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Molecular Characterization and Expression of p23 (OspC) from a North American Strain of *Borrelia burgdorferi*

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We have found that sera from patients with early stages of Lyme disease contain predominant immunoglobulin M reactivity to a major 23-kDa protein (p23) from *Borrelia burgdorferi* 2591 isolated in Connecticut. To characterize this immunodominant antigen, we cloned and sequenced *p23* and found it to be 83% identical by nucleotide sequence and 75% identical by amino acid sequence to pC (recently renamed OspC), an abundantly expressed protein on the outer surface of PKo, a European strain of *B. burgdorferi* (B. Wilske, V. Preac-Mursic, S. Jauris, A. Hofmann, I. Pradel, E. Soutschek, E. Schwab, G. Will, and G. Wanner, Infect. Immun. 61:2182–2191, 1993). In addition, immunoelectron microscopy localized p23 to the outer membrane, confirming that p23 is the strain 2591 homolog of OspC. The North American strain B31, commonly used in serologic assays for Lyme disease, does not express OspC. Northern (RNA) blot analysis detected low levels of *ospC* mRNA in B31, and DNA sequencing of the *ospC* gene from B31 revealed a 54-bp deletion in the upstream regulatory region, possibly accounting for the low transcriptional activity of *ospC*. The *ospC* coding region from B31 was cloned and antibody-reactive OspC was expressed in *Escherichia coli*. An immunoglobulin M enzyme-linked immunosorbent assay using recombinant OspC as the target antigen shows promise for the serodiagnosis of early stages of Lyme disease.

Lyme disease is a multisystem infection caused by the tick-borne spirochete, *Borrelia burgdorferi* (25). Because of the low yield by both culture and direct visualization techniques for identification of this organism, the diagnosis of Lyme disease has relied on serologic confirmation in patients with characteristic clinical findings. Accurate serodiagnosis, however, has been complicated by a delayed humoral response to the spirochete and by cross-reactions with proteins from other bacteria (13, 16). Arriving at a timely and accurate diagnosis of Lyme disease is clinically important as prompt and appropriate antibiotic treatment can prevent the potentially serious sequelae that affect the central nervous and musculoskeletal systems (7, 26).

In North America, immunoblot studies of sera from patients with early stages of the disease suggested that the first detectable humoral response to *B. burgdorferi* is an immunoglobulin M (IgM) antibody restricted primarily to the 41-kDa flagellar antigen (3, 6). Similar studies in Europe, however, have reported antibodies in sera during early stages of the disease to be predominantly directed to an approximately 20-kDa protein, which was named pC (very recently pC has been renamed outer surface protein C [OspC] to denote its expression on the outer membrane of the spirochete [30]).

At our institution we have found predominant IgM reactivity to a protein with a size of approximately 23 kDa, which we named p23, in sera which tested positive by enzyme-linked immunosorbent assay (ELISA) for IgM reactivity to *B. burgdorferi* (9). For both the immunoblot and ELISA, we used *B. burgdorferi* 2591, an isolate which expresses a major protein at approximately 23 kDa, as the source of antigen.

We now report the molecular characterization of p23 and show that it is the strain 2591 homolog of pC (OspC). We also describe a molecular defect in *B. burgdorferi* B31, a commonly distributed isolate used in North America for serodiagnosis, which may explain its lack of OspC expression and the generally unappreciated reactivity of sera from patients with early stages of Lyme disease with OspC in North America. We also report the use of recombinant OspC for the serodiagnosis of Lyme disease.

MATERIALS AND METHODS

***B. burgdorferi* strains and antigen preparation.** *B. burgdorferi* 2591 was obtained from L. Magnarelli, Department of Entomology, The Connecticut Agricultural Experiment Station, New Haven, Conn. It was initially isolated from a white-footed mouse caught in East Haddam, Conn.; *B. burgdorferi* B31 (type strain) was obtained from the American Type Culture Collection (Rockville, Md.) (ATCC 35210). The spirochetes were grown in BSK II medium in a closed flask at 33°C as previously described (2). After 10 to 14 days of growth, the organisms were washed three times in Dulbecco's phosphate-buffered saline (DPBS) (GIBCO, Grand Island, N.Y.) and sonicated on ice by a cell disruptor (model 185; Branson, Danbury, Conn.) with 10 15-s blasts at 60% of maximum power. The sonicate was cleared by centrifugation at 10,000 × *g* and 4°C for 20 min. The protein concentration in the supernatant was determined by the Bradford method (5).

Genomic DNA isolation. Washed spirochetes were suspended in SET buffer (25% sucrose, 50 mM Tris-HCl [pH 7.5], 5 mM Na_2EDTA) and lysed by adding sodium dodecyl sulfate (SDS, final concentration of 0.5%)–RNase A (0.1 mg/ml)–proteinase K (0.1 mg/ml) for 45 min at 37°C with gentle agitation. The DNA was extracted two times with buffered phenol and one time with phenol–chloroform-isoamyl alcohol (25:24:1) and ethanol precipitated.

PAGE and electroelution. The *B. burgdorferi* sonicate (40

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to 80 μg per well (0.8-mm thickness and 80-mm width) was mixed with an equal volume of sample buffer (0.125 M Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 2% 2-mercaptoethanol, 0.001% bromophenol blue), boiled for 5 min, and subjected to polyacrylamide gel electrophoresis (PAGE) in a discontinuous 0.1% SDS–12% polyacrylamide slab gel with buffers described by Laemmli (15). Molecular mass standards included myosin (200,000 Da), *Escherichia coli* β-galactosidase (116,250 Da), rabbit muscle phosphorylase *b* (97,400 Da), bovine serum albumin (BSA; 66,200 Da), hen egg white ovalbumin (45,000 Da), bovine carbonic anhydrase (29,000 Da), soybean trypsin inhibitor (21,500 Da), and hen egg white lysozyme (14,400 Da). The gels were stained and fixed with 0.25% Coomassie brilliant blue R in 50% methanol–10% acetic acid and destained with 40% methanol–10% acetic acid. For the preparative gel, 250 to 825 μg of protein was added to a well (1.5 mm by 140 mm). The band corresponding to p23 was visualized by precipitation with cold 0.1 M KCl and was cut from the remainder of the gel. The protein was isolated by electroelution and dialyzed successively against 0.02 M ammonium bicarbonate–0.1% SDS for 12 h and 0.1 M ammonium bicarbonate–0.02% SDS for 12 h. The protein concentration was determined by the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, Ill.).

