> -116	5'-CAGAACAAAATGTAAAAAACAAGCAAG-3'	PCR; bases 116-145 (+strand)
<i>ospF</i> -521	5'-CCCAAACATTATAGCACACTGCCAAG-3'	PCR; bases 527-521 (-strand)
<i>bbk2.10</i> -130	5'-TGCAAGAATTATGCAAGTGGTGAAG-3'	PCR; bases 130-155 (+strand)
<i>bbk2.10</i> -253	5'-GTCTTCTGTATTTTGTGGCTCTGCT-3'	PCR; bases 232-253 (-strand)
<i>bbk2.11</i> -198	5'-ATCTAAATTAGATGAAAAAGATACAAAAG-3'	PCR; bases 198-226 (+strand)
<i>bbk2.11</i> -682	5'-CTTTAGATTCTTTCTCTAAC-3'	PCR; bases 663-682 (-strand)
<i>elpA1</i> -272	5'-CAGCAAAGGATAAAGAAGATAATAAACCAAG-3'	PCR; bases 272-301 (+strand)
<i>elpA1</i> -847	5'-AGTCACCCCACTCAGAATAAATAG-3'	PCR; bases 824-847 (-strand)
<i>elpA2</i> -281	5'-ATGCTACAGTATTAACCCCGAG-3'	PCR; bases 281-303 (+strand)
<i>elpA2</i> -725	5'-GCACCCGTAACCCCTATCTTCAAC-3'	PCR; bases 703-725 (-strand)
<i>p21</i> -TL(266)	5'-TAAACGCTGGGGACATTCG-3'	Linkage analysis; bases 266-285 (+strand)
<i>elpB2</i> -TL(275)	5'-CCACTTTCTGACAATACTGATGGAGG-3'	Linkage analysis; bases 250-275 (-strand)
<i>ospE</i> -TL(248)	5'-CTGATACATTCGCAGGATACACCGC-3'	Linkage analysis; bases 248-272 (+strand)
<i>elpB1</i> -TL(61)	5'-AATCTTTCGAAGAGCTTATCAGTGC-3'	Linkage analysis; bases 37-61 (-strand)
<i>elpA1</i> -BamH1	5'-CCC <b>GGATCCT</b> GTAAATATTATTCAACGAATAAAG-3'	Cloning; bases 58-82 (+strand)
<i>elpA1</i> -Xho1	5'-GG <b>CTCGAG</b> TTAATTAATTTCCCTGGTCTTCTAG-3'	Cloning; bases 889-909 (-strand)
<i>elpA2</i> -BamH1	5'-CCC <b>GGATCCT</b> GTAAAGTATTATGCAAGGGATAAAG-3'	Cloning; bases 58-82 (+strand)
<i>elpA2</i> -Xho1	5'-GG <b>CTCGAG</b> CTACTATTACAAATCAACATCATCCG-3'	Cloning; bases 1037-1056 (-strand)
<i>elpB1</i> -EcoR1	5'-GCG <b>GAATTC</b> CTTGCAAGAATTATGCAAGTAGTGAA-3'	Cloning; bases 50-75 (+strand)
<i>elpB1</i> -Xho1	5'-GCG <b>CTCGAG</b> TTAATTAAGTTATATCTAAAATATCTTCTTC-3'	Cloning; bases 1102-1125 (-strand)
<i>elpB2</i> -BamH1	5'-GCC <b>GGATCCT</b> GCAGAATTTTACAACACTAGTAAAG-3'	Cloning; bases 52-76 (+strand)
<i>elpB2</i> -Xho1	5'-GCC <b>CTCGAG</b> TTAATATAAATTAATCTCTCGATG-3'	Cloning; bases 1197-1218 (-strand)
<i>bbk2.10</i> -PE	5'-CCACCAACCAACTCTTCTTTTTTTGCC-3'	Primer extension; bases 126-152 (-strand)

<sup>a</sup> Restriction enzyme sites added to oligonucleotides for cloning are indicated in bold.

<sup>b</sup> +strand, positive strand; -strand, negative strand.

We also reported that an *OspF* homolog (designated BbK2.10) in strain 297 is expressed only during mammalian infection (4). To begin elucidating the potential role(s) of these lipoproteins in disease pathogenesis, we characterized the expression patterns of the cp32/18-encoded *OspE*-related, *OspF*-related, and *Elp* lipoproteins in strain 297 throughout the borrelial enzootic cycle. The combined data clearly demonstrate that factors in the tick and/or mammalian host environment, other than temperature, are critical for regulating expression of the *ospE*-related, *ospF*-related, and *elp* genes and that two of these genes are regulated posttranscriptionally. Rather than undergoing simultaneous upregulation during tick feeding, these genes and the corresponding lipoproteins appear to be subject to progressive recruitment or enhancement of expression as *B. burgdorferi* is transmitted from its tick vector to the mammalian host. These findings underscore the potential relevance of these molecules to the pathogenic events of early Lyme disease.

#### MATERIALS AND METHODS

**Bacterial strains.** Virulent *B. burgdorferi* strain 297, originally isolated from the cerebrospinal fluid of a Lyme disease patient (40), was resurrected from frozen stocks in BSK-H medium supplemented with 6% rabbit serum (Sigma

Chemical Co., St. Louis, Mo.). Spirochetes were cultivated in vitro for no more than three serial passages before experiments were performed. For temperature shift experiments, organisms were cultivated at 23°C to mid-logarithmic phase (approximately  $5 \times 10^6$  to  $1 \times 10^7$  organisms per ml), and  $10^3$  spirochetes per ml were used to seed BSK-H medium prewarmed to 37°C. Both 23°C and temperature-shifted cultures were allowed to grow until cell densities reached approximately  $10^8$  organisms per ml for subsequent RNA and protein analyses. To obtain *B. burgdorferi* in a mammalian host-adapted state, organisms were cultivated in dialysis membrane chambers (DMCs) implanted into rat peritoneal cavities as described previously (2). PCR amplification using primers specific for each of the nine different *ospE*-related, *ospF*-related, and *elp* genes (Table 1) was used to ensure that all cultures contained the seven different plasmids which harbor these loci (Fig. 1) before experimental manipulations were performed. Electrocompetent *Escherichia coli* strain DH5 $\alpha$  (Gibco/BRL Life Technologies, Gaithersburg, Md.) was used for all transformations; all clones and transformants were grown using tryptone-yeast agar or broth supplemented with the appropriate antibiotic.

**Transcriptional linkage analysis of the *ospE/elpB1* and *p21/elpB2* loci.** RNA was isolated from mid-logarithmic-phase *B. burgdorferi* strain 297 cultures grown in BSK-H medium at 23°C or shifted from 23 to 37°C or from spirochetes cultivated within DMCs using Tri-Reagent as specified by the manufacturer (Molecular Research Center Inc., Cincinnati, Ohio). Fifteen micrograms of total RNA was then treated with 10 U of RQ1 RNase-free DNase (Promega, Madison, Wis.) for 1 h at 37°C, followed by a 30-min incubation at 70°C to inactivate the DNase. The final RNA preparations were then precipitated with 100% ice-cold ethanol, resuspended in 50  $\mu$ l of diethyl pyrocarbonate-treated water, and subsequently used for reverse transcriptase reactions to produce cDNA.

