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The *Anaplasma marginale msp5* Gene Encodes a 19-Kilodalton Protein Conserved in All Recognized *Anaplasma* Species

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Immunization with *Anaplasma marginale* outer membranes induced immunity against clinical disease which correlated with antibody titer to outer membrane proteins, including a 19-kDa protein (N. Tebele, T. C. McGuire, and G. H. Palmer, Infect. Immun. 59:3199–3204, 1991). This 19-kDa protein, designated major surface protein 5 (MSP-5), was encoded by a single-copy 633-bp gene. The molecular mass of MSP-5, defined in immunoblots by binding to monoclonal antibody ANAF16C1, was conserved among all recognized species of *Anaplasma*: *A. marginale*, *A. centrale*, and *A. ovis*. Recombinant MSP-5, which absorbed the antibody reactivity of bovine immune serum to native MSP-5, was recognized by anti-*A. marginale* and anti-*A. centrale* immune sera in a competitive inhibition assay with monoclonal antibody ANAF16C1. The presence of antibody to the epitope defined by monoclonal antibody ANAF16C1 in all postinfection sera tested indicates that this epitope is a potential diagnostic antigen for use in identifying persistently infected cattle.

Anaplasmosis is an important vector-borne hemoparasitic rickettsial disease of ruminant livestock in tropical and subtropical regions of the world. The disease, caused by *Anaplasma marginale* (24, 37) and *Anaplasma centrale* (38) in cattle and *Anaplasma ovis* in sheep (8, 18), is characterized by anemia, weight loss, abortion, and death (1). Survivors are lifelong carriers of the rickettsia (35).

A strategy for the development of safe and efficacious vaccines for rickettsial diseases, including anaplasmosis, is to identify antigens capable of inducing protective immunity and to produce recombinant replicas of those antigens for use as subunit immunogens. The use of this strategy for anaplasmosis has resulted in identification of two surface proteins, major surface protein 1 (MSP-1) and MSP-2, that induce protective immunity against challenge (25, 28).

Recently, significant protection against homologous challenge was reported after vaccination of cattle with an outer membrane fraction of the Norton (Zimbabwe) strain of *A. marginale* (36). A correlation between antibody titers to the *A. marginale* outer membrane proteins, which included a 19-kDa protein, designated MSP-5, and protective immunity against clinical disease was demonstrated (36). Monoclonal antibody (MAb) ANAF16C1, which defined the 19-kDa protein in the *A. marginale* outer membrane fraction from the Norton strain (36), was used to obtain and characterize the recombinant 19-kDa protein of this report.

MATERIALS AND METHODS

***Anaplasma* strains.** The eight U.S. *A. marginale* strains used in this study, Florida, Washington-Clarkston (Washington-C), Washington-Okanogan (Washington-O), South Idaho, Virginia, North Texas, Missouri, and Mississippi, were described previously (21). Solubilized initial bodies of Israeli strains of *A. centrale* and *A. marginale* (tailed and nontailed) were obtained from the Kimron Veterinary Institute, Beit-Dagan, Israel. *A. ovis* (Idaho strain) initial bodies were obtained from a blood stabilate (32).

MAb production and characterization. The production and initial screening of MAbs to *A. marginale* were as previously described (10). Briefly, hybridomas were made by fusing X63-Ag8.653 murine myeloma cells (15) with spleen cells from BALB/c mice immunized with partially purified initial bodies of the Florida strain of *A. marginale* (10). Hybridoma supernatants were screened for reactivity to *A. marginale* by an indirect immunofluorescence assay. An immunoglobulin G2a MAb that immunoprecipitated a 19-kDa protein from ^{125}I-surface-radiolabeled solubilized initial bodies (data not shown) was designated ANAF16C1.

Construction of *A. marginale* genomic library. An *A. marginale* (Florida strain) genomic DNA library was previously constructed by ligating ultrasonically sheared *A. marginale* DNA into the *Eco*RI site of lambda ZAP (Stratagene Cloning Systems) by standard procedures (19, 30). The lambda library in *Escherichia coli* Y1090 (14) was screened with MAb ANAF16C1 by an immunoblot assay. Proteins were transferred to nitrocellulose by standard procedures, and recombinant protein was detected by using MAb ANAF16C1 (2 μg/ml), rabbit anti-mouse second antibody, and ^{125}I-protein A (14). Positive plaques were isolated, replated, and rescreened to achieve purity. Recombinant plasmids were excised from the bacteriophages (34) and, after induction with 5 mM isopropyl-1-β-D-thiogalactopyranoside (IPTG) (34), tested for expression by immunoblot assay as described below.

