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# Evidence that RpoS ($\sigma^S$) in *Borrelia burgdorferi* Is Controlled Directly by RpoN ($\sigma^{54}/\sigma^N$)$^\nabla$

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**The alternative sigma factor (RpoN-RpoS) pathway controls the expression of key virulence factors in *Borrelia burgdorferi*. However, evidence to support whether RpoN controls *rpoS* directly or, perhaps, indirectly via a transactivator has been lacking. Herein we provide biochemical and genetic evidence that RpoN directly controls *rpoS* in *B. burgdorferi*.**

Lyme disease, caused by *Borrelia burgdorferi*, is the most commonly reported arthropod-borne disease in both the United States and Europe (32). At the molecular level, certain membrane lipoproteins of *B. burgdorferi* are vital for maintaining the spirochete in its zoonotic transmission cycle between ticks and mammals. For example, the reciprocal regulation of outer surface (lipo)proteins A (OspA) and C (OspC) is the best-studied paradigm of the dramatic alterations in protein expression patterns that ensue as the spirochete transitions from the arthropod vector into mammalian tissues (8, 22, 30, 31). Our lab determined previously that expression of OspC is regulated by the alternative sigma factor RpoS ($\sigma^S$) (15, 40). RpoS, though, must first be activated by another alternative transcription factor, RpoN ($\sigma^{54}/\sigma^N$), resulting in an RpoN-RpoS regulatory network (10, 15). However, the precise mechanism(s) by which RpoN activates *rpoS* has not been determined. RpoN could control *rpoS* expression directly by binding to a region near the *rpoS* open reading frame (ORF). Alternatively, another transactivator induced by RpoN might activate *rpoS* expression.

We have been most attracted to the notion that RpoN binds directly to a region upstream of the *rpoS* ORF to facilitate RpoS expression in *B. burgdorferi*. This is because nucleotides −78 to −63 upstream of the *rpoS* ORF comprise a theoretical, RpoN-dependent consensus −24/−12 promoter (33, 34). Many studies with various bacteria have indicated close contact of RpoN with such a −24/−12 promoter region (5, 6, 21, 33). However, as yet, there have been no experimental data to substantiate this prediction for *B. burgdorferi*. The purpose of this study was to garner additional evidence that would either substantiate or refute the hypothesis that *rpoS* is regulated directly by RpoN.

**Identification of *rpoS* initiation of transcription.** Determination of the initiation of transcription for a given gene can provide strategic information for identifying a gene's nearby promoter. *rpoS* transcripts of *B. burgdorferi* BbAH130 (41) were reverse transcribed in BD SMART-RACE (switching mechanism at 5′ end of RNA transcript-rapid amplification of cDNA ends) reactions (BD Biosciences, San Jose, CA) using two *rpoS*-specific primers (rpoSR422 and rpoSR232) (Table 1), which are 422 and 232 bases, respectively, downstream of the *rpoS* translation start site. The BD SMART IIA primer, which anneals to the BD SMART IIA oligonucleotide linked to the 3′ end of the first-strand cDNA, and an *rpoS*-specific primer upstream of the gene-specific primers used in first-strand cDNA synthesis (rpoSR125) (Table 1) were used to amplify the resulting cDNAs. PCR-amplified inserts cloned into the pGEM-T Easy vector (Promega, Madison, WI) were digested with the restriction endonuclease DraI (New England Biolabs, Ipswich, MA) to verify that they represented *rpoS*. Seventeen of 25 clones contained *rpoS*-specific sequences, as determined by the DraI restriction pattern (not shown). Eight of these 17 clones were selected for further analysis. For five of these eight clones, residue −50 upstream of the *rpoS* ORF represented the transcript start site by RACE analysis, whereas there was no consensus residue for the other three sequences. The identified transcription start site was 14 nucleotides downstream of the conserved GC dinucleotide (Fig. 1). This result correlates well with the putative −24/−12 region representing the promoter for *rpoS*, inasmuch as initiation of transcription from a −24/−12 promoter occurs between 8 and 21 nucleotides downstream of the GC dinucleotide (2).