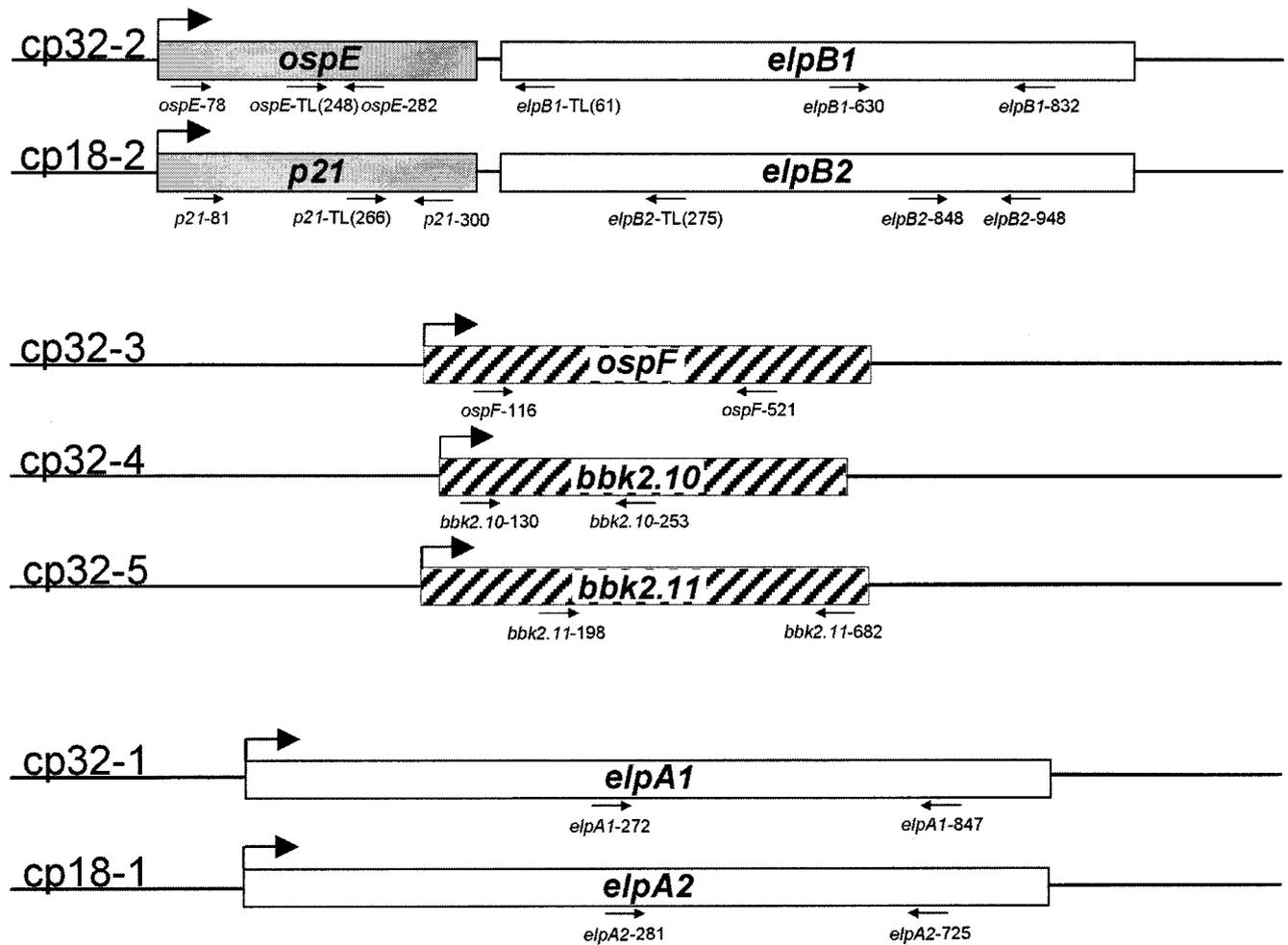


FIG. 1. Schematic representation of the *ospE*-related, *ospF*-related, and *elp* genes characterized in *B. burgdorferi* strain 297. Evolutionarily related genes are shaded similarly. Below each gene, the primers used for the serial dilution RT-PCR and transcriptional linkage analyses are indicated. The cp32 or cp18 plasmid which harbors each locus is indicated at left, and the direction of transcription for each gene or operon is indicated by an arrow.

cDNAs were generated using 2  $\mu$ g of total RNA with 1  $\mu$ M random hexamers, 5 U of SuperScript II reverse transcriptase, 5 mM dithiothreitol, and 1 mM deoxynucleoside triphosphates in a final volume of 20  $\mu$ l, as specified by the manufacturer (Gibco/BRL Life Technologies). Sixty nanograms of each cDNA was then amplified for 45 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s, followed by a final extension step for 7 min at 72°C, using the *ospE/elpB1* and *p21/elpB2* primers described in Table 1. The PCR products were subjected to agarose gel electrophoresis and subsequently stained with ethidium bromide to visualize amplicons. Amplicons were determined to be specific for the *ospE/elpB1* and *p21/elpB2* loci by nucleotide sequence analysis. Nucleotide sequencing was performed at the OUHSC core DNA sequencing facility using an ABI model 373A automated DNA sequencer and PRISM ready reaction DyeDeoxy terminator cycle sequencing kit according to the instructions of the manufacturer (Applied Biosystems Inc., Foster City, Calif.).

**Serial dilution RT-PCR analyses.** Total RNA isolated from spirochetes cultivated at 23°C, temperature shifted, or within DMCs was reverse transcribed as described above to generate cDNA. The final cDNAs were diluted by the addition of 30  $\mu$ l of sterile RNase-free water and serially diluted twofold for subsequent PCR using primers specific for regions of each of the *ospE*-related, *ospF*-related, and *elp* genes and the *flaB*, *ospA*, and *ospC* genes (Table 1). Given the high degree of sequence similarity between some of these genes, primer pairs generated for the reverse transcription (RT)-PCRs were determined to be gene specific by performing nine separate amplification PCRs with each primer set and 20 ng of the *ospE*-related, *ospF*-related, or *elp* plasmid construct as template; amplicons were produced only when the cloned gene and matching primer pair

were included in the same PCR. All PCRs were performed for 45 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s, followed by a final extension step for 7 min at 72°C. Ten microliters of each amplification reaction was then subjected to agarose gel electrophoresis and subsequently stained with ethidium bromide to visualize amplicons.

