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Borrelia burgdorferi bba74 Is Expressed Exclusively during Tick Feeding and Is Regulated by Both Arthropod- and Mammalian Host-Specific Signals▽†

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Although BBA74 initially was described as a 28-kDa virulence-associated outer-membrane-spanning protein with porin-like function, subsequent studies revealed that it is periplasmic and downregulated in mammalian host-adapted spirochetes. To further elucidate the role of this protein in the *Borrelia burgdorferi* tick-mammal cycle, we conducted a thorough examination of its expression profile in comparison with the profiles of three well-characterized, differentially expressed borrelial genes (*ospA*, *ospC*, and *ospE*) and their proteins. In vitro, transcripts for *bba74* were expressed at 23°C and further enhanced by a temperature shift (37°C), whereas BBA74 protein diminished at elevated temperatures; in contrast, neither transcript nor protein was expressed by spirochetes grown in dialysis membrane chambers (DMCs). Primer extension of wild-type *B. burgdorferi* grown in vitro, in conjunction with expression analysis of DMC-cultivated wild-type and *rpoS* mutant spirochetes, revealed that, like *ospA*, *bba74* is transcribed by σ^{70} and is subject to RpoS-mediated repression within the mammalian host. A series of experiments utilizing wild-type and *rpoS* mutant spirochetes was conducted to determine the transcriptional and translational profiles of *bba74* during the tick-mouse cycle. Results from these studies revealed (i) that *bba74* is transcribed by σ^{70} exclusively during the larval and nymphal blood meals and (ii) that transcription of *bba74* is bracketed by RpoS-independent and -dependent forms of repression that are induced by arthropod- and mammalian host-specific signals, respectively. Although loss of BBA74 does not impair the ability of *B. burgdorferi* to complete its infectious life cycle, the temporal compartmentalization of this gene's transcription suggests that BBA74 facilitates fitness of the spirochete within a narrow window of its tick phase. A reexamination of the paradigm for reciprocal regulation of *ospA* and *ospC*, performed herein, revealed that the heterogeneous expression of OspA and OspC displayed by spirochete populations during the nymphal blood meal results from the intricate sequence of transcriptional and translational changes that ensue as *B. burgdorferi* transitions between its arthropod vector and mammalian host.

Lyme disease, the most common arthropod-borne infection in the United States, is caused by the spirochete *Borrelia burgdorferi* (87). In nature, *B. burgdorferi* has an obligate biannual enzootic cycle involving small mammalian host reservoirs, typically *Peromyscus leucopus*, and an *Ixodes* tick vector (51, 96). To successfully complete this life cycle, *B. burgdorferi* must adapt to and propagate within two markedly different physiologic milieus (67, 75, 96). A number of investigators have reported that manipulation of parameters, such as temperature, pH, DNA supercoiling, cell density, and partial O_2 pressure, during in vitro cultivation can trigger changes in gene and protein expression resembling those that occur when spirochetes adapt to their mammalian host (4, 20, 21, 44, 45, 65, 74, 77, 78, 89, 102). It also is now evident, however, that spirochetes must be exposed to as yet unidentified mammalian host-specific signals to induce the full range of transcriptional and translational changes that occur during infection (1, 13, 17, 19, 39, 97). Gene regulation during the tick phase of the enzootic cycle has come to be recognized as equally important to the spirochete's survival strategy (24, 29, 32, 53, 67, 73, 74, 91, 92, 96). In this regard, recent evidence obtained from expression profiling of *p66* and *ospD* points to the existence of arthropod-specific signals that modulate *B. burgdorferi* gene expression at various times during the tick phases of the cycle (24, 91).

Studies of differential gene expression in *B. burgdorferi* have given rise to several regulatory paradigms (90). The two most extensively investigated are the reciprocal regulation of *o*uter *s*urface *p*rotein A (*ospA*) and *ospC* as spirochetes alternate between the arthropod vector and mammalian host (25, 29, 41, 61, 64, 68, 76, 77, 106) and the temperature- and blood meal-dependent induction of the *ospE*, *ospF*, and *elp* (*erp*) genes (3, 6, 28, 39, 58, 88). An important advance in our understanding of the molecular mechanisms underlying these paradigms was the discovery from the genomic sequence that Lyme disease spirochetes coordinate their physiologic adaptations and pathogenic programs with just three sigma factors: the housekeeping sigma factor, σ^{70} (RpoD), and the alternate

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sigma factors RpoN and RpoS (33). Seminal studies by Norgard and coworkers (42, 83, 103) demonstrated that expression of RpoS is regulated by RpoN in concert with the response regulator protein Rrp2. Expression of RpoS, in turn, is essential for the induction of the established virulence factors *ospC*, *dbpBA*, and *bbk32* as well as numerous other genes thought to be required to establish mammalian infection (18, 19, 42, 59, 79, 80, 100). Analysis of *rpoS* and RpoS-dependent genes within infected ticks has demonstrated that induction of the RpoS regulon begins during the nymphal blood meal prior to spirochete transmission (i.e., the RpoS-ON state) (19, 35, 36, 39, 41, 76, 77). RpoS is also required for repression of *ospA* and other tick-phase genes (e.g., *bba62* and *lp6.6*) in response to mammalian host-specific signals (17, 19) although it is not known if RpoS-dependent repression occurs during nymphal tick feeding or only after spirochetes have transited to their murine host. Because *ospA* is transcribed by σ^{70} (84), the repression mechanism most likely involves blockage of transcription by an RpoS-dependent *trans*-acting factor; we along with others have proposed that the poly(T) tract upstream of the *ospA* promoter also contributes to repression (13, 17, 19, 84). Downregulation of *ospA* may also be influenced by environmental factors, such as pH (102), and or DNA topology (4). Expression of genes downregulated by RpoS within the mammalian host is thought to resume once spirochetes are acquired by naïve larvae (i.e., the RpoS-OFF state) (19).

Although the presence of homologous upstream regions and highly similar expression profiles initially suggested that the *ospE*, *ospF*, and *elp* genes are controlled by a common regulatory mechanism (2, 28, 58, 89), expression of *ospF* has been shown to be RpoS dependent while the *ospE* and *elp* paralogs are transcribed by σ^{70} (17, 18, 26, 27). Promoter mapping studies revealed that sequences in the −10 regions of the *ospE*, *ospF*, and *elp* promoters play a critical role in determining recognition by σ^{70} or RpoS (27). Transcription of *ospE* and *elp* paralogs by σ^{70} would make the corresponding proteins available at points within the enzootic cycle when RpoS is not present. By analogy with regulatory mechanisms identified in other bacteria (14, 37), differential expression of σ^{70}-dependent genes in *B. burgdorferi* presumably involves *trans*-acting factors that interact directly or indirectly with RNA polymerase to enhance or diminish the efficiency of gene transcription. Along these lines, Babb et al. (5) have reported that the Erp-binding factor, chromosomal (EbfC), a borrelial YbaB ortholog (55), binds to a region upstream of the *ospE* paralogs although it is unclear what, if any, effect EbfC binding has on transcription of these genes.

BBA74 initially was described as a 28-kDa virulence-associated outer-membrane-spanning protein with porin-like function (81, 82). More recently, we demonstrated that recombinant BBA74 lacks the physical properties typical of porins and that the native protein is located in the periplasmic space of *B. burgdorferi* (63). Bioinformatics analysis, however, has been unable to elucidate a possible function for BBA74 as it has no known orthologs. A clue that BBA74 functions within the arthropod vector was provided by microarray studies of spirochetes cultivated within dialysis membrane chambers (DMCs), which showed that *bba74* is downregulated in response to mammalian host-specific signals (13, 19). To extend these results, we performed a more thorough characterization of *bba74* in comparison to the paradigmatic genes *ospC*, *ospA*, and *ospE*. These analyses revealed that *bba74* is transcribed by σ^{70} exclusively during the larval and nymphal blood meals and that this novel expression pattern is a result of RpoS-independent and -dependent forms of repression that are induced by arthropod host- and mammalian host-specific signals, respectively. Although loss of BBA74 does not impair the ability of *Borrelia* to complete its infectious life cycle (8, 36, 91), the complex regulation of this gene is consistent with the notion that BBA74 facilitates fitness of the spirochete within a narrow window of its tick phase. Our analysis of *bba74* expression during the enzootic cycle also provided an opportunity to reexamine the *ospA-ospC* regulatory paradigm (75, 90, 96). Contrary to the prediction of strictly reciprocal expression of these genes and their corresponding proteins, spirochetes in the midguts of fed nymphs express large amounts of OspA as well as OspC. Our findings suggest that the heterogeneous expression of OspA and OspC by spirochete populations within fed nymphs results from the intricate sequence of transcriptional and translational changes that ensue as spirochetes transition from the RpoS-OFF to RpoS-ON state.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *B. burgdorferi* strains B31-MI (33), B31-A3 (36), 297 wild-type clone CE162, and the *rpoS* mutant CE174 (18) were grown in Barbour-Stoenner-Kelly H (BSK-H) medium supplemented with 6% rabbit serum (7). Standard temperature shift experiments were performed as previously described (19). Mammalian host-adapted spirochetes were obtained by cultivation in DMCs implanted into the peritoneal cavities of rats as previously described (1, 19).