**Assessing RpoN binding to the *rpoS* promoter.** Ideally, direct binding of the *B. burgdorferi* RpoN protein to the *rpoS* promoter sequence would provide the most compelling evidence that the *rpoS* promoter in *B. burgdorferi* is RpoN dependent. However, numerous attempts to produce soluble recombinant forms of *B. burgdorferi* RpoN were unsuccessful. This was true for either the full-length protein or a truncated recombinant that preserved the predicted DNA-binding domain. We also employed lysates generated from *B. burgdorferi* cultures, grown under conditions in which RpoN is active (15, 41), in electrophoretic mobility shift assays (EMSAs). We were unable to demonstrate an interaction of *rpoS* sequences with borrelial cell lysates in EMSAs.

Previous studies have shown functional homology between RpoN proteins from different bacterial species. For example,

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TABLE 1. Oligonucleotides used in this study

| Name | Sequence (5′–3′) | Experiment(s) |
|---|---|---|
| rpoSR422 | TTGGGCGATTTTTCTTCTTC | Transcript initiation |
| rpoSR232 | ATCCCAAGTTGCCTTCTTCA | Transcript initiation, qRT-PCR |
| rpoSR125 | TTTGCTTTTGCATTGCCTCT | Transcript initiation |
| EMSA_rpoS1[a] | ACTTAATTTCCATGCAAAAACTGTGCCAATTTAATTTAAA | EMSA |
| EMSA_rpoS2[a] | TTTAAATTAAATTGGCACAGTTTTTGCATGGAAATTAAGT | EMSA |
| rpoS promoterF[a] | CCTATTTAGTTTAAAACCATTTTTAAATTAAATTGGCACAGTTTTTGCATGGAAATTAAGTAG | EMSA |
| rpoS promoterR[a] | CTACTTAATTTCCATGCAAAAACTGTGCCAATTTAATTTAAAAATGGTTTTAAACTAAATAGG | EMSA |
| rpoS_C-12A[a] | CCTATTTAGTTTAAAACCATTTTTAAATTAAATTGGCACAGTTTTTGAATGGAAATTAAGTAG | Promoter mutants, EMSA |
| rpos_G-12T[a] | CTACTTAATTTCCATTCAAAAACTGTGCCAATTTAATTTAAAAATGGTTTTAAACTAAATAGG | Promoter mutants, EMSA |
| rpoS_T-18G[a] | CCTATTTAGTTTAAAACCATTTTTAAATTAAATTGGCACAGGTTTTGCATGGAAATTAAGTAG | EMSA |
| rpoS_A-18C[a] | CTACTTAATTTCCATGCAAAACCTGTGCCAATTTAATTTAAAAATGGTTTTAAACTAAATAGG | EMSA |
| rpoS_G-24T[a] | CCTATTTAGTTTAAAACCATTTTTAAATTAAATTGTCACAGTTTTTGCATGGAAATTAAGTAG | Promoter mutants, EMSA |
| rpoS_C-24A[a] | CTACTTAATTTCCATGCAAAAACTGTGACAATTTAATTTAAAAATGGTTTTAAACTAAATAGG | Promoter mutants, EMSA |
| rpoS_d-20F[a] | CTACTTAATTTCCATGCAAAAAC-GTGCCAATTTAATTTAAAAATGGTTTTAAACTAAATAGG | Promoter mutants, EMSA |
| rpoS_d-20R[a] | CCTATTTAGTTTAAAACCATTTTTAAATTAAATTGGCAC-GTTTTTGCATGGAAATTAAGTAG | Promoter mutants, EMSA |
| rpoS_d-17/18F[a] | CTACTTAATTTCCATGCAAA-CTGTGCCAATTTAATTTAAAAATGGTTTTAAACTAAATAGG | Promoter mutants, EMSA |
| rpoS_d-17/18R[a] | CCTATTTAGTTTAAAACCATTTTTAAATTAAATTGGCACAG-TTTGCATGGAAATTAAGTAG | Promoter mutants, EMSA |
| rpoS_d-10F[a] | CTACTTAATTTCC-TGCAAAAACTGTGCCAATTTAATTTAAAAATGGTTTTAAACTAAATAGG | EMSA |
| rpoS_d-10R[a] | CCTATTTAGTTTAAAACCATTTTTAAATTAAATTGGCACAGTTTTTGCA-GGAAATTAAGTAG | EMSA |
| flgB promoterF[b] | AAAATACAAATTAACGAACAAAAAGATTTGAATTATTATATAATTTAGTTAAAAGCATTTTAA | EMSA |
| flgB promoterR[b] | TTAAAATGCTTTTAACTAAATTATATAATAATTCAAATCTTTTTGTTCGTTAATTTGTATTTT | EMSA |
| rpoSF30 | CATATATTTAAAATCAGTAAGAGAACACAAGCTAATTACTCACGAAGAAG | qRT-PCR |
| flaBF9 | CAATCATAATACATCAGCTATTAATGCTTCAAG | qRT-PCR |
| flaBR82 | CTTGAGTTTTACTAAGATTAGCAGCGTTAATG | qRT-PCR |