DNA sequencing and sequence analysis. Sequenase (U.S. Biochemical, Cleveland, Ohio) was used in a dideoxynucleotide chain-termination assay (31) to determine the nucleotide sequences of both DNA strands. Nucleotide sequences of insert termini were determined by using primers comple-

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mentary to T3 and T7 promoters and extended by primers synthesized with a 381 DNA synthesizer (Applied Biosystems, Foster City, Calif.). Sequences were analyzed by using the Genetics Computer Group (University of Wisconsin) sequence analysis program.

Subcloning. Cesium chloride-purified DNA of a recombinant plasmid (pAM104) expressing the epitope bound by MAb ANAF16C1 was digested with *Acc*I to subclone the two open reading frames (ORFs) identified from the deduced amino acid sequence. Blunt-end ligation was done by standard techniques (30), and competent *E. coli* XL1-Blue cells (Stratagene) were transformed (12). Restriction endonuclease analysis and nucleotide sequence determination of insert termini were used to demonstrate that subclones with opposite orientations were obtained. The subclone expressing MSP-5 was identified by screening with MAb ANAF16C1 in an immunoblot assay as described below.

Southern blot analysis. DNA was extracted from initial bodies of the Florida strain of *A. marginale* as described previously (7) by using standard procedures (30). After 5 μg of *A. marginale* DNA was digested with *Eco*RI, *Sal*I, and *Bam*HI, DNA fragments were separated by agarose gel electrophoresis. DNA was transferred to Gene-Screen (Du Pont, NEN, Boston, Mass.) as previously described (30) and hybridized at 42°C for 20 h in a formamide buffer (50% deionized formamide, 1% sodium dodecyl sulfate [SDS], 1 M NaCl, and 10% dextran sulfate) with ^{32}P-labeled insert DNA isolated from pAM104. The membrane was washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature for 5 min, 2× SSC with 1% SDS at 65°C for 30 min, and 0.1× SSC at room temperature for 30 min. Kodak X-Omat AR X-ray film was exposed to the membrane for 6 h.

Affinity purification of native MSP-5. Initial bodies of the Florida strain of *A. marginale* were obtained from blood stabilates by differential centrifugation as previously described (27). Initial bodies were solubilized with 1% Nonidet P-40 (28). Purification and coupling of MAb ANAF16C1 to CN-activated Sepharose 4B (Pharmacia LKB Biotechnology, Piscataway, N.J.) were done as described previously (22, 28). Native MSP-5 was eluted from the affinity column with Tris buffer (pH 8.0) containing 0.5% deoxycholate and 2 M potassium thiocyanate (25). The purity of eluted MSP-5 was evaluated by SDS-polyacrylamide gel electrophoresis (PAGE) followed by silver staining. Reactivity of eluted MSP-5 with MAb ANAF16C1 was determined by the immunoblot assay described below. Protein concentrations were determined with a bicinchoninic acid kit (Pierce, Rockford, Ill.).

Adsorption of anti-MSP-5 antibody from immune sera. To demonstrate antigenic similarity between native and recombinant MSP-5, the ability of recombinant MSP-5 to adsorb antibody reactivity to native MSP-5 from bovine immune serum was tested. *E. coli* XL1-Blue bacterial lysates in PI buffer (50 mM Tris [pH 8.0], 5 mM EDTA, 5 mM iodoacetamide, 0.1 mM *N*-α-*p*-tosyl-L-lysine · chloromethyl ketone 1 mM phenylmethylsulfonyl fluoride, 1 mg of lysozyme per ml, 1% Nonidet P-40) containing either pAM104 or pBluescript were prepared from 300 ml of LB broth with 50 μg of ampicillin per ml (11). Nitrocellulose membranes were immersed in bacterial lysates containing pAM104 or pBluescript for 20 min and washed three times for 5 min in phosphate-buffered saline. Bovine preinfection or immune serum (1:500 dilution in a Tris-Tween 20 buffer with 1% polyvinylpyrrolidone) to the Washington-O or Virginia strain of *A. marginale* was incubated four consecutive times with membranes containing bacterial lysate for 30 min at room temperature.