DNA sequence analysis. The *bba74* coding sequence was PCR amplified from strains B31-MI and B31-A3 using the gene-specific PCR primers pS73F and p74comR (see Table S1 in the supplemental material), and the resulting amplicons were sequenced in the Davis Sequencing Facility (CA) using primer p74comF (see Table S1 in the supplemental material). The *bba74* upstream regions from strains B31-A3 and 297 were PCR amplified using primers pSP73F and pSP74R (see Table S1 in the supplemental material), and the resulting amplicons were sequenced using these same primers (Genewiz, Inc., South Plainfield, NJ).

Mouse infection experiments. All animal experiments were performed according to protocols approved by the New York Medical College and University of Connecticut Health Center Animal Care and Use Committees. Four-week-old female C3H/HeJ mice were infected with either B31-MI or B31-A3 strains by intradermal inoculation in doses of 1×10^2, 1×10^3, or 1×10^4 spirochetes. Ear punch biopsies were collected 14 days after inoculation, and mice were sacrificed by CO_2 asphyxiation at 21 days postinoculation. Various tissue samples (joints, hearts, and urinary bladders) were collected for culture and RNA isolation (see below). To culture *B. burgdorferi*, ear punch and tissue samples were transferred to 4 ml of BSK-H medium (Sigma-Aldrich, St. Louis, MO) containing an antibiotic mixture of fosfomycin (2 mg/ml), rifampin (5 mg/ml), and amphotericin B (250 μg/ml) (Sigma-Aldrich). All cultures were maintained at 34°C and examined for the presence of spirochetes every 5 to 7 days by dark-field microscopy beginning 5 days after inoculation. The 50% infectious dose (ID_{50}) values were calculated using the algorithm provided by the National Center for Biotechnology Information (85).

Preparation of *B. burgdorferi*-infected *Ixodes scapularis* ticks. To generate *B. burgdorferi*-infected ticks, approximately 300 to 400 pathogen-free *I. scapularis* larvae (Oklahoma State University, Stillwater, OK) were placed on infected C3H/HeJ mice 2 to 3 weeks after inoculation by syringe, allowed to feed to repletion, and collected over water. Fed larvae were stored over a supersaturated K_2SO_4 solution in an environmental incubator maintained at 22°C with a 16:8-h light-dark photoperiod until they had molted to the nymphal stage. To obtain fed nymphs, naive mice were each infested with 10 to 12 *B. burgdorferi*-infected flat *I. scapularis* nymphs confined within a capsule placed on their shaved backs. Nymphs were allowed to feed to engorgement (~72 h postattachment) and were then removed using forceps.

Immersion-fed larvae were generated according to the method described by

Policastro and Schwan (72). Briefly, cultures of wild-type and *rpoS* mutant isolates were grown to late-logarithmic density in BSK-H medium at 33°C, gently pelleted at 4,000 × *g* for 10 min, and then resuspended in fresh medium to a final density of 1×10^8 spirochetes per ml. The resulting spirochete suspension was mixed gently with 200 to 300 naïve flat *I. scapularis* larvae for 1.5 h at room temperature. Following incubation, immersion-fed larvae were washed twice with cold phosphate-buffered saline (PBS) and left to rest for 24 h over a solution of supersaturated K_2SO_4 before being fed on naïve C3H/HeJ mice as described above.

SDS-PAGE and immunoblot analysis. Spirochetes were harvested by centrifugation at 7,000 × *g* and washed three times with PBS (pH 7.4) at 4°C. Each pellet was resuspended in 100 μl of 50 mM Tris-HCl (pH 8.0) containing 0.3% sodium dodecyl sulfate (SDS) and 10 mM dithiothreitol (DTT). Total protein lysates were separated by 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) and either silver stained (62) or transferred to nitrocellulose membranes (GE-Healthcare, Milwaukee, WI). Membranes were blotted with antiserum directed against BBA74 (diluted 1:1,000) (63), OspC (1:5,000) (23), OspA (1:30,000) (23), OspE (1:1,000) (1), and FlaB (1:10,000) (17) in conjunction with horseradish peroxidase-conjugated goat anti-rat or anti-rabbit immunoglobulin G (IgG) (1:30,000) (Southern Biotechnology Associates, Birmingham, AL). Chemiluminescent detection was performed using Supersignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL). For serological experiments, total-protein lysates (B31-MI) and 5 ng of purified recombinant BBA74 (63), OspA (23), OspC (23), or OspE (1) was separated on 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (GE-Healthcare). The membranes were probed with immune serum (1:1,000) obtained from either needle-inoculated (1×10^4 spirochetes per mouse) or tick-inoculated mice collected at 3 weeks postinoculation. Horseradish peroxidase-conjugated goat anti-mouse IgG (1:20,000) (Southern Biotechnology Associates) was used as the secondary antibody.

RNA isolation. RNAs were isolated from in vitro and DMC-cultivated organisms using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Tissues from infected mice were homogenized in TRIzol reagent using silicon carbide beads (Beadbeater; Biocore, MD) immediately following isolation. Pools of 150 flat or 30 fed *B. burgdorferi*-infected nymphs were homogenized in TRIzol using a glass homogenizer and centrifuged at 500 × *g* for 2 min to remove tick debris. Contaminating genomic DNA was removed from isolated RNA samples by treatment with 10 U of RNase-free TurboDNA free (Ambion, Austin, TX), followed by phenol-chloroform extraction and ethanol precipitation. DNA-free RNAs were stored at −80°C.

Primer extension. A 25-mer oligonucleotide primer (A74ext) (see Table S1 in the supplemental material) located 80 bp downstream of the predicted *bba74* ATG start codon (plasmid lp54 coordinates 51718 to 51742) was end labeled with [γ-^{33}P]ATP using T4 polynucleotide kinase. Ten picomoles of primer was incubated with 7,500 Ci/mmole of [γ-^{33}P]ATP in the presence of 10 units of polynucleotide kinase in 1× polynucleotide kinase buffer (70 mM Tris-HCl, 10 mM $MgCl_2$, and 5 mM DTT, pH 7.6). The reaction mixture was incubated at 37°C for 1 h and terminated by the addition of 0.5 ml of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.6). The end-labeled primer was phenol-chloroform extracted, ethanol precipitated, and resuspended in 100 μl of TE buffer. Approximately 40 μg of DNase I-treated RNA isolated from B31-MI was mixed with 2.5×10^7 cpm of labeled probe and ethanol precipitated. The pellet was resuspended in 8 μl of TE buffer containing 1.25 μmol of KCl, and annealing was carried out at 55°C for 15 min. Ten units of Superscript II reverse transcriptase (RT) (Invitrogen) was added to the reaction mixture, and the mixture was incubated at 42°C for 2 h in RT buffer (10 mM $MgCl_2$, a 1.6 mM concentration of the deoxynucleoside triphosphates [dNTPs], and 0.1 M DTT). The samples were treated with RNase A (5 U) at room temperature for 15 min, extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and ethanol precipitated. The pellets were resuspended in 20 μl of 95% formamide, 1 mM EDTA, 0.25% bromophenol blue, and 50% glycerol. The transcriptional start site was identified by comparing the primer extension product to the region upstream of *bba74* sequenced using the dideoxy chain termination method (USB Corp., Cleveland, OH) as per the manufacturer's instructions. Briefly, the *bba74* locus, including 500 bp of upstream region, was PCR amplified using primers pS73F and p74comR (see Table S1 in the supplemental material) and cloned into the pGEM-T vector for sequencing reactions. Heat-denatured template was annealed at 55°C with primer A74ext in 200 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, and 250 mM NaCl. DNA sequencing reactions were carried out by adding labeling master mix (0.1 M DTT, 1,500 Ci/mmole [γ-^{33}P]ATP, 10 U DNA polymerase, and 7.5 μM dNTPs) containing appropriate dideoxy NTPs to heat-denatured template. Reaction mixtures were incubated at 37°C for 5 min and quenched by the addition of 4 μl of stop buffer (20 mM EDTA, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol). The sequencing reaction and primer extension products were denatured by heating at 75°C for 2 min and immediately resolved on a 6% acrylamide (19:1, acrylamide-bisacrylamide) sequencing gel containing 8 M urea. Gels were dried and exposed to Kodak XAR (Rochester, NY) film overnight at room temperature.