**Construction of GST fusion proteins.** Fusion constructs for OspE, p21, OspF, BbK2.10, BbK2.11, OspA, OspC, and FlaB were generated as previously described (2, 4, 12, 24). Glutathione S-transferase (GST) fusion constructs for ElpA1, ElpA2, ElpB1, and ElpB2 were generated by directionally cloning PCR-amplified products for the mature form of each gene into pGEX-4T-2, using the forward and reverse primers described in Table 1. All GST fusion constructs were sequenced to confirm that the inserted DNA was in frame and that no errors were incorporated during PCR amplification (performed at the OUHSC core DNA sequencing facility as described above).

**Immunological reagents.** GST fusion constructs for OspE, p21, BbK2.10, ElpA1, ElpA2, ElpB1, ElpB2, and OspC were grown in tryptone-yeast broth supplemented with 100  $\mu$ g of ampicillin. When the cultures reached an optical density at 600 nm of 0.7, IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) was added to a final concentration of 1 mM to induce production of the respective GST fusion proteins. Following induction, cultures were pelleted at 6,000  $\times$  g for 20 min, and the fusion proteins were affinity purified using an agarose-glutathione matrix as instructed by the manufacturer (Amersham Pharmacia, Piscataway, N.J.). GST fusion proteins were subsequently cleaved from the GST moiety with thrombin protease, and the final purified proteins were used to hyperimmunize Sprague-Dawley rats to generate monospecific polyclonal antisera as described

previously (4). When necessary, 1-ml aliquots of each antiserum preparation were diluted in 2 ml of phosphate-buffered saline (PBS) containing 0.02% Tween 20 (PBS-T), and antibodies were further purified by overnight incubation with strips of nitrocellulose membranes impregnated with 10 µg of purified protein. Membranes were then washed extensively in PBS, and the final affinity-purified antibodies were eluted from the nitrocellulose strips by incubation with 100 mM glycine (pH 2.0) for 15 min at room temperature. The final solution of each purified antibody was then neutralized with 0.1 volume of 1 M Tris base (pH 8), and antibodies were subsequently renatured by overnight dialysis in PBS. Monoclonal antibodies (MAbs) directed against FlaB (1H6-33) and OspA (14D2-27) and a cross-reactive OspF/BbK2.11 MAb (10D7-42) used for the immunoblot analyses have previously been described (2). To determine which protein products corresponded to BbK2.11 and OspF in Fig. 2B, rat antiserum specific for BbK2.11 (QEAKQKFEELRVQVESTTGG) and OspF (ANSLGLGVSYSSSTGTDSNE) peptides was generated and used for immunoblot analysis of *B. burgdorferi* whole-cell lysates. Sera from C3H/HeJ mice syringe inoculated ( $10^3$  organisms per mouse) or tick infested (five ticks per mouse) with *B. burgdorferi* 297 were collected by tail bleed at 1, 2, 3, 4 (tick infested) or 6 (syringe inoculated) and 8 weeks after syringe inoculation or tick infestation. Sera from five mice at each time point were pooled for enzyme-linked immunosorbent assay (ELISA) as described below. Tick infection and rearing and infestation of mice were performed as described previously (51); prior to infestation of mice, spirochetes used to infect ticks were confirmed to contain all seven plasmids carrying the *ospE*-related, *ospF*-related, and *elp* genes by PCR amplification using the locus-specific primers described in Table 1.

**SDS-polyacrylamide gel electrophoresis and immunoblot analysis.** Whole-cell lysates from  $5 \times 10^6$  *B. burgdorferi* strain 297 organisms cultivated in vitro at 23°C, shifted from 23 to 37°C, or within rat peritoneal DMCs were boiled for 10 min in final sample buffer (62.5 µM Tris-HCl [pH 6.8], 10% [vol/vol] glycerol, 5% [vol/vol] 2-mercaptoethanol, 5% sodium dodecyl sulfate [SDS], 0.001% bromophenol blue) before electrophoresis through 2.4% stacking and 12.5% separating gels. Gels were then either stained with silver according to the procedure described by Morrissey (29) or transferred electrophoretically to 0.2-µm-pore-size nitrocellulose (Schleicher & Schuell, Keene, N.H.) for immunoblot analysis. Prior to immunoblot analysis, an enhanced chemiluminescence immunoblot procedure (Amersham Pharmacia) was used to titrate all antibodies, which indicated that at least 50 pg of recombinant protein could be detected by each polyclonal antibody preparation or MAb utilized. Immunoblots were incubated with 1:50 dilutions of MAbs or 1:100 dilutions of affinity-purified sera. To analyze the specificity of the *OspE*-related, *OspF*-related, and *Elp* antisera, strips of nitrocellulose membrane were impregnated with 100 ng of each of the recombinant proteins using a Bio-Rad (Hercules, Calif.) dot blot apparatus; *OspC* also was used in this experiment as an additional control for specificity. All transferred membranes and membrane strips were then blocked for 2 h at room temperature in PBS-T containing 5% fetal bovine serum before primary antibodies or MAbs were added and incubated for 1 h at room temperature. Membranes subsequently were washed and incubated for 45 min with a 1:5,000 dilution of rabbit anti-rat or goat anti-mouse immunoglobulin G (IgG)-horseradish peroxidase (HRP) conjugate (Zymed, San Francisco, Calif.). Membranes then were washed three times for 10 min each in PBS, followed by three 10-min washes in Tris base (pH 7.6) before chemilumigrams were exposed and developed using the Enhanced Chemiluminescence Plus (Amersham Pharmacia) Western blotting detection system according to the manufacturer's instructions.

**ELISA.** Recombinant FlaB, *OspA*, *OspC*, *OspE*, *p21*, *OspF*, BbK2.10, BbK2.11, *ElpA1*, *ElpA2*, *ElpB1*, and *ElpB2* (50 µl of each at a concentration of 5 µg/ml) in PBS were plated in 96-well Maxisorp Nunc-Immuno plates (Nalge Nunc International, Naperville, Ill.) and allowed to coat overnight at 4°C. PBS was determined to be the optimal coating buffer in preliminary studies by comparing PBS with borate-buffered saline (pH 8.5) and carbonate-buffered saline (pH 10.5). After coating, plates were blocked with PBS-T containing 5% fetal bovine serum for 1 h at 37°C and subsequently washed three times with PBS-T. Triplicate samples of pooled sera from syringe-inoculated or tick-infested C3H/HeJ mice, including uninfected mouse serum as a control, were diluted 1:1,000 in PBS-T containing 5% fetal bovine serum, added to the plates, and allowed to incubate for 1 h at 37°C. The plates were subsequently washed three times with PBS-T. Goat anti-mouse IgG-IgA-IgM (heavy- and light-chain specific) conjugated to HRP was then added at a dilution of 1:1,000 in PBS-T containing 5% fetal bovine serum and incubated 1 h at 37°C. Plates were then washed five times in PBS-T before 100 µl of 0.1 mM ABTS [2,2'-azino-di(3-ethyl)benzothiazoline sulfonic acid] containing 0.3% hydrogen peroxide was added to each well and allowed to incubate at room temperature for 10 min. The reactions were stopped by the addition of 100 µl of 5% SDS, and plates were read on a Spectra Max 340 plate reader (Molecular Devices, Sunnyvale, Calif.) at an optical density of 405