The reactivity of adsorbed sera was tested in an immunoblot assay. Native MSP-5 (2 μg per well) was subjected to SDS-PAGE and transferred to nitrocellulose as described previously (39). Membranes were blocked in Tris-Tween 20 buffer containing 5% milk (20, 36). Native MSP-5 was detected with bovine immune serum or MAb ANAF16C1 (2 μg/ml) and then peroxidase-conjugated rabbit anti-bovine antibody or donkey anti-mouse antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, Pa.) and then subjected to enhanced chemiluminescence (ECL kit) (Amersham International plc, Amersham, United Kingdom). Controls included an isotype MAb and preinfection bovine serum.

Conservation of MSP-5. Solubilized initial body lysates from *A. marginale* Mississippi, Washington-O, North Texas, Missouri, Florida, Washington-C, South Idaho, Virginia, and Israel (tailed and nontailed), *A. centrale*, and *A. ovis* were separated by SDS-PAGE and transferred to nitrocellulose. Protein reactivity was tested by the immunoblot assay described above with either MAb ANAF16C1 or isotype control MAb (2 μg/ml).

Antibody reactivity to MSP-5 epitope defined by MAb ANAF16C1. The presence in bovine immune sera of antibody to the MSP-5 epitope defined by MAb ANAF16C1 was tested in a competitive inhibition enzyme-linked immunosorbent assay (CI ELISA).

Control sera. Forty-nine bovine serum samples that were negative for *A. marginale* were used in this study. Ten preinoculation serum samples from cattle used in a vaccine trial and shown to be negative for *A. marginale* by immunoblotting (19) were used as controls in the CI ELISA. In addition, 34 serum samples from northern Canada were determined to be negative for anti-*A. marginale* antibodies by immunoblotting (21). Five preinoculation serum samples were from animals later infected with the Virginia (four samples) and Washington-O (one sample) strains of *A. marginale* (21).

Anti-*Anaplasma* sera. Thirty-seven postinfection serum samples from cattle that were immune to anaplasmosis were used. Three reference serum samples from the Food and Agricultural Organization were tested. These sera were developed by infecting *Anaplasma*-negative cattle with three *A. marginale* strains (Argentina, Venezuela, and Florida). In addition, we tested 18 serum samples from cattle infected with the Florida (6 samples), Virginia (4 samples), South Idaho (4 samples), Northern Texas (2 samples), and Washington-O (2 samples) strains of *A. marginale* as previously described (21). Also, we tested sera from 10 cattle immunized with one of two membrane fractions of the Norton *A. marginale* strain (Zimbabwe) as described previously (36). Six serum samples from cattle immunized by infection with frozen *A. centrale* live blood vaccine were obtained from the Kimron Veterinary Institute. Finally, one sample of rabbit serum prepared against *A. centrale* initial bodies was also obtained from the Kimron Veterinary Institute.

CI ELISA. A CI ELISA with recombinant MSP-5 was performed as previously described (16). Recombinant MSP-5 was prepared from a 50-ml overnight culture of *E. coli* XL1-Blue containing pAM104 in LB broth with 50 μg of ampicillin per ml. A bacterial lysate was prepared with PI buffer.

The bacterial lysate, containing recombinant MSP-5 in phosphate-buffered saline with 20 mM $MgCl_2$, was coated on individual wells of flat-bottomed plates (Immulon 2;

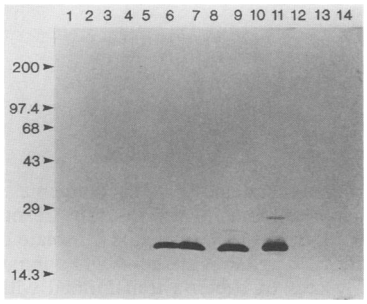

FIG. 1. Demonstration of subclones expressing MSP-5. Lysates of bacteria were transformed with subclone 104A, 104B, or 104C. Immunoblots were probed with MAb ANAF16C1. Lanes 5, 8, 10, and 12 do not contain lysate. Lanes: 1 and 2, subclone 104B without and with IPTG induction, respectively; 3 and 4, subclone 104C without and with IPTG induction, respectively; 6 and 7, subclone 104A without and with IPTG induction, respectively; 9, pAM104 with IPTG induction; 11, affinity purified native MSP-5 (Florida strain); 13 and 14, IPTG-induced pBluescript and gp55 of *B. bigemina*, respectively. Molecular mass markers are presented in kilodaltons.

