

## DNA-Binding Proteins Possibly Involved in Regulation of the Post-Logarithmic-Phase Expression of Lipoprotein P35 in *Borrelia burgdorferi*

KARL J. INDEST AND MARIO T. PHILIPP\*

Department of Parasitology, Tulane Regional Primate Research Center, Tulane University Medical Center, Covington, Louisiana

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Previously, we have shown that the transcription of *p35*, a lipoprotein gene of *Borrelia burgdorferi*, is upregulated or initiated during the post-logarithmic bacterial growth phase in vitro. To identify potential regulatory factors, we examined the formation of DNA-protein complexes by electromobility shift assay, using a 157-bp DNA fragment that spans the *p35* promoter region and cell-free extracts of spirochetes harvested from both logarithmic and stationary growth phases. The binding properties of the extracts with the promoter region of the *flaB* gene, a constitutively expressed, growth-phase-independent gene, were also compared. The results from these experiments demonstrate that *B. burgdorferi* stationary-phase cell-free extracts have a growth-phase-dependent DNA binding protein that interacts specifically with the *p35* promoter region. We show, in addition, that a segment from the 157-bp *p35* promoter region which contains both a T-rich stretch and an inverted repeat is able to compete off the stationary-phase-specific complex when the segment is present in molar excess.

*Borrelia burgdorferi*, the spirochete that causes Lyme disease, is able to survive in two very dissimilar host environments: ixodid ticks, which act as its transmission vector, and vertebrates such as rodents, which serve as its most common reservoir host. It has been postulated that *B. burgdorferi*'s adaptation to the tick and mammalian environments likely involves the expression of different surface components (12). In support of this hypothesis, several lipoproteins have been identified whose expression varies depending on the spirochete's milieu (3, 13, 18). Mechanisms of modulation of lipoprotein expression are unknown in *B. burgdorferi*. An inspection of the *B. burgdorferi* genome sequence revealed few homologs of other bacterial regulatory proteins, including only two response-regulator two-component systems (4). Only one DNA binding activity which is specific for a region upstream of the *ospAB* operon has been reported for *B. burgdorferi* (10). The development of an in vitro model in which regulatory mechanisms of differentially expressed proteins could be easily studied is desirable as a first step toward dissecting *B. burgdorferi* gene regulation. The observations that *B. burgdorferi* is able to modulate gene expression in vitro in response to changes in temperature (16, 17) and cell density or growth phase (8, 11) could be further explored to develop such a model. Previously, we demonstrated that the expression of P35, P7.5, OspC, BmpD, and several unidentified *B. burgdorferi* antigenic proteins is upregulated during post-logarithmic (post-log) spirochetal growth (8, 11). This regulation was shown to occur at the level of transcription for P35, P7.5, OspC, and BmpD (8, 11). P35 was chosen to further investigate this phenomenon. It is possible that the alteration in the rate of transcription or transcriptional initiation is positively or negatively controlled by DNA binding proteins. To identify such potential regulatory factors, we investigated the formation of DNA-protein com-

plexes by electromobility shift assays (EMSA) by using a DNA fragment that encompassed the *p35* promoter region and cell-free *B. burgdorferi* extracts that were harvested from both log and stationary phases.