nm. The background reactivity obtained using normal mouse serum for each antigen was subtracted from the final readings. The serum dilution and recombinant antigen concentrations used for ELISAs were optimized by identifying the point at which antigen reactivity for normal mouse serum was less than 3 standard deviations from that of the HRP conjugate alone. To ensure that specific antibody responses were identified for the highly homologous *OspE/p21* and *OspF/BbK2.11* proteins, infected sera used to determine the reactivity for *p21* and *OspE* were preadsorbed overnight at 4°C with 10 µg of recombinant *OspE* or *p21*, respectively, per ml. Likewise, infected sera used to determine reactivity for *OspF* and BbK2.11 were preadsorbed overnight at 4°C with 10 µg of recombinant BbK2.11 or *OspF*, respectively, per ml. The preadsorption step was optimized and confirmed to result in a complete loss of reactivity for each antigen adsorbed, using the ELISA procedure described above.

**Indirect immunofluorescence assays.** For indirect immunofluorescence experiments with *B. burgdorferi*-infected ticks, midguts were dissected out of *B. burgdorferi* strain 297-infected ticks (nymphal stage) prior to feeding ( $n = 20$ ) or after feeding to repletion ( $n = 15$ ). Extracted midguts were diluted in 20 µl of PBS before being disrupted by repeated pipetting. Additionally, *B. burgdorferi* strain 297 spirochetes from in vitro temperature-shifted cultures were washed once in PBS and diluted to  $5 \times 10^6$  cells per ml in PBS. Ten microliters of each sample (midguts from unfed or fed ticks and temperature-shifted organisms) was spotted onto fluoro slides (Erie Scientific). Slides containing the various suspensions were fixed in acetone and blocked for 30 min at room temperature in PBS containing 0.2% bovine serum albumin (BSA) before rat polyclonal antiserum directed against *OspA*, *OspC*, *OspE*, *ElpB1*, *p21*, *ElpB2*, *OspF/BbK2.11*, BbK2.10, *ElpA1*, or *ElpA2* was added and allowed to incubate for 1 h at room temperature. Also included with each of the primary antibody incubations was polyclonal rabbit anti-FlaB diluted 1:500. The primary antibody incubations were washed three times in PBS-0.2% BSA before Alexa 488-labeled goat anti-rat IgG (heavy- and light-chain specific) and Alexa 568-labeled goat anti-rabbit IgG (heavy- and light-chain specific; Molecular Probes, Eugene, Oreg.) diluted 1:1,000 in PBS-0.2% BSA were added and allowed to incubate 45 min at room temperature. Slides then were washed three times in PBS-0.2% BSA followed by three washes in deionized water and subsequently allowed to air dry. One drop of buffered glycerol mounting medium (Becton Dickinson, Cockeysville, Md.) was then added, and coverslips were placed over each sample. Slides were visualized using an Olympus (Melville, N.Y.) BX-60 fluorescence microscope. For each protein analyzed, multiple fields were viewed before images of representative fields were captured with a Spot digital camera and associated software (Diagnostic Instruments, Sterling Heights, Mich.). One-way analysis of variance (ANOVA) with the Bonferroni multiple-comparison post test was performed using the Prism 3.0 software package (GraphPad, San Diego, Calif.) to determine statistical significance between the percentages of spirochetes expressing individual lipoproteins in the various environments analyzed.

**Primer extension.** RNA for primer extension analysis was isolated as indicated above from *E. coli* cultures transformed with a *bbk2.10*-PhoA fusion construct described previously (4). Primer extension was performed as described elsewhere (33, 48).

**Computer analysis.** All nucleotide and deduced amino acid sequence analyses, restriction enzyme digestion profiles, and selection of primers for serial dilution RT-PCR and transcriptional linkage analysis were performed using the MacVector version 6.5.3 software package (Oxford Molecular Group, Campbell, Calif.). Multiple sequence alignments and phenogram analyses were performed using the ClustalW multiple sequence alignment program of the MacVector version 6.5.3 software package.

## RESULTS

**The *ospE/elpB1* and *p21/elpB2* genes are transcriptionally linked.** Previous DNA sequence analysis (3, 8) indicated that the *ospE/elpB1* and *p21/elpB2* gene pairs located on plasmids cp32-2 and cp18-2, respectively, constitute bicistronic operons (Fig. 1). To interpret subsequent expression studies, it was necessary at the outset to confirm experimentally that the genes in each pair are indeed transcriptionally linked. cDNA amplicons of the expected size were obtained for *ospE/elpB1* by RT-PCR from spirochetes grown at 23°C or temperature shifted and from organisms cultivated within DMCs. In contrast, amplicons for *p21/elpB2* were obtained only from temperature-shifted organisms and DMC-cultivated spirochetes

(data not shown). Nucleotide sequencing confirmed that the PCR products were derived from the genes of interest.

**Expression patterns during in vitro and DMC cultivation.**

**(i) Serial dilution RT-PCR.** After confirming the specificity of each primer pair using cloned genes as templates (Table 1), we determined the highest dilution of cDNA which resulted in a visible amplification product from organisms cultivated in vitro in BSK-H medium at 23°C, following temperature shift, and following cultivation within DMCs. Of the nine *ospE*-related, *ospF*-related, and *elp* homologs, only *elpA2* was transcribed at an appreciable level when organisms were cultivated at 23°C (Fig. 2A). Temperature shift increased the transcriptional activity from 2-fold (for *p21* and *elpB2*) to 32-fold (for *elpA1* and *elpB1*); all other genes fell somewhere in between these induction levels. With the exception of *elpA1*, all genes were transcriptionally upregulated an additional two- to eightfold in the DMC-cultivated organisms compared to temperature-shifted spirochetes. Consistent with prior studies (2, 11, 28, 37, 51), transcription of *flaB* did not change under the various conditions. As expected, transcription of *ospA* was dramatically downregulated during cultivation within DMCs, while *ospC* expression was upregulated by temperature shift and further increased by cultivation within DMCs.

**(ii) Immunoblot analysis.** Immunoblot analysis was performed on whole cells taken from the same cultures and phase of growth used for isolation of RNA. For these experiments, we generated monospecific polyclonal antibodies for seven of the nine lipoproteins and utilized a well-characterized MAb (10D7-42) which recognizes both OspF and BbK2.11 (data not shown) (2). As shown in Fig. 2B, the protein expression profiles of OspE, p21, OspF (lower band), BbK2.11 (upper band), ElpB1, ElpB2, and ElpA2 correlated well with the transcriptional data obtained by RT-PCR (compare Fig. 2A and B) and our prior studies (2, 4). In contrast, no protein was detected for either BbK2.10 or ElpA1 under any of the conditions analyzed (Fig. 2B), although mRNA was identified for *elpA1* and *bbk2.10* following temperature shift and cultivation within DMCs (Fig. 2A). As expected, no apparent differences in protein expression were observed for FlaB, while expression of OspC or OspA was dramatically upregulated or downregulated, respectively, in DMC-cultivated organisms.