Dynatech Laboratories, Chantilly, Va.). The appropriate dilution of bacterial lysate was determined by titration with MAb ANAF16C1. All incubations were done at room temperature. Immune sera (1:10 dilution) and MAb ANAF16C1 (0.14 μg per well) were added, and then bound MAb was detected with an avidin alkaline phosphatase detection system (Vector Laboratories, Burlingame, Calif.) and *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo.).

Ten preinfection bovine serum samples were used as negative controls to determine the percent inhibition as previously described (5) with the following equation: 100 − [(optical density in the presence of test serum/optical density in the absence of test serum) × 100].

Nucleotide sequence accession number. The sequence reported in this paper has been deposited in the GenBank data base with accession number M93392.

RESULTS

Identification of a recombinant clone expressing an epitope defined by MAb ANAF16C1. An immunoglobulin G2a MAb that immunoprecipitated a 19-kDa protein from ^{125}I-surface-radiolabeled (27), solubilized initial bodies (data not shown) was designated ANAF16C1. MAb ANAF16C1 had previously identified a 19-kDa protein in an outer membrane fraction of the Norton (Zimbabwe) *A. marginale* strain (36). Bacterial clone 104, containing plasmid pAM104 expressing the epitope defined by MAb ANAF16C1, was isolated from an *A. marginale* genomic DNA library. In an immunoblot assay, pAM104 in *E. coli* XL1-Blue expressed a 19-kDa protein that comigrated with a 19-kDa protein, designated MSP-5, of the Florida *A. marginale* strain (Fig. 1, lanes 9 and 11). Expression of the recombinant 19-kDa protein did not require IPTG induction. An isotype control MAb did not bind to protein expressed by pAM104 (data not shown).

Nucleotide sequence and subcloning of pAM104 insert. The nucleotide sequences of both strands of the pAM104 insert (2.3 kb) were determined. Two ORFs of 210 and 320 amino acids were identified. Both ORFs were in reverse orientation relative to the *lacZ* promoter. To identify the ORF expressing MSP-5, the pAM104 insert was digested with *Acc*I, which removed a 1.2-kb fragment containing the 320-amino-acid ORF. The 210-amino-acid ORF remained with pAM104

