Microbiology (2010), **156**, 2194–2204 **DOI** 10.1099/mic.0.036608-0

Two regulatory elements required for enhancing *ospA* expression in *Borrelia burgdorferi* grown *in vitro* but repressing its expression during mammalian infection

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Received 20 November 2009
Revised 4 March 2010
Accepted 8 April 2010

During cycling between the tick vector and a mammal, the Lyme disease spirochaete *Borrelia burgdorferi* must coordinate expression of outer-surface proteins (Osps) A and B to quickly respond to environmental changes. The pathogen abundantly produces OspA/B in the tick, but represses their expression during mammalian infection. This paper reports a regulatory structure, consisting of two sequences flanking the *ospAB* promoter, that is required for enhancing *ospA* expression in *B. burgdorferi* grown *in vitro*, but repressing its expression during murine infection. Deletion or replacement of either the upstream or downstream sequence of the *ospAB* promoter caused a significant decrease in *ospA* expression *in vitro*, but a dramatic increase during murine infection. Fusion of either sequence with the *flaB* reporter promoter led to increased expression of an *ospA* reporter gene *in vitro*, but a decrease in the murine host. Furthermore, simultaneous fusion of both sequences with the reporter promoter showed a synergistic effect in enhancing expression of the *ospA* reporter *in vitro*, but repressing its expression during murine infection. Taken together, the results demonstrate that the regulatory structure functions oppositely in the two different environments and potentially provides *B. burgdorferi* with a molecular mechanism to quickly adapt to the distinct environments during its enzootic life cycle.

INTRODUCTION

Outer-surface proteins (Osps) A and B, encoded by a two-gene operon (Howe *et al.*, 1986), are the surface antigens most abundantly produced by the Lyme disease spirochaete *Borrelia burgdorferi* in engorged and unfed *Ixodes* ticks (de Silva *et al.*, 1996; Ohnishi *et al.*, 2001; Schwan *et al.*, 1995; Schwan & Piesman, 2000). In response to a fresh blood meal, *B. burgdorferi* downregulates OspA/B and upregulates OspC and other Osps, a process that prepares the spirochaete for infection of a mammal (Fingerle *et al.*, 2007; Grimm *et al.*, 2004; Pal *et al.*, 2004b; Stewart *et al.*, 2006). Repressing *ospAB* expression during mammalian infection is critical for *B. burgdorferi* to evade the immune system, cause persistent infection and maintain the enzootic cycle, as OspA and OspB, even expressed at a low level, may ultimately induce a strong specific humoral response owing to their high immunogenicity. The specific response can impose tremendous pressure on the pathogen or clear the infection (Strother *et al.*, 2007; Xu *et al.*, 2008a). Even though the anti-OspA/B response may not effectively target spirochaetes with low OspA/B production in mammalian tissues, once acquired by the tick vector, the pathogen dramatically upregulates OspA/B and becomes extremely vulnerable to anti-OspA/B antibodies in a blood meal (de Silva *et al.*, 1997; Tsao *et al.*, 2001, 2004), potentially leading to the eradication of the organism and consequently to discontinuation of the enzootic cycle.

Expression of the *ospAB* operon is driven by a σ^{70}-dependent promoter (Sohaskey *et al.*, 1999); thus, *B. burgdorferi* cannot shut off this major sigma factor in order to achieve the downregulation of OspA/B. Sohaskey *et al.* (1999) showed that deletion of the T-rich region immediately upstream of the *ospAB* promoter resulted in greatly reduced expression of a chloramphenicol acetyltransferase reporter gene in *B. burgdorferi* grown *in vitro*. Caimano *et al.* (2005) showed that the downregulation of OspA requires RpoS production and proposed a model for RpoS-dependent *in vivo* repression of *ospA*, which involves an unknown repressor protein or an unknown cofactor that can bind RpoS and an unidentified sequence within the *ospAB* promoter (Caimano *et al.*, 2005). Later, the group further suggested that the T-rich region immediately

Abbreviations: DR, direct repeat; Osp, outer-surface protein; SCID, severe combined immunodeficiency; RT, reverse transcription; qPCR, quantitative PCR.

Two supplementary tables with details of the constructs, clones and plasmids used in this study are available with the online version of this paper.

upstream of the *ospAB* promoter may serve in this capacity (Caimano *et al.*, 2007).

In spite of this progress, it remains largely unknown how *B. burgdorferi* can so effectively down- and upregulate *ospAB* during its enzootic cycle. The present study focused on sequences both upstream and downstream of the *ospAB* promoter, and successfully identified two *cis* elements, which are involved in *ospAB* regulation by *B. burgdorferi* grown *in vitro* as well as during murine infection.

METHODS

Previously generated strains and constructs used in the current study. Clones 13A and 13A/E22/C and the *ospA* mutant, Δ*ospA*, were generated previously (Xu *et al.*, 2007a, b, 2008b). The constructs pBBE22-*CpospA* and pBBE22-*ospA′* were constructed in an earlier study (Xu *et al.*, 2008b). The shuttle vector pBBE22 and the *B. burgdorferi* B31 clone 5A11 were kindly provided by S. Norris (Purser & Norris, 2000; Purser *et al.*, 2003). The features of these clones and constructs are summarized in Supplementary Table S1, available with the online version of this paper.

Creation of the constructs pBBE22-*ospA1* to pBBE22-*ospA7*. The seven constructs were generated as illustrated in Fig. 1. Briefly, three amplicons, *ospA1* (1085 bp), *ospA*2 (958 bp) and *ospA*3 (925 bp), were generated by PCR using forward primers P1F, P2F and P3F (Supplementary Table S2), respectively, and a common reverse primer, P1R, with borrelial DNA as template. The resulting products were digested with the restriction enzymes *Bam*HI and *Xba*I, purified, and cloned into the shuttle vector pBBE22, digested with the same enzymes. The inserts and their flanking regions were sequenced to ensure they were arranged as designed. The three constructs were designated pBBE22-*ospA1*, pBBE22-*ospA2* and pBBE22-*ospA3*.

As illustrated in Fig. 2(A), the fragment *ospA4* was directly amplified from the construct pBBE22-*CpospA* with use of primers P4F and P1R (Supplementary Table S2). The long primer, P4F, incorporated the −35 and −10 regions of the *ospAB* promoter, and the sequence between them (Supplementary Table S2).

To generate the fragment *ospA5* (Fig. 2B), two amplicons were generated from the construct pBBE22-*ospA1* with use of primers P5F and P5R, and from the construct pBBE22-*ospA′* with primers P6F and P6R (Supplementary Table S2), respectively. The resulting amplicons were fused via blunt-end ligation and further amplified with use of primers P1F and P1R to introduce *Bam*HI and *Xba*I restriction enzyme sites at the ends.

To construct *ospA6* (Fig. 2C), two amplicons were generated from the construct pBBE22-*ospA′* with the use of primer pairs P5F and P7R, and P7F and P6R (Supplementary Table S2), respectively. The resulting amplicons were digested with *Ase*I, ligated and amplified using primers P8F and P1R, to introduce *Bam*HI and *Xba*I restriction enzyme sites at the ends.

To create the fragment *ospA7* (Fig. 2D), two amplicons were generated from the construct pBBE22-*ospA1* with the use of primers P5F and P5R, and from the construct pBBE22-*ospA6* using primers P6F and P6R, respectively. The resulting amplicons were fused via blunt-end ligation and further amplified with the use of primers P1F and P1R to introduce *Bam*HI and *Xba*I restriction enzyme sites at the ends.