**Lipoprotein expression patterns in vitro and in *B. burgdorferi*-infected *Ixodes scapularis* ticks.** We next performed indirect immunofluorescence analysis on spirochetes within tick midguts before and after feeding to repletion and, for comparative purposes, also on temperature-shifted spirochetes. In these preliminary studies, we noted that individual lipoproteins often were not uniformly expressed within a population of organisms. For this reason, we determined the percentage of spirochetes expressing each protein. The results for these experiments are summarized in Table 2.

OspE and ElpB1 were expressed by almost all of the temperature-shifted spirochetes, while few of the same organisms expressed p21 and ElpB2. OspF and/or BbK2.11 was expressed by all temperature-shifted organisms. By contrast, ElpA2 was expressed by only 64% of the temperature-shifted organisms, while neither BbK2.10 nor ElpA1 was detected. Although ElpA2 was the sole lipoprotein expressed by spirochetes within unfed ticks, only a minute percentage of organisms expressed this protein. Within fed ticks, only OspE, ElpB1, OspF, and/or

BbK2.11 was detected, although at a significantly reduced number as compared to the temperature-shifted organisms (Table 2). ElpA1, ElpA2, p21, ElpB2, and BbK2.10 were not expressed in midguts from fed ticks. It was interesting that in some instances there was an overall heterogeneity in the amount of fluorescence, which may indicate that certain lipoproteins can be expressed at different levels in some organisms. As a control for these experiments, expression of OspA and OspC in fed and unfed tick midguts also was analyzed. The expression patterns for these two antigens were highly similar to those recently reported by Schwan and Piesman for *B. burgdorferi* strain B31 (36).

**Antibody response following syringe inoculation or tick infestation of C3H/HeJ mice.** To gain additional information about the temporal patterns of expression of the nine lipoproteins during mammalian infection, we also analyzed the antibody responses generated following syringe inoculation or tick infestation of C3H/HeJ mice; the combined ELISA results are shown in Fig. 3. Tick-infested mice produced specific antibodies within 2 weeks against OspE, ElpB1, and p21 and within 4 weeks against OspF, BbK2.11, and BbK2.10. Specific antibody responses for ElpA1, ElpA2, and ElpB2 did not appear until 4 to 6 weeks postinfestation. Syringe-inoculated mice mounted similar antibody responses against OspE, ElpB1, OspF, BbK2.11, and ElpA1, whereas the appearance of antibodies against p21, ElpB2, ElpA2, and BbK2.10 was delayed by at least an additional 4 weeks. Specific antibodies produced against FlaB, OspC, and OspA also were assessed. As expected, no antibodies to OspA were generated following either tick or needle inoculation, whereas antibodies specific for FlaB and OspC appeared by 3 weeks postinfection.

**Comparison of the upstream promoter regions of the *ospE*-related, *ospF*-related, and *elp* loci.** We next asked whether sequence relationships among the *ospE*-related, *ospF*-related, and *elp* promoter regions could be correlated with the gene expression patterns described above. As shown in Fig. 4A, we performed primer extension analysis to identify the transcriptional start site for *bbk2.10* to help identify the putative promoter regions for the various genes analyzed. This analysis indicated that the transcriptional start site is 14 bp upstream of the start codon for *bbk2.10*. Based on this start site, we then performed a multiple lineup of the upstream regions for all seven loci and identified correctly spaced and positioned RNA polymerase binding sites (i.e., -10 and -35 hexamers) closely related to the *E. coli* consensus  $\sigma^{70}$  binding site for all of the loci analyzed (Fig. 4B). As previously noted, the upstream regions of these genes are essentially identical (3, 8, 10, 27); however, several nucleotide transitions/transversions and insertions/deletions were observed between the start codons and consensus -10 and -35 hexamers. The multiple sequence alignment revealed that the consensus -10 and -35 sites for *bbk2.10* and *elpA1* were identical, the *ospF* and *bbk2.11* upstream regions differed by 4 nucleotides, and the *ospE* and *p21* upstream regions differed by 11 nucleotides with two located within the -10 hexamer. Additionally, three conserved residues found only in the *bbk2.10* and *elpA1* promoters were identified. These consisted of G residues at -36 and -42 as well as a T residue at position -39, all of which were found in the spacer region between the -10 and -35 sites.

