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Posttranscriptional Self-Regulation by the Lyme Disease Bacterium's BpuR DNA/RNA-Binding Protein

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Bacteria require explicit control over their proteomes in order to compete and survive in dynamic environments. The Lyme disease spirochete *Borrelia burgdorferi* undergoes substantial protein profile changes during its cycling between vector ticks and vertebrate hosts. In an effort to understand regulation of these transitions, we recently isolated and functionally characterized the borrelial nucleic acid-binding protein BpuR, a PUR domain-containing protein. We now report that this regulatory protein governs its own synthesis through direct interactions with *bpuR* mRNA. *In vitro* and *in vivo* techniques indicate that BpuR binds with high affinity and specificity to the 5′ region of its message, thereby inhibiting translation. This negative feedback could permit the bacteria to fine-tune cellular BpuR concentrations. These data add to the understanding of this newly described class of prokaryotic DNA- and RNA-binding regulatory proteins.

Global regulatory factors that bind nucleic acids act on diverse targets to modulate bacterial physiology and pathogenesis. Due to the inherent biochemical features of nucleic acid-binding proteins, self-regulation is a common theme (1–7). This mode of action affords precise feedback regulation, creating rheostat-like control over the protein's regulon. It could be surmised that this feature is a critical component of bacterial physiology to ensure that cellular concentrations do not surpass a critical threshold, which might result in deleterious effects (5, 8–11).

In addition to transcription initiation, accumulating evidence indicates that, like their multicellular counterparts, bacteria regulate their proteomes at the posttranscriptional level. Strategies that they use to do so are diverse and include antisense RNAs, secondary structures that respond to small molecules (riboswitches), and mRNA-binding proteins that can stabilize or promote transcript degradation (12–16). Known functions of bacterial RNA-binding proteins include roles in virulence, cellular physiology, DNA replication, and molecular trafficking (17–29). As knowledge of the diverse targets of RNA-binding proteins continues to expand, the need to understand how these factors are regulated becomes more pressing.

We recently biochemically characterized *Borrelia burgdorferi* BpuR, a novel type of prokaryotic nucleic acid-binding protein (19). BpuR shares significant structural and sequence identity with eukaryotic PUR-domain proteins, which are critical pre- and posttranscriptional regulatory factors (30–36). BpuR is a transcriptional regulatory factor in the Lyme disease spirochete *Borrelia burgdorferi* and its own production is controlled by the bacterium (19). We now demonstrate that BpuR can bind with high affinity to its own mRNA and inhibit its own translation.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *B. burgdorferi* was cultured in Barbour-Stoenner-Kelly II medium (37, 38). When appropriate, kanamycin, gentamicin, or both were added to cultures of transformed bacteria at final concentrations of 200 μg/ml and 70 μg/ml, respectively. For transformation studies, a clonal derivative of *B. burgdorferi* type strain B31, known as B31e2, was used. RNA immunoprecipitation and primer extension experiments were performed using the infectious clonal strain B31 MI-16 (39).

5′ RACE to determine the *bpuR* transcriptional start site. *B. burgdorferi* B31 MI-16 was grown at either 23 or 34°C to mid-exponential growth phase (approximately 5×10^7 cells/ml). Cells were harvested, and RNA was isolated as previously described (40). Purified RNA was treated with DNase to remove contaminating DNA and reverse transcribed into cDNA following the manufacturer's recommended procedures (Roche). Primer extension was performed on each RNA preparation using 5′ rapid amplification of cDNA ends (RACE; Invitrogen, Carlsbad, CA). Controls consisted of reaction mixtures that lacked reverse transcriptase, hexameric oligonucleotide primers, or template DNA. The resulting fragments were cloned into pCR2.1 (Invitrogen). Twelve clones were selected at random, and the inserts were sequenced. The sequences of the products were aligned, compared to the *B. burgdorferi* B31 genome sequence, and aligned with the sequences of other Lyme disease-causing spirochetes using the Geneious program.

***In vivo* production of BpuR or EbfC from inducible promoter constructs.** We previously described plasmid constructs in which either *bpuR* or *ebfC* is under the transcriptional control of the inducible P*ost* promoter system (19, 41–43). Each plasmid was individually introduced into *B. burgdorferi* B31e2. To evaluate the dose-dependent response to inducer, anhydrotetracycline (ATc) was added to early-exponential-phase cultures (approximately 10^5 bacteria/ml) of each transformed strain at a final concentration of 0, 0.2, 0.4, or 0.8 μg/ml. After cultivation to a final density of approximately 10^7 bacteria/ml, bacteria were harvested by centrifugation, washed, and lysed and proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE). Total proteins were detected by Coomassie brilliant blue staining to assess them for equal loading.

Immunoblot analyses. EbfC was identified by immunoblotting using monospecific antiserum (41). The constitutively expressed FlaB protein

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TABLE 1 EMSA probes and oligonucleotide primers used in this study[a]

