INFECTION AND IMMUNITY, Apr. 1997, p. 1165–1171 Vol. 65, No. 4
0019-9567/97/$04.00+0

Cell-Density-Dependent Expression of *Borrelia burgdorferi* Lipoproteins In Vitro

KARL J. INDEST,[1] RAMESH RAMAMOORTHY,[1] MÓNICA SOLÉ,[1] ROBERT D. GILMORE,[2] BARBARA J. B. JOHNSON,[2] AND MARIO T. PHILIPP[1]*

Department of Parasitology, Tulane Regional Primate Research Center, Tulane University Medical Center, Covington, Louisiana,[1] and Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado[2]

Received 29 October 1996/Returned for modification 5 December 1996/Accepted 15 January 1997

Previously, we had identified non-OspA-OspB surface proteins of *Borrelia burgdorferi* that are targeted by the antibody-dependent complement-mediated killing mechanism. Here we demonstrate by Western blotting that one of these proteins, P35, is upregulated at the onset of stationary phase in vitro. Northern analysis revealed that the upregulation of P35 is at the level of transcription. In addition, the expression of an open reading frame (ORF) located downstream of the *p35* gene was found to be regulated in the same fashion as that of P35. This ORF encodes a 7.5-kDa lipoprotein. The transcriptional start sites for both of these genes were determined, to aid in the identification of the putative promoter regions. Additional sequencing of the 5′ flanking region of the *p35* gene revealed a region of dyad symmetry 52 bp upstream of the transcription start site. Southern analysis demonstrated that the expression of these genes was not due to a cell-density-dependent rearrangement in the genome of *B. burgdorferi*. These findings provide an in vitro model for studying mechanisms of gene regulation in *B. burgdorferi*.

Borrelia burgdorferi, the spirochete that causes Lyme disease, is able to survive in two very dissimilar host environments, the ixodid ticks that act as its transmission vector and vertebrates such as rodents, which serve as its most common reservoir host. The natural history of *B. burgdorferi* encompasses intervals of quiescence interspersed with periods of active cell division. When larval *Ixodes scapularis* ticks feed on a chronically infected mouse, the spirochetes are taken up from the skin, where they are present in densities much lower than those during the acute phase of infection (3) and probably are dividing at a very low rate, if at all. This putatively quiescent period is followed by one of active multiplication, for the spirochetal population in fed larvae increases considerably during the first 6 days after engorgement (20).

Tick survival, however, dictates that unrestrained cell division of the spirochete must come to an end. The ensuing stationary phase, probably caused either because key nutrients become exhausted within the tick or because an appropriate signal is elicited, likely persists through transtadial transmission. Active cell division is detectable again upon engorgement of the infected nymph (6, 20) and must occur also during the early localized and disseminated phases of infection in the reservoir host. In contrast, during the chronic phase of the infection, spirochetal sparsity (3) could be maintained either by a lower rate of division or other mechanisms involving host immune surveillance.

During a survey of *B. burgdorferi* antigens that appeared to be targets of antibody-dependent, complement-mediated killing of the spirochete in vitro (2), we noticed serendipitously that one of these antigens, a 35-kDa molecule, was detectable on Western blots of whole spirochetal antigens when the organisms were harvested during the stationary phase of growth in vitro but not when they were harvested in mid-log phase. It appeared as though expression of this antigen was regulated in vitro in a cell-density-dependent manner. In view of the spirochete's natural history, the potential significance of the correlation between expression of P35 and the bacterial growth phase prompted us to investigate this phenomenon further. The *p35* gene was cloned (9), a monoclonal antibody specific for this antigen was produced (11), and with the aid of these tools we determined whether the onset of expression of P35 in vitro was regulated at the level of transcription. Here we present the results of this study.

MATERIALS AND METHODS

Spirochete strains, culture conditions, and antigen preparation. Low passages of *B. burgdorferi* B31, JD1, or HB19 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 μg of amphotericin per ml, 48 μg of rifampin per ml, and 192 μg of phosphomycin per ml. Cultures were incubated at 34°C in a tri-gas incubator set at 3% CO_2, 5% O_2, and the rest N_2. Spirochetes were counted under dark-field microscopy as described previously (19). To investigate growth-dependent expression, spirochetes were grown as described above, starting at a concentration of 10^3 cells/ml. At designated time intervals, samples were removed for antigen preparation. Briefly, approximately 10^9 spirochetes were centrifuged at 10,000 × *g* for 10 min, and the supernatant was removed. The cell pellet was then washed three times with phosphate-buffered saline (PBS) (pH 7.4) and resuspended in a minimal amount (<60 μl) of sodium dodecyl sulfate (SDS) lysis buffer (50 mM Tris-HCl [pH 7.5], 1% SDS, 1% glycerol, 2.2% 2-mercaptoethanol, 0.002% bromophenol blue). Samples were boiled for 5 min and centrifuged for 5 min at 10,000 × *g* in a microcentrifuge to remove cell debris. Prior to protein determination, the supernatant was diluted 1:400 such that the SDS concentration did not exceed 0.0025% and the 2-mercaptoethanol concentration did not exceed 0.0055% (0.025 M). The protein concentration in the supernatant was determined by the Bradford method (4).