Fragments *ospA4*, *ospA5*, *ospA6* and *ospA7* were cloned into pBBE22, to complete the creation of the constructs pBBE22-*ospA4*, pBBE22-*ospA5*, pBBE22-*ospA6* and pBBE22-*ospA7* (Fig. 1B). The inserts and flanking regions were sequenced to ensure that the constructs were created as designed.

Transformation of *B. burgdorferi* and selection of transformants. Constructs were electroporated into the Δ*ospA* mutant; the resulting transformants were screened and analysed for plasmid content as described previously (Xu *et al.*, 2005). Restoration of OspA production was verified using immunoblots probed with a mixture of FlaB and OspA mAbs, as described in an earlier study (Xu *et al.*, 2008b).

Constructs were also electroporated into clone 13A spirochaetes; the resulting transformants were screened and selected as described above, but immunoblotting was not performed because overwhelming OspA production from the native *ospA* copy potentially masked the contribution from introduced constructs.

Mouse infection. Severe combined immunodeficiency (SCID) mice (BALB/c background; age 4–8 weeks; provided by the LSU Division of Laboratory Animal Medicine) were given a single intradermal/subcutaneous injection of 10^4 spirochaetes. Animals were examined for the development of arthritis at 2 day intervals, starting at day 7, and sacrificed 1 month post-inoculation. If no significant joint swelling was noted, heart, joint and skin samples were subjected to spirochaetal isolation as described previously (Xu *et al.*, 2005). If joint swelling was apparent, skin, joint and heart specimens were collected for RNA preparation. RNA was converted to cDNA by reverse transcription (RT) and quantified for the mRNA copy numbers of *flaB* and *ospA* by quantitative PCR (qPCR) as described previously (Liang *et al.*, 2004a). The animal procedures described here were approved by the Institutional Animal Care and Use Committee at Louisiana State University.

Statistical analysis. Data were first analysed by using a one-way analysis of variance when an experiment involved more than two groups. A two-tailed Student *t* test was used to compare two treatments and calculate *P*-values. Calculated *P*-values of $\leqslant 0.05$ were considered significant.

RESULTS

Generation of constructs

A total of seven constructs, pBBE22-*ospA1* to pBBE22-*ospA7*, were generated from the shuttle vector pBBE22. pBBE22 was modified from pBSV2 by inserting a copy of *bbe22*, a gene that codes for a nicotinamidase essential for the basic survival of *B. burgdorferi* in mammalian hosts (Purser *et al.*, 2003; Stewart *et al.*, 2001). Because the *B. burgdorferi* B31 clone 13A, which lacks lp25 and lp56 (Xu *et al.*, 2007a), was used as the parental strain in the current study, constructs harbouring *bbe22* were required for restoration of infectivity of its derivatives.

Deletion of the direct repeats and the T-rich region leads to decreased *ospA* expression *in vitro* but increased expression in mice

Three constructs, pBBE22-*ospA1* to pBBE22-*ospA3*, were first electroporated into Δ*ospA*; between 11 and 20 transformants were obtained from transformation with each construct. Plasmid content analyses led to the selection of two clones containing each construct. The six clones, Δ*ospA*/*ospA1*/1, Δ*ospA*/*ospA1*/2, Δ*ospA*/*ospA2*/1,