As noted earlier, detailed phylogenetic comparisons of these

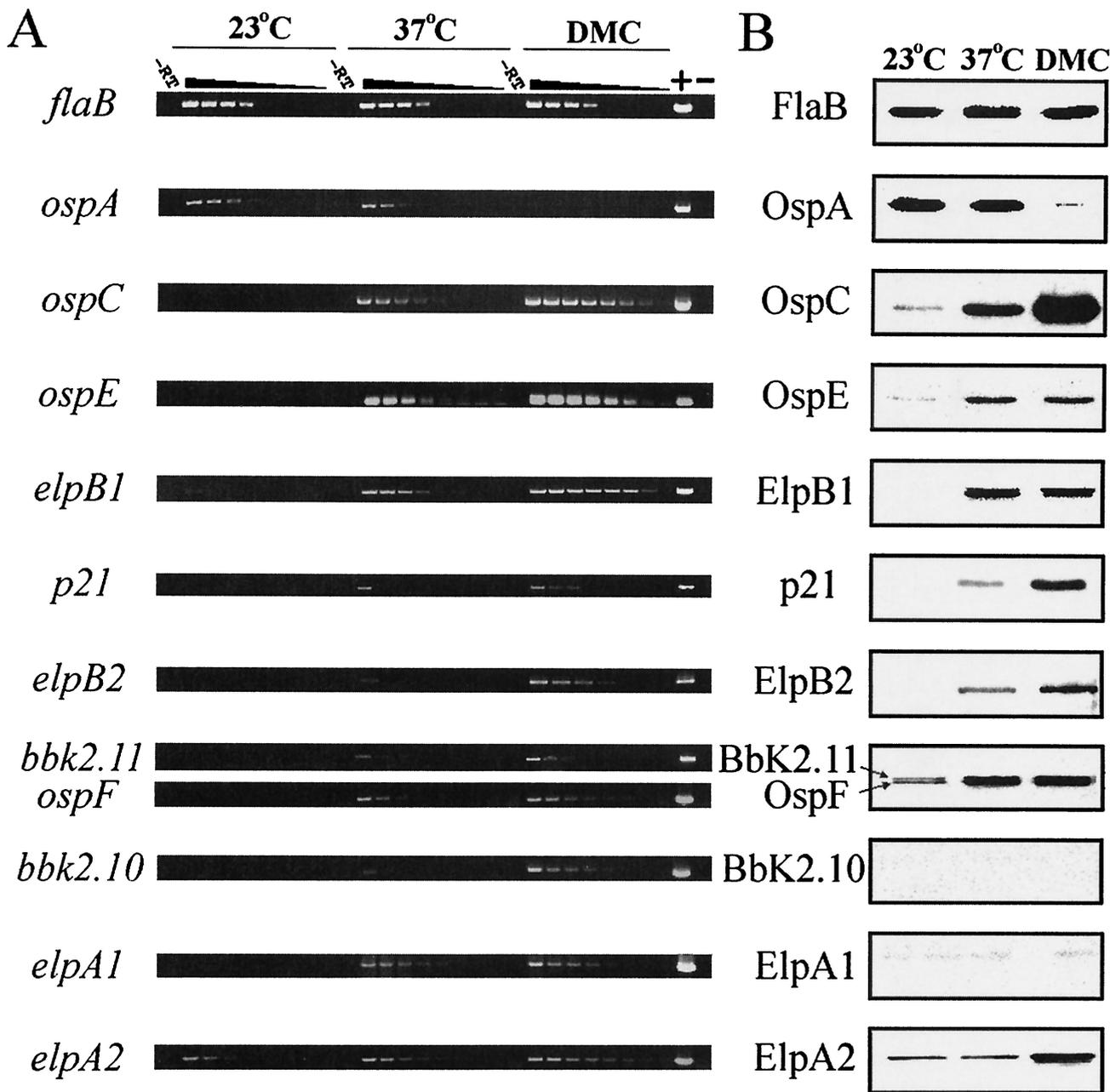


FIG. 2. Serial dilution RT-PCR and immunoblot analyses of the *ospE*-related, *ospF*-related, and *elp* genes and proteins. (A) Total RNA was isolated and processed as indicated in Materials and Methods from spirochetes cultivated in vitro at 23°C, shifted from 23 to 37°C, or cultivated within rat peritoneal DMCs. Twofold serial dilutions of cDNA were amplified for 45 cycles by PCR with the specific primers indicated in Table 1 to detect mRNA abundance under the various conditions. The final amounts of cDNA used were from 5 pg to 39 fg for *flaB*, 313 to 2.4 pg for *ospA* and *ospC*, and 20 ng to 156 pg for the various *ospE*-related, *ospF*-related, and *elp* genes. -RT indicates reaction mixtures lacking reverse transcriptase; + and - indicate control PCRs using 10 ng of total *B. burgdorferi* strain 297 genomic DNA and water, respectively. (B) *B. burgdorferi* whole-cell lysates probed with 1:100 dilutions of affinity-purified rat anti-OspC, -OspE, -ElpB1, -p21, -ElpOs2, -BbK2.10, -ElpA1, and -ElpA2 or MAbs 10D7-42 for OspF/BbK2.11, 1H6-33 for FlaB, and 1402-27 for OspA. Lysates were generated from organisms cultivated in vitro at 23°C, shifted from 23 to 37°C, or cultivated within rat peritoneal DMCs. Protein expression was detected using the Enhanced Chemiluminescence Plus Western blotting system as described in Materials and Methods.

lipoproteins from strain 297 revealed that they fall into three distinct families comprised of OspE-related, OspF-related, and Elp proteins (Fig. 4C, right) (3, 8). To determine if the phylogenetic relationships between the upstream promoter regions were similar to those found for the protein families, we trans-

formed the multiple sequence alignment into a phenogram. As shown in Fig. 4C (left), the resulting phenogram was strikingly different from that of the lipoproteins. Rather than corresponding to the similarities of the lipoprotein families, the promoters segregated into three branches according to their

TABLE 2. Expression of OspE-related, OspF-related, and Elp proteins from spirochetes temperature shifted in vitro and from fed or unfed *I. scapularis* midguts

Antibody	% of spirochetes expressing protein <sup>a</sup>		
	In vitro <sup>b</sup>	Tick midguts <sup>c</sup>	
		Unfed	Fed
OspA	74*	97	52†
OspC	78*	30	96†
OspE	91*	0	63†
ElpB1	93*	0	61†
p21	9*	0	0
ElpB2	11*	0	0
OspF/BbK2.11	100*	0	90†
BbK2.10	0	0	0
ElpA1	0	0	0
ElpA2	64*	7	0

<sup>a</sup> Standard deviations were less than 7% for all samples. \*, significant difference between number of organisms expressing lipoprotein in temperature-shifted organisms compared to organisms analyzed from unfed or fed tick midguts (significance [ $P \leq 0.05$ ], determined using ANOVA with the Bonferroni multiple comparison post test); †, significant difference in expression of lipoprotein between fed and unfed tick midguts, determined using ANOVA with the Bonferroni multiple comparison post test ( $P \leq 0.001$ ).

<sup>b</sup> Determined from the total number of organisms counted from 20 separate microscopic fields ( $n = 376$  to 633 spirochetes). Spirochetes were cultivated in BSK-H medium in vitro and shifted from 23 to 37°C.

<sup>c</sup> Determined from the total number of organisms counted from 50 separate microscopic fields ( $n = 142$  to 312 spirochetes).

overall patterns of expression during the borrelial enzootic cycle. One branch consisted of *ospF*, *bbk2.11*, and *ospE*, genes apparently regulated by temperature, while the *p21* and *elpA2* promoters comprised a second group primarily expressed within the mammalian host. The third group consisted of *bbk2.10* and *elpA1* which were found to be induced transcriptionally by temperature and host factors but appear to be translated only in the mammalian host environment.

## DISCUSSION

Plasmid-encoded proteins appear to play critical roles in *B. burgdorferi* virulence and Lyme disease pathogenesis (6, 13–17, 35, 38, 41). Although prior studies have investigated the differential expression patterns of one or more of the plasmid-encoded OspE, OspF, and Elp orthologs during in vitro cultivation (41, 50), this is the first time a comprehensive analysis has been performed for all known orthologs from a single infectious isolate throughout the borrelial enzootic life cycle. Furthermore, in contrast to prior analyses, we used monospecific DNA and antibody reagents that allowed us to discern between the various paralogs from each of the lipoprotein families examined. The combined data indicate that specific mammalian and/or tick factors, in addition to temperature, are critical in the overall regulation of these lipoproteins. Supporting this notion were the findings that (i) transcription was enhanced by organisms cultivated within a mammalian host environment (i.e., DMCs) as compared to temperature-shifted organisms cultivated in vitro, (ii) the overall amount of protein expressed and profile of expression during the various in vitro cultivation conditions did not mimic the patterns observed within DMCs or midguts from unfed or fed ticks, (iii) the proportions of spirochetes expressing individual OspE, OspF,