Oligonucleotide name	Modification	Sequence (5′→3′)	Target/purpose
BioRNAbpuRp-F (RNA)	5′ biotin	CUUAAAUGUAGUCAAGUACAAAAACUUGUGUGGAGGAAAUU GAUGGGAGAGAGAGGGGAAGUAUACUCU	*bpuR* TSS to nt +30/EMSA
BioRNAbpuRORF (RNA)	5′ biotin	AAACUAUUUACAGAGUCUGAGAGAACUUAUUUUUUUAAUGU CAAGGAAAAUAGAAAAGGAGAUUAUUUU	*bpuR* nt +31 to +100/EMSA
BioRNA61-F (RNA)	5′ biotin	AAUGGAGAGAUUUUGGGGGAGUUGUUUAAAAUUACAUUUG CGUUUUGUUAAAAUG	*erp* operator/EMSA
bpuRp-12	None	GCTAGCTAAAAATAACATTAC	*bpuR* NCD/EMSA-dsDNA
BiobpuRp-13	5′ biotin	GTAATGTTATTTTTAGCTCGA	*bpuR* NCD/EMSA-dsDNA
bpuRp-14	None	CCACACAAGTTTTTGTACTTGAC	*bpuR* NCD/EMSA-dsDNA
Bioerp 129-F	5′ biotin	GAGACGGGGAGTTGTTAAATT	*erp* operator/EMSA-dsDNA
A69-R	None	GTAACAGCTGAATGTAAC	*erp* operator/EMSA-dsDNA
GFP-5	None	GTGACAAGTGTTGGCCATGGAAC	*gfp* mRNA
GFP-6	None	CACTGGAGTTGTCCCAATTCTTGTTG	*gfp* mRNA
BpuR-1	None	GGAGAGAGAGGGGAACTATAC	*bpuR* RIP-PCR
BpuR-2	None	GCCTTGCAAAGGAGCCAACG	*bpuR* RIP-PCR
Fla-3	None	GGGTCTCAAGCGTCTTGG	*flaB* RIP-PCR
Fla-4	None	GAACCGGTGCAGCCTGAG	*flaB* RIP-PCR

[a] All nucleic acids were DNA, except as noted. NCD, noncoding DNA region; ORF, open reading frame; TSS, transcription start site.

served as a loading control (44, 45). Antiserum directed against BpuR was produced commercially in New Zealand White rabbits by NeoPeptide (Cambridge, MA). A polypeptide corresponding to the BpuR sequence VESKRSPSGDFERH was used for vaccination. Antibodies were affinity purified from serum using the vaccinogen polypeptide.

Lysates from transformed strains were boiled, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were blocked with Sea Block blocking buffer (Thermo Fisher, Hudson, NH). Primary antibody detection was accomplished by secondary donkey anti-rabbit (EbfC and BpuR) or goat anti-mouse (FlaB) IgG conjugated to horseradish peroxidase and detected by SuperSignal West Pico chemiluminescent substrate (Thermo Fisher, Hudson, NH). Band intensities were normalized to the band intensity of FlaB by densitometric analysis and graphed using GraphPad Prism (version 5.0) software (46).

Recombinant proteins. Recombinant BpuR was expressed and purified as previously described (19). Purified proteins were dialyzed against a buffer compatible with electrophoretic mobility shift assay (EMSA) (1 nM dithiothreitol, 25 mM Tris [pH 7.5], 5 mM NaCl, 0.01% [vol/vol] Tween 20, 10% glycerol, 0.1% [vol/vol] phenylmethanesulfonyl fluoride) or compatible with *in vitro* transcription/translation reactions (25 mM Tris [pH 7.5], 5 mM NaCl, 1% [vol/vol] glycerol) (19, 41, 42). Protein concentrations were determined by Bradford analysis (Bio-Rad, Hercules, CA). Protein preparation purity was determined by SDS-PAGE and staining with Coomassie brilliant blue. Aliquots of purified protein were stored at −80°C.

EMSA. Nucleic acids used as probes or PCR primers are described in Table 1. For double-stranded DNA (dsDNA) probes, one oligonucleotide primer was 5′ end labeled with biotin and annealed as previously described (Integrated DNA Technologies [IDT], Coralville, IA) (47). Single-stranded RNA probes labeled with biotin at the 5′ end were synthesized chemically by IDT. All probe concentrations were determined spectrophotometrically.

EMSAs were performed essentially as previously described (48, 49). Protein-nucleic acid combinations were subjected to electrophoresis using 10% nondenaturing polyacrylamide gels (Invitrogen). Following transfer to Biodyne nylon membranes (Thermo Pierce) and UV cross-linking (Stratalinker 1800; Stratagene, San Diego, CA), biotin-labeled DNAs were visualized using nucleic acid detection kits (Thermo Pierce) and autoradiography.

For RNA-binding assays, all equipment was treated with diethyl pyrocarbonate prior to use, and RiboGuard RNase inhibitor (Epicenter, Madison WI) was added to each reaction mixture to a final concentration of 1 U/ml.

RNA immunoprecipitation (RIP). Culture conditions, cross-linking, and soluble fraction preparation have been previously described, with the exception that cross-linking time was reduced to 4 min (41, 42). We modified the immunoprecipitation (IP) buffer to contain 5 U/ml of RiboGuard. To shear the bacterial RNA, lysates were sonicated using a Branson 102C sonicator (Branson Ultrasonics, Danbury, CT) with 10 pulses of 15 s each at 15% amplitude.

Anti-BpuR or anti-IgG control (Santa Cruz) antibodies or phosphate-buffered saline (PBS) alone (bead control) was incubated with equal amounts of cleared lysate overnight at 4°C. Protein G resin particles were added to each IP or IP control reaction for 2 h at 4°C in the presence of RiboGuard. Target antigen-bead complexes were washed 5 times as previously described with the addition of 2 U/ml of RiboGuard (41, 42). The formaldehyde cross-link was reversed in RNase-free TE (Tris-EDTA) at 75°C for 10 min, RNA was purified and DNase treated, and cDNA synthesis was performed as described previously (50).

Oligonucleotides specific for the *bpuR* or *flaB* open reading frame (ORF) were used in separate PCRs, with BpuR IP eluates, the bead control, or the IP control serving as the template cDNA (Table 1). Amplicons were separated by agarose gel electrophoresis, stained with ethidium bromide, and imaged.