[a] The putative −24/−12 region of the *rpoS* promoter is underlined, and nucleotides deleted are shown with dashes.
[b] The −35 and −10 promoter sequences are underlined.

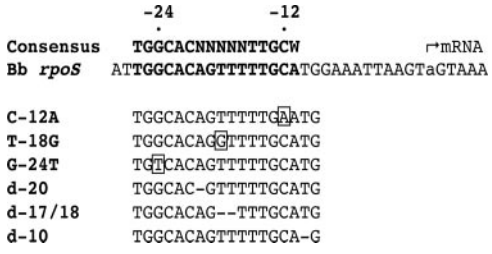


FIG. 1. *B. burgdorferi* (Bb) *rpoS* promoter region. The top line indicates the consensus −24/−12 RpoN-binding site (34), which is highlighted in bold in the *rpoS* promoter region beneath it. The lowercase letter "a" indicates the transcript start site (mRNA) experimentally determined by 5′-RACE analysis in this study. The relevant sequence changes in double-stranded oligonucleotides designed for this study are shown below the consensus region. The boxes highlight base substitutions, whereas the dashes identify base deletions.

*Escherichia coli* RpoN recognizes *Caulobacter crescentus flbG* and *flaN* promoters in an *E. coli* cell-free transcription system (25), and *Klebsiella pneumoniae* RpoN recognizes the *nifH* promoter sequence of *Rhizobium meliloti* (4). For this reason, as an alternative heterologous approach, we performed experiments to determine whether *E. coli* RpoN was capable of binding to the *B. burgdorferi rpoS* promoter region.

A 40-bp oligonucleotide encoding the predicted −24/−12 region of *B. burgdorferi rpoS* was end labeled with $^{32}$P and mixed with graded amounts of purified recombinant *E. coli* RpoN (16) in binding buffer (19). Resulting DNA-protein complexes were resolved by electrophoresis on 4.5% polyacrylamide and visualized by autoradiography. These EMSAs (Fig. 2) showed that *E. coli* RpoN bound to the *rpoS* target in a dose-dependent fashion. In addition, unlabeled *rpoS* promoter DNA could compete for binding, but sheared salmon sperm DNA had no competing effect. No shift was observed when bovine serum albumin was added as a nonbinding protein in place of *E. coli* RpoN. These data constitute strong

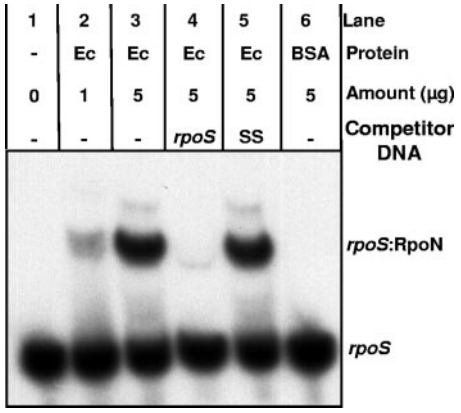


FIG. 2. EMSAs demonstrating binding of *E. coli* RpoN to the *B. burgdorferi rpoS* promoter region (40-bp oligonucleotide). Lanes 1, 2, and 3 represent increasing amounts (μg) of *E. coli* RpoN protein (Ec); lane 4 contains a 50-fold excess of unlabeled double-stranded oligonucleotide (*rpoS*); lane 5 contains a 50-fold excess of sheared salmon sperm DNA (SS); and lane 6 contains 5 μg of bovine serum albumin (BSA) in place of *E. coli* RpoN.