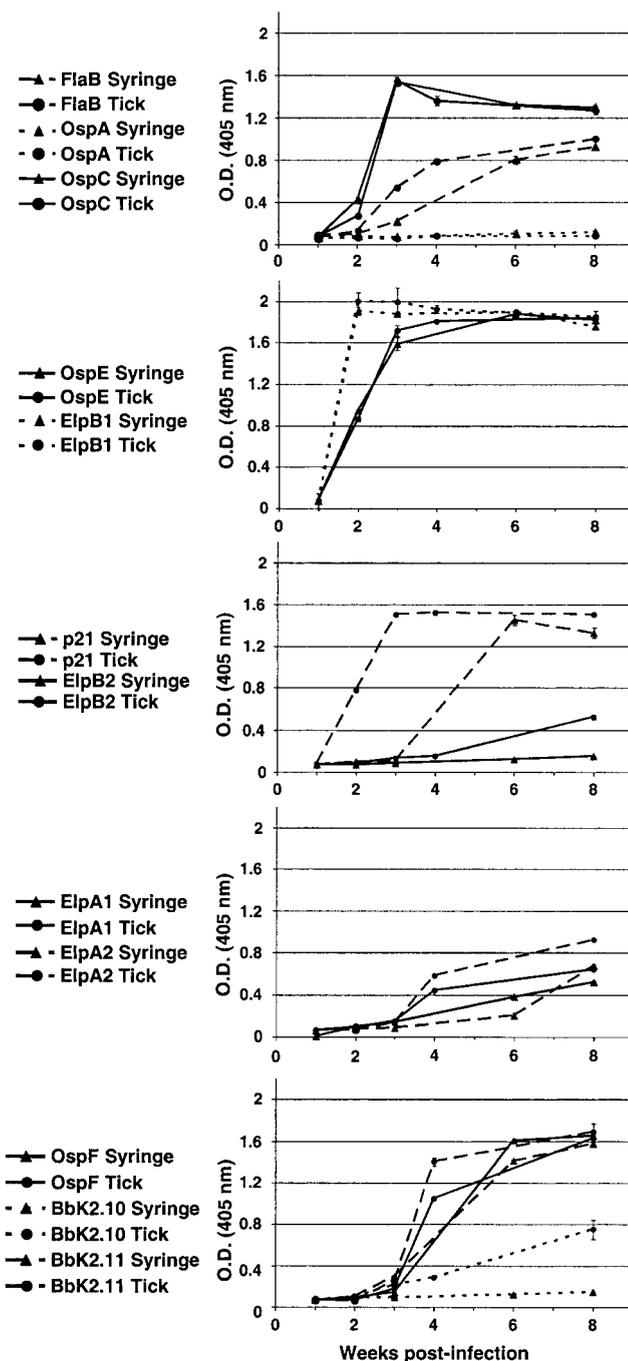


FIG. 3. Ontogeny of the antibody response against the OspE-related, OspF-related, and Elp proteins following syringe inoculation or tick infestation of C3H/HeJ mice. Pooled sera collected over an 8-week period from five C3H/HeJ mice either syringe inoculated or tick infested were analyzed for reactivity against the cleaved, recombinant OspE-related, OspF-related, and Elp proteins by ELISA. Additionally, antibody reactivity to the constitutively expressed FlaB protein, temperature-induced OspC lipoprotein, and OspA (which is downregulated or repressed during infection) was included in the analysis. To ensure monospecificity for the highly homologous p21/OspE and OspF/BbK2.11 proteins, sera from all time points were preadsorbed overnight at 4°C with the potentially cross-reactive paralog prior to ELISA analysis.

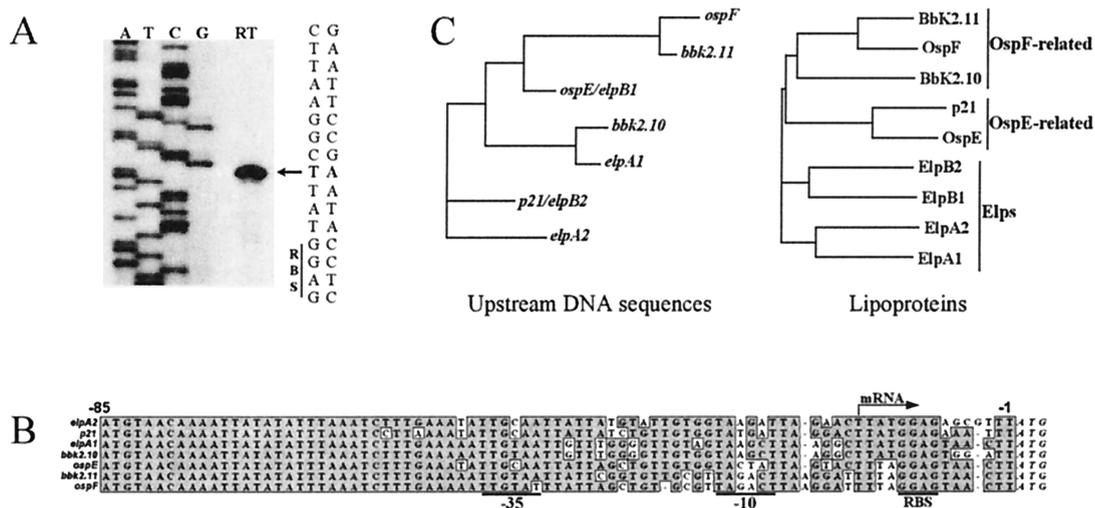


FIG. 4. Primer extension, multiple sequence alignment, and phylogenetic analysis of the *ospE/ospF/elp* promoter regions. (A) Primer extension analysis showing the product of the *bbk2.10* mRNA generated with reverse transcriptase (lane RT) and the sequencing ladder of the homologous region of the *bbk2.10* gene. Lanes loaded with individual dideoxy sequencing reaction mixtures are labeled A, T, C, and G. The arrow indicates the position of the primer extension product in relation to the sequencing ladder. RBS indicates the putative ribosomal binding site. Also shown are ClustalW alignment (B) and phenogram analysis (C) of the upstream 85 bp of the putative promoter regions for the *ospE*-related, *ospF*-related, and *elp* loci (left); for comparison, a phenogram of the full-length lipoproteins also is shown (right).

and Elp proteins in unfed and fed tick midguts did not correlate with that observed from organisms temperature-shifted during in vitro cultivation, and (iv) two lipoproteins, BbK2.10 and ElpA1, appeared to be expressed exclusively during mammalian infection. In addition to upregulating expression, it was also noteworthy that specific tick or mammalian factors also appear capable of repressing protein synthesis. Along these lines, OspE/ElpB1, p21/ElpB2, OspF, BbK2.11, and ElpA2 were found to be expressed by temperature-shifted organisms in vitro, while all were downregulated or not expressed in fed ticks. This finding is consistent with prior observations by us and others that host-specific signals can override the positive stimulus provided by elevated temperatures (2, 16). It appears that these signals also affect the overall heterogeneity of spirochetes expressing a given protein as well as the apparent amount expressed by an individual spirochete during tick feeding. A similar phenomenon was recently reported by Ohnishi et al. (32) with regard to the expression of OspA and OspC.