***bpuR*::*gfp* transcriptional/translational fusions and flow cytometry.** The plasmid containing a promoterless green fluorescent protein (GFP) gene (pBLS590) is described elsewhere and served as a background fluorescence control (51). In addition, this construct served as the backbone to splice the noncoding region of *bpuR* DNA into a site immediately 5′ of *gfp*, generating pGJ1. Derivative pBLJ370 was produced by site-directed mutagenesis of pGJ1 to add the first 30 bp of *bpuR* in frame with the beginning of *gfp*.

These three constructs were individually introduced into *B. burgdorferi*. In addition, *B. burgdorferi* strains that contained pBLJ307, which constitutively expresses high levels of *bpuR*, plus either pGJ1 or pBLJ370, were produced. pBLJ307 confers resistance to gentamicin, whereas pGJ1 and pBLJ370 confer resistance to kanamycin. Each strain was cultured at 34°C to mid-exponential growth phase. Bacteria were independently harvested, washed in PBS, and resuspended in PBS at approximately 10^6 cells/ml. The mean GFP fluorescence per bacterial cell was analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA), with excitation at 488 nm and detection at 530 nm. Each experiment involved measuring a minimum of 50,000 individual bacteria. The results reported represent a mean of three independent experiments.

***In vitro* coupled transcription and translation.** A linear DNA fragment was produced by PCR from template pGJ1 or pBLJ370 using oligonucleotide primers M13 Forward (5′-GTAAAACGACGGCCAG-3′) and

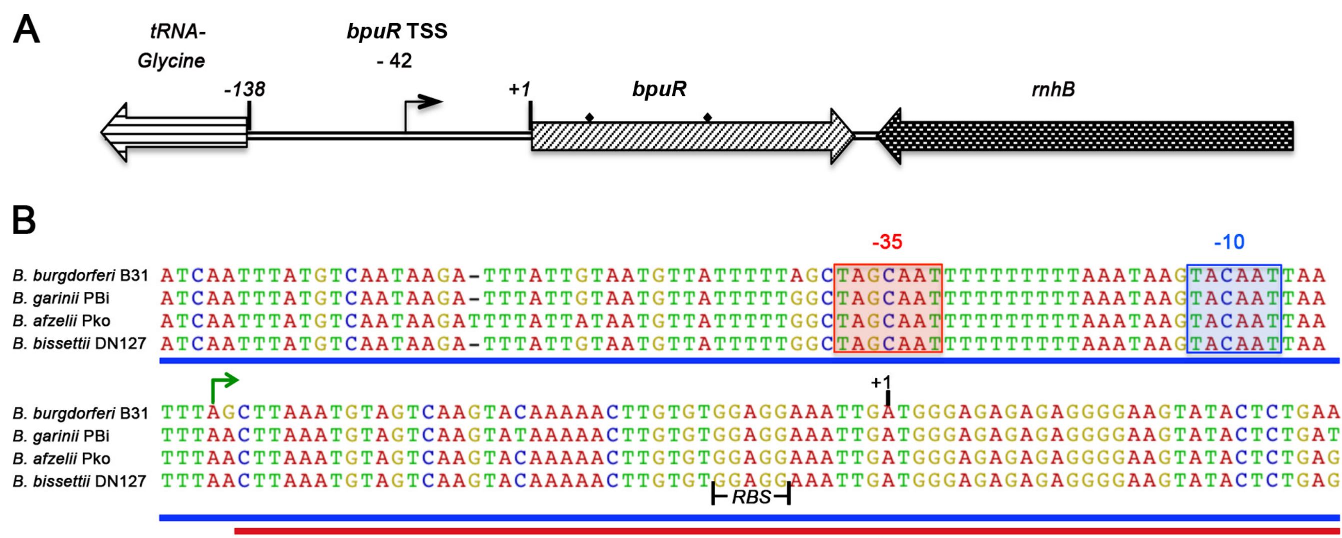


FIG 1 Mapping the *bpuR* transcriptional start site. (A) Schematic of the *bpuR* operon and neighboring loci in Lyme disease spirochetes. 5′ RACE analyses located the *bpuR* transcriptional start site 42 nt upstream of the first translational start codon (+1). Diamonds, locations of primers used for RIP-PCR. (B) Nucleotide sequence alignment of the *bpuR* operons indicated conservation in Lyme disease-causing spirochetes. Green arrow, site of the transcription initiation nucleotide; shaded boxes, predicted −10 and −35 regions; solid vertical black lines, *bpuR* ribosome-binding site (RBS); blue line, DNA sequence used for the *bpuR* operon fusion pBLJ370; red line, the corresponding DNA of pBLJ370 used for RNA; black line, the *bpuR* mRNA required for negative autoregulation shown at the level of DNA.

M13 Reverse (5′-CAGGAAACAGCTATGAC-3′). Coupled *in vitro* transcription and translation reactions, using *Escherichia coli* S30 extracts (Promega) were performed as previously described, with slight modifications (19, 41). Each reaction mixture was identical, with the exception that the final BpuR concentration was 0, 2, 4, or 8 nM. In addition, 1 U of RNase inhibitor was added to all reaction mixtures to prevent mRNA decay, which could influence the total amount of RNA present.