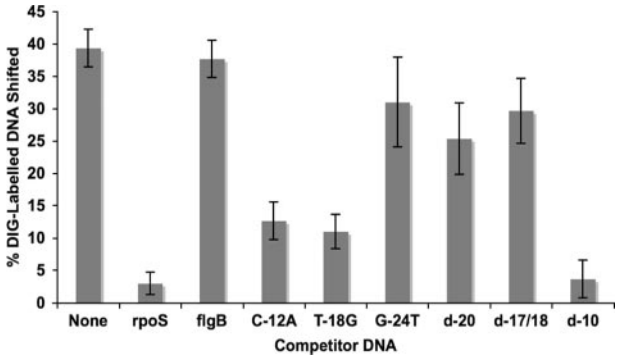


FIG. 3. Quantitative competitive EMSAs demonstrating that the affinity of *E. coli* RpoN for dsDNA is sequence dependent. Proportions of dsDNA shifted by the binding of *E. coli* RpoN to the labeled *rpoS* promoter are shown for the absence and presence of a 125-fold excess of unlabeled competitor dsDNA ($n = 3$). Competitor dsDNAs consisted of the unlabeled *rpoS* promoter, a $\sigma^{70}$-specific *flgB* promoter, and *rpoS* promoters with the base pair substitutions and deletions noted. EMSAs were performed with a 63-bp digoxigenin (DIG)-labeled target of the *rpoS* promoter mixed with a lysate from a strain of *E. coli* that overexpresses *E. coli* RpoN.

evidence that the *rpoS* promoter region contains a binding site for RpoN.

**Site-directed mutagenesis of key residues in the proposed RpoN-binding site upstream of *rpoS*.** The −24 (GG region) and −12 (GC region) regions are highly conserved among RpoN-dependent promoters (2); removal of these dinucleotides in *C. crescentus*, for example, eliminates transcription of *flbG* and *flaN* (23). Promoter function is also essentially abolished by deleting one or more nucleotides between the −24/−12 regions of the 4521 gene in *Myxococcus xanthus* (18), *nifH* in *K. pneumoniae* (3), and *flbG* and *flaN* in *C. crescentus* (23, 24). The preponderance of information thus indicates that there is a stringent requirement for these motifs to be located on the same face of the DNA helix (2).

Double-stranded oligonucleotides with single-base-pair substitutions (C-12A and G-24T) or 1 (d-20)- or 2 (d-17/18)-bp deletions (Fig. 1 and Table 1) in the *rpoS* promoter region were used as competing DNAs in EMSAs. A mutation downstream of the −24/−12 region (d-10), which theoretically should have no effect on RpoN binding, and a base change in a less-conserved area of the −24/−12 region (T-18G) were also included. A 63-bp oligonucleotide carrying the predicted *rpoS* promoter was 3′ digoxigenin-11-dUTP labeled (DIG gel shift kit, second generation; Roche Applied Science, Mannheim, Germany) and incubated with a lysate from RpoN-overproducing *E. coli* LCA7 (1). When quantitation was desired, X-ray film was exposed for various times, and relative units of chemiluminescence were determined using a Gel Logic 200 imaging system (Kodak Scientific Imaging Systems, New Haven, CT).

Incubation of a cell lysate with labeled double-stranded DNA (dsDNA) and resolution by EMSA resulted in two shifted bands, both of which were supershifted when a monoclonal anti-$\sigma^{54}$ antibody (W0005; NeoClone Biotechnology International, Madison, WI) was added to the reaction mixture (not shown). The observed supershifts confirmed that both bands represented *E. coli* RpoN bound to the labeled dsDNA. It is known that the DNA-bound holoenzyme has reduced mobility compared to the RpoN-DNA complex (4). Therefore, the two bands visible in the gel are a reflection of RpoN bound to the DNA with (upper band) and without (lower band) the core RNA polymerase.