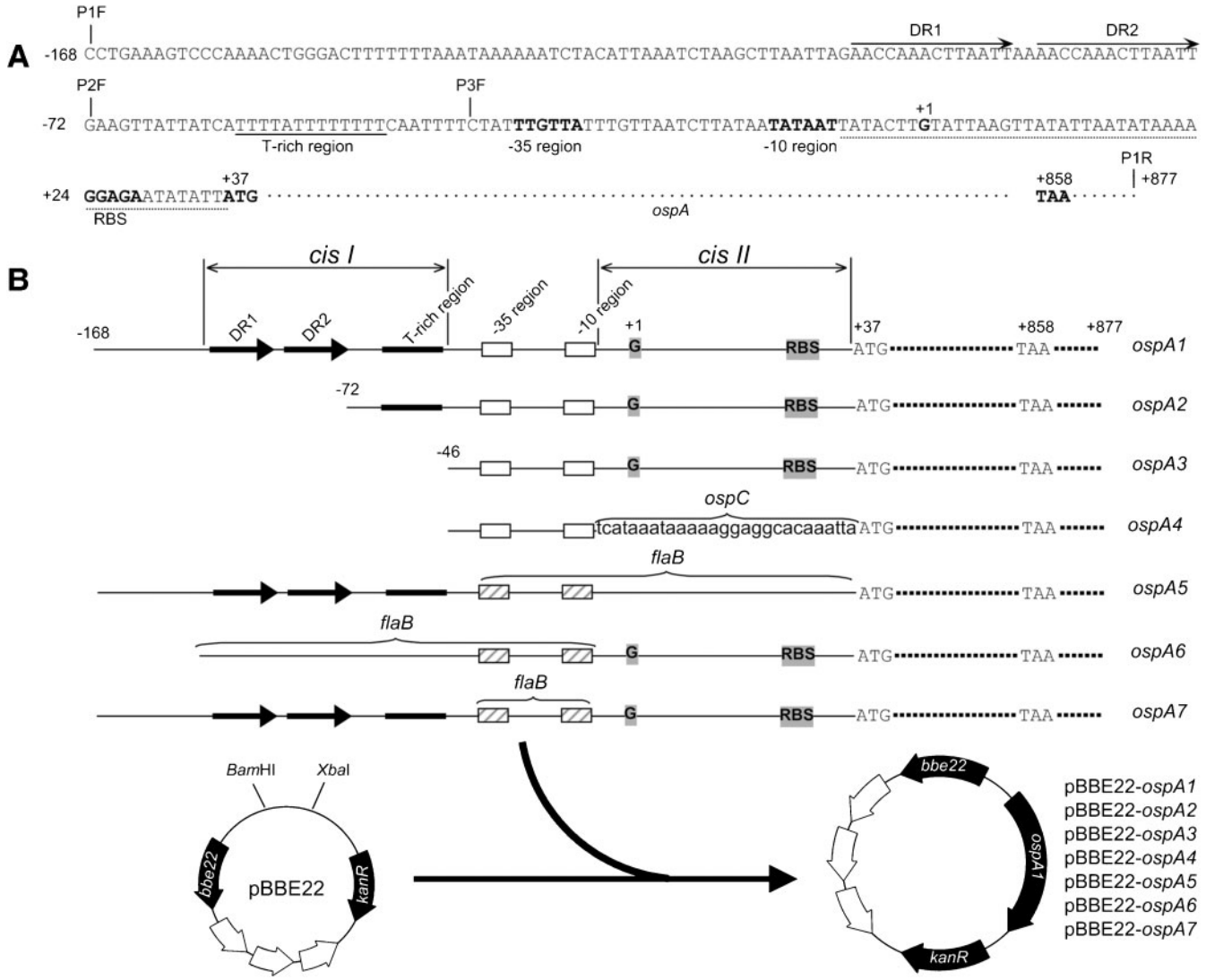

Fig. 1. Generation of constructs. (A) Potential regulatory sequences upstream of the coding region of the *ospAB* operon, including a pair of direct repeats, DR1 and DR2 (labelled with long arrows), a T-rich region (underlined with a solid line), and a sequence between the −10 region and the start codon ATG (underlined with a dotted line). The −35 and −10 regions, putative ribosome-binding site (RBS), start codon ATG, and stop codon TAA (all in bold type) are indicated. The +1 marks the previously identified transcriptional initiation site (Jonsson *et al.*, 1992). The amplification start sites of primers P1F, P2F, P3F and P1R, designed for amplifying the fragments *ospA1*, *ospA2* and *ospA3*, are marked with vertical lines. (B) Generation of constructs pBBE22-*ospA1* to pBBE22-*ospA7*. Three amplicons, *ospA1*, *ospA2* and *ospA3*, were generated by PCR using forward primers P1F, P2F and P3F, respectively, and a common reverse primer P1R, with borrelial DNA as a template, and cloned into the shuttle vector pBBE22. The fragments *ospA4*, *ospA5*, *ospA6* and *ospA7* were created as illustrated in Fig. 2 and were cloned into pBBE22. The DRs and the T-rich region are collectively designated *cisI*. *cisII* represents the sequence between the −10 region and the start codon.

Δ*ospA*/*ospA2*/2, Δ*ospA*/*ospA3*/1 and Δ*ospA*/*ospA3*/2, shared the same plasmid content as Δ*ospA*, which had lost cp9, lp5, lp21, lp28-1, lp25 and lp56 (Xu *et al.*, 2008b). An immunoblot analysis showed that all transformants abundantly produced OspA (Fig. 3A).

To precisely compare the *ospA* expression activity driven by each construct, the Δ*ospA*/*ospA1*/1, Δ*ospA*/*ospA1*/2, Δ*ospA*/*ospA2*/1, Δ*ospA*/*ospA2*/2, Δ*ospA*/*ospA3*/1 and Δ*ospA*/*ospA3*/2 spirochaetes were harvested at late-exponential phase (10^8 cells ml^{-1}). RNA was prepared and analysed for *ospA* and *flaB* mRNA copies by RT-qPCR. As shown in Fig. 3(B), the pBBE22-*ospA1* spirochaetes accumulated 17 % and 29 % more *ospA* transcripts than the genotypes pBBE22-*ospA2* ($P<0.05$) and pBBE22-*ospA3* ($P=9.2\times10^{-3}$), respectively. Moreover, the genotype pBBE22-*ospA2* produced

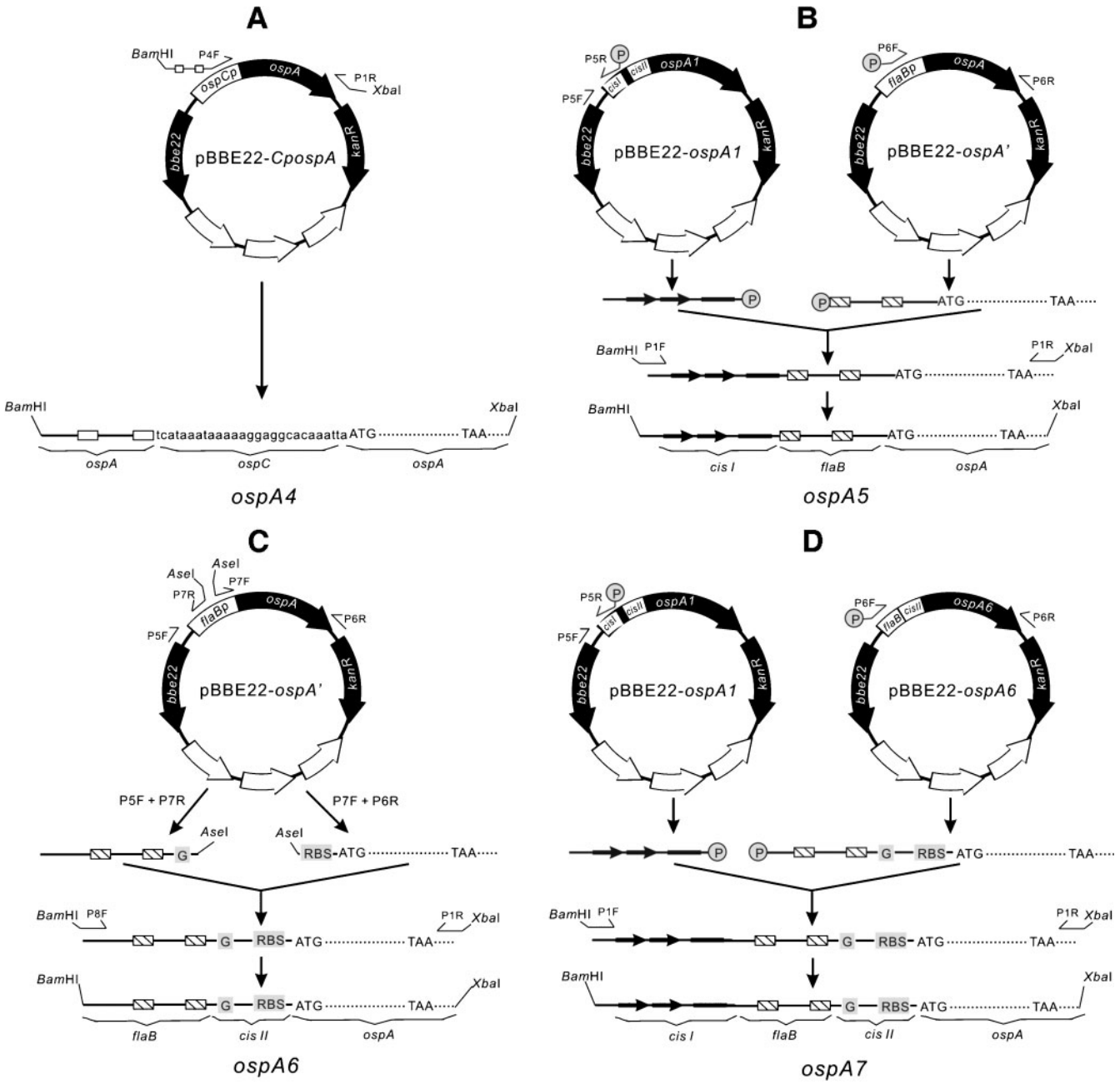

Fig. 2. Creation of fragments *ospA4* (A), *ospA5* (B), *ospA6* (C) and *ospA7* (D). Details of the constructions are described in Methods.

14 % more *ospA* mRNA than the genotype pBBE22-*ospA3* (P=0.02). These data indicate that the presence of the T-rich region increases *ospA* expression and that the inclusion of the sequence of direct repeats (DRs) further enhances expression in *B. burgdorferi* grown *in vitro*. The current study was unable to rule out whether the T-rich region and DRs functioned as a single regulatory element or two independent units, so they were collectively designated *cisI*.

Next, groups of three SCID mice were inoculated with clone Δ*ospA*/*ospA1*/1, Δ*ospA*/*ospA1*/2, Δ*ospA*/*ospA2*/1, Δ*ospA*/*ospA2*/2, Δ*ospA*/*ospA3*/1 or Δ*ospA*/*ospA3*/2. Only 2 of the 18 inoculated animals were infected when mice were euthanized 1 month post-inoculation (data not shown). The inability to fully restore infectivity of Δ*ospA* prevented us from using its derivatives in animal studies.