Immunoblot analysis. Proteins separated by SDS-PAGE were transferred to nitrocellulose and incubated with sera or supernatants containing monoclonal antibody (MAb) by a modification of the method described by Towbin et al. (28). Transfer of the proteins to nitrocellulose (Bio-Rad Laboratories, Hercules, Calif.) was done in a Trans-Blot cell (Bio-Rad Laboratories) containing 192 mM glycine, 25 mM Tris base, and 20% methanol at 0.5 A for 1 h with cooling. The transferred proteins were visualized by staining the nitrocellulose membrane with 0.5% Ponceau S in 1% glacial acetic acid. Nonspecific binding to the blots was blocked by incubation for 1 h at 20°C in TBS (20 mM Tris-HCl [pH 7.5], 150 mM NaCl) with 1% BSA. The blots were washed three times with TBST (TBS with 0.05% Tween-20) and then incubated with patient's sera (1:100 in TBS–1% BSA) or hybridoma supernatant (1:5 in TBS–1% BSA) for 1 h at 20°C. After the blots were washed four times with TBST, they were incubated with goat anti-mouse IgM and IgG (heavy and light chains) conjugated to alkaline phosphatase (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) or goat anti-human γ chain and anti-human μ chain conjugated to peroxidase (Sigma, St. Louis, Mo.). The blots were then washed four times with TBST, and substrate (nitroblue tetrazolium–BCIP [5-bromo-4-chloro-3-indolylphosphate toluidinium] for the alkaline phosphatase conjugate or 3,3′-diaminobenzidine–hydrogen peroxide for the peroxidase conjugate) was added.

Partial amino acid sequence determination of p23. Trypsin digestion was performed as previously described (22). Briefly, the electroeluted protein was lyophilized, resuspended in 20% trichloroacetic acid, and precipitated at 4°C for 12 h. After centrifugation, the pellet was resuspended in 0.5 ml of cold 0.2% HCl in acetone and incubated at −20°C for several days. After two washes with cold acetone, the protein (approximately 25 μg) was dried at 20°C and digested with 0.25 nmol of trypsin (Worthington, Freehold, N.J.) in 2.3 M urea–0.1 M Tris-HCl [pH 8.06]–0.2 M ammonium bicarbonate for a maximum of 24 h at 20°C. The peptide mixture was resolved by reverse-phase high-performance liquid chromatography (HPLC) (23). The sequence analysis of the peptides was carried out on an Applied Biosystems model 470A gas-phase sequencer equipped with a model 120A PTH analyzer according to instructions from the manufacturer.

Isolation of the gene encoding p23. Degenerate oligonucleotide primers were synthesized on the basis of the amino acid sequence of two trypsin-digested peptide fragments. The polymerase chain reaction (PCR) was used to amplify the intervening segment of DNA between the two primers: upstream primer, 5′-GT(AT) AAG GAG GT(AT) GA(AG) AC-3′, and downstream primer, 5′-CC GTT (TC)TG (AG)TT (GATC)GC (GATC)CC-3′. Amplification was performed in a volume of 100 μl in a thermal controller (MJ Research, Watertown, Mass.) under the following conditions: 94°C for 5 min, 40°C for 1 min, 72°C for 1 min, 94°C for 1 min, 39°C for 1 min, 72°C for 1 min; 94°C for 1 min, 38°C for 1 min, 72°C for 1 min for 30 cycles; and 72°C for 5 min for extension. Each primer was used at a final concentration of 0.5 μM, and 50 ng of genomic DNA was used as the template. The amplification buffer included 50 mM KCl, 20 mM Tris-HCl (pH 8.4) (at 25°C), 2 mM $MgCl_2$, 0.1 mg of BSA per ml, 0.125 nM (each) deoxynucleoside triphosphate, and 2.5 U of *Taq* DNA polymerase. The amplified DNA was radiolabeled by the random primer technique and used to probe a Southern blot of genomic *B. burgdorferi* DNA separately restricted with eight different restriction enzymes. Genomic DNA was cut with the appropriate restriction enzyme, and fragments with corresponding sizes were isolated from low-melt agarose and cloned into pBS (Stratagene, La Jolla, Calif.) and transformed into DH5α (Bethesda Research Laboratories, Gaithersburg, Md.). The radiolabeled PCR-amplified fragment was used to probe the selected library by colony hybridization, and positive colonies were grown. The cloned insert was sequenced in both orientations by dideoxy chain termination with Sequenase version 2.0 (U.S. Biochemicals, Cleveland, Ohio).

Northern (RNA) blot analysis and transcriptional start site. Total cellular RNA was obtained from the spirochetes in the presence of diethylpyrocarbonate as previously described (27). RNA (15 μg per lane) was electrophoresed in a 0.66 M formaldehyde-MOPS (morpholinepropanesulfonic acid)–1% agarose denaturing gel, transferred to a nylon membrane (Nytran; Schleicher & Schuell, Keene, N.H.), and hybridized to a synthetic 17-mer oligonucleotide (5′-CTTTCCCT GAATTATTA-3′), complementary to a sequence which is identical in strains 2591 and B31. The oligonucleotide was 3′ labeled with digoxigenin, and prehybridization and hybridization were performed at 42°C as recommended by the manufacturer (Genius System; Boehringer Mannheim Corp., Indianapolis, Ind.). The membrane was washed in 6× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate)–0.05% PP_i twice at 20°C for 5 min and twice at 42°C for 15 min. Immunodetection of the oligonucleotide with an alkaline phosphatase-conjugated antidigoxigenin antibody and visualization by chemiluminescence were performed according to the manufacturer's recommendations.