Western blot analysis. Antigen preparations were subjected to SDS-polyacrylamide gel electrophoresis in a discontinuous 0.1% SDS–10% polyacrylamide slab gel with gel buffers as described by Laemmli (15). A total of 20 μg of protein per lane was loaded and separated overnight at 50 V with a Protean II xi slab cell (Bio-Rad Laboratories, Hercules, Calif.). Proteins were transferred to nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.) in a Trans-Blot electrophoretic transfer cell (Bio-Rad) at 50 V for 1 hour with cooling. The composition of the transfer buffer was 48 mM Tris base, 39 mM glycine, and 20% methanol. Following transfer, nonspecific binding to the nitrocellulose was blocked with 3% fat-free powdered milk prepared in PBS, containing 0.05% Tween 20 (Integrated Separation Systems) (PBS-T), for 2 h at room temperature. A mouse monoclonal

* Corresponding author. Mailing address: T. R. P. R. C., 18703 Three Rivers Rd., Covington, LA 70433. Phone: (504) 892-2040. Fax: (504) 893-1352. E-mail: philipp@tpc.tulane.edu.

antibody directed against P35 and the antiflagellin monoclonal antibody H9724 (Symbicom, Umea, Sweden) were allowed to react at the appropriate dilution in PBS with the nitrocellulose membrane for 1 h with slow shaking. The nitrocellulose was washed three times with PBS-T for a total of 15 min. After the washes, the membrane was incubated for 1 h with biotinylated anti-mouse immunoglobulin M (μ-chain-specific) and immunoglobulin G (γ-chain-specific) antibodies (Vector Laboratories, Burlingame, Calif.) diluted 1/200 in PBS-T. After washing as described above, a biotinylated horseradish peroxidase-avidin complex (Vector) was used to probe the biotinylated antibodies. The blot was developed with hydrogen peroxide and the chromogen 4-chloro-1-naphthol (Sigma) at concentrations of 0.015% and 2.8 mM, respectively, in PBS with 16% methanol. The color reaction was stopped by washing the membrane with distilled water.

RNA isolation and Northern analysis. Total cellular RNA was isolated from *B. burgdorferi* B31 with hot acidic phenol (8). Prior to extraction, spirochetes were rapidly chilled in a dry-ice–ethanol bath. After extraction, nucleic acids were resuspended in 40 mM Tris-HCl (pH 7.9)–6.0 mM $MgCl_2$–10 mM KCl–10 mM $CaCl_2$–400 U of RNasin/ml and treated with 40 U of RQ1 RNase-free DNase (Promega Biotech, Madison, Wis.) for 15 min at 37°C. Following a phenol-chloroform extraction, the RNA was precipitated with 1/10 volume of 3.0 M sodium acetate–3 volumes of 100% ethanol and dissolved in water. Approximately 20 μg of RNA was electrophoresed through a 1% agarose-formaldehyde gel and transferred to nitrocellulose as previously described (24). The RNA was UV cross-linked to the membrane with a UV Stratalinker 800 (Stratagene Inc., La Jolla, Calif.). Prehybridizations and hybridizations were carried out at 42°C in 5× sodium chloride-sodium citrate (SSC) (0.75 M NaCl, 75 mM $C_6H_5Na_3O_7 \cdot 2H_2O$)–5× Denhardt's solution (0.1% Ficoll 400 [Sigma], 0.1% polyvinylpyrrolidone [Sigma], 0.1% bovine serum albumin [Pentax fraction V; Sigma])–1% SDS–100 μg of salmon sperm DNA per ml–50% formamide. Approximately 10^6 cpm of ^{32}P-labeled probe per ml was added to the prehybridization mix, and the membrane was incubated overnight. The membranes were subsequently washed for 20 min at 25°C in 1× SSC–0.1% SDS, followed by two washes at 55°C for 20 min each in 0.2× SSC–0.1% SDS. Blots were exposed to XAR-5 film (Kodak Inc., Rochester, N.Y.) overnight.

Southern analysis. Total genomic DNA was isolated from *B. burgdorferi* B31 with Qiagen (Chatsworth, Calif.) genomic tips. Approximately 2 μg of DNA was digested with the appropriate restriction enzyme and separated through a 4× Tris-acetate-EDTA (160 mM Tris-acetate, 8 mM $Na_2EDTA \cdot 2H_2O$, pH 8.5) agarose gel. Prior to transfer of the DNA to nitrocellulose, the gel was treated for 10 min with 0.25 N HCl. After the transfer, the DNA was UV cross-linked to the membrane, and prehybridizations and hybridizations were carried out as described above for Northern analysis.

Cloning and sequencing of the upstream region of the *p35* gene. A library of randomly sheared DNA from *B. burgdorferi* JD1 had been generated previously in the bacteriophage λ ZAPII (22). The upstream region of *p35* was identified by probing approximately 10^5 plaques with a radiolabeled *Eco*RI-*Sac*I DNA fragment from a recombinant *p35* gene clone (9). The resulting positive plaques were isolated, resuspended in 400 μl of SM medium (100 mM NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris-HCl [pH 7.5], 0.01% gelatin), and screened by PCR for inserts containing regions of DNA upstream from the *p35* gene. Amplifications of DNA were carried out in 50-μl reaction volumes containing 2.5 U of *Taq* DNA polymerase (Promega), 1 μM each primer, 200 μM each deoxyribonucleoside triphosphate, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, and 0.1% Triton X-100. Reactions were performed in a Perkin-Elmer Cetus DNA thermal cycler at an annealing temperature of 55°C for 2 min, an extension temperature of 72°C for 3 min, and a denaturation temperature of 94°C for 2 min, for a total of 25 cycles. In vivo excision of recombinant pBluescript plasmid from the chosen phage was carried out by simultaneous infection of recombinant XL1-Blue *Escherichia coli* cells with ExAssist helper phage (Stratagene). Plasmid DNA for sequencing was prepared with the Qiagen Plasmid Midi Kit. The nucleotide sequence was determined by sequencing both DNA strands by the dideoxy chain termination method (25), using a modified T7 DNA polymerase (Sequenase version 2.0; United States Biochemical Corp., Cleveland, Ohio) and α-^{35}S-dATP (NEN DuPont, Boston, Mass.). The oligonucleotide primers used for sequencing and PCR were 5′TCTTTGACTTCCAGAGAGCA3′, which hybridizes to positions 171 to 190 of the *p35* gene (9), and Stratagene's KS primer.