Quantitative reverse transcription-PCR (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA) were used to evaluate the influence of BpuR on *gfp* transcript and protein levels, respectively. Briefly, after completion, the reaction mixtures were separated into equal volumes and *gfp* transcript or GFP protein levels were evaluated as described previously (19, 41). For transcript production, total RNA was extracted using an Epicenter MasterPure RNA extraction kit following the manufacturer's recommended procedure. Contaminating DNA was removed by DNase treatment, and RNA was reverse transcribed into cDNA as previously described (50). Quantitative PCRs (qPCRs) were performed as previously described (42, 52). ELISAs were performed using standard methods, as previously described (41, 53). GFP detection utilized anti-GFP–horseradish peroxidase conjugate (MACSmolecular; Miltenyi Biotec, Auburn, CA).

Statistical analyses. Statistical significance between samples was determined by Student's *t* test, assuming unequal variance. Protein level differences were evaluated densitometrically using ImageJ software and normalized against the levels for the loading controls. Each experiment was performed at least twice, unless otherwise described in the text.

RESULTS

Defining the *bpuR* transcriptional unit. Extending our observations that *B. burgdorferi* controls cellular levels of the BpuR protein (19), we sought to determine the basis of this regulation. The *bpuR* gene is flanked on either side by divergently transcribed genes, indicating that *bpuR* forms a monocistronic operon (54) (Fig. 1A). Mapping by 5′ RACE detected a single transcriptional start site 42 bp upstream of the first coding methionine (Fig. 1B). Identical results were obtained from analyses of RNA purified from bacteria cultured under conditions that yielded either high or low levels of BpuR production, suggesting that *bpuR* is transcribed from only a single promoter (19, 46).

BpuR binds *bpuR* mRNA. The first clue about BpuR autoregulation came from studies of *B. burgdorferi* strains that contain a construct that should enhance cellular concentrations of BpuR. In order to evaluate the effects of BpuR levels on *B. burgdorferi* physiology, the *bpuR* ORF was placed under the control of the inducible P*ost* promoter (19, 43). This TetR-repressible promoter permits the experimenter to precisely regulate transcription by titrating the inducer molecule anhydrotetracycline (ATc) into culture medium (43). In all previously tested P*ost*-regulated chimeras, increasing concentrations of ATc induced protein in a dose-dependent manner (41–43, 47). For example, *B. burgdorferi* containing a P*ost*::*ebfC* transcriptional fusion produced steadily increasing concentrations of EbfC protein as ATc was titrated into the culture medium (Fig. 2) (41, 42). However, equivalent titration of ATc into cultures of *B. burgdorferi* carrying the P*ost*::*bpuR* construct initially increased BpuR levels approximately 1.8-fold, but BpuR levels did not increase further, regardless of how much ATc was added (Fig. 2). Noting that this construct lacks the native *bpuR* promoter, the observed regulation of BpuR protein production must be an intrinsic property of BpuR and/or the *bpuR* ORF.

BpuR binds RNA with a 10-fold higher affinity than dsDNA (19). The protein exhibits sequence specificity with both substrates, preferentially interacting with guanine-rich sequences (Fig. 3; see Fig. 5) (19). The initial nucleotides of *bpuR* mRNA include an extensive stretch of purines (Fig. 1). Centering on the translational start site, 18 of 34 (53%) nucleotides (nt) of *bpuR* mRNA are guanine, an oddity in this bacterium that overall contains less than 30% G+C (54).

To test whether BpuR can bind its own mRNA, EMSAs were performed using labeled RNAs derived from the transcriptional

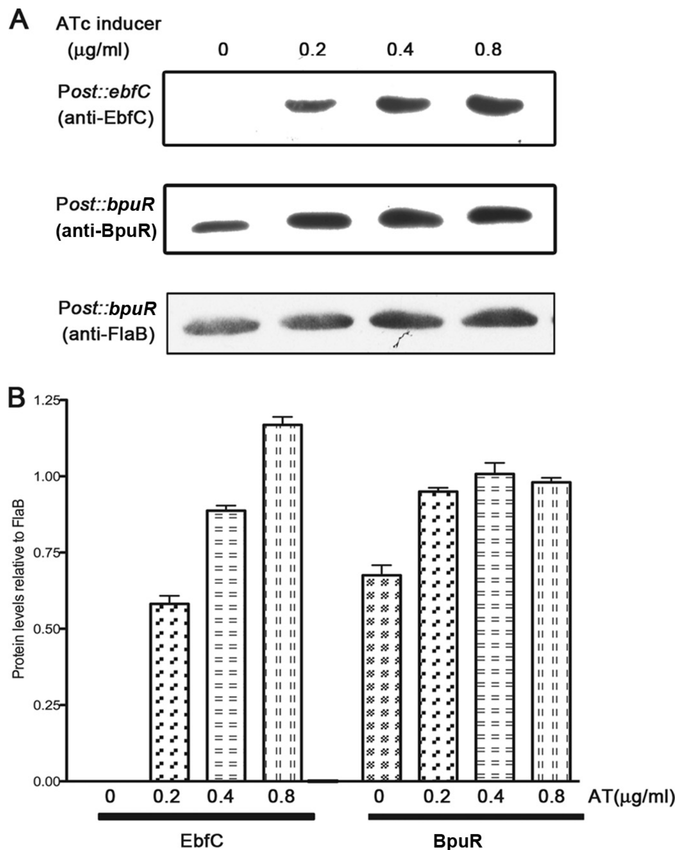


FIG 2 Autogenous regulation of BpuR occurs independently of the *bpuR* promoter. (A) *B. burgdorferi* was independently transformed with plasmids carrying P*ost*::*ebfC* or P*ost*::*bpuR*. The levels of EbfC and BpuR were assessed by immunoblotting following induction with 0, 0.2, 0.4, or 0.8 µg/ml ATc. Levels of FlaB (flagellin), which served as a loading control, were determined by immunoblotting. Illustrated are FlaB immunoblotting results for the bacteria carrying P*ost*::*bpuR*. (B) Densitometric analysis of P*ost*::*ebfC* and P*ost*::*bpuR* immunoblotting results normalized to the FlaB data for each strain. Note that the anti-BpuR antibody detected BpuR produced by the bacterium's native *bpuR* locus (lane with no ATc added).