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        Acc I
1   GTATACTCAGTTGCGCCTGGCGCTTGACCAACCTGGGCATAGGTGCTACGATCG   54
55  CGCCTGCTCGTTTTGCCGTCCGGCAATGTGGCGCATTTTTGAGTGTTCGTTGGG  108
109 GTGTGATAGATGAGAATTTTCAAGATTGTGTCTAACCTTCTGCTGTTCGTTGCT  162
            M  R  I  F  K  I  V  S  N  L  L  L  F  V  A
163 GCCGTGTTCCTGGGGTACTCCTATGTGAACAAGAAAGGCATTTTCAGCAAAATC  216
    A  V  F  L  G  Y  S  Y  V  N  K  K  G  I  F  S  K  I
217 GGCGAGAGGTTTACCACTTCCGAAGTTGTAAGTGAGGGCATAGCCTCCGCGTCT  270
    G  E  R  F  T  T  S  E  V  V  S  E  G  I  A  S  A  S
271 TTCAACAATTTGGTTAATCACGAGGGGGTCACCGTCAGTAGCGGCGATTTTGGC  324
    F  N  N  L  V  N  H  E  G  V  T  V  S  S  G  D  F  G
325 GGCAAGCACATGTTGGTAATATTCGGCTTCTCAGCCTGTAAGTACACGTGCCCT  378
    G  K  H  M  L  V  I  F  G  F  S  A  C  K  Y  T  C  P
379 ACCGAGTTAGGCATGGCTTCTCAGCTCCTAAGTAAACTAGGCGACCATGCCGAT  432
    T  E  L  G  M  A  S  Q  L  L  S  K  L  G  D  H  A  D
433 AAGTTGCAAGTTGTGTTCATAACTGTTGATCCGAAAAATGACACCGTAGCCAAG  486
    K  L  Q  V  V  F  I  T  V  D  P  K  N  D  T  V  A  K
                                  EcoRI
487 CTTAAAGAGTACCACAAGTCTTTTGATGCGAGAATTCAGATGCTCACAGGCGAA  540
    L  K  E  Y  H  K  S  F  D  A  R  I  Q  M  L  T  G  E
541 GAAGCAGACATAAAGAGCGTGGTTGAAAACTACAAGGTGTATGTGGGCGACAAG  594
    E  A  D  I  K  S  V  V  E  N  Y  K  V  Y  V  G  D  K
595 AAGCCAAGTGATGGTGATATCGACCACTCAACGTTCATGTACCTCATCAATGGG  648
    K  P  S  D  G  D  I  D  H  S  T  F  M  Y  L  I  N  G
649 AAAGGCAGGTATGTCGGGCATTTTGCGCCAGATTTTAACGCGTCTGAGGGCCAA  702
    K  G  R  Y  V  G  H  F  A  P  D  F  N  A  S  E  G  Q
703 GGCGAGGAGCTGTTTAAGTTTGTCAGCGGTCACATGCTTAATTCTTAGTTAAGC  756
    G  E  E  L  F  K  F  V  S  G  H  M  L  N  S  *
757 ATGGCAGTGGTACAGTTTCGTGTGTCGGTCGTCCTTGTGAGGCAGTAGAAAGTA  810
811 TGGGGCTTTGGGGGCTTTCCTTTGTGGCGTTTGTCGCGCTTGCGTTAGGAGCTG  864
865 GGGCTGACCAGATCAGGGTGGTTGGCTCTTCCACCGTGTTCCCATTTATCTCTT  918
919 CTGTTGCCGAAGAGTTTGGTAGATTCTCCGCCTATAGAACCCCCGTCATAGAGT  972
973 CCGTGGGAAGTGGCATGGGCTTTAACATGTTTTGCGCTGGCAGCAGCAGTGATA 1026
1027 CACCAGACATAGCCATGTCCTCTAGGCGCATCAAGGATGCAGAAGTCGAACTTT 1080
1081 GCGGCATGAATGGCGTGAAGGACATGATCGAGATAGGTCTGGGCTACGACGGCA 1134
1135 TAGCCCGAATTC                                          1146
     EcoRI
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FIG. 2. Nucleotide and deduced amino acid sequences of the *msp5* gene. Within the flanking sequences, the predicted −35 and −10 loci, ribosomal binding site, and termination site are underlined, and selected restriction enzyme sites are indicated above the nucleotide sequence. The deduced amino acid sequence is indicated below the nucleotide sequence, and the stop codon is indicated with an asterisk.

in a 1.1-kb fragment. After religation, the plasmid containing the 210-amino-acid ORF was designated pAM104A. The 1.2-kb *Acc*I fragment containing the 320-amino-acid ORF was ligated into pBluescript in both orientations, and the plasmids were designated pAM104B and pAM104C. Only pAM104A expressed a 19-kDa protein that reacted with MAb ANAF16C1 (Fig. 1, lanes 6 and 7). Expression did not require IPTG induction, and MAb ANAF16C1 did not bind to proteins in XL1-Blue lysates containing pBluescript or an insert encoding gp55 of *Babesia bigemina* (19) (Fig. 1, lanes 13 and 14).

The nucleotide sequence of the pAM104A insert and the deduced amino acid sequence of the 210-amino-acid ORF are shown in Fig. 2. Prokaryotic gene elements similar to *E. coli* consensus sequences and in reverse orientation relative to the *lacZ* promoter were identified from the nucleotide sequence of the pAM104 insert. Promoter sequence motifs, identified as possible −10 (TACGAT) (bp 47 through 52) and −35 (TTGACC) (bp 24 through 29) elements upstream from

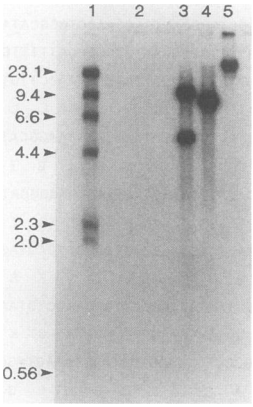

FIG. 3. Hybridization of ^{32}P-labeled pAM104 insert to *A. marginale* Florida and bovine DNA after digestion with restriction endonucleases. Lanes: 1, standards in kilobases; 2, *Eco*RI-digested bovine DNA; 3 through 5, *A. marginale* DNA digested with *Eco*RI, *Sal*I, and *Bam*HI, respectively.

the start codon, differed minimally from *E. coli* −10 (TATAAT) and −35 (TTGACA) consensus sequences (13). The two promoter sequences were separated by 17 bp, the distance between *E. coli* promoter sequences indicative of a strong promoter (13). The expression of MSP-5 from both pAM104 and pAM104A inserts, in reverse orientation relative to the *lacZ* promoter, indicates that MSP-5 expression was probably directed by *A. marginale* regulatory elements.