Primer extension. Transcriptional start site analysis was performed by way of primer extension to aid in identifying the putative promoter regions of the *p35* gene and the *p7.5* gene. Two primers were used for this purpose: 5′TCTTTGACTTCCAGAGAGCA3′ for the *p35* transcript and 5′CATCTCATCTGAAATTCTTG3′, corresponding to nucleotides 154 to 173 (see Fig. 4), for *p7.5*. Approximately 3 pmol of the appropriate end-labeled oligonucleotide was mixed with 35 μg of *B. burgdorferi* RNA and annealing buffer (10 mM Tris-HCl [pH 8.3], 150 mM KCl, 1 mM EDTA) in a total volume of 30 μl. Samples were incubated for 1.5 h at 65°C and then allowed to cool slowly to room temperature. After annealing, the samples were precipitated with 3.0 M sodium acetate (pH 5.5), and 100% ethanol. The pellet was washed with 70% ethanol and resuspended in 20 μl of 1× first-strand synthesis buffer (Bethesda Research Laboratories, Gaithersburg, Md.) supplemented with 0.01 M dithiothreitol, 0.46 mM deoxynucleoside triphosphates, 1 μg of actinomycin D, and 200 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories). Primer extension was carried out for 1 h at 42°C, and the mixture was heat treated for 10 min at 65°C. Five micrograms of RNase A (Qiagen) was added to the reaction mixture and incubated for 30 min at 37°C. The samples were extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), ethanol precipitated, washed with 70% ethanol, and resuspended in Sequenase stop solution (United States Biochemicals). Samples were run through a 6% polyacrylamide-urea sequencing gel alongside a set of DNA sequencing reactions.

Other procedures and reagents. Restriction enzymes were purchased from New England Biolabs, Inc. (Beverly, Mass.) and used as recommended by the manufacturer. Double-stranded DNA probes were radiolabeled by random priming with [α-^{32}P]dATP by using the Prime-A-Gene system (Promega) and passed through a Microspin G-50 column (Pharmacia Biotech Inc., Piscataway, N.J.) to remove unincorporated radioactivity. Oligonucleotide probes were end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs) and purified by passage through a Microspin G-25 column (Pharmacia). Oligonucleotide primers for PCR and sequencing reactions were synthesized at the Louisiana State University Medical Center Core Laboratories, New Orleans.

Nucleotide sequence accession numbers. The nucleotide sequences for *p7.5* and the 5′ flanking region of the *p35* gene can be found in the GenBank database under the accession numbers U59487 and U88867, respectively.

RESULTS

Cell-density-dependent expression of P35. *B. burgdorferi* B31 was grown in vitro, and samples of the culture were taken at selected time points (Fig. 1A). The bacteria were lysed as described in Materials and Methods, and equal amounts of protein from each lysate were analyzed by Western blotting with a mouse monoclonal antibody that is specific for the P35 antigen as a probe. Samples taken during the mid-log phase (Fig. 1A, point 1) showed no detectable P35 protein (Fig. 1B, lane 1). However, as the cells approached stationary phase (Fig. 1A, point 2), the level of P35 protein increased, with expression being first detected at 3.8×10^7 cells/ml (Fig. 1B, lane 2). To internally control for the amount of protein in each lane, these samples were simultaneously probed with a monoclonal antibody to *B. burgdorferi* flagellin. The same amount of flagellin can be detected in each sample, confirming that equal amounts of protein are present in each lane. The same growth-dependent expression of P35 was also observed for *B. burgdorferi* JD1 and HB19 (data not shown).

Northern blot analysis of the *p35* transcript. Northern analysis was performed to determine if the *p35* transcript was expressed in a growth-dependent manner. The cell density (time) points chosen to monitor *p35* gene transcription were the same as those chosen previously to monitor protein expression (8.3×10^6, 3.8×10^7, 7×10^7, and 1.1×10^8 cells/ml). RNA samples were probed with a 2-kb *Eco*RI-*Eco*RI* DNA fragment, which contains the *p35* coding region and additional DNA sequence downstream of the *p35* gene (9) (Fig. 2C). A transcript of approximately 1 kb was detected in each of the RNA samples (Fig. 2A). The size of this transcript correlates well with the predicted 909-bp coding region of the *p35* gene (9). In addition, a second, smaller transcript of approximately 300 bases was detected (Fig. 2A). This region had been cloned and sequenced previously (9). DNA sequence analysis of the region downstream from the *p35* gene indicated that there is a small (204-bp) open reading frame (ORF) that is transcribed in the opposite direction and on the other DNA strand relative to the *p35* gene (Fig. 3A). To confirm that this ORF is transcribed, RNA samples were probed with a 570-bp *Sac*I-*Eco*RV fragment (Fig. 2C, probe B). A single transcript of approximately 300 bases was detected (data not shown). This small ORF codes for a putative protein with a predicted molecular mass of 7,762 Da and has been designated *p7.5* (see Discussion). A homology search with *p7.5* in several protein databases by using the BLAST algorithm revealed 100% identity to a putative 7.5-kDa lipoprotein from *B. burgdorferi* B31 and 297 (GenBank accession numbers U59859 and U59857, respectively) and 98% identity to a protein from *B. burgdorferi* N40