qRT-PCR. Quantitative RT-PCR (qRT-PCR) was performed using Superscript III RT (Invitrogen, Carlsbad, CA) in reaction mixtures containing 2 μg of total RNA, 10 mM of each dNTP, and 250 ng of random hexamer primers in first-strand buffer (250 mM Tris-HCl, 375 mM KCl, and 15 mM $MgCl_2$,) and 0.1 M DTT. Reaction mixtures were incubated at 42°C for 2 h. The resulting cDNA was amplified in an iCycler thermal cycler (Bio-Rad, Hercules, CA) using the gene-specific primer pairs listed in Table S1 in the supplemental material. Amplification of cDNAs was carried out using 1× iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions with the annealing temperature and concentration of magnesium chloride optimized for each primer pair. Control reactions (no RT and without template) were included for each assay. Amplicons corresponding to each gene target were cloned into pCR2.1-TOPO (Invitrogen), and purified recombinant plasmid DNAs were diluted (10^7 to 10^2 copies/μl) to generate standard curves. Transcript copy numbers were calculated using the iCycler postrun analysis software based on internal standard curves and normalized against copies of *flaB* present in the same cDNA.

Indirect immunofluorescence. Infected flat nymphs were individually crushed in 10 μl of CMRL medium (US Biological, Swampscott, MA), smeared on poly-L-lysine-coated glass slides (Sigma-Aldrich) and air dried. Pools of five fed nymphs or 10 larvae were eviscerated in 0.5 ml of cold CMRL medium, and the midguts were pelleted by centrifugation at 500 × *g* for 5 min at 4°C. The pellets were washed twice with 0.5 ml of ice-cold CMRL medium and resuspended in 0.05 ml of CMRL medium; 10-μl aliquots of washed midgut contents were smeared onto Poly-prep slides (Sigma-Aldrich) and air dried. All slides were fixed by immersion in ice-cold acetone for 10 min at −20°C. The slides were incubated overnight in blocking buffer (PBS supplemented with 10% naïve goat serum [NGS] and 0.2% bovine serum albumin) at 4°C. The slides were gently washed twice in PBS supplemented with 1% NGS, air dried, and incubated with rabbit anti-FlaB antiserum (1:200) in combination with rat antiserum directed against BBA74 (1:200), OspA (1:400), OspC (1:200), or OspE (1:200) in PBS with 10% NGS for 1 h at room temperature. After incubation, the slides were washed twice in PBS supplemented with 1% NGS and incubated with Alexa Fluor 488-labeled goat anti-rabbit IgG (1:750) and Alexa Fluor 594-labeled goat anti-rat IgG (1:750) antibodies (Molecular Probes, Invitrogen) in PBS with 10% NGS for 1 h in the dark. The slides were washed twice with PBS with 1% NGS, followed by two washes with PBS alone and two washes with ultrapure water. Slides were air dried in the dark, and the labeled smears were mounted in VectaShield medium containing 4′,6′-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). Each double-labeling experiment was performed in triplicate using three independently prepared pools of fed midguts or whole engorged larvae. For each antigen, the percentage of labeling compared to FlaB was evaluated by three individuals, two of whom were blinded; each person counted a minimum of 1,000 FlaB-labeled organisms per antigen tested. The mean percentage ± standard deviation for each antigen was calculated based on the number of FlaB-labeled organisms.

Statistical analysis. To determine the statistical significance of differences observed in immunofluorescence assay (IFA) and qRT-PCR studies, percent labeling and normalized transcript copy number values, respectively, were compared within Prism, version 5.0 (GraphPad Software, San Diego, CA), using an unpaired *t* test with two-tailed *P* values and a 95% confidence interval.

RESULTS

Transcription and translation of *bba74* correlate in spirochetes cultivated in DMCs but not in vitro. At the outset, we compared the transcriptional profiles of *bba74*, *ospA*, *ospC*, and *ospE* in spirochetes cultivated in vitro (at 23°C and following a temperature shift to 37°C) and within DMCs. Expression of *bba74* increased nearly threefold ($P = 0.016$) in response to elevated temperature but downshifted markedly in DMC-grown organisms (13, 19, 74); this expression pattern is very similar to that observed for *ospA* (Fig. 1A). Both *ospC* and *ospE* were expressed negligibly at 23°C but were induced strongly upon a temperature shift to 37°C; transcript levels for *ospC* also showed a further twofold enhancement ($P = 0.004$)

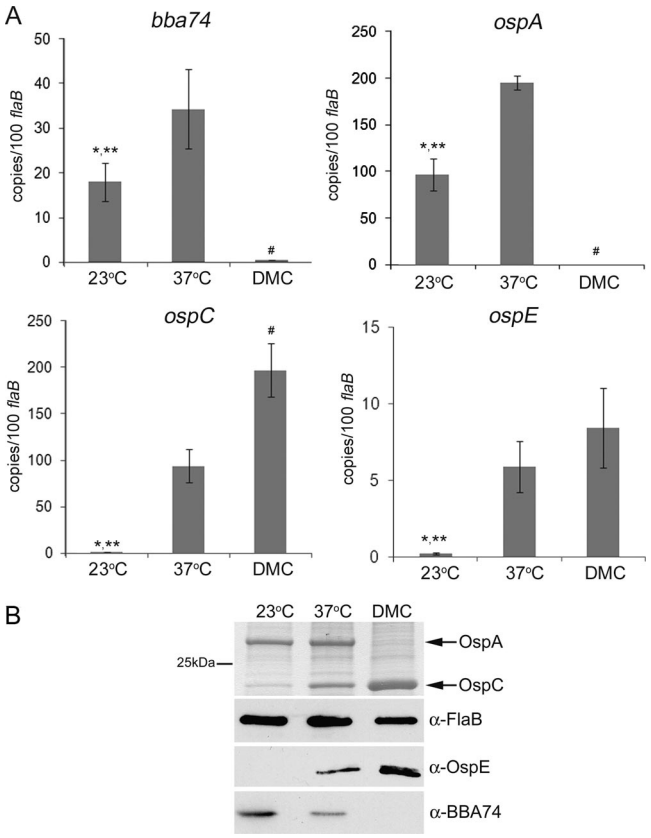

FIG. 1. Transcription and translation of *bba74* correlate in vivo but not in vitro. (A) qRT-PCR analyses of *bba74*, *ospA*, *ospC*, and *ospE*. Values represent the average copy number (± standard deviation) for each gene normalized per 100 copies of *flaB*. Statistical significance was determined using an unpaired *t* test; average values are considered significantly different when P is ≤0.05. The single asterisk (*), double asterisks (**), and pound symbol (#) indicate significantly different values for 23°C versus 37°C, 23°C versus DMCs, and 37°C versus DMCs, respectively. (B) *B. burgdorferi* B31-MI whole-cell lysates were separated by SDS-PAGE and then silver stained to assess expression of OspA and OspC or immunoblotted using specific antisera directed against OspE and BBA74. Reactivity against FlaB was used to confirm that equivalent amounts of lysate were loaded per lane. α, anti.

during DMC cultivation (Fig. 1A). Expression of OspA, OspC, and OspE in vitro and within DMCs correlated well with the qRT-PCR results for each respective gene (Fig. 1B). As expected, given the lack of *bba74* transcript, BBA74 was undetectable in spirochetes grown within DMCs. In contrast, and despite the increase in transcript copy numbers observed following temperature shift, production of BBA74 decreased in vitro at the higher temperature. In each case, the same expression profile was observed in both strains 297 and B31-MI (data not shown) (18, 19, 39). These experiments, therefore, revealed a dichotomy between the in vitro transcriptional and translational behavior of *bba74* that does not occur within DMCs.