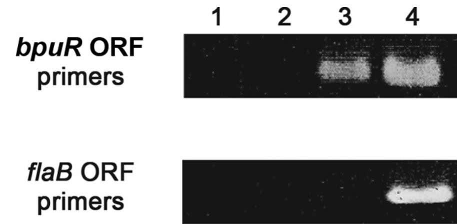


FIG 4 RIP demonstration that BpuR binds *bpuR* mRNA *in vivo*. Live *B. burgdorferi* bacteria were cross-linked, fixing BpuR to mRNAs within the cell. IP was conducted with BpuR-specific antibodies. BpuR-bound mRNAs were eluted, DNase treated, and reverse transcribed. PCR was performed using oligonucleotides specific for the *bpuR* or *flaB* ORF (top and bottom panels, respectively). Lane 1, control reactions of protein A beads alone; lane 2, control reactions of protein A beads plus nonspecific IgG; lane 3, RIP reactions of protein A beads plus BpuR-specific antibodies; lane 4, PCR of purified *B. burgdorferi* genomic DNA, serving as a control.

start site through the first 30 nt of the *bpuR* ORF, or nt 31 to 100 of the *bpuR* ORF. In addition, an RNA molecule consisting of the high-affinity BpuR-binding sequence in the *erp* operator served as a positive control (19). BpuR bound to the RNA that consisted of the *bpuR* ribosome-binding site and the first 30 nucleotides of the open reading frame (Fig. 3A). The affinity for this labeled RNA was similar to that displayed for the control probe, which has a dissociation constant (K_d) of 13 nM (Fig. 3A and C) (19). In contrast, BpuR did not bind to the RNA sequence derived from the downstream region of its own open reading frame (Fig. 3B). Since each RNA probe was approximately the same size (~70 nt), those differences cannot be attributed to a probe length bias (55).

Extending our *in vitro* analysis, we next directly tested whether BpuR binds its native mRNA in live *B. burgdorferi*, using **R**NA **i**mmuno**p**recipitation (RIP) (56–58). Much like chromatin immunoprecipitation (ChIP), RIP utilizes cross-linking of proteins to nucleic acids in live bacteria, followed by immunoprecipitation of complexes that contain a specific protein. However, RNA rather than DNA is purified from the immunoprecipitate. The RNA was reverse transcribed and then subjected to PCR with oligonucleotide primers specific for *bpuR* mRNA.

BpuR-specific RIP yielded a single amplicon of the same size as a genomic DNA control (Fig. 4, top, lanes 3 and 4, respectively). RIP control reactions included a mock immunoprecipitation using an irrelevant IgG affixed to protein G beads or protein G beads alone. Those reactions failed to produce PCR products (Fig. 4, top, lanes 1 and 2). Since molecular crowding and absolute transcript levels can cause transient, nonspecific interactions between proteins and nucleic acids, the *flaB* mRNA was tested as a control to confirm RIP specificity (59, 60). *flaB* expression is constitutive, and cellular mRNA levels are abundant relative to the levels of other mRNAs in the borrelial cell, making it a suitable control

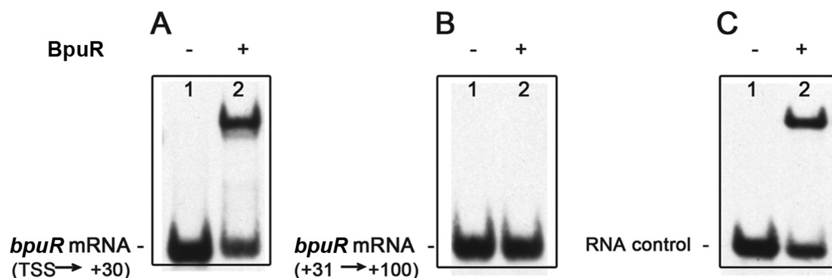


FIG 3 BpuR binds specifically to *bpuR* mRNA *in vitro*. EMSAs were performed with recombinant BpuR and a labeled *bpuR* RNA transcript. (A) Labeled RNA (2 nM) corresponding to the *bpuR* transcriptional start site to position +30 of the *bpuR* ORF; (B) labeled RNA (2 nM) corresponding to positions +31 to +100 of the *bpuR* ORF; (C) labeled *erp* operator sequence (2 nM), as RNA, serving as a positive control (19). Each probe was incubated either with 10 nM BpuR protein (lanes +) or without added BpuR (lanes −).