The transcriptional start site for the *p23* gene was determined by primer extension analysis as previously described (14, 21). The 17-mer primer used in the Northern blot analysis was 5′ labeled with [γ-^{32}P]ATP (Amersham, Arlington Heights, Ill.) and T4 polynucleotide kinase (Bethesda Research Laboratories) and separated from unincorporated label with a G25 spin column (Select-D; 5Prime→3Prime, Boulder, Colo.). Three pmoles of the labeled primer was mixed with 15 μg of RNA in 3 μl of hybridization buffer (100 mM KCl, 50 mM Tris-HCl [pH 8.3]), heated to 90°C for 5 min, annealed at 42°C for 10 min, and placed on ice for 15

min. One microliter of 5× reverse transcriptase buffer (250 mM Tris-HCl [pH 8.3], 200 mM KCl, 15 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM (each) deoxynucleoside triphosphate) and 1 μl of RNase H^- reverse transcriptase (Superscript; Bethesda Research Laboratories) were added to the annealed reaction and incubated at 42°C for 1 h. Five microliters of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) was added, and half of the volume was loaded onto a 6% polyacrylamide sequencing gel. The sizes of the extended products were determined by comparison with a DNA sequencing ladder obtained with the 17-mer oligonucleotide primer and a plasmid containing the *p23* gene.

Expression of p23 as a fusion protein. Genomic DNA was used as template for PCR amplification of p23 with primers based on the sequenced DNA. The product was cloned into the *SmaI* site of the expression vector pGEX-2T (Pharmacia-LKB, Piscataway, N.J.) for expression as a fusion protein with glutathione *S*-transferase at the amino terminus to facilitate affinity purification. The cloned gene was sequenced to confirm that it had been inserted in the appropriate reading frame. Colonies were grown overnight in 2 ml of superbroth (32 g of tryptone, 20 g of yeast extract, 5 g of NaCl, 5 ml of NaOH per liter), on the next day isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma) was added to 0.1 mM, and the culture was grown for an additional 2 h. The cells were pelleted, resuspended in cold DPBS, and sonicated. The supernatant was cleared by centrifugation, and 50 μl of 50% (wt/vol) glutathione-agarose beads (Sigma) was added to the supernatant and mixed gently at 20°C for 10 min. The beads were washed three times with DPBS, resuspended in SDS-PAGE sample loading buffer, and run in an SDS–12% PAGE.

Use of recombinant p23 in an ELISA. Large-scale preparation of the p23 fusion protein was performed as described above, with the additional step of elution of the protein from the beads with 5 mM reduced glutathione (Sigma) in 50 mM Tris-HCl (pH 8.0). Sixty microliters of the fusion protein (5 μg/ml) in DPBS was added to alternate wells of a flat-bottom microdilution plate (Nunc-Immunoplate; Marsh Biomedical Products, Rochester, N.Y.) for 12 h at 4°C. An equimolar amount of the carrier protein in DPBS was added as a control antigen to the remaining wells. The plates were blocked for 1 h at 37°C with 200 μl of DPBS containing 0.05% horse serum and 0.01% dextran sulfate. The plates were washed six times with DPBS with 0.05% Tween-20 (DPBST). Patients' sera were serially diluted twofold from 1:20 to 1:1,280 in DPBST. Positive and negative control sera were included on each plate. After the addition of sera, the plates were incubated for 1 h at 37°C and then washed six times in DPBST. The secondary antibody used was goat anti-human IgM (μ chain specific) conjugated to peroxidase (Sigma) diluted in DPBST. For screening the hybridoma supernatants for MAbs the secondary antibody used was goat $F(ab')_2$ anti-mouse IgG and IgM conjugated to peroxidase (Tago, Burlingame, Calif.). Sixty microliters of chromogen substrate [equal volumes of 2,2'-anzino-di-(3-ethylbenzthiazoline sulfonate) and hydrogen peroxide; Kirkegaard and Perry] was added to each well. The plates were checked spectrophotometrically at 414 nm until the optical density reading of the 1:160 dilution of the positive control on the fusion protein-containing wells minus the background on the carrier protein-containing wells was equal to 0.5. The plates were then read immediately. A serum dilution was considered positive if the net absorbance (fusion protein well minus carrier protein well) was 3 standard deviations or more above the mean absorbance of the negative serum wells.

MAb to p23. A MAb was produced by fusion of splenic cells from an immunized female BALB/c mouse (4 to 8 weeks old) to NSO/1. The mouse was initially immunized with 200 μg of the fusion protein in DPBS emulsified in an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) supplemented with 5 mg of desiccated *Mycobacterium tuberculosis* (Difco) per ml. The mouse received five booster injections of 100 μg of fusion protein in DPBS intraperitoneally every 2 weeks. Three days after the final injection, the spleen was harvested, and a single-cell suspension of splenocytes was obtained over a fine-mesh stainless-steel screen. Hybridomas were obtained essentially as previously described (8). The fusion was performed with 50% (wt/vol) polyethylene glycol 1500 (Boehringer Mannheim) in 75 mM HEPES (*N*-hydroxylethylpiperazine-*N'*-2-ethanesulfonic acid [pH 8.01]) at a spleen-myeloma cell ratio of 5:1. The cells were initially plated out at 5×10^4 myeloma cells per well of a 96-well flat-bottom cluster tray (Costar, Cambridge, Mass.). Hybridomas were selected by growth for 14 days in complete medium (Dulbecco's modified Eagle medium with 4,500 mg of D-glucose per liter [GIBCO] per liter, 2 mM L-glutamine, 100 U of penicillin G per ml, 100 μg of streptomycin per ml, 10% NCTC 109, 5×10^{-5} M 2-mercaptoethanol, and 10 mM HEPES) supplemented with 20% (vol/vol) of fetal calf serum (GIBCO) and HAT (10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, 1.6×10^{-5} M thymidine; Sigma). Subsequently, the cells were grown in complete medium with 20% fetal calf serum and HT (HAT medium without aminopterin; Sigma) for 7 days and then in complete medium with 20% fetal calf serum. Supernatants from wells containing growing hybridomas were screened for selective reactivity with the fusion protein and not with the carrier protein in an ELISA. Hybridomas from antibody-positive wells were cloned twice by limiting dilution in 96-well trays with BALB/c thymus cells (one thymus per 60 wells) as a feeder layer.