Like many other bacterial pathogens, *B. burgdorferi* is thought to adapt to the various environments it encounters by differentially expressing genes and proteins (2, 4, 13, 16, 19, 28, 32, 37, 41, 52). During this study, we observed a progressive induction of the OspE, OspF, and Elp proteins as *B. burgdorferi*-infected ticks engorged on blood and were subsequently transmitted to the mammalian host. Interestingly, a representative from each lipoprotein family was expressed in the tick midgut after feeding, while other members from each family were expressed only at later points during transmission or infection. For example, while OspF, BbK2.11, OspE, and ElpB1 were expressed in the tick midgut during feeding, ElpA1, ElpA2, ElpB2, and the OspE and OspF homologs p21 and BbK2.10, respectively, were all expressed at some point after feeding or during dissemination. The observation that members of each lipoprotein family were induced at different points in the borrelial enzootic cycle leads us to speculate that

individual members of each protein family have evolved so that they are expressed in specific environmental niches.

All of the *ospE*-related, *ospF*-related, and *elp* orthologs identified to date have highly similar upstream regions (4, 10, 25, 27, 47). This observation, combined with the finding that all of the orthologs in strain B31 appear to be regulated by temperature in vitro, has led Stevenson and coworkers to propose that the same DNA-binding protein(s) regulates all of these genes (41). Presently, Hbb and Gac are the only two DNA-binding proteins identified in *B. burgdorferi* (22, 23). Hbb seems unlikely to regulate these loci, since no specific Hbb-binding sites were identified around any of the *ospE*, *ospF*, and *elp* genes in strain 297 or in the completely sequenced plasmids which harbor orthologs in strain B31 (data not shown). However, a role for the nonspecific DNA-binding protein Gac (21) or some unidentified protein cannot be ruled out. On the other hand, it is not known if DNA-binding proteins are involved in the regulation of these loci, and it is just as plausible that alterations in the overall topology of the promoters in different environments result in the differential expression patterns observed.

It should be noted that single base changes in promoters are known to influence both RNA polymerase binding and strand separation, which can dramatically change mRNA expression levels (20, 34, 39). In this regard, it is interesting that the majority of the differences observed in the upstream regions were found in the putative -10 and -35 hexamers and spacer region between, all of which are critical for RNA polymerase recognition and binding. Therefore, it is possible that the minor sequence differences observed between the promoters result in an altered alignment of the -10 and -35 sites in these promoters under different environmental conditions, which, in turn, may alter the affinity of RNA polymerase for these promoters. Future studies can now be performed on the upstream regions of these genes to systematically address whether DNA-

binding proteins, topological changes, or a combination regulate transcription at these loci.

The expression patterns for the *ospE*, *ospF*, and *elp* genes correlated with the similarities found between the upstream promoter regions rather than the OspE, OspF, and Elp protein family groupings (3, 8). It appears that the variation in the promoter sequences has occurred both by duplication of a common ancestral upstream region and by prior recombinatorial events. While the function of the lipoproteins encoded at these loci is not known, the data presented here suggest that the point at which they are expressed during transmission and dissemination is important in the parasitic strategy of this pathogen. If this contention is correct, it would indicate that prior recombination events resulted in specific promoters and lipoproteins being fused together and that subsequent selection generated the most advantageous expression patterns for each lipoprotein during the borrelial enzootic cycle. This also would suggest that not only is there a constraint placed on the lipoprotein-encoding gene(s) which conserves the functionality of each family, but there also must be a similar constraint placed on the upstream promoter regions which preserves their expression patterns in various environments.

One of the most exciting outcomes of this study was the identification of a potential posttranscriptional mechanism of regulation for *bbk2.10* and *elpA1*. Recent studies have indicated that some pathogenic microorganisms utilize posttranscriptional regulatory mechanisms as a method for quickly responding to different environmental stimuli encountered during infection (1, 5). In this study, we observed that although mRNAs for *bbk2.10* and *elpA1* were present, neither protein could be detected in temperature-shifted organisms, in DMC-cultivated organisms, or within tick midguts, using highly specific and sensitive antibody reagents, suggesting that they are regulated posttranscriptionally. This observation was further supported by the observation that BbK2.10- and ElpA1-specific antibodies were produced by *B. burgdorferi*-infected mice, indicating that they are transcribed and translated in the mammalian host. Additionally, we previously reported that a recombinant protein consisting of the first 87 amino acids of BbK2.10 fused to alkaline phosphatase (BbK2.10-PhoA) is abundantly expressed in *E. coli* (4). However, a plasmid construct containing the native *bbk2.10* gene, including several hundred base pairs of upstream and downstream sequence, is transcribed but not translated in *E. coli* (D. R. Akins and J. D. Radolf, unpublished data). The combined observations suggest that either specific *trans*-acting factors which regulate *bbk2.10* are shared by *B. burgdorferi* and *E. coli* or, more likely, the mRNA is folded in a manner that inhibits ribosomal binding and efficient translation. If the latter is correct, it would suggest that the *bbk2.10* mRNA structure is dynamic and can be maintained in an untranslatable fold in some environments (e.g., within tick midguts, in DMCs, and during *in vitro* cultivation in *B. burgdorferi* and *E. coli*) but that this fold can be altered within the mammalian host such that translation is allowed to proceed. Although the signal(s) regulating translation of BbK2.10 and ElpA1 is not known, our data would indicate that it must be either (i) more than 8,000 Da (the molecular mass cutoff of the dialysis tubing used to cultivate organisms in DMCs), (ii) a mammalian signal attached to a host cell surface

requiring direct contact by *B. burgdorferi*, or (iii) not found in the rat peritoneal cavity environment.

In summary, the differential patterns of antigen expression observed in this study for the OspE-related, OspF-related, and Elp orthologs indicates that they are not regulated solely by temperature. Rather, there appears to be a temporal pattern of expression that begins during tick feeding and proceeds throughout the early stages of infection. Furthermore, the expression patterns observed for the various lipoproteins appear to be closely linked to the promoter sequences rather than to the function of an individual lipoprotein family. The fact that all of these proteins are expressed during tick feeding and transmission or soon after dissemination further suggests that these lipoproteins and the corresponding plasmids which encode them are important during the early pathogenic events of Lyme disease. Delineating the profiles of expression for these lipoproteins throughout the borrelial enzootic cycle has laid the foundation for future studies aimed at determining the mechanisms responsible for regulating these genes and identifying the cellular location of these proteins. The significance of the expression patterns observed, the overall genetic diversity of these hypervariable loci, and the influence of circular plasmid-encoded proteins and their role in *B. burgdorferi* virulence and Lyme disease pathogenesis represent an important area of research to be addressed in the future.

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