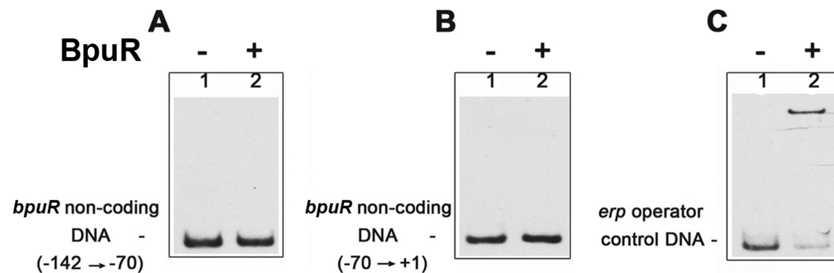


FIG 5 BpuR does not bind *bpuR* promoter DNA. EMSAs of recombinant purified BpuR and *bpuR* 5′ noncoding DNA. (A) Labeled dsDNA (1 nM) corresponding to positions −142 to −71 relative to the *bpuR* translational start site; (B) labeled dsDNA (1 nM) corresponding to positions −70 to +1 relative to the *bpuR* translational start site; (C) labeled *erp* operator dsDNA (1 nM) serving as a positive control (19). Each probe was incubated either with 60 nM BpuR protein (lanes +) or without added BpuR (lanes −).

locus for RIP evaluation (44, 45). Control or BpuR IP PCR of immunoprecipitated mRNA did not yield a *flaB* amplicon (Fig. 4, bottom). Thus, these RIP assays may be concluded to be specific, demonstrating that BpuR binds to its own mRNA in live *B. burgdorferi*.

BpuR does not bind *bpuR* promoter DNA. Many nucleic acid-binding proteins control their own production by interacting with DNA adjacent to their own promoter (5, 61, 62). Noting that BpuR can bind double-stranded DNA (19), we could not disregard that possibility for BpuR. Moreover, eukaryotic PUR-domain proteins may govern their own transcription through direct interactions with noncoding DNA (35). To that end, EMSAs were performed with recombinant BpuR protein and two dsDNA probes that span the *bpuR* 5′ noncoding region. BpuR did not bind to either of these probes (Fig. 5A and B). Since both dsDNA probes were the same length as the *bpuR* RNA probes described above, we could confidently discount probe length as a variable influencing BpuR-nucleic acid interactions (55). Furthermore, controls with a labeled *erp* operator dsDNA probe confirmed that BpuR was active and bound to that high-affinity dsDNA sequence (Fig. 5C) (19). While it is possible that BpuR might bind dsDNA outside the *bpuR* 5′ noncoding region, additional studies described below demonstrated that BpuR does not detectably affect transcription from its own promoter.

BpuR is a negative autoregulator. To test the effect of BpuR binding to mRNA in live borreliae, two GFP reporter constructs were created: pGJ1, which consists of the *bpuR* promoter driving transcription of *gfp*, and pBLJ370, which consists of the *bpuR* promoter and the first 30 bp of *bpuR* fused in frame to *gfp* (Fig. 6A). *B. burgdorferi* was transformed with pGJ1 or pBLJ370 to produce strains BJ26 and BJ27, respectively. Each of those plasmids was additionally transformed into a *B. burgdorferi* strain that carries a second plasmid, pBLJ307, which caused maximal, constitutive expression of BpuR. Thus, strain BJ30 carries pGJ1 and pBLJ307 and strain BJ31 carries pBLJ370 and pBLJ307. This strategy provided a means for comparison between increased BpuR production and the potential effect on both the transcription and translation of the *bpuR* gene. Each construct harbors a different selectable marker, so incubation with both kanamycin and gentamicin ensured maintenance of both plasmids.

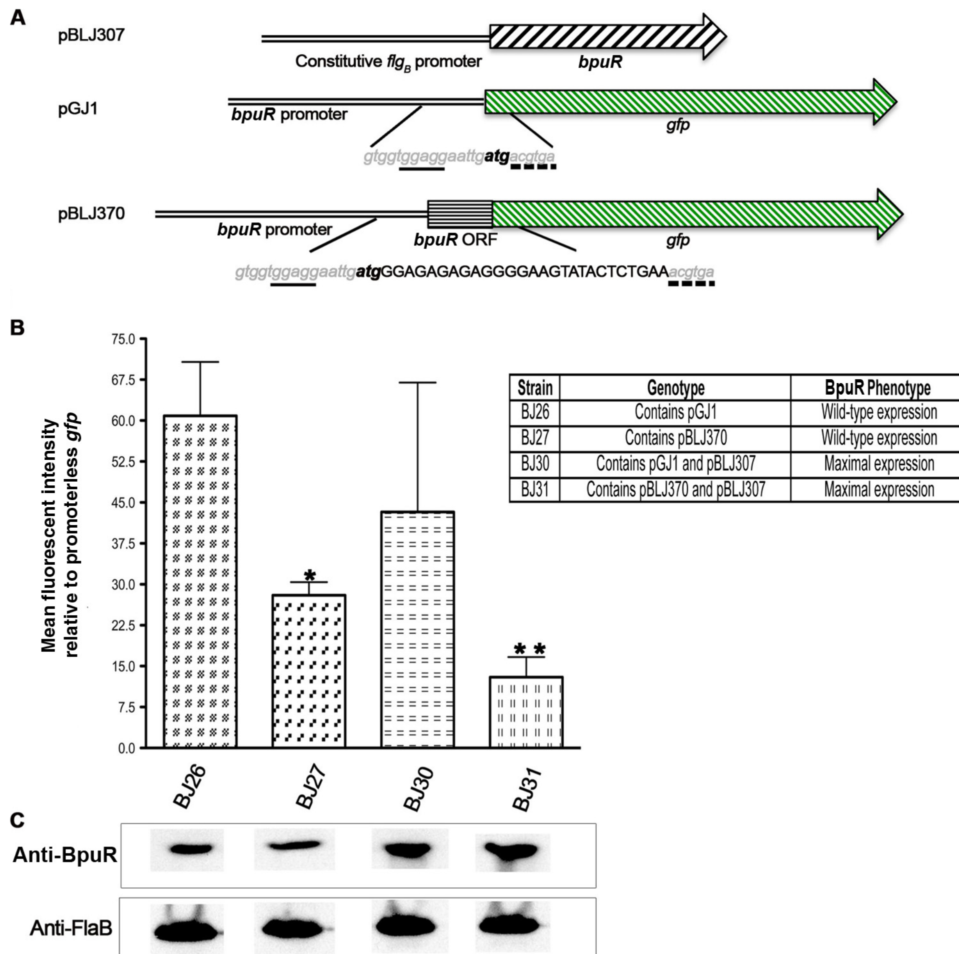
There were no significant differences between GFP expression by BJ26 and BJ30, which carry the wild-type *gfp* gene and do not and do express high levels of BpuR, respectively (Fig. 6B and C). However, addition of the first 30 bp of *bpuR* to *gfp* reduced GFP levels by 50% in bacteria that produced wild-type levels of BpuR (strain BJ27) and by 75% in bacteria that expressed higher levels of BpuR (strain BJ31) (Fig. 6B and C).