Low-passage isolates of *B. burgdorferi* B31 were routinely grown in 1-liter bottles of BSK-H medium (Sigma Chemical Co., St. Louis, Mo.) supplemented with 10% heat-inactivated young rabbit serum (Pel-Freez, Roger, Ark.), 7.5  $\mu$ g of amphotericin per ml, 48  $\mu$ g of rifampin per ml, and 192  $\mu$ g of phosphomycin per ml. Cultures were incubated at 34°C in a trigas incubator set at 3% CO<sub>2</sub>, 5% O<sub>2</sub>, and the rest of N<sub>2</sub>. Spirochetes were counted under a dark-field microscope. Approximately 10<sup>10</sup> spirochetes were harvested from log-phase (10<sup>6</sup> cells/ml) and stationary-phase cultures (10<sup>8</sup> cells/ml) and used to prepare cell-free extracts for EMSA analysis. Cell-free extracts were prepared as described by Margolis and Samuel (10), and their protein concentrations were determined by the Bradford method (1). EMSA were essentially carried out as described by Chodosh (2). All EMSA presented herein were performed at least twice with the same results. EMSA substrates containing the *p35* promoter region were generated by PCR with primer sequences 5' CGCTCTAGAACTAGTGG ATC 3' and 5' GTAATTTATATTAATTAATAATTTAAT TACCC 3' and plasmid DNA from a *p35* clone described previously (8). PCRs were performed at an annealing temperature of 45°C for 1 min, an extension temperature of 72°C for 1 min, and a denaturation temperature of 94°C for 1 min for a total of 30 cycles. The DNA fragment that contained the *flaB* promoter region was generated by PCR as described previously (14). EMSA substrates were digested with appropriate restriction enzymes (New England Biolabs Inc., Beverly, Mass.) and labeled with digoxigenin (DIG)-11-ddUTP and terminal transferase (Boehringer Mannheim, Indianapolis, Ind.) according to the manufacturer's instructions. A typical binding reaction mixture consisted of 23 fmol of EMSA substrate, 2  $\mu$ g of nonspecific competitor DNA [poly(dI-dC); Sigma], 300  $\mu$ g of bovine serum albumin per ml (final concentration), 20 mM HEPES (pH 7.9), 40 mM KCl, 1 mM EDTA, 1 mM dithio-

\* Corresponding author. Mailing address: T. R. P. R. C., 18703 Three Rivers Rd., Covington, LA 70433. Phone: (504) 871-6221. Fax: (504) 871-6390. E-mail: philipp@tpc.tulane.edu.