***bba74* is transcribed by σ^{70} but is subject to RpoS-mediated repression during mammalian host adaptation.** Primer extension analysis was used to identify the promoter elements upstream of the *bba74* coding sequence. As shown in Fig. 2A, a transcriptional start site was identified 29 bp upstream of the ATG start codon. Inspection of the upstream DNA revealed excellent matches for the consensus −10 and −35 σ^{70} recognition motifs (38). Previously, we demonstrated by microarray and qRT-PCR analysis that repression of *bba74* in vivo requires RpoS (19). Here, we confirmed these findings at the protein level by immunoblot analysis using lysates prepared from CE174, a well-characterized *rpoS* mutant (18, 26), and CE162, its strain 297 wild-type parent (Fig. 2B). To ensure the applicability to the B31 strain background of *bba74* and BBA74 expression studies performed using the strain 297 *rpoS* mutant, we compared 500 bp of *bba74* upstream DNA sequence from B31-MI with that of strain 297; the resulting alignments revealed only two nucleotide differences between these isolates (data not shown), neither of which was located within the promoter itself. Also note that *bba74* was strongly transcribed at 23°C (Fig. 1A), a growth condition under which *rpoS* and RpoS-dependent genes are not expressed (18, 42). Taken together, these results indicate that *bba74* is transcribed exclusively by σ^{70} and that downregulation of this gene requires both mammalian host-specific signals and RpoS.

***bba74* is induced during tick feeding but is not expressed during murine infection.** To extend the above findings, we next used qRT-PCR to examine expression of *bba74* and BBA74 within flat and fed nymphs at drop-off (72 to 96 h postattachment) and in mouse hearts approximately 17 days postattachment. The data in Fig. 3A show strong induction of *bba74* during tick feeding, whereas virtually no transcripts for *bba74* were detected in either flat nymphs or infected mice. This expression profile differed from that of *ospA*, which was expressed intensely in flat nymphs and continued to be expressed at high levels during feeding but was not detected in infected mouse hearts. *ospC* was induced upon nymphal feeding, and its expression increased further during acute murine infection. *ospE* expression was induced during feeding, albeit at approximately sevenfold-lower transcript copy numbers than *ospC*, and continued to be expressed within infected heart tissue. Antibody responses in mice during acute infection were examined to corroborate the qRT-PCR results at the protein level. As shown in Fig. 3B, tick-inoculated mice produced a strong antibody response to OspC and a weaker response to OspE while antibodies directed against BBA74 were not detected. Thus, the transcriptional profile of *bba74* is unique in that, like *ospE* and *ospC*, it is induced by the mammalian host signals encountered during tick feeding, but, like *ospA*, it is not expressed during infection.

Immunofluorescence microscopy was used to assess expression of BBA74 by individual spirochetes colonizing flat nymphs and within engorged nymphs and larvae. The cumulative results of three independent experiments are presented in Table 1. BBA74 was barely detected in flat ticks but was expressed by more than two-thirds of the spirochetes in both fed nymphal and larval midguts; this result closely resembles the transcriptional pattern observed in flat and fed nymphs (Fig. 3A). OspC was virtually undetectable in flat nymphs and fed larvae but was strongly induced in engorged nymphs; it is important to note, however, that this antigen was detected in less than half of the spirochetes in fed nymphs. OspA, in contrast, was expressed by nearly all of the spirochetes in flat nymphal midguts, by the vast majority of organisms in fed larvae, and by approximately two-thirds of the spirochetes in engorged nymphs. The

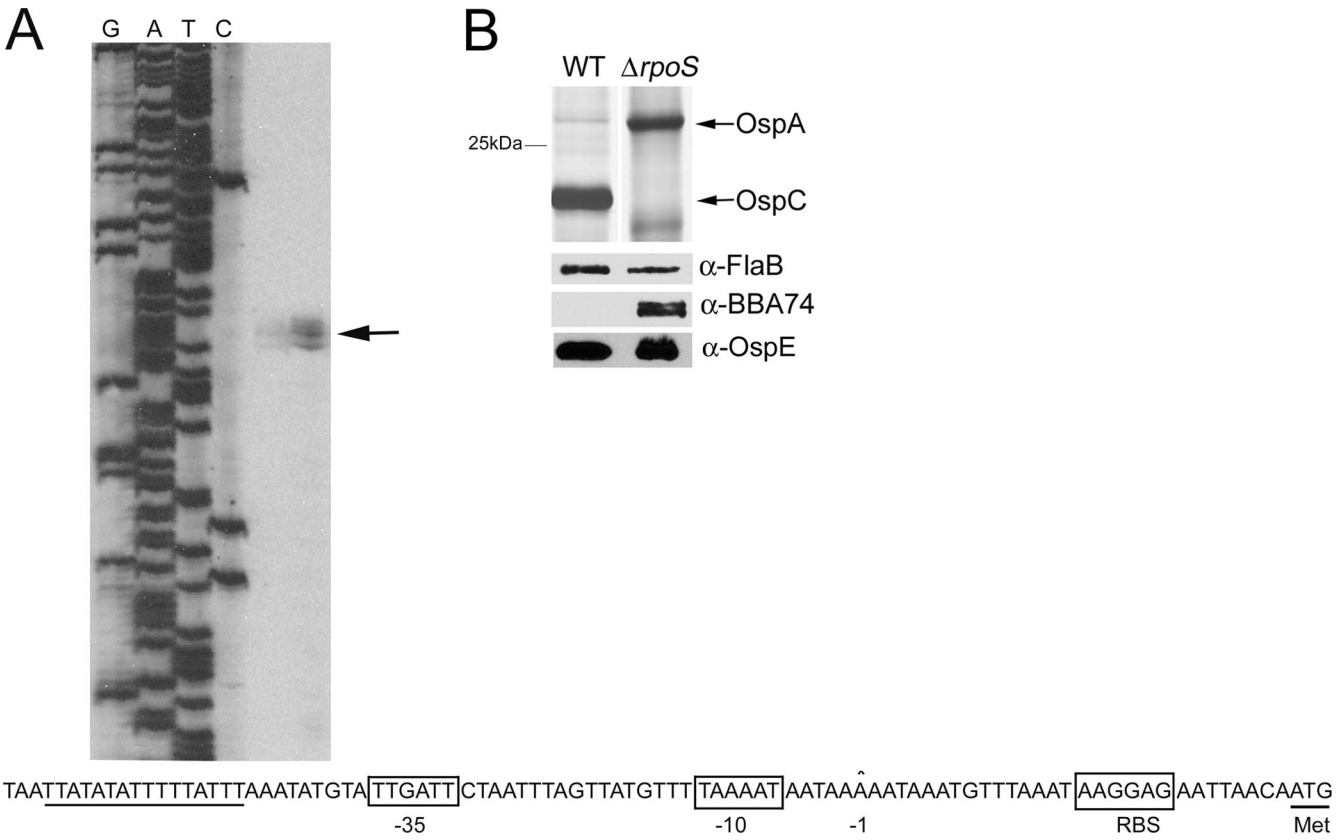

FIG. 2. *bba74* is transcribed by σ^{70} but requires both mammalian host signals and RpoS for downregulation in vivo. (A) The transcriptional start site for *bba74* identified by primer extension analysis is indicated by an arrow. Putative −10 and −35 σ^{70} factor recognition motifs (boxed) as well as a poly(T) tract (underlined) are indicated below. RBS, ribosomal binding site. (B) RpoS-deficient spirochetes fail to downregulate BBA74 during cultivation within DMCs. Whole-cell lysates prepared from wild-type (WT) and *rpoS* mutant (Δ*rpoS*) strain 297 isolates were separated by SDS-PAGE and then silver stained to assess expression of OspA and OspC or immunoblotted using specific antisera directed against OspE and BBA74. Reactivity against FlaB was used to confirm that equivalent amounts of lysate were loaded per lane. α, anti.

decreased labeling for OspA in fed compared to flat nymphal midguts was statistically significant ($P = 0.035$). The labeling pattern for BBA74 most closely resembled that of OspE, which was expressed by very few spirochetes in flat nymphs but was highly expressed in both fed midgut environments; the difference in labeling between fed nymphs and larvae was not significant ($P = 0.09$). As expected, the expression patterns for all four antigens in strain 297 were highly similar to expression in B31-MI (18, 19, 39; also data not shown). The strong parallel between BBA74 and OspE during all tick stages (Table 1) stands in striking contrast to the differences in their expression levels observed at 23°C in vitro (Fig. 1).