Immunoelectron microscopy. Spirochetes were removed from BSK II medium by centrifugation at 7,000 × *g* and 20°C for 20 min and washed two times by centrifugation at 7,000 × *g* and 10°C for 20 min in Hank's balanced salt solution (Bio-Whitaker, Walkersville, Md.). Following the second wash, spirochete pellets were fixed for 30 min in 4% formaldehyde (Elecron Microscopy Sciences, Ft. Washington, Pa.) in 0.1 M sodium cacodylate buffer (pH 7.4) to stabilize their outer membranes. Fixed pellets were washed four times in Ca^{2+}-Mg^{2+}-free PBS and sequentially incubated in 0.1 M glycine (pH 7.4)–PBS containing 1% BSA (PBS-BSA) for 30 min each. Spirochete pellets were incubated with supernatant containing MAb 4D7F5, a control isotype-matched mouse MAb, or PBS-BSA alone for 2 h at 20°C. Following three 5-min washes with PBS, spirochete pellets were incubated with goat anti-mouse IgG–10-nm-diameter gold conjugate (Amersham Life Sciences), diluted 1:20 in PBS-BSA, for 60 min at 20°C. Pellets were washed three times for 5 min with PBS and postfixed with 2.5% glutaraldehyde (Electron Microscopy Sciences) in 0.1 M sodium cacodylate buffer (pH 7.4). The pellets were osmicated, stained en bloc with uranyl acetate, dehydrated, and embedded in Spurr's resin. Thin sections were cut, stained with uranyl acetate-lead citrate, and viewed with a Philips CM 10 transmission electron microscope.

Nucleotide sequence accession numbers. The *ospC* sequences from *B. burgdorferi* 2591 and B31 have been as-

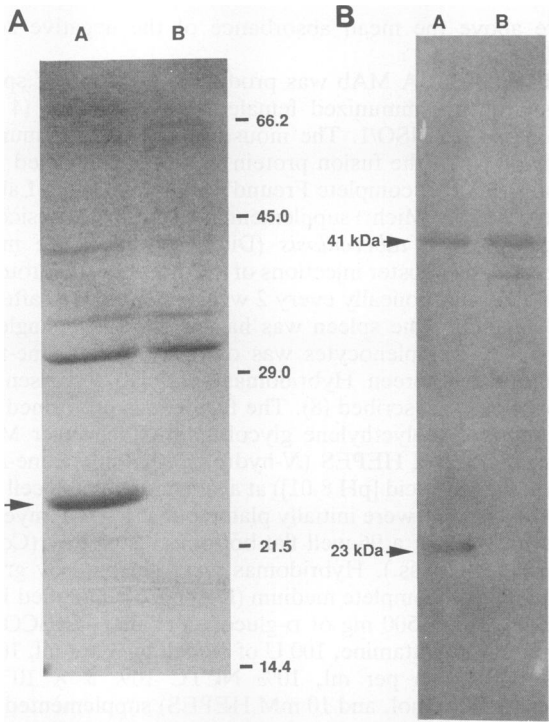

FIG. 1. Coomassie blue-stained polyacrylamide gel (A) and immunoblot (B) of sonicated *B. burgdorferi* 2591 (lane A) and B31 (lane B). In panel A, the arrowhead indicates a protein band corresponding to p23. Molecular mass (in kilodaltons) markers are shown on the right. In panel B, immunoblot reacted with serum from a patient with early Lyme disease. The secondary antibody is goat anti-human IgM. Arrowheads designate bands corresponding to the 41-kDa flagellar protein and the 23-kDa (p23) protein.

signed GenBank accession numbers U01892 and U01894, respectively.

RESULTS

Serologic reactivity with p23. Immunoblot analysis was performed on randomly selected sera from 79 patients who tested positive by ELISA for IgM reactivity to *B. burgdorferi*. The Connecticut strain 2591 was used as the source of antigen for both the ELISA and immunoblot studies. The bands most commonly seen on IgM immunoblot corresponded to proteins with molecular masses of 23, 41, 60, 66, and 31 kDa. The 23-kDa band was present in 98.7% (78 of 79) of the immunoblots. In 15% (12 of 79) of the specimens, the 23-kDa band was found in conjunction with either the 60- or 66-kDa band. The 23- and 41-kDa bands occurred simultaneously in 82% (65 of 79) of the serum samples. The 41-kDa band was never present without the 23-kDa band.

Molecular characterization of p23. Analysis of the strain 2591 sonicate by SDS-PAGE stained with Coomassie blue demonstrated the expression of an abundant protein (p23) with a mobility corresponding to a molecular mass of approximately 23 kDa (Fig. 1A, lane A). Examination of the protein pattern from *B. burgdorferi* B31 isolated in North America demonstrated the lack of a detectable 23-kDa protein (Fig. 1A, lane B). Both strains had been grown under the same conditions. An immunoblot of these electrophoretic patterns with serum from a patient with an early stage of Lyme disease demonstrated strong IgM reactivity to the 41-kDa flagellar antigen and relatively weak reactivity to the 37-, 39-, and 75-kDa antigens from both strains (Fig. 1B). However, only with the strain 2591 sonicate was there detectable IgM reactivity with the 23-kDa protein (Fig. 1B, lane A). No additional antigen reactivity was detected in the lane containing the sonicate of strain B31.

An initial attempt to obtain a partial amino acid sequence of p23 by microsequencing of the protein after transfer to a polyvinylidenedifluoride membrane (19) revealed a blocked amino terminus. Therefore, p23 was purified from a preparative SDS-PAGE by electroelution for cleavage to allow isolation of internal fragments of the protein for sequencing. An initial attempt at chemical fragmentation with cyanogen bromide did not indicate a significant change in p23 mobility on SDS-PAGE. Consequently, the protein was digested with trypsin, and two resultant peptide fragments were isolated by reverse-phase HPLC and sequenced (the sequences of the two peptide fragments, p1 and p2, are shown in Fig. 2). Comparison of the sequences of the two peptide fragments with published sequences of *B. burgdorferi* genes revealed that p23 was homologous to the pC protein isolated from the European *B. burgdorferi* PKo (11). This homology permitted the synthesis of a pair of degenerate oligonucleotides in the appropriate orientation for amplification by PCR of a 104-bp product from strain 2591 genomic DNA. This product was radiolabeled and used as a probe for cloning a 3-kb *Pst*I fragment. DNA sequence analysis of the 3-kb *Pst*I fragment identified an open reading frame encoding a 212-amino-acid protein with an estimated molecular mass of 22,250 Da (Fig. 2). An identical match between the predicted amino acid sequence of this gene and the sequence of both peptides confirmed this sequence to be the gene coding for p23. Comparison of p23 from strain 2591 with pC from strain PKo revealed 83% identity by nucleotide sequence and 75% identity by amino acid sequence (Fig. 3). These results strongly suggested that p23 is a homologous protein to pC first isolated in Europe, with the differences between the two proteins representing interstrain divergence.