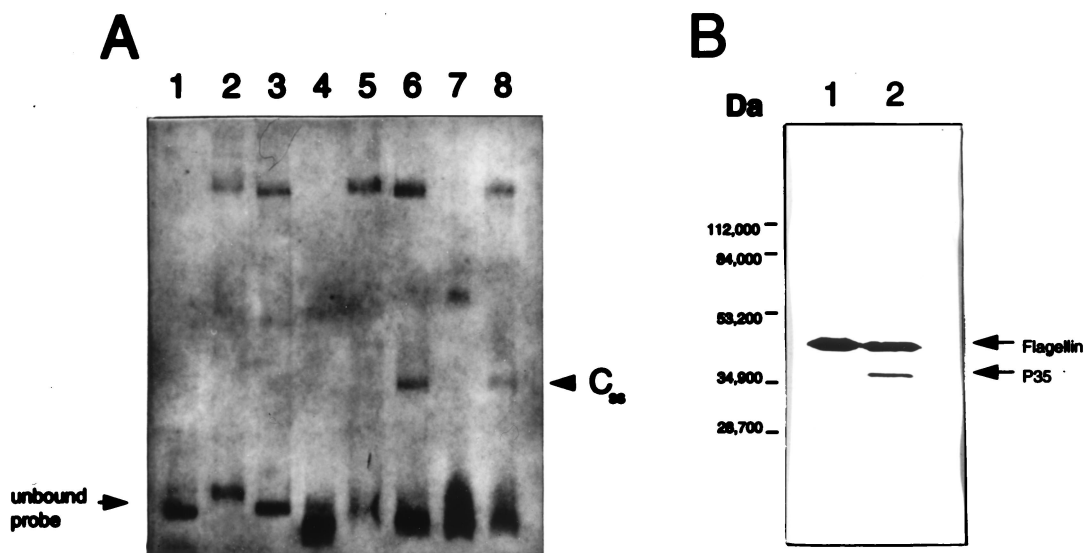


FIG. 1. (A) EMSA analysis of the *B. burgdorferi* *p35* and *flaB* promoter regions. Twenty-three femtomoles of the 175-bp DIG-labeled *flaB* promoter probe was electrophoresed with no cell-free extract added (lane 1) or with 7  $\mu$ g of extract from spirochetes harvested in log phase (lane 2) or in stationary phase (lane 3). Twenty-three femtomoles of the 157-bp DIG-labeled *Bam*HI-*Pac*I *p35* promoter probe was electrophoresed with no cell-free extract added (lane 4), with 7  $\mu$ g of extract from spirochetes harvested in log phase (lane 5) or in stationary phase (lane 6), or with 7  $\mu$ g of stationary-phase extract pretreated with 80  $\mu$ g of proteinase K (lane 7) or heat-inactivated proteinase K (lane 8). (B) Analysis of P35 expression by Western immunoblotting of extracts from cultured spirochetes harvested at log phase (lane 1) and stationary phase (lane 2). The same batches of log- and stationary-phase spirochetes were used to generate cell-free lysates for EMSA analysis. The blot was reacted with a monoclonal antibody specific for P35 (8) and with the flagellin-specific monoclonal antibody H9724. Molecular size markers are indicated on the left.

threitol, 10% glycerol, and 7  $\mu$ g of cell-free extract in a final volume of 15  $\mu$ l. The reaction mixture was incubated for 15 min at 30°C, and the DNA-protein complexes were resolved by electrophoresis through a nondenaturing 6% polyacrylamide gel by using a high-ionic-strength Tris-glycine buffer (250 mM Tris-Cl, 1.9 M glycine, 10 mM EDTA, [pH 8.0]). During competitive binding reactions, cell-free extracts were allowed to preincubate for 5 min at room temperature with the corresponding competitor DNAs added prior to the addition of labeled probe. Double-stranded competitor oligonucleotides were generated by heating a mixture of equal molar amounts of single-stranded complementary oligonucleotides at 95°C in a Perkin-Elmer Cetus 9600 thermal cycler. Double-stranded competitor oligonucleotides were then slowly annealed by allowing the thermal cycler to ramp to 4°C over a 60-min period. Following electrophoresis, resolved components were transferred from the gels to positively charged nylon membranes, probed with an anti-DIG-alkaline phosphatase-labeled antibody, and detected by chemiluminescence as described by the manufacturer (Boehringer). Western blot analysis of *B. burgdorferi* proteins was performed as described previously (8).

A comparison of the results of the EMSA experiments performed with the *p35* promoter region with those of the EMSA experiments with the constitutively expressed, growth-phase-independent *flaB* promoter region indicates that stationary-phase cell-free extracts contain a DNA binding activity that is specific for the *p35* gene. In these experiments, a DIG-labeled 157-bp *Bam*HI-*Pac*I *p35* promoter probe (see Fig. 2A) was used as a substrate for protein binding in the EMSA reaction. This fragment includes several potential *cis* elements which might be involved in DNA-protein interactions, such as a -10/-35 promoter region, a T-rich segment containing an area of dyad symmetry, and a 32-bp AT-rich region. As a negative control for nonspecific binding and growth-phase-independent binding activities, a DIG-labeled 175-bp *flaB* promoter probe (14) was used as a substrate for protein binding in the EMSA

reaction (Fig. 1A, lane 1). The *flaB* promoter probe in the presence of both log-phase (Fig. 1A, lane 2) and stationary-phase (Fig. 1A, lane 3) cell-free extracts generated a shifted band pattern that consisted predominantly of an intense, slow-migrating complex. The *p35* promoter probe (Fig. 1A, lane 4) in the presence of both log-phase (Fig. 1A, lane 5) and stationary-phase (Fig. 1A, lane 6) cell-free extracts generated a similar shifted band pattern as the *flaB* promoter probe, except for an additional prominent, fast-migrating complex which was contributed only by the stationary-phase cell-free extract. This complex, designated C<sub>SS</sub> for stationary (-) phase-specific complex, appears to be specific for the *p35* promoter region. EMSA extracts were treated with proteinase K to verify that the shifted bands observed were the results of a DNA-protein interaction. In the presence of 80  $\mu$ g of proteinase K, the shifted band pattern was altered such that C<sub>SS</sub> and the slow-migrating complex were no longer detectable (Fig. 1A, lane 7). The band observed in lane 7 is believed to be the result of residual binding activity remaining after incomplete proteinase K digestion. To insure that the alteration in banding pattern observed in lane 7 was not due to the interference from the presence of additional protein mass contributed by the proteinase K, the same amount of heat-treated proteinase K (95°C for 10 min) was added to a control EMSA reaction. The result shows that in the presence of heat-treated proteinase K, the stationary-phase complex can be observed (Fig. 1A, lane 8). Western blot analysis of P35 expression was performed with the same log- and stationary-phase cell-free lysates that were used for the EMSA analysis (Fig. 1B, lanes 1 and 2, respectively). The blot was incubated with monoclonal antibodies specific for P35 (8) and flagellin (monoclonal antibody H9724); the latter was included to control for protein mass. The results confirm that the cultured spirochetes used to generate the cell-free extracts were harvested at the appropriate growth phase, i.e., the log-phase cells had not overgrown and begun synthesizing P35. The data from both the EMSA and Western