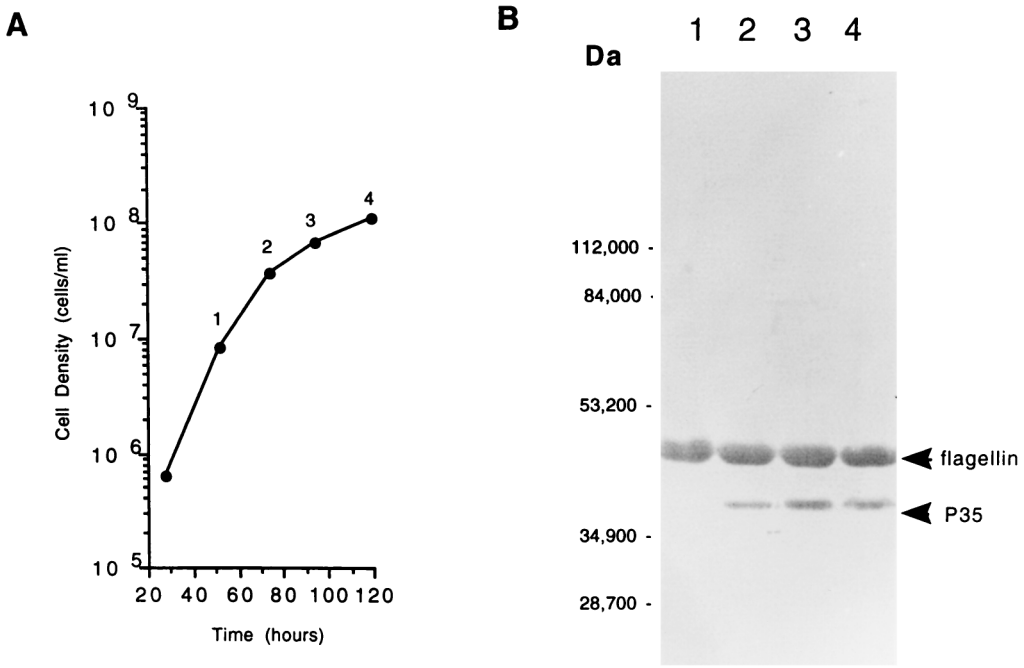

FIG. 1. (A) Growth curve of *B. burgdorferi* B31 grown in vitro. The numbers on the curve correspond to the harvest points of samples shown in lanes 1 to 4 in panel B. (B) Western blot analysis of P35 expression in *B. burgdorferi* B31 as a function of cell density. Samples of cultured spirochetes were taken at 8.3×10^6 cells/ml (lane 1), 3.8×10^7 cells/ml (lane 2), 7×10^7 cells/ml (lane 3), and 1.1×10^8 cells/ml (lane 4), solubilized in SDS-polyacrylamide gel electrophoresis lysis buffer, separated on a 10% acrylamide gel, and transferred to nitrocellulose. Membranes were probed with a MAb specific for P35 and with the antiflagellin antibody H9724.

(GenBank accession number U59858). When RNA samples were probed with a 930-bp *Eco*RI-*Sac*I DNA fragment (Fig. 2C, probe A), a single transcript of approximately 1 kb was detected (data not shown).

Consistent with the Western blot analysis presented above, detection of the *p35* and *p7.5* transcripts varied in a cell-density-dependent fashion. The RNA sample taken during mid-log phase contained very small amounts of the *p35* and *p7.5* transcripts (Fig. 2A, lane 1), whereas samples collected as the cells approached stationary phase contained larger amounts of the *p35* and *p7.5* transcripts (Fig. 2A, lanes 2, 3, and 4). The amount and integrity of the mRNA in each sample were controlled for by probing the same samples with a PCR-generated *B. burgdorferi* flagellin gene fragment (Fig. 2B). With the exception of lane 2, each lane in Fig. 2B contains an equal amount of flagellin mRNA. The flagellin gene was amplified with the primers 5′GCATTAACGCTGCTAATC3′ and 5′TTGCAGGCTGCATTCCAA3′ (30).

Transcriptional start sites of the *p35* and *p7.5* genes. Transcriptional start site analysis was performed by way of primer extension to aid in identifying the putative promoter regions of the *p35* gene and the *p7.5* gene. In each case, a single start site was detected (Fig. 4). The start site for the *p35* gene is 61 bp upstream of the putative TTG translational start codon (Fig. 4A) (9). There are putative −35 and −10 sites, appropriately spaced, which may serve as the promoter for this gene (Fig. 3B). The start site for the *p7.5* gene is 24 bp upstream of a putative ATG translational start codon (Fig. 4B). Approximately 10 bp downstream from this transcriptional start site, there is a purine-rich sequence that could serve as a ribosome binding site. In addition, the region upstream from the transcription start site contains an appropriately spaced −10/−35 sequence that may serve as a promoter for this gene (Fig. 3A).

DNA sequence analysis of the *p35* 5′ flanking region. The upstream region of the *p35* gene was cloned from a randomly sheared total DNA library of *B. burgdorferi* JD1 in an attempt to identify potential *cis*-acting elements that may be involved in the density-dependent regulation of P35 expression. This region upstream from the P35 ORF was sequenced in both directions (Fig. 3B). The region contains a 9-nucleotide (nt) inverted repeat located 52 bp upstream from the transcriptional start site, which may be involved in the regulation of the *p35* gene. Overall, the region between positions −1 and −128 is 80.5% AT rich and includes a 43-nt AT tract from position −86 to −128 (Fig. 3B).

Southern analysis. Southern analysis was performed to determine if the upregulation of *p35* was the result of a rearrangement at this locus in the genome of *B. burgdorferi*. Total genomic DNA was isolated from *B. burgdorferi* B31; digested with *Eco*RI, *Eco*RV, or *Nsp*I; separated on an agarose gel; and transferred to nitrocellulose. The blot was probed with an *Eco*RI-*Sac*I fragment which contains most of the *p35* coding region and about 40 bp of the 5′ flanking sequence (Fig. 2C, probe A). The hybridization patterns were identical for each enzyme digest, regardless of whether the DNA was isolated from log-phase cells or from stationary-phase cells (data not shown). These results indicate that the regulation of the expression of P35 is not mediated by a rearrangement in the genome of the organism.