Fortunately, the native *ospAB* operon is not expressed by *B. burgdorferi* during mammalian infection; therefore its presence should not affect the behaviour of an introduced construct that may drive *ospA* expression. The constructs pBBE22-*ospA1* to pBBE22-*ospA3* were electroporated into

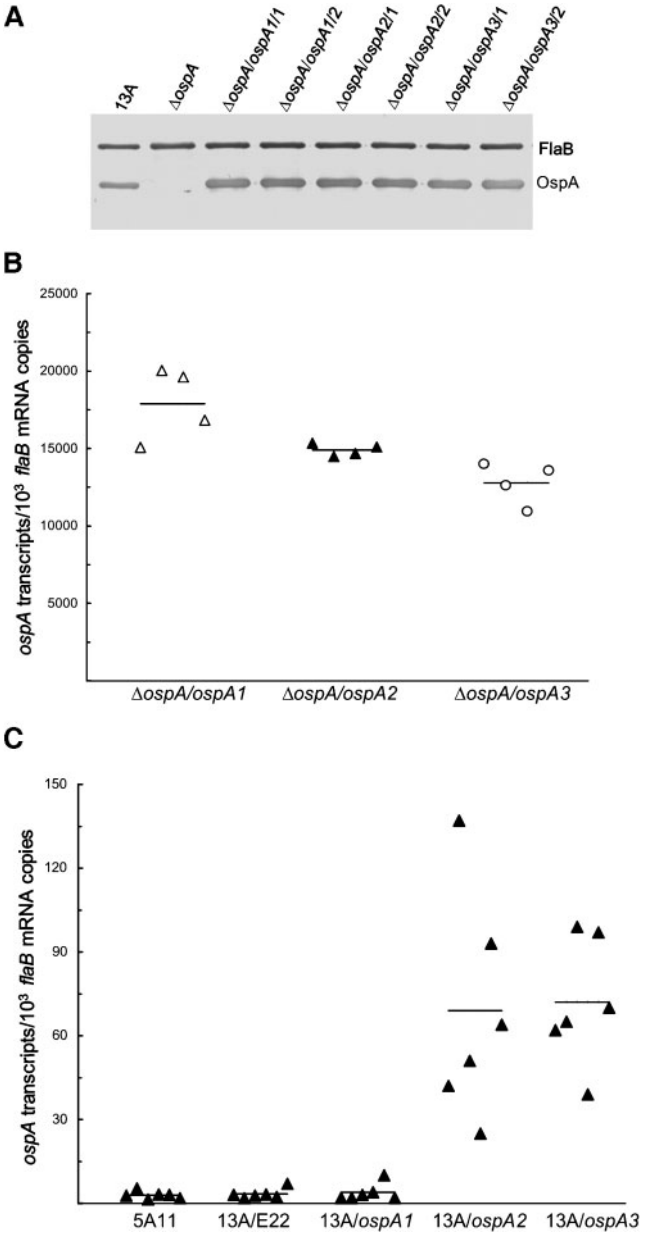

Fig. 3. The presence of DRs and the T-rich region enhances *ospA* expression *in vitro* but reduces expression in mice. (A) Restoration of OspA synthesis resulting from introduction of a construct. 13A, Δ*ospA*, Δ*ospA*/*ospA1*/1, Δ*ospA*/*ospA1*/2, Δ*ospA*/*ospA2*/1, Δ*ospA*/*ospA2*/2, Δ*ospA*/*ospA3*/1 and Δ*ospA*/*ospA3*/2 spirochaetes were harvested at late-exponential phase and subjected to immunoblot analysis, probed with a mixture of FlaB and OspA mAbs. (B) Deletion of *cisI* and the T-rich region causes reduced *ospA* expression *in vitro*. Δ*ospA*/*ospA1*/1, Δ*ospA*/*ospA1*/2, Δ*ospA*/*ospA2*/1, Δ*ospA*/*ospA2*/2, Δ*ospA*/*ospA3*/1 and Δ*ospA*/*ospA3*/2 spirochaetes were harvested at late-exponential phase in duplicate. RNA was prepared and analysed for *ospA* and *flaB* expression by RT-qPCR. The expression activity is presented as *ospA* mRNA copy numbers per 10^3 *flaB* transcripts. For comparison, the subgroups were combined into three groups: Δ*ospA*/*ospA1*/1 and Δ*ospA*/*ospA1*/2, Δ*ospA*/*ospA2*/1 and Δ*ospA*/*ospA2*/2, and Δ*ospA*/*ospA3*/1 and Δ*ospA*/*ospA3*/2. The mean copy numbers (horizontal lines) for each group are presented. (C) The sequence of DRs is involved in repressing *ospA* expression *in vivo*. Subgroups of three SCID mice were inoculated with clone 13A/*ospA1*/1, 13A/*ospA1*/2, 13A/*ospA2*/1, 13A/*ospA2*/2, 13A/*ospA3*/1 or 13A/*ospA3*/2, and an additional 12 mice were infected with clones 5A11 and 13A/E22/C. All animals were euthanized 1 month later. RNA was extracted from skin specimens and quantified for *flaB* and *ospA* mRNAs by RT-qPCR. Data are presented as *ospA* transcripts per 10^3 *flaB* mRNA copies in five groups (genotypes including 5A11, 13A/E22, 13A/*ospA1*, 13A/*ospA2* and 13A/*ospA3*). The means (horizontal lines) for each group are also presented.

13A spirochaetes. Six clones, 13A/*ospA1*/1, 13A/*ospA1*/2, 13A/*ospA2*/1, 13A/*ospA2*/2, 13A/*ospA3*/1 and 13A/*ospA3*/2, were selected for the study as they had the same plasmid content, including having lost cp9, lp21 and lp5, in addition to lp25 and lp56. Subgroups of three SCID mice were inoculated with each of these clones. An additional 12 mice were challenged with clone 5A11 or 13A/E22/C as controls. Clone 5A11 contains 20 plasmids, lacks lp5, and is considered to have a wild-type phenotype (Purser & Norris, 2000); clone 13A/E22/C has the same plasmid content as the six test clones and was generated via introduction of pBBE22 into clone 13A in our previous study (Xu *et al.*, 2007b). The two controls were used to assess whether the lack of cp9, lp21, lp5, lp25 and lp56 affected *ospA* expression in mice. Evidence of joint swelling in all 30 mice occurred within 12 days after inoculation and quickly developed into severe arthritis (data not shown), indicating that all the clones were infectious in SCID mice.

All mice were euthanized 1 month post-inoculation; RNA was prepared from skin specimens and assessed for the relative copy numbers of *ospA* and *flaB* mRNAs by RT-qPCR. As shown in Fig. 3(C), both 5A11 and 13A/E22/C spirochaetes expressed *ospA* at a baseline level ($P=0.67$), indicating that the absence of cp9, lp21, lp5, lp25 and lp56 does not affect *ospA* regulation *in vivo*. There was also no significant difference in *ospA* expression detected in the genotypes 13A/E22 and 13A/*ospA1* ($P=0.74$). When a gene is expressed at an extremely low level, RT-qPCR data may only reflect 'noise'. This could be the reason why the 13A/*ospA1* spirochaetes did not show increased *ospA* expression although they contained a construct-source *ospA* copy in addition to the native gene. Nevertheless, these data indicate that the presence of the native *ospA* copy does not interfere with data interpretation in our system.