An additional 185-bp region upstream of the AUG translation start codon of the p23 open reading frame was also sequenced (Fig. 2). The analysis of this region revealed a putative promoter containing consensus −35 and −10 hexamer sequences from *E. coli* (20) separated by 17 bp, as well as a consensus ribosomal binding site sequence (12) 9 bp upstream of the start codon.

Transcriptional start site. To confirm whether the identified putative promoter sequence was the active site for transcription of *p23*, primer extension analysis was performed to ascertain the transcriptional start site (Fig. 4). We used a 17-mer primer and RNA from strain 2591 and mapped the major *p23* transcript start site 20 bp upstream of the AUG translation start codon and 7 bp downstream from the −10 hexamer (Fig. 2). Longer exposure of the autoradiogram did not identify the presence of any longer transcript, suggesting there were no additional upstream promoters significantly contributing to the transcriptional activity of the *p23* gene.

Expression of the p23 recombinant protein. On the basis of our knowledge of the complete coding sequence of p23, oligonucleotide primers were synthesized to amplify *p23* by PCR from strain 2591 genomic DNA for expression in recombinant form. To avoid potential difficulty during isolation of the expressed protein, primers were selected so that the 20 amino acid residues composing the leader peptide were deleted from the final recombinant product (4, 11). The amplified p23-encoding product was inserted in frame with the carrier protein of the expression vector pGEX-2T. After induction with IPTG, an approximately 46-kDa fusion pro-

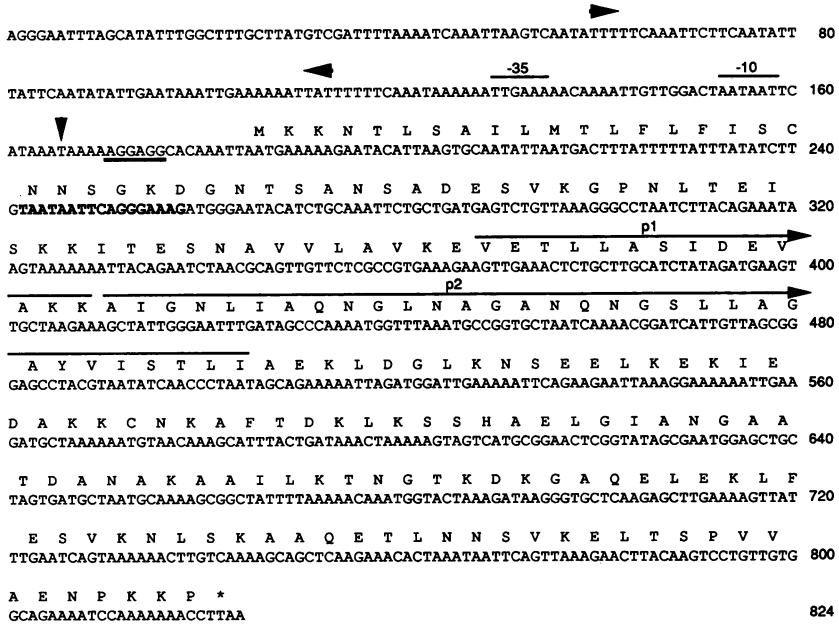

FIG. 2. Nucleotide sequence of *p23* gene from strain 2591. The deduced amino acid sequence in single-letter designation is also shown. The sequences of the two tryptic peptide fragments obtained from 2591 (p1 and p2) are indicated by labeled arrows above the sequences. Consensus −10 and −35 promoter hexamers from *E. coli* and the consensus ribosomal binding site (heavy underline) are shown. The downward-pointing arrowhead designates the transcriptional start site as determined by primer extension analysis. The sequence between the horizontal arrowheads (nucleotide positions 60 to 113) designates the deletion in strain B31. The sequence complementary to the 17-mer oligonucleotide used for primer extension and Northern blot analysis is shown in boldface print (nucleotide positions 242 to 258). The stop codon (TAA) is marked by an asterisk.

tein was obtained. The fusion protein was purified from the *E. coli* lysate by use of glutathione-agarose beads, which bind to the carrier protein. To confirm the identity of immunologic reactivity of native p23 and recombinant p23, patient serum with strong IgM reactivity to p23 from a sonicate on an immunoblot was adsorbed with the fusion protein attached to glutathione beads. For controls, the serum was also adsorbed with glutathione beads alone and beads with only the carrier protein attached. As shown in Fig. 5, only adsorption with beads attached to the fusion protein removed completely the serum's reactivity to p23 on an immunoblot. Adsorption did not affect the serum's reactivity to the other proteins on the blot.