DISCUSSION

We have demonstrated that the expression of *p35* and *p7.5* is either upregulated or initiated at the onset of stationary phase. The expression of these genes does not appear to be the result of a genomic rearrangement, as seen in other *Borrelia* species,

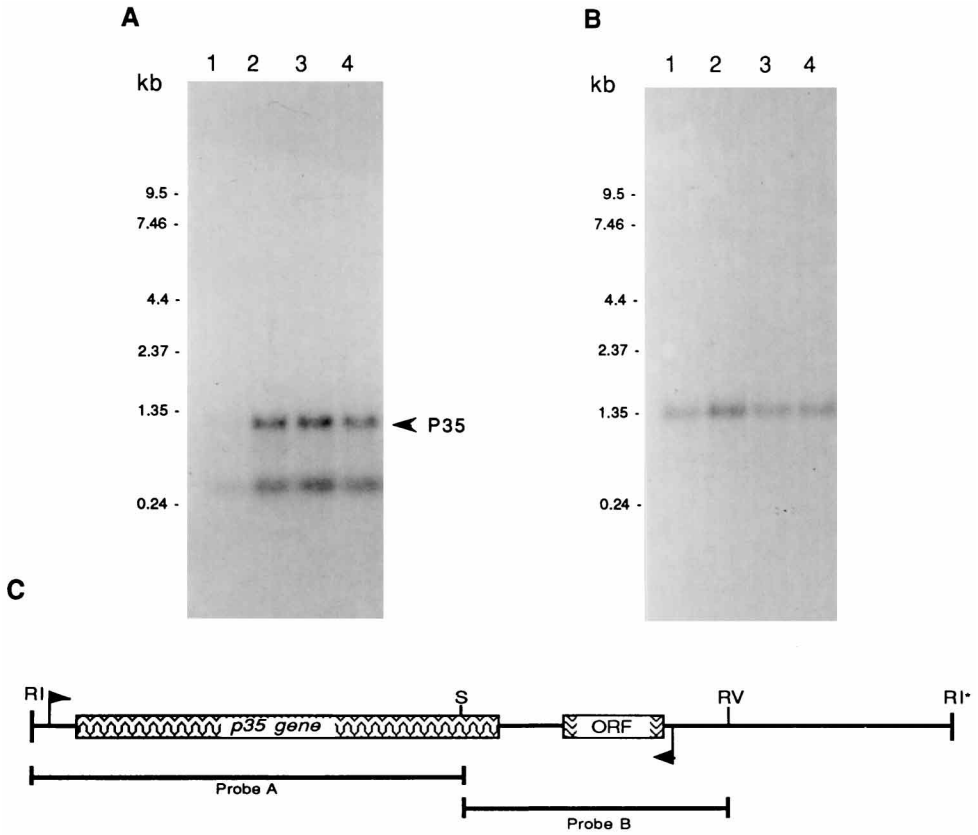

FIG. 2. Northern blot analysis of the *p35* transcript. Twenty-microgram samples of total cellular RNAs isolated from *B. burgdorferi* B31 at 8.3×10^6 cells/ml (lane 1), 3.8×10^7 cells/ml (lane 2), 7×10^7 cells/ml (lane 3), and 1.1×10^8 cells/ml (lane 4) were separated by electrophoresis and transferred to nitrocellulose. The membrane was first hybridized with a ^{32}P-labeled DNA probe that hybridized to both *p35* and *p7.5* (A). The probe is represented in panel C as the fragment between the *Eco*RI (RI) and *Eco*RI* (RI*) sites. The blot was stripped and reprobed with a PCR-amplified, ^{32}P-labeled flagellin DNA probe (B). (C) Restriction endonuclease map of a cloned 1.9-kb *B. burgdorferi* B31 DNA region containing the *p35* gene and the ORF encoding P7.5. Unique *Eco*RI (RI), *Eco*RI* (RI*), *Sac*I (S), and *Eco*RV (RV) restriction sites are shown, as well as probes A and B. Probes A and B were used in experiments described in Results.

such as *Borrelia hermsii* (18), but rather is regulated at the level of transcription. The cloning of a putative *rpoD* gene from *B. burgdorferi* suggests that the mechanisms of transcriptional regulation in *B. burgdorferi* may be similar to mechanisms of transcriptional regulation of *E. coli* σ^{70} promoters (28). A recent survey of 132 *E. coli* σ^{70} promoters concluded that approximately 49% of the promoters are controlled by a single repressor, whereas 25% are controlled by a single activator (10). In addition, most promoters were found to be part of regulons in which they are coregulated by independent binding of multiple regulators. While there are currently no reported mechanisms of gene regulation for *B. burgdorferi*, one likely explanation for the increase in, or initiation of, synthesis of *p35* and *p7.5* is that a DNA binding protein is influencing their transcription. Recently, a sequence-specific DNA-binding activity, upstream of the OspAB operon, was reported for *B. burgdorferi* (17). Interestingly, there is an area of dyad symmetry located 52 bp upstream from the transcriptional start site of the *p35* gene. Such a sequence may be involved in the recruitment of a DNA binding protein(s) that facilitates transcription of the *p35* gene. In addition, the region upstream of *p35* contains a potential integration host factor (IHF) binding site (AAatAAGACCT TG; consensus IHF binding site, WATCAA-N4-TTR) (12) located 79 bp from the transcription start site. IHF is a multifunctional DNA binding and bending protein of *E. coli* that can mediate protein-protein and protein-DNA interactions. Recently, a gene was isolated from *B. burgdorferi* which encodes a protein that is homologous to members of the HU/IHF family of *E. coli* proteins (27a). Directly upstream of the IHF binding site is a stretch of AT-rich sequence. Such AT-rich elements are known to increase binding of IHF to its target sequence (12). In contrast to the case for the *p35* gene, the 5′ flanking region of *p7.5* does not contain any regions of dyad symmetry. The lack of common sequence elements in *p35* and *p7.5* suggests that the regulation of these genes has additional layers of complexity and is mediated not by a single element but perhaps by several elements. It is of interest that other lipoproteins of *B. burgdorferi*, such as OspA, are not regulated in vitro in a cell-density-dependent manner (21).