When the three genotypes harbouring an *ospA* construct were compared for *ospA* expression in skin, the genotype 13A/*ospA1* accumulated *ospA* transcripts at a 17.3- and 18.0-fold lower level than the genotypes 13A/*ospA2* ($P=2.9\times10^{-3}$) and 13A/*ospA3* ($P=2.7\times10^{-5}$), respectively (Fig. 3C); but there was no significant difference in *ospA* expression between the latter two genotypes

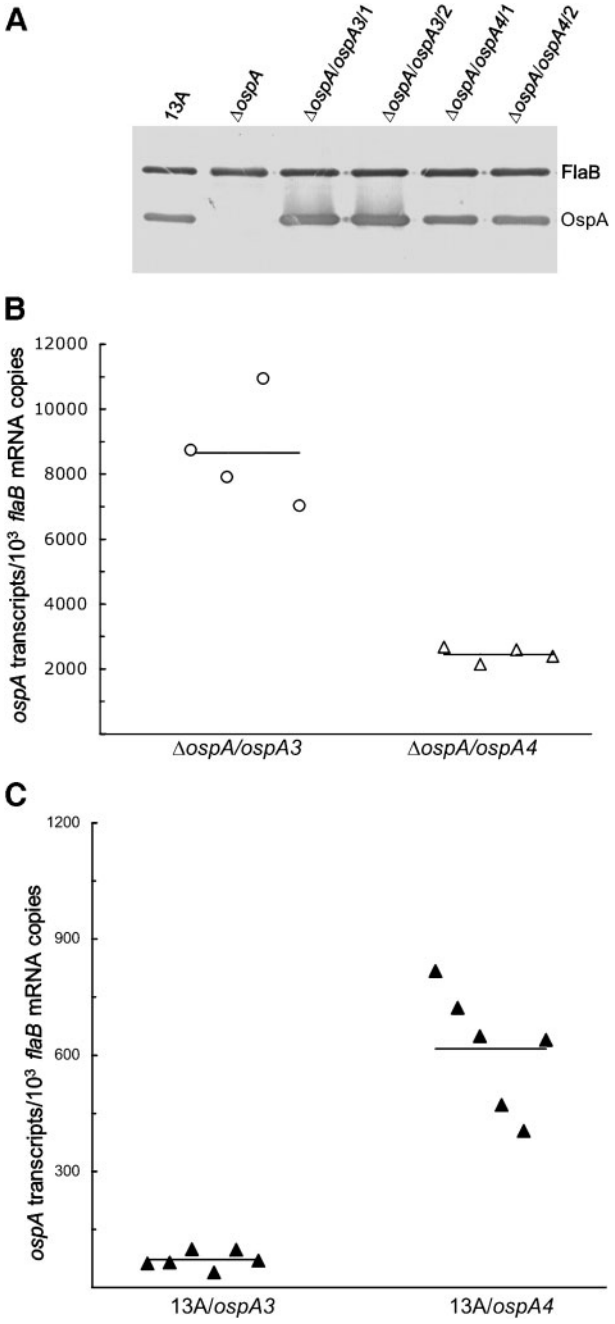

Fig. 4. The sequence *cisII* contributes to increased *ospA* expression *in vitro* but decreased expression in mice. (A) Restoration of OspA synthesis resulting from introduction of pBBE22-*ospA4*. 13A, Δ*ospA*, Δ*ospA*/*ospA3*/1, Δ*ospA*/*ospA3*/2, Δ*ospA*/*ospA4*/1 and Δ*ospA*/*ospA4*/2 spirochaetes were harvested at late-exponential phase and subjected to immunoblot analysis, probed with a mixture of FlaB and OspA mAbs. (B) The sequence *cisII* significantly contributes to *ospA* mRNA accumulation *in vitro*. Δ*ospA*/*ospA3*/1, Δ*ospA*/*ospA3*/2, Δ*ospA*/*ospA4*/1 and Δ*ospA*/*ospA4*/2 spirochaetes were harvested at late-exponential phase in duplicate; RNA was extracted and analysed for *ospA* and *flaB* expression by RT-qPCR. The expression activity is presented as *ospA* mRNA copy numbers per 10^3 *flaB* transcripts in two groups (combining the subgroups), Δ*ospA*/*ospA3*/1 and Δ*ospA*/*ospA3*/2, and Δ*ospA*/*ospA4*/1 and Δ*ospA*/*ospA4*/2. The mean copy numbers (horizontal lines) for each group are presented. (C) The sequence *cisII* contributes to the down-regulation of *ospA* expression *in vivo*. Subgroups of three mice were inoculated with clone 13A/*ospA3*/1, 13A/*ospA3*/2, 13A/*ospA4*/1 or 13A/*ospA4*/2. Mice were euthanized 1 month later; RNA was extracted from skin specimens and quantified for *flaB* and *ospA* expression by RT-qPCR. Data are presented as *ospA* transcripts per 10^3 *flaB* mRNA copy numbers in two groups (genotypes including 13A/*ospA3* and 13A/*ospA4*). The means (horizontal lines) for each group are also presented.

(P=0.86). Similar *ospA* expression patterns were detected for these genotypes when RNA prepared from the heart and joint specimens was analysed (data not shown). These data indicated that the sequence upstream of the *ospAB* promoter, which includes at least DRs, functions as a *cis* element critical for repressing *ospA* expression in mice. Although our data are not indicative of the T-rich region being a part of the regulatory element, our study cannot rule out its involvement, as the deletion of the DRs alone may completely destroy the integrity of the unit. This is a reason why both the T-rich region and the DRs are collectively termed *cisI*.

Replacement of *cisII* with the corresponding sequence of the *ospC* gene leads to decreased *ospA* mRNA accumulation *in vitro* but increased accumulation in mice

Surprisingly, the construct pBBE22-*ospA3* carried the minimum *ospAB* promoter but drove significant *ospA* expression in murine tissues, albeit initiating much lower expression than the *flaB* promoter that was fused with the *ospA* reporter gene (Xu *et al.*, 2008a), leading us to hypothesize that other sequences adjacent to the *ospAB* promoter may also be involved in repression of *ospA* expression. To examine this hypothesis, we focused on the sequence between the minimal *ospAB* promoter and the start codon ATG, which was collectively designated *cisII*. To this end, construct pBBE22-*ospA4* was generated as illustrated in Figs 1 and 2. Within this construct, *cisII* was replaced with the corresponding region of *ospC* (the sequence between the −10 region of the *ospC* promoter and the start codon of the *ospC* gene), which was selected based on a previous study showing its non-involvement in gene regulation (Xu *et al.*, 2007a). The construct was first electroporated into Δ*ospA* to examine whether this modification affected the activity of the *ospA* promoter *in vitro*. Two clones, Δ*ospA*/*ospA4*/1 and Δ*ospA*/*ospA4*/2, which shared the same plasmid content as Δ*ospA*, were selected for the study. An immunoblot analysis showed that the construct conferred OspA production at a level slightly lower than that of pBBE22-*ospA3* (Fig. 4A).

Next, RNA was prepared and analysed for *ospA* and *flaB* expression by RT-qPCR. Clone Δ*ospA*/*ospA3* produced 72 % more *ospA* transcripts than Δ*ospA*/*ospA4*

($P=3.3\times10^{-4}$) (Fig. 4B). Clearly, the replacement of *cisII* with the corresponding sequence of the *ospC* gene led to a significant reduction in *ospA* expression *in vitro*. However, this piece of data alone does not necessarily suggest that the modification reduces the activity of the *ospAB* promoter. Alternatively, *cisII* may play an important role in enhancing *ospA* expression *in vitro*.