A 125-fold excess of unlabeled dsDNA corresponding to the $\sigma^{70}$ promoter from *B. burgdorferi flgB* (13) could not compete with the labeled *rpoS* dsDNA for RpoN binding (Fig. 3), confirming that binding of *E. coli* RpoN was sequence dependent. In addition, a 125-fold excess of dsDNA containing deletions (d-17/18 and d-20) or the G-24T base pair substitution could not compete for *E. coli* RpoN binding (Fig. 3). However, there was a reduction of labeled RpoN-bound DNA that shifted when a 125-fold excess of the dsDNA containing the C-12A or T-18G base pair substitution was added as competitor DNA. These reductions, however, were substantially less than those when the unlabeled *rpoS* promoter was the competing dsDNA (Fig. 3). As expected, a deletion outside the −24/−12 region (d-10) could readily compete with the *rpoS* promoter double-stranded oligonucleotide. The combined competitive EMSA results indicate that *E. coli* RpoN has a greater affinity for the *B. burgdorferi rpoS* −24/−12 region than for dsDNA containing a C-12A or T-18G substitution and has little to no affinity for dsDNA with the G-24T substitution or for sequences containing deletions in the −24/−12 region.

As in other RpoN systems (3, 18, 23, 24), there seems to be a requirement for the −24 and −12 regions to be on the same face of the DNA helix for recognition, as deleting one or two nucleotides impeded RpoN binding. The −24 region of the *glnAP2* promoter is more important than the −12 region for recognition and binding by *E. coli* RpoN (39); this was corroborated in our study in that a dsDNA with a base substitution at position −24 could not compete with the borrelial *rpoS* dsDNA for RpoN binding. A dsDNA with a base pair substitution in a less-conserved residue (T-18G) could compete somewhat with the wild-type promoter, but the binding affinity was markedly lower. The affinity was similar to that for the C-12A base pair substitution, which is a key conserved residue. The −12 element not only contributes to binding affinity, al-

though it is of secondary importance relative to the −24 region, but also determines the level of basal transcription (14, 36, 37). Substitutions of the −12 C nucleotide result in deregulated transcription in vitro (37), although these substitutions do not result in stronger transcription under activating conditions in vivo (36). RpoN binds to DNA in the absence of RNA polymerase, but the holoenzyme binds tighter than RpoN alone, except to early melted DNA, where both bind equally well (7, 12, 38). The spatial relationship between RpoN, the core RNA polymerase, and the −12 promoter element changes when the closed promoter complex becomes an open one (6). The weaker base pairing that results from the C-12A substitution may cause the DNA conformation to be more similar to that of the early melted DNA, thereby still allowing recognition even though there is a base pair change in a conserved region of the −24/−12 promoter.

**Mutations in the predicted RpoN-dependent promoter influence expression of RpoS and OspC.** The results of quantitative competitive EMSAs showed that double-stranded oligonucleotides containing mutations in the putative RpoN-binding site upstream of *rpoS* were partially (C-12A and T-18G) or almost totally (G-24T, d-20, and d-17/18) impeded in the ability to compete with the wild-type promoter sequence for binding by *E. coli* RpoN. By extrapolation, these same mutations should reduce or prevent RpoN-dependent expression of *rpoS* in *B. burgdorferi*. As an initial investigation of this possibility, the mutations in the key conserved residues (C-12A, G-24T, d-20, and d-17/18) were introduced by site-directed mutagenesis (QuikChange II site-directed mutagenesis kit; Stratagene, La Jolla, CA) of plasmid pXY240. Plasmid pXY240 contains a 4.2-kb region of *B. burgdorferi* strain 297 DNA, including 1.9 kb upstream and 1.5 kb downstream of *rpoS*, cloned into the shuttle vector pJD7, derived from pKFSS1 (11). The erythromycin-resistant, RpoS-deficient mutant *B. burgdorferi* BbAH206 (15) was made electrocompetent and transformed with sequence-verified plasmids (28) containing either a wild-type or mutated *rpoS* promoter. Three transformants for each plasmid, selected by streptomycin resistance, were chosen for further studies. Desired plasmid sequences in *B. burgdorferi* transformants were confirmed by specific digestion patterns obtained after treatment with pertinent restriction enzymes.