Considering that constitutive BpuR expression might alter reporter plasmid copy numbers, which could explain the reduction in GFP production, qPCR was performed on strains BJ27 and BJ31. Plasmid amplicons from each strain were determined quantitatively, and their amounts were normalized to the relative amount of the *B. burgdorferi* main linear chromosome present. The relative ratio of the amount of pBLJ370 in strain BJ27 to that in strain BJ31 was 1.095, indicating that elevated BpuR levels did not impact reporter plasmid copy number.

As a further, independent approach to determining the effects of BpuR on its own translation, we used an *in vitro* cell-free transcription/translation system (41). Two different linear templates were generated: the *bpuR* promoter fused to *gfp* (pGJ1 template) and the *bpuR* promoter fused to the first 30 nt of the *bpuR* ORF in frame with *gfp* (pBLJ370 template) (Fig. 6A). Purified BpuR was titrated into *E. coli* S30 transcription/translation reaction mixtures containing each template, and then qRT-PCR and ELISA were performed to determine the relative levels of mRNA and protein produced, respectively. The addition of purified BpuR did not have any significant effects on transcription from either template DNA (Fig. 7A). BpuR did not significantly affect the synthesis of GFP from the pGJ1 *bpuR* promoter fusion. In contrast, addition of 8 mM BpuR significantly inhibited translation from the pBLJ370 *bpuR*::*gfp* chimera (Fig. 7B). Collectively, these findings indicate that BpuR inhibits its own translation but does not significantly affect its transcription.

DISCUSSION

In vitro and *in vivo* studies indicated that BpuR binds *bpuR* mRNA and, consequently, inhibits its own translation. The BpuR binding site includes a region proximal to its ribosome-binding site. Thus, BpuR could effectively compete with 30S binding to nascent mRNA, since the calculated K_d for ribosome binding is higher than that of BpuR (19, 63). Physical boundaries for the translational machinery on mRNA extend from positions −20 to +15 relative to the translational start site, which constitutes a considerable portion of BpuR's high-affinity binding site on *bpuR* mRNA (64, 65). Alternatively, since ribosome occupancy has been proposed to include the first 12 nt from the charged tRNA P site, BpuR may act as a roadblock to prevent productive translation following ribosome loading (15, 66). It is also possible that *bpuR* transcription and translation are not directly coupled but are instead slightly delayed, creating a larger window for newly synthe-



Strain	Genotype	BpuR Phenotype
BJ26	Contains pGJ1	Wild-type expression
BJ27	Contains pBLJ370	Wild-type expression
BJ30	Contains pGJ1 and pBLJ307	Maximal expression
BJ31	Contains pBLJ370 and pBLJ307	Maximal expression

FIG 6 BpuR transcriptional and translational fusions. (A) Schematic of the plasmids transformed into *B. burgdorferi*. pBLJ307 carries a gentamicin resistance cassette and the *bpuR* locus driven by the constitutively expressed *flgB* promoter. pGJ1 possesses the *bpuR* promoter and 5′ noncoding DNA fused to *gfp*. pBLJ370 additionally contains the first 30 bp of the *bpuR* ORF fused to *gfp*. Both pGJ1 and pBLJ370 carry kanamycin resistance markers. Nucleotide sequences displayed inside vertical lines show the similarities (gray, lowercased, and italicized nucleotides) and differences (uppercased and black nucleotides) between pGJ1 and pBLJ370. The same ribosome-binding site (underlined nucleotides) and start codon (bold, lowercased nucleotides) were present in each construct. For continuity, the 2nd and 3rd codons of the GFP ORF are shown (broken lines). (B) Mean GFP expression assessed by flow cytometry from three independent experiments (arbitrary units). All values were normalized against the values obtained from a strain that carried a promoterless *gfp* construct (51). *, statistically significant difference between BJ26 and BJ27; **, statistically significant difference between BJ27 and BJ31. (C) Immunoblot analyses of each *B. burgdorferi* strain to assess cellular levels of BpuR and the constitutively expressed FlaB protein (loading control).

sized BpuR to adhere to the *bpuR* message and exert its inhibitory effect (67, 68). Any or all of these mechanisms of translational inhibition could be at play, and further molecular and atomic analyses will be required to determine the kinetics and limits of BpuR-mRNA binding *in vivo*.

Bacterial Hfq proteins also exhibit self-limiting, posttranscriptional autoregulation (14, 69). However, the mechanisms differ from what appears to occur with BpuR, with Hfq binding to the 5′ untranslated leader of *hfq* mRNA. BpuR's posttranscriptional autoregulation is reminiscent of the effect of *E. coli* Hfq on *ompA* mRNA, where binding adjacent to the ribosome-binding site reduces OmpA translation (15, 70). In that BpuR controls its own translation, it is possible that BpuR may similarly be a posttranscriptional regulator of other *B. burgdorferi* genes.