MAb against p23. Splenocytes from a mouse immunized with the p23-containing fusion protein was used to obtain hybridomas producing MAb to p23. Hybridoma supernatants were screened by ELISA for selective reactivity to the fusion protein and lack of binding to the carrier protein. Antibody from several of the hybridoma clones demonstrated particularly strong binding to both the fusion protein and native p23 on immunoblot. To determine the localization of p23, supernatant from one of these clones, 4D7F5, was

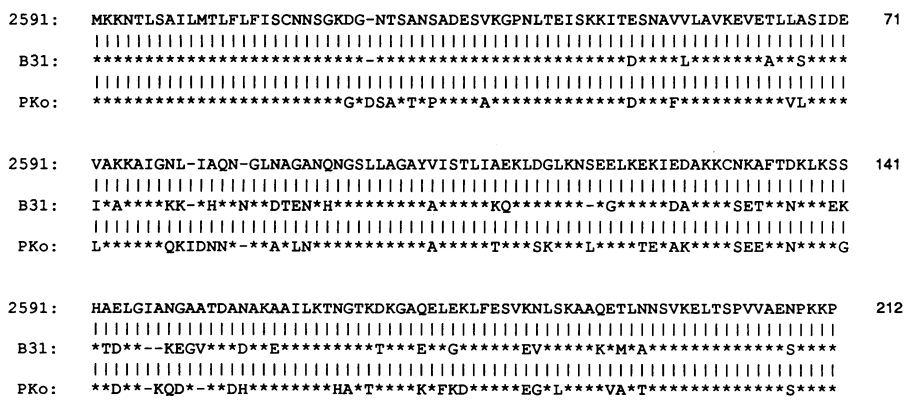

FIG. 3. Comparison of the amino acid sequences of OspC (p23 and pC) from three different *B. burgdorferi* isolates. Single-letter representation of the deduced amino acid sequence of OspC is based on sequencing of genomic DNA from strains 2591 (this paper), B31 (this paper), and PKo (10). The identity of the amino acid sequence in B31 and PKo with 2591 is indicated by an asterisk below the designated position. Amino acid substitutions are represented by the placement of an appropriate letter below the position. Spaces (−) have been included to optimize the match among the proteins.

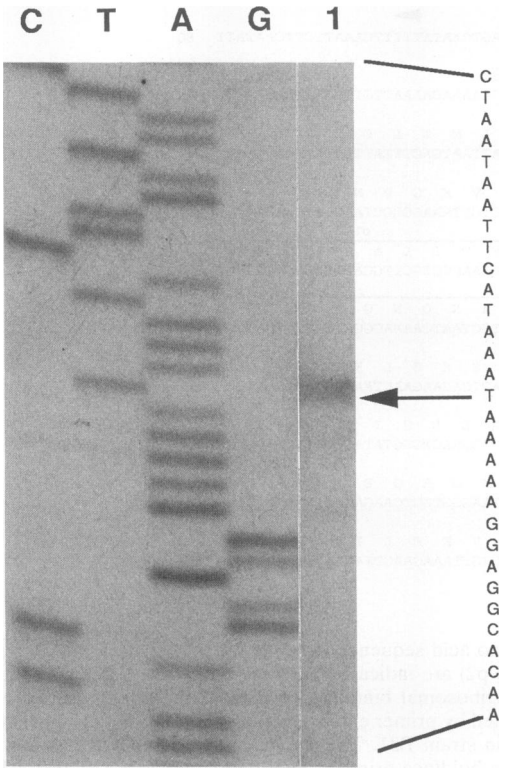

FIG. 4. Determination of the 5′ end of *p23* mRNA from *B. burgdorferi* 2591 by primer extension analysis. The size of the reverse transcriptase primer extension product of *p23* mRNA was compared to a DNA sequencing ladder of *p23* from strain 2591. The analysis was performed as described in Materials and Methods. Lanes C, T, A, and G represent DNA sequencing reactions with the appropriate dideoxynucleotide triphosphate by using the same 17-mer oligonucleotide primer used for primer extension of mRNA. Lane 1 shows the primer extension product. The sequence to the right of lane 1 is the sequence in the region of the 5′ end of p23 mRNA, and the arrow indicates the residue at the 5′ end of the extended product.

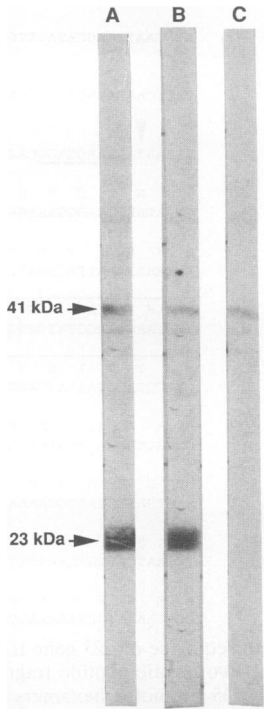

FIG. 5. Removal of IgM anti-p23 reactivity from serum by adsorption with recombinant p23. The IgM immunoblot of sonicate of *B. burgdorferi* 2591 with patient serum after adsorption on glutathione beads alone (lane A) revealed strong reactivity with native p23. Lane B shows reactivity of serum after adsorption on carrier protein attached to glutathione beads. Lane C shows complete removal of serum's reactivity after adsorption with p23 fusion protein attached to glutathione beads. Reactivity with other proteins including the 41-kDa flagellar protein was not affected.

selected for use in immunoelectron microscopy of strain 2591. As shown in Fig. 6, p23 was found to be expressed on the outer surface of the spirochete. A control isotype-matched antibody did not bind (data not shown). In addition, MAb 4D7F5 did not label strain B31, confirming the lack of expression of p23 by this strain (data not shown).

Molecular defect in p23 gene from strain B31. The lack of reactivity of the sera and the MAbs to both the B31 sonicate and whole organism led us to investigate the molecular reason for the lack of expression of p23 in this strain. Northern blot analysis for *p23* mRNA revealed the strong expression of an approximately 700-bp transcript from strain 2591 and a very weak signal of the same size from strain B31 (Fig. 7). Examination of the RNA stained with ethidium bromide had revealed that an equivalent amount of RNA from each strain was loaded in the gel and that there was no appreciable degradation of the RNA. In addition, primer extension analysis also suggested a significantly reduced level of *p23* mRNA in strain B31 (data not shown). Also, the size of the *p23* primer extension termination product from strain B31 was found to be 1 base longer than the transcript from strain 2591 (data not shown). To gain a better understanding of the reason for the relative lack of p23 expression by strain B31, we proceeded to clone and sequence the *p23* gene from strain B31 in the same fashion as we had done previously for strain 2591. Comparison of the deduced amino acid sequences of p23 from strains 2591 and B31 as well as pC from PKo is shown in Fig. 3. p23 from strain B31 was found to contain 210 amino acids, 2 less than the other two homologs, and shares 80% amino acid sequence identity and 85% nucleotide sequence identity with p23 from strain 2591. Comparison of the proteins from the three strains showed conservation of sequence at the 5′ (mostly leader peptide sequence) and 3′ ends of the protein, with increased variability in the central region.