RpoS-mediated repression begins during nymphal tick feeding. Because RpoS-dependent genes also are induced during nymphal feeding (19, 36, 69, 76, 77), we could not state unequivocally that expression of *bba74* during this stage is dependent solely on σ^{70}. Moreover, the data presented thus far left open the question of whether the RpoS-mediated repression of *bba74* observed in DMC-cultivated spirochetes is induced by signals conveyed by the blood meal or occurs only after spirochetes have been transmitted to their mammalian host. To address these issues, we infected naïve *I. scapularis* larvae with wild-type or *rpoS* mutant *B. burgdorferi* using the immersion feeding method developed by Policastro and Schwan (72); once fed on a naïve mouse, *rpoS* mutant spirochetes survived the larval molt and persisted in flat nymphs at levels comparable to the wild-type parent (data not shown). Flat nymphs infected by immersion with either wild-type or *rpoS* mutant spirochetes were fed on naïve mice and examined by qRT-PCR and IFA. As shown in Fig. 4, the normalized transcript copy numbers for both *bba74* and *ospA* increased dramatically (4.75-fold and 2.3-fold, respectively) in the *rpoS* mutant compared to its wild-type counterpart while *ospE* was expressed at similar levels by both isolates. Transcripts for *ospC* were detected only in ticks infected with the wild-type isolate, thereby confirming that the absolute RpoS dependence of this lipoprotein observed in other systems (19, 26, 42, 105) also pertains in ticks. IFAs revealed that a significant proportion of wild-type strain 297 spirochetes in fed nymphs were negative for BBA74 (26%) and OspA (23%), whereas virtually all of the *rpoS* mutant spirochetes expressed these two antigens (Table 2). OspE, on the other hand, was detected at comparable levels in both isolates during feeding. Consistent with the qRT-PCR data, OspC was not detected in the RpoS-deficient organisms.

Loss of *bba74* does not appear to affect spirochetal infectivity or transit between tick and mouse. In an attempt to functionally characterize BBA74, we took advantage of the seren-

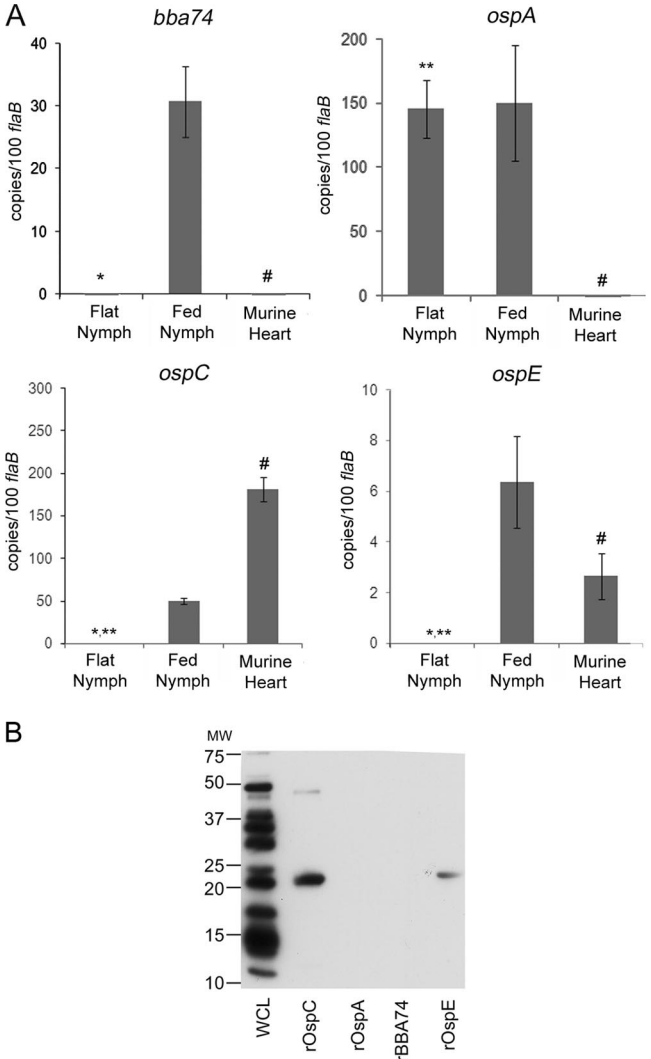

FIG. 3. *bba74* is expressed during nymphal feeding but not during murine infection. (A) qRT-PCR analysis of *bba74*, *ospA*, *ospC*, and *ospE* in flat and fed nymphal ticks and in murine hearts. RNAs used to generate cDNAs were obtained from pools of 150 flat or 30 fed B31-MI-infected nymphs at 72 h postattachment and from hearts isolated from three tick-infected C3H/HeJ mice at 17 days postattachment. Values represent the average copy number for each gene (± standard deviation) normalized per 100 copies of *flaB*. Statistical significance was determined using an unpaired *t* test; average values are considered significantly different when P is ≤0.05. The single asterisk (*), double asterisks (**), and pound symbol (#) indicate significantly different values for flat versus fed nymphs, flat nymphs versus murine tissue, and fed nymphs versus murine tissue, respectively. (B) Serological responses in mice 3 weeks following infestation with B31-MI-infected *I. scapularis* nymphs were assessed by immunoblot analysis using B31-MI whole-cell lysates (WCL) or purified recombinant (r) antigens. MW, molecular weight in thousands.

dipitous observation that B31-A3 contains a point mutation at position 118 of the coding sequence, resulting in a mature polypeptide truncated at amino acid 40 (data not shown). Immunoblot analysis confirmed that B31-A3 does not express full-length BBA74, whereas a polypeptide of the appropriate size (apparent molecular mass of 25 kDa) was readily detected in its isogenic parent, B31-MI (data not shown). The virulence

TABLE 1. Immunofluorescent labeling of *B. burgdorferi* organisms within infected *Ixodes* ticks

Antigen	Percentage of antigen-labeled spirochetes in population of FlaB-labeled spirochetes in[a]:		
	Flat nymphs	Fed nymphs[b]	Fed larvae[c]
BBA74	1.77 ± 0.6	78.72 ± 7.18[d]	63.4 ± 8.37[d]
OspA	92.28 ± 6.69[e]	67.75 ± 11.96[d,e]	89.12 ± 9.21[d]
OspE	3.21 ± 0.46	81.18 ± 7.58[d]	69.84 ± 4.43[d]
OspC	ND	43.68 ± 5.30	ND

[a] A minimum of 1,000 FlaB-labeled organisms were counted for each double-labeling experiment. Values presented are the average of three biologically independent experiments ± standard deviations. ND, no labeled spirochetes detected.
[b] Fed nymphs were collected at 72 h postattachment.
[c] Fed larvae were collected within 24 h of repletion.
[d] Values for fed larvae versus fed nymphs labeled for the same antigen are not significantly different.
[e] $P = 0.035$ for flat versus fed nymphs.

of this natural mutant was compared to that of B31-MI following syringe inoculation. As shown in Table 3, the ID_{50} values for B31-A3 and B31-MI were highly similar (890 and 1,450, respectively). Because *bba74* is induced in fed ticks, it was, of course, possible that the gene is involved in transmission and/or acquisition of spirochetes. However, B31-A3 isolates containing this point mutation (K. Tilly, personal communication) have been taken through the tick-mouse infectious cycle without any obvious defect (8, 91, 95).