Functional similarities between BpuR and Hfq are not limited to autoregulation. BpuR was isolated due to its affinity for *erp* operator dsDNA (19, 71), and *E. coli* Hfq has been reported to interact with dsDNA (72–74). Hfq hexamers have two distinct RNA-binding faces, one of which interacts with purine-rich regions of RNA (75). PUR-domain proteins are widespread throughout the *Spirochetes* and *Bacteroidetes* phyla, but recognizable homologues are absent from most other bacterial genomes. In contrast, Hfq is almost ubiquitous throughout the *Eubacteria* and yet absent from the *Bacteroidetes* (76). An intriguing possibility is that bacterial PUR-domain proteins may be functionally equivalent to Hfq. *B. burgdorferi* has the most complex known prokaryotic genome and encodes both an Hfq and BpuR, which may together coordinate the different genetic elements (23, 54, 77).

In summary, *B. burgdorferi* BpuR binds to its own mRNA and inhibits translation. As demonstrated through the use of inducible transcription constructs, this prevents cellular BpuR concentrations from exceeding a certain level. BpuR is a transcriptional and posttranscriptional regulator, binding to both DNA and RNA (19;

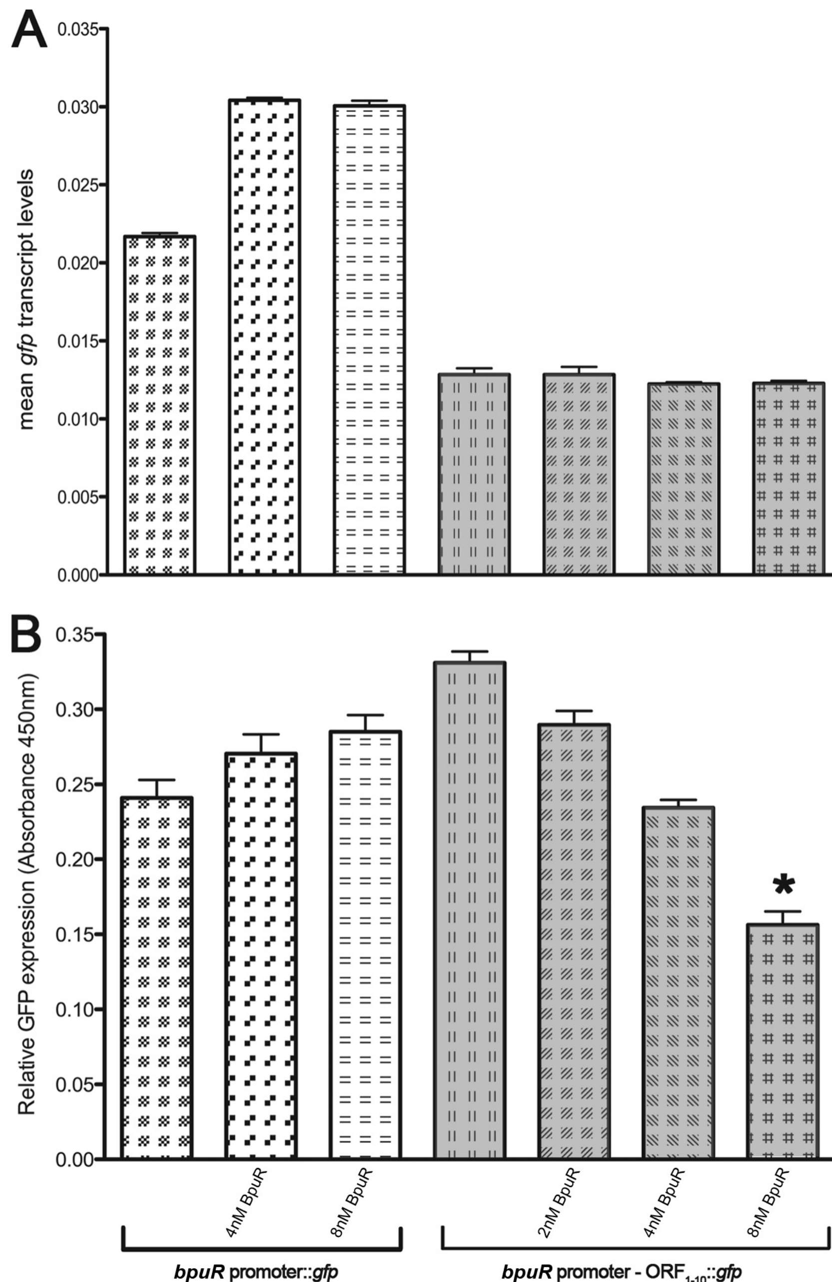


FIG 7 *In vitro* transcription and translation reactions indicating that BpuR inhibits its own translation. Note that the use of two different templates for these analyses means that absolute numbers cannot be compared between templates. (A) Mean qRT-PCR results for the *gfp* transcript produced from DNA templates consisting of either the *bpuR* promoter fused to *gfp* (unshaded bars) or the *bpuR* promoter plus the first 30 bp of the *bpuR* ORF fusion in frame to *gfp* (shaded bars). Reactions were conducted either without added BpuR protein or in the presence of 2, 4, or 8 nM recombinant BpuR. None of the differences in results without and with BpuR were statistically significant. (B) Mean ELISA values of GFP production levels from the same reactions. Values were normalized to those for a reaction mixture containing no template DNA. *, statistically significant difference ($P < 0.05$).

this study). This novel, multifunctional protein thus presents itself as a significant model for exploring the burgeoning field of bacterial posttranscriptional regulation.

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