Cultures of transformants were grown at 34°C in complete Barbour-Stoenner-Kelly (BSK-H) medium (Sigma-Aldrich, St. Louis, MO) at pH 6.8 to a high cell density ($>1 \times 10^8$ bacteria/ml). Complementation of *rpoS* in the RpoS-deficient mutant was determined at the protein level by immunoblot detection of RpoS and OspC, which is under the control of RpoS (9, 15, 42), utilizing the SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology, Inc., Rockford, IL). To confirm that the numbers of spirochetes loaded into the gel lanes were substantially equivalent, immunoblotting for the flagellar protein FlaB was performed. RpoS was detected with rat polyclonal antibodies, generated according to previously published protocols (20). Chicken immunoglobulin Y anti-FlaB antibody was a gift from Kayla Hagman (University of Texas Southwestern Medical Center, Dallas). A monoclonal antibody directed against OspC, 1B2-105, was generated by immunizing BALB/c mice with the corresponding fusion proteins according to previously published protocols (29). The wild-type *B. burgdorferi* strain BbAH130 and an RpoN-deficient mutant, BbJSB18-B2, were included as positive and negative controls, respectively. The 297M3 *rpoN*::*aadA* insertion mutant, BbJSB18-2, was generated as previously described (15), except that the erythromycin resistance marker (*ermC*) in the mutagenesis construct pALH394 was exchanged for a cassette conferring resistance to streptomycin (*aadA*) (11).

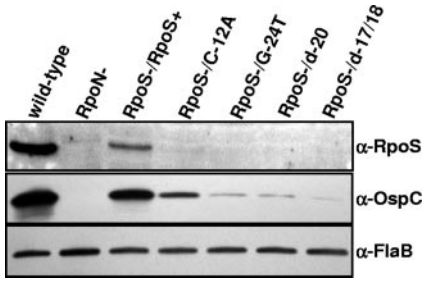


FIG. 4. Mutations in the *rpoS* −24/−12 region influence the rescue of OspC expression in an RpoS-deficient background. Whole-cell lysates from *B. burgdorferi* grown in BSK-H medium at pH 6.8 to a high density ($>1 \times 10^8$ bacteria/ml) were loaded at $\sim 5 \times 10^7$ cells per well for chemiluminescence immunoblot detection of FlaB and OspC. About $10^8$ cells per well were loaded for the detection of RpoS. Immunoblots of an RpoN-deficient strain (RpoN−) and an RpoS-deficient strain (RpoS−) complemented with the wild-type *rpoS* promoter or promoter mutants are depicted.

In immunoblots, RpoS and OspC were not detected in RpoS-deficient (not shown) or RpoN-deficient (Fig. 4) mutants of *B. burgdorferi*, as expected (15). In the *rpoS*-complemented RpoS-deficient strain, RpoS expression was restored, albeit to a lesser degree than the level of wild-type expression (Fig. 4). RpoS was below chemiluminescence immunoblot detection limits in all promoter mutant complements. Given its low abundance, RpoS is difficult to detect even by sensitive chemiluminescence immunoblotting. However, OspC is highly abundant when induced in *B. burgdorferi* strain 297. OspC expression was thus detectable in the promoter mutant complements (Fig. 4), and the relative levels of OspC expression in the promoter mutant complements corresponded to the relative binding affinities of *E. coli* RpoN (Fig. 3). Elevated levels of OspC were present in the C-12A promoter complement compared to those in the G-24T and deletion mutants (d-17/18 and d-20), which had little to no binding affinity for *E. coli* RpoN. In other words, in the three cases where the double-stranded oligonucleotide mutations G-24T, d-20, and d-17/18 failed to compete with the native *rpoS* sequence (i.e., those mutations that adversely affected RpoN binding) (Fig. 3), the cognate *B. burgdorferi* complemented stains barely expressed OspC, if at all (Fig. 4). Similarly the C-12A mutation, which only partially competed with native *rpoS* in EMSAs (Fig. 3), allowed more robust OspC production in the borrelial complemented strain (Fig. 4).

It has been reported that cultivation of *B. burgdorferi* in the presence of whole blood can induce the upregulation of a number of genes, including *ospC* and other genes governed by the RpoN-RpoS pathway (35). Therefore, *B. burgdorferi* strains were cultivated in BSK-H medium with 6% whole blood as described previously (35), except that fresh heparinized rabbit blood with the buffy coat removed was used as an alternative to