Analysis of the B31 DNA sequence 5′ to the coding region of p23 revealed a 54-bp deletion upstream to the consensus −10 and −35 promoter sequences found to be the active promoter region in strains 2591 and B31. The finding of this deletion in close proximity to the functional promoter region suggested the loss of an enhancing element which results in low transcriptional activity of *p23* and lack of expression of the p23 protein in strain B31.

To confirm that the *p23* gene from B31 could encode antigenic protein, the coding region of *p23* was cloned into an expression vector and expressed in *E. coli* as a fusion protein. The recombinant p23 protein derived from strain B31 was as strongly reactive with sera and the MAbs as the native and recombinant forms of p23 from strain 2591 (data not shown).

Use of recombinant p23 for testing of sera. On the basis of

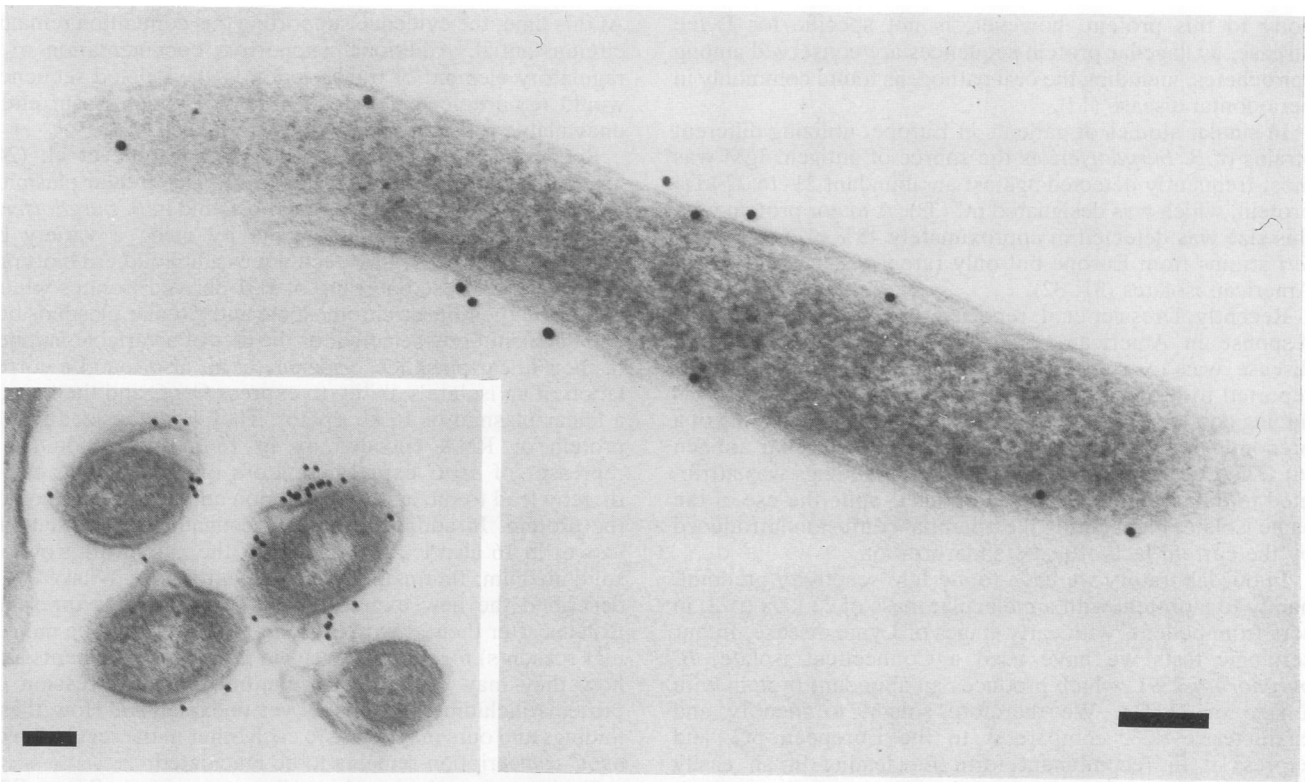

FIG. 6. Immunoelectron micrograph of thin-sectioned *B. burgdorferi* 2591 labeled with 4D7F5, an MAb with reactivity to p23. Localization of the MAb was detected with goat anti-mouse IgG conjugated to 10-nm-diameter gold beads. The immunogold is seen along the outer surface (see insert of cross-section view). Bar, 0.1 μm.

our observation that IgM reactivity to p23 may be a significant serologic marker for early stages of Lyme disease, we proceeded to test the feasibility of using recombinant p23 in an ELISA. We initially tested sera from 15 patients with clinically suspected Lyme disease and positive IgM immunoblots with a minimum of three positive bands including reactivity to p23. All 15 of these serum samples were strongly positive by the recombinant p23 ELISA. Sera from five patients with syphilis, 10 patients with high-titered rheumatoid factor, 5 patients with Epstein-Barr virus infection, and 10 patients with high-titer antinuclear antibodies all tested negative with this assay. All of these sera also tested negative when examined by immunoblot with a sonicate from strain 2591.

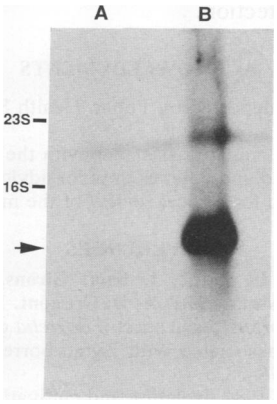

FIG. 7. Northern blot analysis of *p23* transcript. Total cellular RNA (15 μg per lane) was isolated from *B. burgdorferi* B31 (lane A) and 2591 (lane B), fractionated in a formaldehyde denaturing gel, transferred to a nylon membrane, and hybridized to a 17-mer oligonucleotide based on the sequence of p23. The arrowhead designates the *p23* transcript. The positions of 23S and 16S rRNA are indicated.