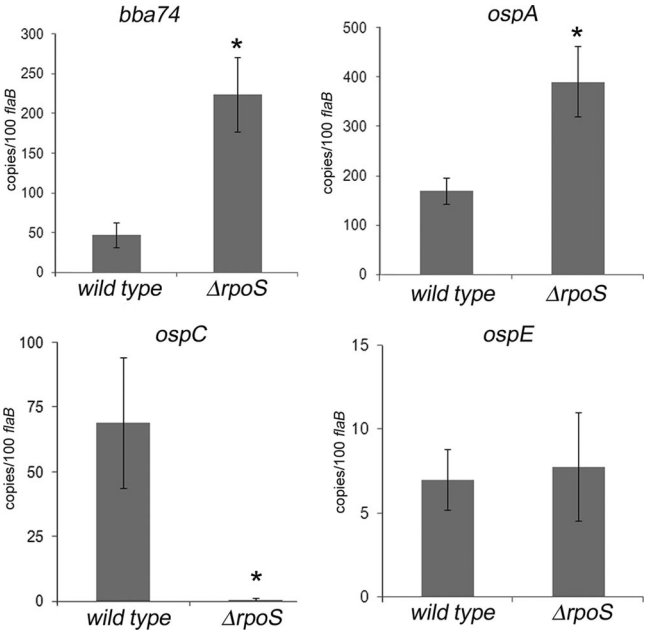

FIG. 4. qRT-PCR analysis of *bba74*, *ospA*, *ospC*, and *ospE* in fed nymphal ticks infected with either wild-type or *rpoS* mutant *B. burgdorferi* strain 297. RNAs used to generate cDNA for each isolate were obtained from pools of 30 fed nymphs ~72 h postattachment. Values represent the average copy number for each gene (± standard deviation) normalized per 100 copies of *flaB*. Statistical significance was determined using an unpaired *t* test; average values are considered significantly different when P is ≤0.05 (indicated by asterisks).

TABLE 2. Immunofluorescent labeling of wild-type and *rpoS* mutant *B. burgdorferi* organisms within fed *Ixodes* nymphs

Antigen	Percentage of antigen-labeled spirochetes in the indicated population of FlaB-labeled spirochetes[a]		P^b
	Wild type	*rpoS* mutant	
BBA74	74.13 ± 6.43	91.18 ± 5.48	0.002
OspA	77.33 ± 2.91	94.22 ± 3.11	0.004
OspE	53.90 ± 4.94	51.27 ± 72.12	NS
OspC	49.43 ± 2.25	0.6 ± 1.07	0.005

[a] A minimum of 1,000 FlaB-labeled organisms were counted for each double-labeling experiment. Values presented are the average of three biologically independent experiments ± standard deviations.
[b] NS, not significant.

TABLE 3. Infectivity of isolates B31-A3 and B31-MI

Isolate and dose (no. of spirochetes/ mouse [ID_{50}])[a]	No. of culture-positive samples/ total no. of samples[b]			
	Ear	Joint	Bladder	All sites
B31-A3 (890 ± 155)				
10^4	3/3	2/3	3/3	8/9
10^3	2/3	2/3	2/3	6/9
10^2	0/3	0/3	0/3	0/9
B31-MI (1,450 ± 150)				
10^4	3/3	3/3	2/3	8/9
10^3	1/3	1/3	1/3	3/9
10^2	0/3	0/3	0/3	0/9

[a] C3H/HeJ mice (three per group) were needle inoculated with each isolate at the dosage indicated. B31-A3 harbors a truncated BBA74 protein while isolate B31-MI expresses the full-length protein. ID_{50} values were calculated using an algorithm provided by the National Center for Biotechnology Information.
[b] Based on the presence of spirochetes 3 to 5 weeks following culture in BSK medium.

DISCUSSION

The complex changes that *B. burgdorferi* undergoes in response to either arthropod- or mammalian host-specific signals are mediated by only two transcriptional pathways. The first utilizes the constitutively expressed sigma factor, σ^{70}, while the second is controlled by the Rrp2-RpoN/RpoS signaling network (17–19, 32, 33, 83, 103, 105). The majority of the genes controlled by σ^{70} encode proteins that are related to cell maintenance and general metabolism and, therefore, are likely to be required throughout the enzootic cycle. In addition to these housekeeping genes, σ^{70} also controls the differential expression of genes involved in host adaptation. Some of the earliest and best-studied examples of σ^{70}-dependent upregulation are the *ospE* alleles (also referred to as *erpA*, *erpC*, and *erpP* in B31-MI). Differential expression of these genes is regulated in a temperature-dependent manner in vitro that is mirrored by their expression profiles during the enzootic cycle (1, 3, 35, 39, 60, 89, 93). σ^{70} also transcribes a subset of genes whose expression is downregulated in response to either mammalian host-specific (i.e., *ospA*) (1, 35, 41, 71, 77) or arthropod-specific (i.e., *ospD* and *p66*) (24, 91) signals. As is well recognized in other bacteria, changes in promoter recognition by σ^{70} in *B. burgdorferi* likely involve the binding of *trans*-acting factors. The borrelial genome encodes at least four putative DNA binding proteins, Hbb (48, 94), Gac (47), BosR/Fur (10, 46), and the putative YbaB ortholog, EbfC (5, 55), which could contribute either positively or negatively to transcription. The constitutive nature of σ^{70} enables the spirochete to modulate the expression of genes transcribed by this sigma factor at any point during the enzootic cycle.

The second transcriptional pathway in *B. burgdorferi* involves the spirochete's two alternate sigma factors, RpoN and RpoS. Unlike σ^{70}, these sigma factors must be either activated (RpoN) or transcribed de novo (RpoS) in order to modulate gene expression. Although Fisher et al. (32) contended that RpoN controls the expression of a large number of genes independently of RpoS, more recent data demonstrating a substantial overlap between the RpoN and RpoS regulons (9, 66) argues that the contribution of RpoN to borrelial gene regulation is largely confined to the induction of *rpoS*, in line with the model originally proposed by Hubner et al. (42). Microarray analyses by us (19) and others (9, 32, 66) have identified more than 100 genes whose expression is absolutely dependent on RpoS (19). The strict requirement for RpoS ensures that these genes are transcribed only under conditions when this alternate sigma factor is present. RpoS-dependent gene expression begins during the nymphal blood meal (19) and continues within the mammalian host (34, 40, 43, 52, 54, 104); we have designated this period within the enzootic cycle as the RpoS-ON state (Fig. 5). RpoS also is required for the downregulation of 33 σ^{70}-dependent genes in response to mammalian host signals, among which are the tick-phase genes *ospA*, *bba62*, and *lp6.6* (17, 19). Relief from RpoS-mediated repression occurs almost immediately following acquisition of spirochetes by feeding naïve larvae (16, 19, 35, 76), thereby giving rise to a period within the enzootic cycle that we have designated as the RpoS-OFF state (Fig. 5). By synchronizing the reciprocal regulation of tick- and mammalian host-phase-specific genes with these alternating RpoS states, *B. burgdorferi* has evolved a facile means of precisely coordinating the transcriptome changes required for transitioning between its mammalian host and arthropod vector. The demonstration here that RpoS-deficient spirochetes survive and replicate at levels comparable to their wild-type counterparts within flat and fed nymphs infected as larvae by immersion provides further support for our contention that RpoS is not required for physiological adaptation during the enzootic cycle but, rather, controls the expression of key virulence determinants involved in cycling between the arthropod and mammalian hosts (18, 19).