To examine the influence of *cisII* on *ospA* expression *in vivo*, pBBE22-*ospA4* was electroporated into 13A spirochaetes. Two clones, 13A/*ospA4*/1 and 13A/*ospA4*/2, shared the same plasmid content as the genotypes 13A/*ospA1*, 13A/*ospA2* and 13A/*ospA3*, and were inoculated into six SCID mice. An additional six mice were inoculated with clone 13A/*ospA3*/1 or 13A/*ospA3*/2 as a control. Mice were euthanized 1 month post-inoculation, and RNA was prepared from skin specimens and analysed. As shown in Fig. 4(C), the genotype 13A/*ospA4* produced 8.6-fold more *ospA* transcripts than the genotype 13A/*ospA3* ($P=6.5\times10^{-6}$). Similar *ospA* expression patterns were noted for these genotypes when heart and joint RNA preparations were analysed (data not shown). The data indicated that the *ospA* promoter can drive active gene expression in the murine host once *cisII* is substituted, thus suggesting that *cisII* contributes to the downregulation of *ospA* expression in the murine host.

Confirmation of the role of both *cisI* and *cisII* in enhancing *ospA* expression *in vitro* but reducing its expression in mice by using the *flaB* reporter promoter

In a previous study, we created a construct, pBBE22-*ospA′*, by fusing the *flaB* promoter with a promoterless *ospA*, demonstrating that it drove active *ospA* expression both *in vitro* and *in vivo* (Xu *et al.*, 2008a). In the current study, we used the same system to confirm the role of *cisI* and *cisII* in *ospA* regulation through generation of three constructs, pBBE22-*ospA5* to pBBE22-*ospA7*. In pBBE22-*ospA5*, *cisI* was directly fused with the *flaB* promoter, aimed at confirmation of the influence of *cisI* on the activity of a downstream promoter. In pBBE22-*ospA6*, *cisII* was inserted between the *flaB* promoter and the *ospA* reporter gene, a construct that would allow us to further examine whether *cisII* can influence downstream gene expression. In pBBE22-*ospA7*, *cisI* and *cisII* were, respectively, inserted immediately upstream and downstream of the *flaB* reporter promoter, in order to investigate whether the two *cis* elements have a synergistic effect on gene regulation.

These three constructs were first electroporated into Δ*ospA* to investigate whether the two *cis* elements contribute to *ospA* regulation *in vitro*. As a control, pBBE22-*ospA′* was also electroporated into Δ*ospA*. Eight clones, namely Δ*ospA*/*ospA5*/1, Δ*ospA*/*ospA5*/2, Δ*ospA*/*ospA6*/1, Δ*ospA*/*ospA6*/2, Δ*ospA*/*ospA7*/1, Δ*ospA*/*ospA7*/2, Δ*ospA*/*ospA′*/1 and Δ*ospA*/*ospA′*/2, were selected for the study as they had the same plasmid content as Δ*ospA*. An immunoblot analysis indicated that all three *flaB* promoter derivatives drove abundant OspA production (Fig. 5A).

RNA was prepared from spirochaetes harvested at late-exponential phase and analysed by RT-qPCR. As shown in Fig. 5(B), the genotype Δ*ospA*/*ospA′* accumulated 28 % and 141 % less *ospA* transcripts than the genotypes Δ*ospA*/*ospA5* ($P=0.008$) and Δ*ospA*/*ospA6* ($P=4.1\times10^{-6}$), respectively, confirming the role of both *cis* elements in enhancing *ospA* expression *in vitro*. Moreover, the genotype Δ*ospA*/*ospA6* produced 88 % more *ospA* mRNA than the genotype Δ*ospA*/*ospA5* ($P=2.1\times10^{-5}$), indicating that *cisII* increases *ospA* accumulation more effectively than *cisI*. Finally, the genotype Δ*ospA*/*ospA7* accumulated 189 %, 126 % and 20 % more *ospA* mRNA than the genotypes Δ*ospA*/*ospA′* ($P=9.3\times10^{-6}$), Δ*ospA*/*ospA5* ($P=2.7\times10^{-5}$) and Δ*ospA*/*ospA6* ($P<0.02$), respectively, indicating that *cisI* and *cisII* function synergistically in enhancing *ospA* expression in cultivated *B. burgdorferi*.

To confirm the role of both *cisI* and *cisII* in *ospA* regulation *in vivo*, the four constructs were electroporated into 13A spirochaetes. Eight clones, 13A/*ospA5*/1, 13A/*ospA5*/2, 13A/*ospA6*/1, 13A/*ospA6*/2, 13A/*ospA7*/1, 13A/*ospA7*/2, 13A/*ospA′*/1 and 13A /*ospA′*/2, were selected to challenge subgroups of three SCID mice. These clones contained the same plasmids as the genotypes 13A/*ospA1*, 13A/*ospA2*, 13A/*ospA3* and 13A/*ospA4*. Mice were euthanized 1 month post-inoculation; skin RNA was prepared and analysed. As shown in Fig. 5(C), the genotype 13A/*ospA5* accumulated twofold less *ospA* transcripts than the control 13A/*ospA′* ($P=2.9\times10^{-9}$), thus confirming the role of *cisI* in repressing *ospA* expression *in vivo*. The genotype 13A/*ospA6* accumulated 7.3 times less *ospA* transcripts than the control 13A/*ospA′* ($P=2.2\times10^{-9}$), confirming the contribution of *cisII* to the downregulation of *ospA* expression. When the genotypes 13A/*ospA5* and 13A/*ospA6* were compared, the former expressed 4.1-fold higher *ospA* transcripts than the latter ($P=9.7\times10^{-8}$), indicating that *cisII* more effectively contributes to *ospA* repression than *cisI*.

When both *cisI* and *cisII* were simultaneously fused with the *flaB* promoter, upstream and downstream, respectively, their contribution to *ospA* repression was further confirmed (Fig. 5C): the genotype 13A/*ospA7* produced 64 times less *ospA* transcripts than the genotype 13A/*ospA5* ($P=6.1\times10^{-8}$). It expressed 18-fold less *ospA* mRNA than the genotype 13A/*ospA6* ($P=2.0\times10^{-6}$). Overall, the collective effect of the two *cis* elements reduced *ospA* expression by 129-fold ($P=3.5\times10^{-10}$), when 13A/*ospA′* and 13A/*ospA7* were compared.