The nucleotide sequence of *p7.5* revealed that it codes for a putative 7.5-kDa lipoprotein. Our nucleotide sequence of *p7.5* is identical to the sequence in the database (GenBank accession number U59859) for *B. burgdorferi* B31, with the exception that our sequence contains an additional 73 bp upstream of the gene. This additional sequence is important because it contains the transcription start site and the putative promoter region of the *p7.5* gene (Fig. 3A). Interestingly, a translated DNA sequence (nucleotides 606 to 806) from the GenBank database, corresponding to the upstream flanking DNA region of the previously identified D6 protein of *Borrelia garinii* (strain VS102; GenBank accession number U50840), revealed significant identity (56%) to P7.5. Further inspection of the DNA sequence from *B. garinii* revealed the presence of a second ORF, downstream and on the opposing DNA strand from the

A

```
TTTTTTTTAT TTAGAACAAA AAACCAATTG ATATTGATTA TTTTAAGATT      50

ATATTTTAAT ATAATATCAG TTTTAAAGAA GGAGAATAAA CA ATG ACA      98
                                                  Met Thr

AAA TTA ATG TAC GCT ATA TTT TTG AGT GCA ATA TTA TTT GTT     140
Lys Leu Met Tyr Ala Ile Phe Leu Ser Ala Ile Leu Phe Val

GCT TGC GAA ACT ACA AGA ATT TCA GAT GAG ATG GAA AAT ACT     182
Ala Cys Glu Thr Thr Arg Ile Ser Asp Glu Met Glu Asn Thr

AGC GAT GAA GAT TCA AAA GTT ACA GCT CCA ATG ACA GAT AAA     224
Ser Asp Glu Asp Ser Lys Val Thr Ala Pro Met Thr Asp Lys

GAT ATG ATG AAG TCA ATG CCA GAC AAA AAT ACC AAA TCA ATG     266
Asp Met Met Lys Ser Met Pro Asp Lys Asn Thr Lys Ser Met

AAG CAA CCT ATG ACA AAG TCA ATG AAA AAG TAA TAATACTTGC     309
Lys Gln Pro Met Thr Lys Ser Met Lys Lys ***

TTGTAAATTA ATTTAAATAC AGTACTAATA ACTATTTATT ATTTTGATTT     359

AGAAAAATTA AATTAATATT AAAATTAAAT ATAAAAAAGG GGAAAGAGGT     409

TTTTAAACCC CTCTCCCCCC TT      431
```

B

```
CCGACTTCAC TTAGACACAT TATTTAAATT TAATAATAAT TAAAAAATAA      50

TTAAAAAATA AGACCTTGTA CCCAAAGAGA GCGCATGCTC TCTTTTTTTT      100

TTGAATTCTT CTTGTACAAT AACAATTTTG AATTAAAGTT ATTAATGGGG      150

TAATTAAAAT TATTAATTAA TATAAATTAC TTAAAATTTT TATTGAAGGG      200

TAACATTTTG      210
```

FIG. 3. (A) Nucleotide and deduced amino acid sequences of *p7.5* and flanking regions. The putative promoter sequence (double underlined), the Shine-Dalgarno sequence (underlined), and the stop codon (asterisks) are marked. The transcription start site is indicated by an arrow. A putative signal peptidase II cleavage site is boxed. The sequence of the primer used to determine the transcription start site for *p7.5* is designated under the long arrow. (B) Nucleotide sequence of the region upstream of the *p35* gene. The sequence has been extended to include the leucine start codon. An inverted repeat located at positions −53 to −74 is indicated by the converging arrows. The transcriptional start site is indicated by an arrow. The putative promoter sequence is double underlined. A 43-nt AT tract is boxed.

P7.5 homolog, which has significant identity (>75%) to P35. It appears that the linear order of the *p35*, *p7.5*, and *D6* genes is conserved in *B. garinii* and *B. burgdorferi* sensu stricto.

The putative P7.5 protein is unusually small compared to lipoproteins characterized thus far for *B. burgdorferi*. The amino terminus of P7.5 contains a hydrophobic leader, as well as a motif for a signal peptidase II cleavage site (LXXXC) (31). Serendipitously, a monoclonal antibody (MAb) that binds to a low-molecular-weight lipoprotein from *B. burgdorferi* (MAb 240, Biodesign Corporation, Kennebunkport, Maine)

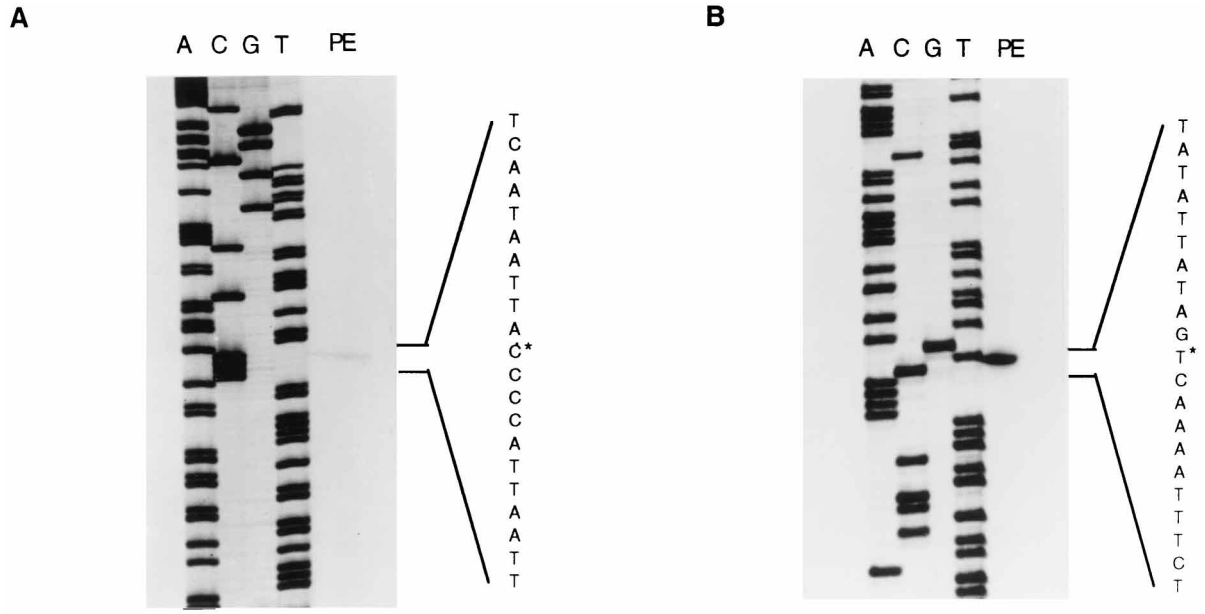

FIG. 4. Primer extension analysis of the *p35* (A) and *p7.5* (B) genes by reverse transcription of *B. burgdorferi* B31 RNA. The transcription start sites are indicated by the asterisks. The extension products (PE) were examined in parallel with a corresponding DNA sequencing ladder. See Materials and Methods for further details.