C<sub>SS</sub> binding suggest that the protein(s) interacting with this sequence may activate transcription. The -35 promoter region of the *p35* gene is poorly defined, and protein binding near this region could facilitate enhanced RNA polymerase promoter binding. Such a mechanism may require direct contact of the activator with RNA polymerase and/or may require additional protein-protein interactions. Transcriptional activation is a plausible mechanism whereby *B. burgdorferi* could control gene expression. Thus far, only one other DNA-binding activity has been reported for *B. burgdorferi* (10).

The identity of the protein(s) that binds to the upstream promoter region of *p35* cannot be inferred from our results. However, a T-rich region similar to that described herein was recently identified by Sohaskey et al. as a possible regulatory element in *B. burgdorferi* (15). Through promoter deletion analyses, these authors identified a T-rich region, located upstream of the *ospA* gene's -35 promoter region, which positively influenced *ospA* transcription (15). Presence of this T-rich region may be necessary but is not sufficient for growth-phase-dependent regulation, for *ospA* is not regulated by growth phase in vitro (11). On the other hand, it is possible that the inverted repeat that is also present on the K2 fragment is, alone or in concert with the adjacent T-rich segment, responsible for the observed DNA binding and the correlated up-regulation. Of the three additional genes which are regulated in a growth-phase-dependent fashion whose DNA sequences are known, namely *p7.5*, *bmpD*, and *ospC*, the first two exhibit appropriately located regulatory *cis* elements, as the one mentioned herein (Indest and Philipp, unpublished). *p7.5* and *bmpD* both contain T-rich sequences upstream from their -35 promoter regions, and *bmpD*, but not *p7.5*, also has an inverted repeat in this region. On the other hand, these elements are not easily identifiable in the promoter region of the *ospC* gene. It is possible that other factors or mechanisms other than the one we suggest here are involved in growth-phase-dependent gene regulation. It is perhaps significant that the 157-bp *p35* promoter region contains a portion of a putative integration host factor (IHF) binding site (AAAtATAAGACCTTG; consensus IHF binding site, WATCAA-N4-TTR) (7). The latter is located 79 bp upstream from the transcription start site of *p35*. IHF is a multifunctional DNA binding and bending protein which is found in bacteria and which can mediate protein-protein and DNA-protein interactions. IHF has been shown to positively modulate gene expression in the context of multiple regulatory factors (5, 9). The mechanism by which IHF modulates gene expression is poorly understood. It is assumed that this protein acts as a molecular scaffold of sorts, configuring the DNA in a manner that facilitates the assembly of other protein-DNA complexes (5). AT-rich elements are associated with IHF binding sites and are thought to increase binding of IHF to its target sequence (7). Recently, a gene was isolated from *B. burgdorferi* which encodes a protein that is homologous to members of the IHF/histone utilization family of *E. coli* proteins (19).

Currently, we are trying to further characterize the DNA binding activity identified herein. Attempts at cloning this DNA binding activity by screening *B. burgdorferi* genomic DNA expression libraries with appropriate DNA probes have been unsuccessful. The possibility that this protein(s) may bind to DNA upon acquiring a particular quaternary structure has compelled us to employ DNA-affinity chromatography meth-

ods for purification. It is our goal to specifically dissect the mechanism by which the *p35* gene is regulated and thus hopefully gain an understanding of how *B. burgdorferi* regulates gene expression as it shuttles through diverse environments.

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