The expression profile of *bba74* illustrates the complexity of gene regulation that the spirochete can achieve by integrating elements of both the σ^{70}- and RpoN/RpoS-dependent regulatory networks. *bba74* is transcribed when *rpoS* is either not expressed (i.e., at 23°C in vitro and within fed larvae) or not present (i.e., *rpoS* mutant). Based on these findings, we conclude that transcription of *bba74* is strictly σ^{70} dependent, and, therefore, differential expression of this gene likely involves modulating promoter recognition by the housekeeping sigma factor. Like *ospA*, *bba74* is not expressed during murine infection, i.e., during the RpoS-ON state. The avirulence of RpoS-deficient spirochetes (18) precludes a direct examination of mechanism(s) controlling downregulation of *bba74* and *ospA* during infection; using DMCs as a surrogate for infection, however, we have demonstrated, here and elsewhere (17, 19),

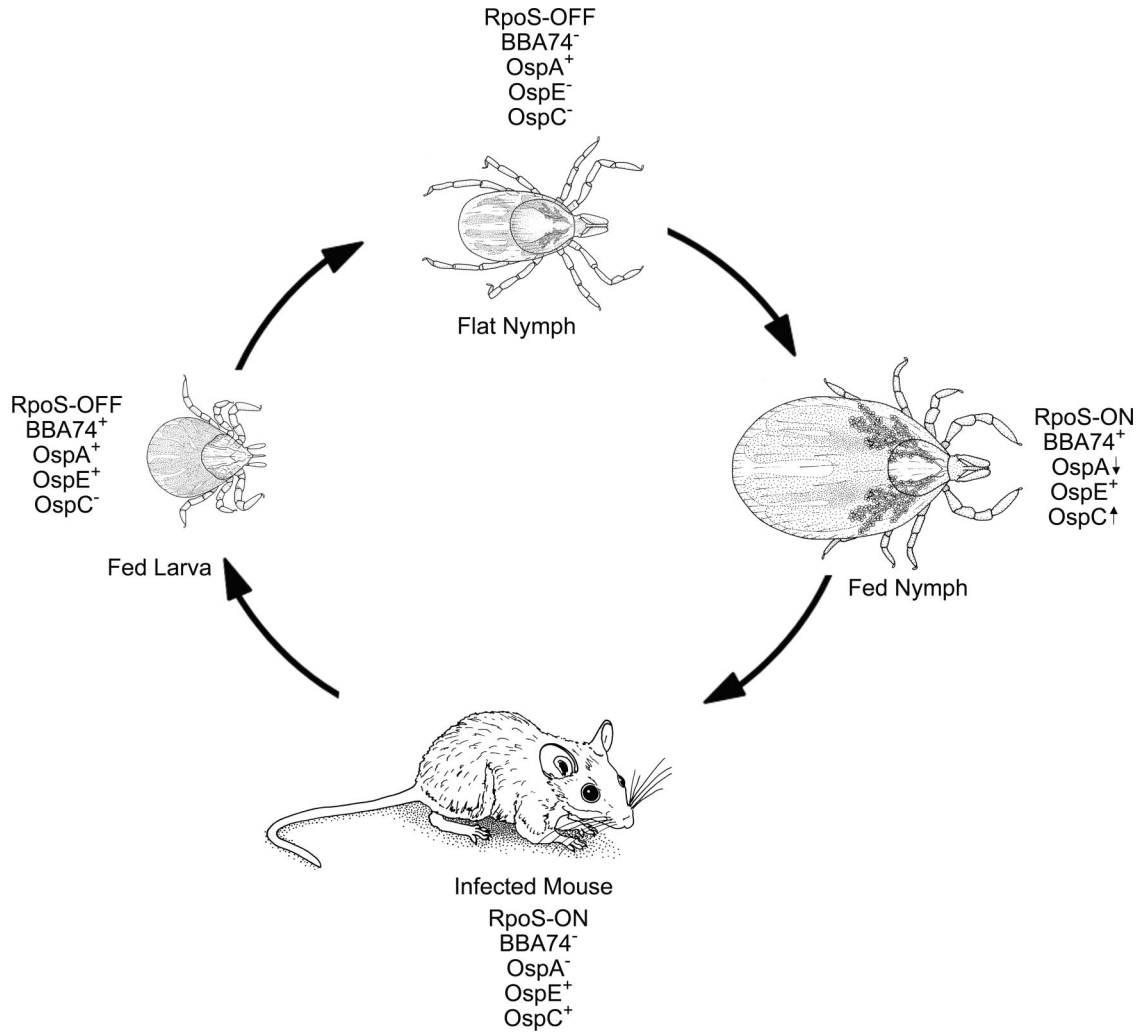

FIG. 5. Expression profiles of BBA74, OspA, OspE, and OspC in relation to the on and off states of RpoS during the enzootic cycle. Expression of each antigen is based on qRT-PCR and IFA data. Up and down arrows are used to indicate the decreasing and increasing expression of OspA and OspC, respectively, by spirochetes during the nymphal blood meal.

that repression of both of these genes within the mammalian host is mediated by RpoS. Expression of *bba74* and *ospA* resumes during the larval blood meal, when newly acquired spirochetes assume the RpoS-OFF state. In contrast to *ospA*, expression of *bba74* declines to virtually undetectable levels following the larval molt when *rpoS* is no longer expressed (19). Based on the observation that *bba74* is well expressed at 23°C in vitro (Fig. 1A), we hypothesize that the lack of expression of this gene within flat nymphs is due to an RpoS-independent form of suppression induced by arthropod-specific signals. Release from this suppression would then be triggered by the influx of the next blood meal as demonstrated by the high levels of BBA74-positive spirochetes in fed nymphs. As feeding progresses and spirochetes prepare for transmission to the mammalian host, expression of *bba74* once again becomes subject to RpoS-mediated repression, as is evident by the marked increase in BBA74 positivity in fed nymphs infected with *rpoS* mutant spirochetes. By bracketing the transcription of *bba74* between two forms of repression that are synchronized with the spirochete's RpoS-ON and RpoS-OFF states, *B. burgdorferi* is able to restrict its expression to the larval and nymphal blood meals. The interrelationship between the σ^{70} and RpoS transcriptional networks discerned from our analyses of *bba74* also provides a fresh perspective on *ospE*, the other σ^{70}-dependent gene examined herein. Like *bba74*, *ospE* is not expressed within flat nymphs and is induced during nymphal feeding. Because it is not subject to RpoS-mediated repression, *ospE* continues to be transcribed during murine infection and larval acquisition (Fig. 5). This expression pattern ensures that OspE is present during all stages of the enzootic cycle when the spirochete requires its complement-inactivating (11, 49) and plasminogen-binding (12) activities.

A comparison of the upstream sequences for *bba74* and the three paradigmatic genes examined herein helps to explain how spirochetes achieved the versatile transcriptional programs involved their expression (Fig. 6). Mutagenesis studies in *B. burgdorferi* (26, 27), as well as in *Escherichia coli* (98), have demonstrated that the −10 motif is a critical determinant of sigma factor recognition and/or promoter selectivity. Consistent with their σ^{70} dependence, the promoters for *bba74*,

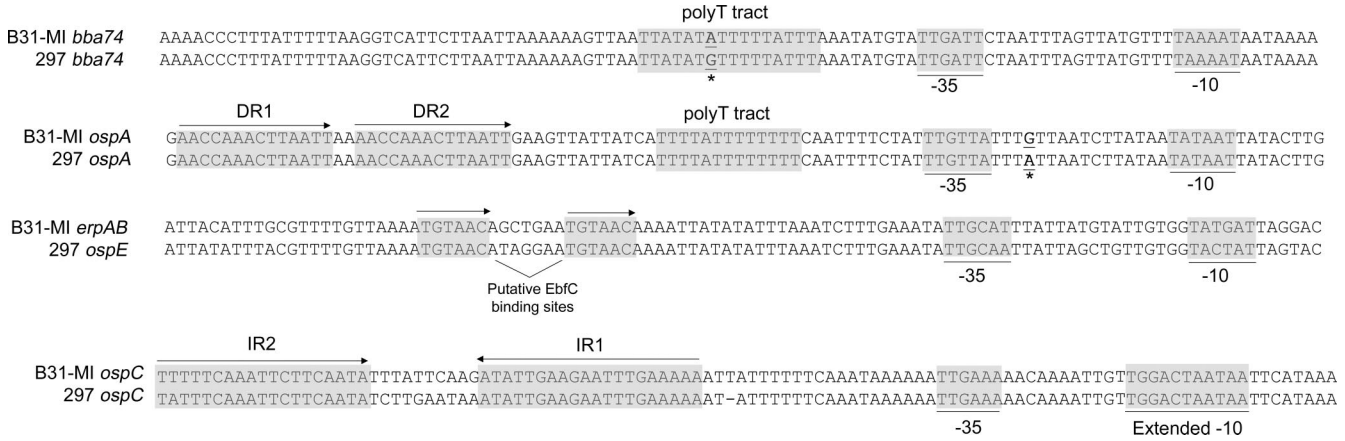

FIG. 6. Alignment of the *bba74*, *ospA*, *ospE*, and *ospC* upstream sequences from strains B31-MI and 297. The σ^{70} consensus −35 and −10 sites for *ospA*, *bba74*, and *ospE* are based on those of *E. coli* (50, 99) while the RpoS-dependent extended −10 for *ospC* is from Eggers et al. (26). The *ospA* direct repeats (DR1 and DR2) and *ospC* operator region (IR1 and IR2) are based on Sohaskey et al. (84) and Xu et al. (101), respectively. Poly(T) tracts upstream of *ospA* and *bba74* are based on Caimano et al. (19). Putative EbfC binding sites are based on Babb et al. (5). Transcriptional start sites (+1) for *ospA* (84), *ospC* (57), and *ospE* (39) are based on previously published reports while the start site for *bba74* is based on primer extension analyses presented here. Asterisks are used to indicate nucleotide polymorphisms between the *ospA* and *bba74* genes of strains B31-MI and 297, confirmed by sequencing.