(14) reacted strongly with a 7.5-kDa protein in whole-cell lysates of an *E. coli* recombinant clone containing a plasmid which encodes both P35 and P7.5 (our unpublished data). This same MAb did not cross-react with any protein in wild-type *E. coli* lysates. These results indicate very strongly that the region downstream from the *p35* gene codes for the previously described 7.5-kDa lipoprotein (9). If so, the *p7.5* gene is, like *p35* (6), also likely to be expressed in vivo, because MAb 240 reacts with *B. burgdorferi* in tissue sections from infected rhesus monkeys (19, 23).

The induction of gene expression at the onset of stationary phase in prokaryotes is a well-documented phenomenon (16). In this context, it is instructive to consider how *B. burgdorferi* populations are maintained in their natural zoonotic cycle, as they undergo periods of active cell division interspersed with intervals of quiescence. For example, upon attachment of a flat nymph or larva to an appropriate host, spirochetes can grow with doubling times of close to 4 h, reaching a mean of 7,848 within 15 h of attachment (6). Tick survival, however, dictates that the increase in spirochete numbers must come to an end. In fact, spirochetal burdens have been found to stabilize at approximately 50,000 at day 75 to 135 in replete nymphs (20). Yet somehow the spirochete persists, confined to the midgut of the nymph until later in the year when the tick, now an adult, attaches to another host, thus completing the cycle of infection.

Because spirochete density has been shown to fluctuate in the tick, as illustrated above, we wondered if the pattern of cell-density-dependent expression of P35 (and P7.5) in the tick would mimic the pattern of expression observed in vitro. By applying reverse transcriptase PCR, using *p35*- and *p7.5*-specific primers to RNA isolated from *B. burgdorferi*-infected *I. scapularis* nymphs at different time points during the feeding and postfeeding periods, it should be possible to determine whether the correlation between the expression of P35 or P7.5 and the onset of stationary phase is also valid in vivo. If the correlation was maintained in the tick, then the observations that (i) serum from rhesus monkeys infected with *B. burgdorferi* react with purified recombinant P35 (our unpublished data) and (ii) anti-P7.5 MAb 240 reacts with *B. burgdorferi* present in the tissues of infected monkeys (19, 23) could be construed as evidence that during chronic infection, *B. burgdorferi* persists in stationary phase. Furthermore, if it was possible to demonstrate that the expression of *p35* and *p7.5* is regulated in a cell-density-dependent manner within the tick, it would be reasonable to infer that the regulatory mechanisms of this phenomenon that are at work in vitro and in vivo are similar. This similarity would be important in relation to several recent observations on the modulation of expression of lipoprotein genes in vivo. For example, the expression of OspC increases in the midgut of infected ticks after ingestion of a blood meal (26), whereas the expression of OspA, which is plentiful in the tick's midgut, is suppressed or downregulated when the spirochetes reach the tick's salivary glands, on the brink of infection (6, 7, 26). In addition, several *B. burgdorferi* genes which are expressed only upon infection of the vertebrate host have been described (1, 5, 27, 29). Modulation of surface lipoprotein expression, a likely adaptive strategy for the spirochete, is a phenomenon whose mechanisms are entirely unknown. We submit that the phenomenon of upregulation of P35 and P7.5 in response to changes in cell density could serve as the basis to develop an in vitro model in which the mechanisms of initiation or upregulation of *B. burgdorferi* lipoprotein gene expression could be easily investigated. As with all models, such a system could be an oversimplification of gene regulation as it occurs in a natural infection. Nonetheless, it should be much easier to handle experimentally than a system that requires in vivo cues to set off regulatory stimuli, for no large number of spirochetes can be made available ex vivo from infected arthropod or vertebrate tissues (3, 6). Thus, the difficulties of isolating, e.g., DNA binding proteins that are involved in gene regulation in vivo could be circumvented. In fact, in a preliminary attempt to dissect the mechanism(s) of upregulation of the *p35* gene at the onset of stationary phase, we identified a DNA-binding activity specific for the region immediately upstream of the *p35* gene (13).

ACKNOWLEDGMENTS

This work was supported by a grant from SmithKline Beecham Biologicals, by grants U50/CCU606604 from the Centers for Disease Control and Prevention and AI 35027 from the National Institutes of Health (all three to M.T.P.), and by NCRR-NIH grant RR00164.

The excellent secretarial help of Christie Trew and the photographic skill of Murphy Dowouis are acknowledged with thanks.