DISCUSSION

Although controversies surround the exact functions of OspA and OspB in the tick, abundant production of these surface lipoproteins is crucial for *B. burgdorferi* to colonize the vector during its natural life cycle (Battisti *et al.*, 2008;

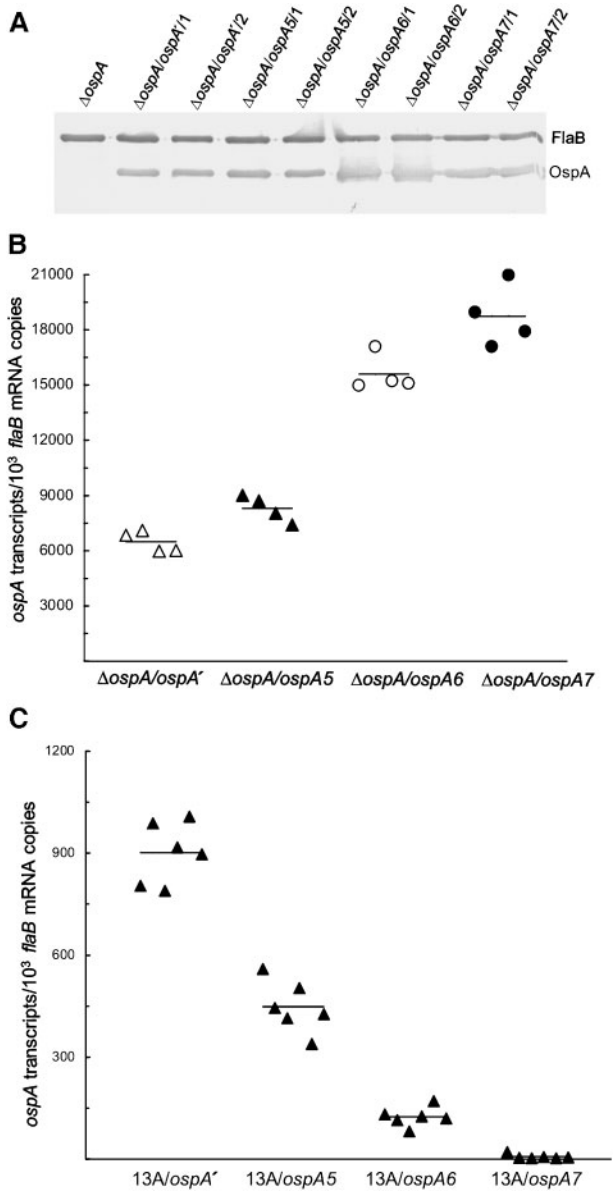

Fig. 5. Confirmation of the role of *cisI* and *cisII* in enhancing *ospA* expression *in vitro* but reducing expression *in vivo*. (A) Restoration of OspA synthesis resulting from introduction of pBBE22-*ospA5*, pBBE22-*ospA6* or pBBE22-*ospA*7. Δ*ospA*, Δ*ospA*/*ospA′*/1, Δ*ospA*/*ospA′*/2, Δ*ospA*/*ospA5*/1, Δ*ospA*/*ospA5*/2, Δ*ospA*/*ospA6*/1, Δ*ospA*/*ospA6*/2, Δ*ospA*/*ospA*7/1 and Δ*ospA*/*ospA*7/2 spirochaetes were harvested at late-exponential phase and subjected to immunoblot analysis, probed with a mixture of FlaB and OspA mAbs. (B) Confirmation of the role of *cisI* and *cisII* in enhancing *ospA* expression *in vitro*. Δ*ospA*/*ospA′*/1, Δ*ospA*/*ospA′*/2, Δ*ospA*/*ospA5*/1, Δ*ospA*/*ospA5*/2, Δ*ospA*/*ospA6*/1, Δ*ospA*/*ospA6*/2, Δ*ospA*/*ospA*7/1 and Δ*ospA*/*ospA*7/2 spirochaetes were harvested at late-exponential phase in duplicate. RNA was extracted and analysed for *ospA* and *flaB* mRNA accumulation by RT-qPCR. The expression activity is presented as *ospA* mRNA copy numbers per 10^3 *flaB* transcripts and compared in four groups by combining the subgroups Δ*ospA*/*ospA′*/1 and Δ*ospA*/*ospA′*/2, Δ*ospA*/*ospA5*/1 and Δ*ospA*/*ospA5*/2, Δ*ospA*/*ospA6*/1 and Δ*ospA*/*ospA6*/2, and Δ*ospA*/*ospA*7/1 and Δ*ospA*/*ospA*7/2. The mean copy numbers (horizontal lines) for each group are also presented. (C) Confirmation of the contributions of *cisI* and *cisII* to the downregulation of *ospA* expression *in vivo*. Subgroups of three mice were inoculated with clone 13A/*ospA′*/1, 13A/*ospA′*/2, 13A/*ospA5*/1, 13A/*ospA5*/2, 13A/*ospA6*/1, 13A/*ospA6*/2, 13A/*ospA*7/1 or 13A/*ospA*7/2 and euthanized 1 month later. RNA was extracted from skin specimens and quantified for *flaB* and *ospA* expression by RT-qPCR. Data are presented as *ospA* transcripts per 10^3 *flaB* mRNA copies in four groups (genotypes including 13A/*ospA′*, 13A/*ospA5*, 13A/*ospA6* and 13A/*ospA*7). The means (horizontal lines) for each group are also presented.

Neelakanta *et al.*, 2007; Pal *et al.*, 2004a; Yang *et al.*, 2004). Moreover, the downregulation of the *ospAB* operon is critical for the pathogen to effectively evade the immune system, cause persistent infection, and maintain the enzootic cycle (Strother *et al.*, 2007; Tsao *et al.*, 2001, 2004; Xu *et al.*, 2008a). The molecular mechanisms by which *B. burgdorferi* dramatically changes OspA/B expression during cycling between the two distinct hosts remained largely unknown, until this study carefully analysed the elements surrounding the *ospAB* promoter, showing involvement of sequences both upstream and downstream of the promoter in the regulation of *ospA* expression.

During the past 3 years, we have generated three *ospAB* mutants, and all lack the expected infectivity following complementation. These mutants were thinner and less motile than the wild-type, growing very poorly while aggregating at the bottom of culture tubes during *in vitro* cultivation, essentially consistent with a report by Sadziene *et al.* (1995) describing severe growth defects of OspAB-deficient *B. burgdorferi*. In contrast, we successfully inactivated *ospC*, *dbpAB*, and *rpoS* without difficulty using the same system, readily restoring these mutants to full infectivity (Shi *et al.*, 2008; Xu *et al.*, 2007a). Because both OspA and OspB are such highly produced outer-surface lipoproteins in cultivated *B. burgdorferi*, inactivation of the *ospAB* operon may cause a severe compromise in the integrity of the outer-surface lipoprotein layer, consequently severely affecting spirochaete survival. The *ospAB* mutants selected most likely underwent dramatic changes in gene expression to compensate for the loss of OspA/B. To date, three groups have generated infectious *ospAB* or *ospB* mutants (Battisti *et al.*, 2008; Neelakanta *et al.*, 2007; Yang *et al.*, 2004), but concluded differing roles for OspA/B during the life cycle of *B. burgdorferi* in the tick vector. While different tick-feeding techniques used in their studies could be an explanation for these disparities, as discussed by Battisti *et al.* (2008), the fact that each group used different mutants should also be considered, as the current study shows derivatives of the *ospAB* mutant producing inconsistent results following inoculation into mice. A recent study by He *et al.* (2008) reported two

phenotypes of the *ospA* mutant: one with constitutive *ospC* expression and the other having lost the ability to induce *ospC* expression in response to environmental changes. Our *ospAB* mutant exhibits a third phenotype, the *ospC* expression pattern of wild-type *B. burgdorferi* (Xu *et al.*, 2008b).