ospA, and *ospE* all contain similar −10 consensus motifs that are distinct from the extended −10 sequence [TG(G/A)(G/A) ATA(T/A)ATT] required for promoter recognition of *ospC* and other RpoS-dependent genes (19). The high degree of similarity between the *bba74* and *ospA* −10 motifs compared to the same region of *ospE* could account for the markedly higher transcript levels for these two genes in vitro and within fed ticks. The *ospA* upstream sequence contains two motifs, a poly(T) tract and the direct repeats DR1 and DR2, which could function as *cis*-acting elements for the downregulation of this gene (Fig. 6). If one assumes that the RpoS-dependent downregulation of *bba74* and *ospA* occurs via a common *trans*-acting factor, then DR1 and DR2 cannot be involved because they are not present upstream of *bba74*. We (17, 19) along with others (13, 84) have proposed that the poly(T) tracts shared by both *bba74* and *ospA* represent a candidate repressor binding site. *ospE* is thought to be regulated, in part, by EbfC, a chromosomally encoded YbaB DNA binding protein ortholog (55) induced during tick feeding and murine infection (5, 59). The absence of EbfC recognition sites (TGTAACA) within the *bba74* upstream region leads us to predict that *bba74* and *ospE* are regulated differently despite the fact that both are downregulated within flat nymphs and induced during the nymphal blood meal. The observation that *ospE* displays a classic temperature-inducible expression pattern in vitro (1, 2, 28, 39, 89) while *bba74* does not (Fig. 1A) supports this supposition.

The reciprocal expression of OspA and OspC as spirochetes alternate between the arthropod vector and mammal host is a central paradigm of differential gene regulation by *B. burgdorferi* (75, 90). Numerous studies demonstrating that spirochetes within flat nymphs express abundant amounts of OspA and no OspC, while the converse pattern holds during mammalian infection, are often interpreted to mean that expression of these two lipoproteins is mutually exclusive. On the other hand, qRT-PCR and IFA studies, including those presented here, have shown that spirochetal populations within fed nymphs express both antigens simultaneously (31, 64, 76, 77), while Ohnishi et al. (64) demonstrated by double labeling that this is true for individual organisms as well. How can one explain the ostensible dichotomy created by the findings that strict reciprocal expression of OspA and OspC does not occur during all stages of the enzootic cycle? The heterogeneity in OspA and OspC expression levels observed during nymphal feeding could also be viewed as problematic for our concept of RpoS as the coordinator of the *ospA* ↔ *ospC* transcriptional switch. One potential explanation is that upregulation of OspC occurs during the nymphal blood meal while RpoS-dependent downregulation of OspA occurs within the mammalian host. Two lines of evidence, however, argue strongly that these two transcriptional changes occur concurrently: (i) IFAs revealing that a substantial proportion of spirochetes within fed nymphs are $OspA^{-}/OspC^{+}$ (30, 64, 76) (Tables 1 and 2) and (ii) our demonstration herein that expression of *ospA* is markedly enhanced in *rpoS* mutant spirochetes during the nymphal blood meal (Fig. 4 and Table 2).

We propose that the "deviation"' from the OspA/OspC paradigm results instead from the concatenation of transcriptional and translational events that ensue when spirochetes transition from the RpoS-OFF to -ON state (Fig. 5). The substantial proportion of spirochetes within engorged nymphs that remain $OspC^{-}$ (~50 to 80%) (64, 76) (Tables 1 and 2) indicates that, for unknown reasons, many organisms either do not initiate the RpoS program or do so very slowly. For those organisms that do demonstrate an RpoS-ON state, one would predict that the appearance of OspC, which is dependent solely on the transcription and translation of *ospC* mRNA, would occur much more rapidly than the downregulation of OspA, which requires the synthesis and/or activation of an unidentified repressor, the turnover of residual *ospA* mRNA, and the dilution of the large amounts of OspA lipoprotein present prior to the burst of replication that accompanies the blood meal. Srivastava and de Silva (86) proposed a similar scenario based on the use of flow cytometry to study the expression of OspA and OspC by wild-type and *rpoN* mutant spirochetes following a temperature

shift in vitro. This sequence of events serves equally well to explain the heterogeneity of BBA74 expression within fed nymphs. A key question that remains unresolved is how spirochetes achieve the high degree of $OspA^{-}/OspC^{+}$ uniformity that is characteristic of acute infection. Ohnishi et al. (64) concluded that the selectivity for $OspA^{-}/OspC^{+}$ spirochetes does not occur within the tick but within the dermis of the infected murine host. Previous reports (32, 36) demonstrating that a lack of OspC does not impair the ability of spirochetes to traverse the midgut and penetrate the salivary glands are in accord with this conclusion. A recent study by Battisti et al. (8) suggests that, in addition to its role as a TROSPA ligand, OspA serves a critical antibody-shielding role within nature, where *B. burgdorferi*-infected nymphal ticks may feed on reservoir hosts that have been previously infected; in this case, the continued expression of OspA during the nymphal blood meal would provide a survival advantage to spirochetes that would not be reflected within laboratory-infected nymphs fed on naïve hosts. Given the minute fraction of organisms that reach the salivary glands (22, 32, 36, 56, 69, 70; also our unpublished findings), it is reasonable to presume that the transcriptional events initiated by feeding do generate an infection-competent phenotype that is denoted by an as yet undetermined antigen expression pattern, including downregulation of BBA74.

We took advantage of the serendipitous discovery that B31-A3 is a natural BBA74 mutant to investigate the biological function of the corresponding gene product. The virulence of B31-A3 by needle inoculation is in line with our findings that *bba74* is not expressed during murine infection. In light of the high levels of expression of BBA74 within both fed larvae and nymphs, we were surprised to learn, however, that B31-A3 is able to successfully complete the tick-mouse cycle (8, 36, 91). We cannot dismiss the possibility that *bba74* encodes either a nonessential gene product or that its loss is compensated for by another borrelial gene. Alternatively, it is possible that insertional inactivation of *bba74* within a clonal wild-type background may yield results that differ from those comparing B31-A3 and B31-MI. Moreover, naturally infected ticks collected from sites of endemicity often have substantially lower spirochete burdens (15) than those generated experimentally. Therefore, a comparison of wild-type- and Δ*bba74* mutant-infected ticks containing spirochete burdens closer to those found in nature may reveal a difference in survival rates between the two isolates. Lastly, BBA74 may be required for adaptation within a tick microenvironment that cannot be readily detected using qRT-PCR and IFA. Stewart et al. (91) recently reported that *ospD* exhibits a blood meal-restricted expression profile similar, though not identical, to that of *bba74*. As with BBA74, OspD-deficient spirochetes did not exhibit an obvious phenotype and were able to complete the tick-mouse infectious cycle, leading the authors to propose that OspD may have a nonessential, but nevertheless important, role in intercellular cell signaling and/or nutrient scavenging. Unlike the OspD lipoprotein, BBA74 is located entirely within the periplasm and, therefore, would not function at the host-pathogen interface. *bba74* and *ospD* point to the existence of a class of gene products required by the spirochete for adaptation to the common physiological demands and/or environmental stresses imposed by the blood meal during both stages of the tick life cycle.

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