REFERENCES

1. **Akins, D. R., S. F. Porcella, T. G. Popova, D. Shevchenko, S. I. Baker, M. Li, M. V. Norgard, and J. D. Radolf.** 1995. Evidence for *in vivo* but not *in vitro* expression of a *Borrelia burgdorferi* outer surface protein F (OspF) homologue. Mol. Microbiol. **18:**507–520.
2. **Aydintug, M. K., Y. Gu, and M. T. Philipp.** 1994. *Borrelia burgdorferi* antigens that are targeted by antibody-dependent, complement-mediated killing in the rhesus monkey. Infect. Immun. **62:**4929–4937.
3. **Barthold, S.** 1996. Lyme borreliosis in the laboratory mouse. J. Spirochetal Tick-Borne Dis. **3:**22–44.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
5. **Champion, C. I., D. R. Blanco, J. T. Skare, D. A. Haake, M. Giladi, D. Foley, J. N. Miller, and M. A. Lovett.** 1994. A 9.0-kilobase-pair circular plasmid of *Borrelia burgdorferi* encodes an exported protein: evidence for expression only during infection. Infect. Immun. **62:**2653–2661.
6. **de Silva, A. M., and E. Fikrig.** 1995. Growth and migration of *Borrelia burgdorferi* in *Ixodes* ticks during blood feeding. Am. J. Trop. Med. Hyg. **53:**397–404.
7. **de Silva, A. M., S. R. Telford, L. R. Brunet, S. W. Barthold, and E. Fikrig.** 1996. *Borrelia burgdorferi* OspA is an arthropod-specific transmission-blocking Lyme disease vaccine. J. Exp. Med. **183:**271–275.
8. **Gabain, A., K. G. Belasco, J. L. Schottel, C. Y. Chang, and S. N. Cohen.** 1983. Decay of mRNA in *Escherichia coli*: investigation of the fate of specific transcripts. Proc. Natl. Acad. Sci. USA **80:**653–657.
9. **Gilmore, R. D., Jr., K. J. Kappel, and B. J. B. Johnson.** 1997. Molecular characterization of the 35-kilodalton protein of *Borrelia burgdorferi*, an antigen of diagnostic importance in early Lyme disease. J. Clin. Microbiol. **35:**86–91.
10. **Gralla, J. D., and J. Collado-Vides.** 1996. Organization and function of transcription regulatory elements, p. 1232–1245. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, D.C.
11. **Gu, Y., and M. T. Philipp.** Unpublished data.
12. **Hales, L. M., R. I. Gumport, and J. F. Gardner.** 1994. Determining the DNA sequence element required for binding integration host factor to two different target sites. J. Bacteriol. **176:**2999–3006.
13. **Indest, K. I., and M. T. Philipp.** Unpublished data.
14. **Katona, L. I., G. Beck, and G. S. Habicht.** 1992. Purification and immunological characterization of a major low-molecular-weight lipoprotein from *Borrelia burgdorferi*. Infect. Immun. **60:**4995–5003.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
16. **Loewen, P. C., and R. Hengge-Aronis.** 1994. The role of the sigma factor σ^s (KatF) in bacterial global regulation. Annu. Rev. Microbiol. **48:**53–80.
17. **Margolis, N., and D. S. Samuel.** 1995. Proteins binding to the promoter of the operon encoding the major surface proteins OspA and OspB of *Borrelia burgdorferi*. Mol. Biol. Rep. **21:**159–164.
18. **Meier, J. T., M. I. Simon, and A. G. Barbour.** 1985. Antigenic variation is associated with DNA rearrangements in a relapsing fever *Borrelia*. Cell **41:**403–409.
19. **Philipp, M. T., M. K. Ayintug, R. P. Bohm, F. B. Cogswell, V. A. Dennis, H. N. Lanners, R. C. Lowrie, E. D. Roberts, M. D. Conway, M. Karacorlu, G. A. Peymen, D. J. Gubler, B. J. Johnson, J. Piesman, and Y. Gu.** 1993. Early and early disseminated phases of Lyme disease in the rhesus monkey: a model for infection in humans. Infect. Immun. **61:**3047–3059.
20. **Piesman, J., J. R. Oliver, and R. J. Sinsky.** 1990. Growth kinetics of the Lyme disease spirochete (*Borrelia burgdorferi*) in vector ticks (*Ixodes dammini*). Am. J. Trop. Med. Hyg. **42:**352–357.
21. **Ramamoorthy, R., and M. T. Philipp.** Unpublished data.
22. **Ramamoorthy, R., L. Povinelli, and M. T. Philipp.** 1996. Molecular characterization, genomic arrangement, and expression of *bmpD*, a new member of the *bmp* class of genes encoding membrane proteins of *Borrelia burgdorferi*. Infect. Immun. **64:**1259–1264.
23. **Roberts, E. D., R. P. Bohm, F. B. Cogswell, H. N. Lanners, R. C. Lowrie, L. Povinelli, J. Piesman, and M. T. Philipp.** 1995. Chronic Lyme disease in the rhesus monkey. Lab. Invest. **72:**146–160.
24. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
26. **Schwan, T. G., J. Piesman, W. T. Golde, M. C. Dolan, and P. A. Rosa.** 1995. Induction of an outer surface protein of *Borrelia burgdorferi* during tick feeding. Proc. Natl. Acad. Sci. USA **92:**2909–2913.
27. **Suk, K., S. Das, W. Sun, B. Jwang, S. W. Barthold, R. A. Flavell, and E. Fikrig.** 1995. *Borrelia burgdorferi* genes selectively expressed in the infected host. Proc. Natl. Acad. Sci. USA **92:**4269–4273.

27a. **Tilly, K., J. Fuhram, J. Campbell, and D. Scott Samuels.** 1996. Isolation of *Borrelia burgdorferi* genes encoding homologues of DNA-binding protein HU and ribosomal protein S20. Microbiology **142:**2471–2479.

28. **Tsai, C., and M. Pan.** 1996. Sequence of a gene encoding a putative sigma factor from *Borrelia burgdorferi* strain B31. Gene **168:**123–124.
29. **Wallich, R., C. Brenner, M. D. Krames, and M. M. Simon.** 1995. Molecular cloning and immunological characterization of a novel linear-plasmid-encoded gene, *pG*, of *Borrelia burgdorferi* expression only in vivo. Infect. Immun. **63:**3327–3335.
30. **Wise, D. J., and T. L. Weaver.** 1991. Detection of the Lyme disease bacterium, *Borrelia burgdorferi*, by using polymerase chain reaction and a nonradioisotopic gene probe. J. Clin. Microbiol. **29:**1523–1526.
31. **Wu, H. C., and M. Tokunaga.** 1986. Biogenesis of lipoproteins in bacteria. Curr. Top. Microbiol. Immunol. **125:**127–157.

Editor: J. G. Cannon