Deletion of the DRs resulted in decreased *ospA* expression *in vitro*, and further removal of the T-rich region led to an additional reduction, suggesting that both sequences are involved in positive regulation of *ospA* expression *in vitro*. However, our study could not determine whether DRs and the T-rich region function independently or act collectively as a single regulatory element; hence they were designated *cisI*. The identification of the *cis* element predicts the existence of regulatory protein(s) called activator(s). Further identification of these unknown regulator(s) should help solve the puzzle of whether DRs and the T-rich region constitute a single regulatory unit. To confirm the regulatory function of *cisI*, it was used to replace the upstream sequence of the *flaB* promoter and was shown to significantly increase expression of an *ospA* reporter gene *in vitro*.

Exactly opposite to what was observed *in vitro*, deletion of the DRs caused a significant increase in *ospA* expression in the murine host, indicating that the sequence is involved in repressing *ospA* expression *in vivo*. Although further removal of the T-rich region did not affect *ospA* expression in murine tissues, our study could not rule out whether the region and DRs collectively form a regulatory element because deletion of DRs alone may destroy its function. The regulatory function of *cisI*, which includes both the DRs and the T-rich region, in mammalian hosts was further confirmed by using the *flaB* reporter promoter. *cisI* is likely to function as an operator, whose identification predicts the existence of a regulator named repressor. Further identification of the unknown regulator should help determine whether the T-rich region is a part of the operator of *B. burgdorferi* during mammalian infection.

Sohaskey *et al.* (1999) showed that deletion of the T-rich region results in a great reduction in expression of a chloramphenicol acetyltransferase reporter gene *in vitro*, while Caimano *et al.* (2007) suggested that this sequence may be involved in the downregulation of *ospA/B* in the mammalian host. The current study clearly confirmed the findings of Sohaskey *et al.* (1999) when *B. burgdorferi* was grown *in vitro*. However, we could not confirm the T-rich region as a part of the regulatory element of this spirochaete during mammalian infection.

Because the downstream sequence, collectively called *cisII*, of the *ospAB* promoter contains the transcriptional initiation site and ribosome-binding site, it cannot simply be deleted in order to investigate its regulatory function. The replacement of *cisII* caused a significant decrease in *ospA* expression *in vitro*, indicating a role of the sequence in enhancing expression. This role was further confirmed via fusion of *cisII* with the *flaB* reporter promoter. An opposite function of *cisII* was revealed in *B. burgdorferi* during murine infection. Like *cisI*, *cisII* may function as an operator interacting with an as-yet-unidentified repressor and contribute to *ospAB* downregulation in the mammalian host. Given that activator sites are usually located upstream of the −10 region (Collado-Vides *et al.*, 1991) and that *cisII* is sited between the −10 region and the start codon, with most of its sequence being transcribed into mRNA, it may be involved in post-transcriptional rather than transcriptional regulation (Lucchetti-Miganeh *et al.*, 2008), when *B. burgdorferi* is grown *in vitro*. The RNA sequence transcribed from *cisII* may interact with an as-yet-unidentified RNA-binding protein, and consequently lead to protection of *ospAB* transcripts when *B. burgdorferi* is grown *in vitro*. RNA-binding protein may also have an opposite function, which is to facilitate RNA turnover and subsequently cause gene downregulation (Lucchetti-Miganeh *et al.*, 2008). From this point of view, *cisII* does not necessarily function as an operator in *B. burgdorferi* during murine infection, but instead its RNA sequence may interact with a specific RNA-binding protein and, as a consequence, facilitate mRNA degradation. Interestingly, our previous study noted that the 3′ sequence (*ospB* portion) of *ospAB* mRNA is more stable than the 5′ (*ospA*) portion in murine tissues but less stable when spirochaetes are grown *in vitro* (Liang *et al.*, 2004a). It remains to be addressed whether this differential degradation of *ospA* and *ospB* portions of the bicistronic mRNA is due to a function of *cisII*.

The identification of the regulatory structure provided insights into the complexity of *ospAB* regulation. During *in vitro* cultivation, this currently unidentified activator is induced, which may enhance *ospAB* expression through an interaction with *cisI*. To further increase expression, the *in vitro* environment may also induce an unknown RNA-binding protein, which binds to the specific RNA sequence transcribed from *cisII* and protects *ospAB* mRNA. In contrast, the mammalian environment may induce two unidentified repressors, which bind to *cisI* and *cisII*, respectively, and shut off the *ospAB* operon. The mammalian milieu may also induce an unknown RNA-binding protein, which binds to the RNA sequence of *cisII* and facilitates mRNA degradation to further minimize OspA/B production. One could speculate that the unknown activator and repressor may be the same regulators, whose function is environment-dependent, and that the same RNA-binding protein may protect *ospAB* mRNA *in vitro* but facilitate decay during mammalian infection. Future studies should help to resolve these interesting issues.

A previous study reported that the downregulation of *ospA* is induced by IgG molecules (Hodzic *et al.*, 2005), in contrast to our earlier study showing that *B. burgdorferi* effectively represses *ospA* expression during infection of both SCID and immunocompetent mice (Liang *et al.*, 2004a). The current study clarified the issue of whether IgG is required for the induction of the downregulation. When *cisI* was removed and *cisII* was replaced, the *ospA* promoter efficiently initiated *ospA* expression as the *flaB* promoter

drove the *ospA* reporter gene in SCID mice. Moreover, when one of the elements was fused with the core *flaB* promoter, expression of the *ospA* reporter gene was dramatically reduced, and when both sequences were fused with the promoter, the reporter was essentially shut off. Given that IgG molecules were absent under each of these situations, the current study allowed us to conclude that the mammalian environment alone sufficiently induces downregulation of the *ospAB* operon.

Both OspA and OspB are highly immunogenic, so even a low level of expression may stimulate a significant humoral response. Although the responses may not effectively target spirochaetes with low OspA and OspB production in mammalian tissues, once the organisms are acquired by a tick vector, the surface antigens are greatly upregulated and consequently become effective targets in a blood meal. Thus, concealing OspA and OspB from the adaptive immune system is crucial for maintaining the enzootic cycle. The current study demonstrated that this ability depends on the newly identified *cis* elements. Even more importantly, the dual functions of these regulatory sequences can potentially provide *B. burgdorferi* with a greater advantage for quick adaptation to diverse environments during its enzootic life cycle travelling between the two distinct hosts. During persistent infection of a mammal, many outer-surface lipoproteins, including both RpoS- and σ^{70}-dependent gene products, are actively produced (Liang *et al.*, 2002, 2004b; Miller & Stevenson, 2006). Abundant surface lipoprotein expression is required for stabilizing the outer membrane of *B. burgdorferi* against innate defences (Xu *et al.*, 2008b). Once acquired by the vector, however, *B. burgdorferi* quickly downregulates *rpoS* (Caimano *et al.*, 2007) and consequently RpoS-dependent genes, such as *dbpA*, *dbpB*, *ospF* and *bbk32* (Eggers *et al.*, 2004; He *et al.*, 2007; Hubner *et al.*, 2001), as well as some σ^{70}-dependent genes such as *vlsE* and some *erp* genes (Bykowski *et al.*, 2006). A simple shift of *cisI* and *cisII* from the repressing mode to the activating function would allow *B. burgdorferi* to boost OspA/B production, effectively maintain the integrity of its outer-surface lipoprotein layer, and ultimately adapt to the new environment.

ACKNOWLEDGEMENTS

The authors would like to thank S. Norris for providing pBBE22 and B31 5A11. This work was supported in part by AI077733, AR053338, and RR020159 from the National Institutes of Health. F. T. L. is the recipient of an Arthritis Foundation Investigators Award.

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Edited by: P. van der Ley