DISCUSSION

Establishing the diagnosis of Lyme disease continues to rely on serologic confirmation of exposure to the causative agent, *B. burgdorferi*, in the setting of characteristic clinical findings. Recognizing Lyme disease in the early stages can be difficult, however, because these patients may not manifest the characteristic rash or may have only nonspecific flu-like symptoms (25). This difficulty of diagnosis is compounded by the delayed emergence of a humoral response to the spirochete as detected by available serologic tests (25). These tests, which currently lack standardization, also do not readily distinguish between reactivity to *B. burgdorferi* proteins and cross-reactive proteins from commensual or other pathogenic organisms (13, 16). Delay in establishing the diagnosis of Lyme disease in its early stages is clinically important because timely institution of appropriate antibiotic treatment can prevent the serious sequelae from this potentially chronic infection (7, 26).

Initial studies with immunoblot analysis of patients from North America with the early manifestations of Lyme disease found IgM reactivity predominantly against the 41-kDa flagellar protein of *B. burgdorferi* (3, 6). Detection of anti-

body to this protein, however, is not specific for Lyme disease, as flagellar protein sequences are conserved among spirochetes, including the oral pathogens found commonly in periodontal disease (17).

In similar studies of patients in Europe, utilizing different strains of *B. burgdorferi* as the source of antigen, IgM was most frequently detected against an abundant 21- to 22-kDa protein, which was designated pC (32). A major protein with this size was detected in approximately 45% of *B. burgdorferi* strains from Europe but only rarely observed in North American isolates (31, 32).

Recently, Dressler et al. reported the most prominent IgM response in American patients with early stages of the disease was to a 21-kDa protein (10). This protein was reported to be reactive with an MAb specific for pC. This finding contrasts with this same group's previous finding of a predominant early response to the 41-kDa flagellar antigen (6). The discrepancy between these two findings was attributed to different antigen preparations despite the use of the same isolate, highlighting the potential confusion introduced by the current lack of test standardization.

In our laboratory we have found IgM reactivity predominantly to a protein with a molecular mass of 23 kDa (p23) in sera from patients with early stages of Lyme disease. In our serologic tests we have used a Connecticut isolate, *B. burgdorferi* 2591, which produces an abundant protein with a size of 23 Da. We therefore sought to identify and characterize p23, compare it to the European pC, and express it in recombinant form for testing in an easily standardized assay for the detection of a serologic response in early stages of Lyme disease.

We cloned and sequenced the *p23* gene from strain 2591 and predicted the protein to contain 212 amino acids with a molecular mass of 22,250 kDa. Comparison of the nucleic acid and predicted amino acid sequences of p23 with those of pC from the European isolate PKo revealed a high degree of homology (11). Furthermore, as recently reported for pC, we have localized p23 to the outer surface of 2591 by immunoelectron microscopy (30). Thus, p23 and pC are homologs of the same outer surface protein, OspC.

During the course of these studies, we confirmed the lack of expression of OspC by strain B31, an isolate which has been commonly used as the source of antigen for commercially available serologic assays in North America. Strain B31, as well as strain 2591, belongs to genospecies I, *B. burgdorferi* sensu stricto, whereas strain PKo belongs to genospecies III, group VS461 (1, 29, and unpublished results). We could not detect expression of OspC in a B31 sonicate by SDS-PAGE and immunoblot and on the whole organism by immunoelectron microscopy. We did confirm however that the *ospC* gene is present in B31 and identified a deletion of 54 bp in the 5′ noncoding region of the gene located just upstream of −35 and −10 consensus *E. coli* promoter sequences. Northern blot analysis of B31 revealed a low level of *ospC* mRNA, and primer extension studies showed a transcription start site for *ospC* 1 base upstream of that found for strain 2591. Recently, Marconi et al. have reported that *ospC* transcription in some strains may be under the control of one or more promoters in addition to the major functional promoter that we have described in this paper (18). We could not detect in strains 2591 and B31, however, any evidence of longer primer extension termination products by overexposure of the autoradiographs.

We propose that the deleted 54-bp segment contains an enhancer element(s) required for increased *ospC* transcriptional activity and detectable levels of expression of OspC. At this time, the evidence supporting this contention remains circumstantial. Additional supportive documentation of a regulatory element of transcription in the deleted sequence would require gene transfer technology which is currently unavailable for *Borrelia* species.

Recently, Marconi et al. (18) and Sadziene et al. (24) localized the *ospC* gene to a 26- to 27-kDa circular plasmid, the first gene mapped to a circular plasmid in *B. burgdorferi*. Marconi et al. mapped the gene by using a variety of electrophoretic separation techniques and Southern blotting. Sadziene et al. used a group of B31-derived isolates which contained their linear chromosome and circular plasmids but had been antibody selected for the loss of a variable number of their linear plasmids. Sadziene et al. also found a correlation of an isolate's ability to express OspC and the loss of a linear plasmid of 16 kb (lp 16). They hypothesized that a protein or RNA encoded by lp 16 may function as a repressor of *ospC* expression. Loss of this plasmid could thereby lead to the loss of repression and new expression of the protein. In addition, these investigators noted that the loss of lp 16 also led to a failure of the mutant to grow on solid medium. Interestingly, in a recent report, Wilske et al. described the new expression of OspC by nonexpressing isolates after their growth on solid medium (30). The molecular reason(s) for these changes in growth requirements and how they may affect the regulation of the expression of proteins including OspC is as yet unexplained. How these findings and ours may relate to each other in the regulation of *ospC* transcription remains to be elucidated.

To determine whether the coding region of *ospC* from B31 is capable of encoding an immunologically detectable protein, we expressed recombinant OspC from B31 in *E. coli*. We found that patients' sera and MAbs reactive with p23 (OspC) from strain 2591 were strongly reactive with recombinant OspC from strain B31. Because of the observation that p23 from strain 2591 is an early immunodominant target of the humoral response in Lyme disease, we produced a recombinant form of the protein for testing in diagnostic assays. Preliminary studies with recombinant p23 suggest that it may be a sensitive and specific antigen for serologic detection early in disease. In addition, immunoassays which utilize recombinant forms of antigens have the added advantage of being easily standardized. Currently, we are extending these studies and are optimizing the assay to assess the limits of early detection.

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