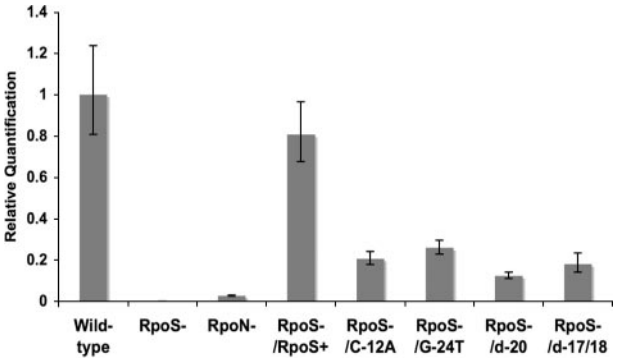


FIG. 5. Complementation of an RpoS-deficient strain with plasmids containing mutations in key residues of the *rpoS* −24/−12 region influences transcript levels of *rpoS*. *B. burgdorferi* cells were harvested at ~1 × $10^8$ bacteria/ml from BSK-H medium containing 6% rabbit blood, and *rpoS* transcript levels relative to *flaB* (flagellar protein) levels were determined by qRT-PCR. Results for RpoN-deficient (RpoN−) and RpoS-deficient (RpoS−) strains and for the RpoS-deficient strain complemented with the wild-type *rpoS* promoter or promoter mutant are presented compared to wild-type *rpoS* expression ($n = 3$, except for d-17/18, where $n = 2$).

citrated human blood. Under these cultivation conditions, RpoS protein levels were below the limits of chemiluminescence immunoblot detection in the *rpoS*-complemented strain (not shown). OspC protein was detected in the *rpoS*-complemented strain, but OspC protein was barely detectable, if at all, in the promoter mutant-complemented strains (not shown).

Because RpoS was not detectable in immunoblots of complemented strains, further assessment of *rpoS* transcription was accomplished by quantifying *rpoS* transcripts with SYBR green one-step quantitative reverse transcription-PCR (qRT-PCR), using the *flaB* gene as an internal control (Applied Biosystems 7500 real-time PCR system). qRT-PCR confirmed that *rpoS* transcription was restored in an *rpoS*-complemented strain to about 80% of the wild-type level (Fig. 5). For the promoter mutants, *rpoS* transcription was reduced to 12 to 26% of the wild-type level, with no significant differences between them. This level of induction is slightly higher than the levels measured by galactosidase activity for similar mutations in an *M. xanthus* RpoN-dependent promoter (18), which were just below 10% of wild-type activity. The RpoN-deficient strain exhibited *rpoS* transcript levels with <10% of wild-type activity. Based on many studies (3, 18, 23, 24) and our EMSA results, one might predict that *rpoS* transcription would be abolished, especially in the deletion mutants. However, if there is excess RpoN present in the cell, even low-affinity promoters may be significantly occupied in vivo prior to activation. For example, in *E. coli* there is a larger number of RpoN molecules in the cell (close to 100) than the number of promoters (fewer than 20) (5, 17), and a similar ratio of RpoN molecules to promoters may be present in *B. burgdorferi.*

**Summary and implications.** It is now generally accepted that the alternative sigma factor regulatory network, the RpoN-RpoS pathway (10, 15), controls virulence factor expression in *B. burgdorferi.* However, evidence has been lacking as to whether RpoN controls *rpoS* expression directly or possibly via a transactivator induced by RpoN. The transcription start site of *rpoS* was determined to be 14 nucleotides downstream of the conserved GC element of a putative RpoN-dependent −24/−12 promoter. EMSAs indicated that *E. coli* RpoN affinity was higher for a wild-type *B. burgdorferi rpoS* promoter sequence than for *rpoS* promoter sequences in which essential bases were replaced or deleted. Complementation of an RpoS-deficient strain of *B. burgdorferi* indicated that mutation of key conserved residues in the −24/−12 region reduced *rpoS* transcription and protein expression as well as the expression of OspC, which is controlled by an RpoS-dependent promoter (9, 15, 42). These results provide compelling evidence that RpoN directly controls *rpoS* expression in *B. burgdorferi* by binding to the −24/−12 sequence upstream of the *rpoS* ORF. In addition, RpoN-mediated transcription is a tightly controlled process, inasmuch as the promoter-bound RpoN holoenzyme remains inactive until it is activated by an enhancer-binding protein (26, 27, 38). The response regulator Rrp2, the only enhancer-binding protein yet identified in *B. burgdorferi*, is essential for activation of the RpoN-RpoS regulatory network (41). Therefore, we also predict that there is a requisite Rrp2 enhancer-binding site in the vicinity of the *B. burgdorferi rpoS* gene.

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