Additional potential gene or protein structural features identified were a sequence (bp 107 through 112) similar to the *E. coli lacI* ribosomal binding site (17) and an untranslated leader sequence (17) of 65 bp preceding the start ATG (bp 119) of the 210-amino-acid ORF. Also, a predictive hydrophilicity plot identified 22 hydrophobic amino acids at the amino-terminal end that could constitute a signal peptide (40), including a potential cleavage site between amino acids 16 and 17. Using the GCG sequence analysis program, a putative transcription terminator sequence was identified between bp 805 and 819 (underlined in Fig. 2).

Genomic characterization of *msp5*. In the Southern blot assay, the pAM104 insert hybridized to two bands of 4.8 and 10 kb from *Eco*RI-digested genomic *A. marginale* DNA (Florida strain) (Fig. 3, lane 3) and to one band of *Sal*I (8 kb) and *Bam*HI (30 kb) digests (Fig. 3, lanes 4 and 5, respectively). An *Eco*RI site at 624 bp in the pAM104 insert was identified from the restriction enzyme map; however, no *Sal*I or *Bam*HI site was identified (Fig. 2). Bovine calf thymus DNA did not hybridize to the pAM104 insert (Fig. 3, lane 2).

Antigenic similarity of native and recombinant MSP-5s. Recombinant MSP-5 depleted anti-native-MSP-5 antibody reactivity from bovine immune sera to the *A. marginale* Washington-O (Fig. 4) and Virginia (data not shown) strains. In immunoblots, bovine immune sera adsorbed with bacterial lysates containing recombinant MSP-5 did not react with affinity purified native MSP-5 (2 μg/lane) (Fig. 4, lane 2). Immune sera adsorbed with protein equivalent bacterial lysates that did not contain recombinant MSP-5 (Fig. 4, lane 2) reacted with native MSP-5. Negative controls that did not react with native MSP-5 included bovine preinfection serum and an isotype control MAb (data not shown).

Molecular mass conservation of MSP-5 among *Anaplasma* strains and species. To determine the presence and size of MSP-5 in *Anaplasma* strains and species, the total initial

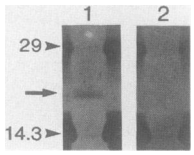

FIG. 4. Immunoblots demonstrating antigenic similarities between native and recombinant MSP-5s. The reaction of immune serum (1:500 dilution) to the Washington-O isolate of *A. marginale* with native MSP-5 (2 μg/lane) is shown. The immune serum was absorbed with lysates of bacteria transformed with either pBluescript (lane 1) or pAM104 (lane 2). Molecular mass standards are indicated in kilodaltons. The arrow indicates the location of native MSP-5.

body lysates of eight U.S. and two Israeli *A. marginale* (tailed and nontailed) strains, an Israeli *A. centrale* strain, and a U.S. *A. ovis* strain were transferred to nitrocellulose and reacted with MAb ANAF16C1. A 19-kDa protein containing the epitope defined by MAb ANAF16C1 was identified in all *A. marginale* strains and species tested (Fig. 5). The immunoblots of the Florida, South Idaho, Virginia, and Washington-C strains of *A. marginale* are not shown.

Antibody reactivity in bovine immune sera to the recombinant MSP-5 epitope defined by MAb ANAF16C1. The presence of antibody in bovine immune sera to the recombinant MSP-5 epitope defined by MAb ANAF16C1 was tested in a CI ELISA (Fig. 6). With 10 negative bovine serum samples as standards, inhibition ranging from 0.5 to 27% (9.6% ± 8.3%) was obtained. The inhibition obtained with five preinfection serum samples is shown (Fig. 6A, lane 6). Forty percent inhibition, a value of greater than three times the standard deviation above the mean percent inhibition of the 10 negative serum samples used as standards, was scored as positive.

The Food and Agricultural Organization reference sera inhibited 99.5% of MAb binding (Fig. 6B, lane 3). Inhibition of MAb binding by U.S. anti-*A. marginale* immune sera ranged from 63 to 92% (Fig. 6A, lanes 1 through 5). In Fig. 6B, lane

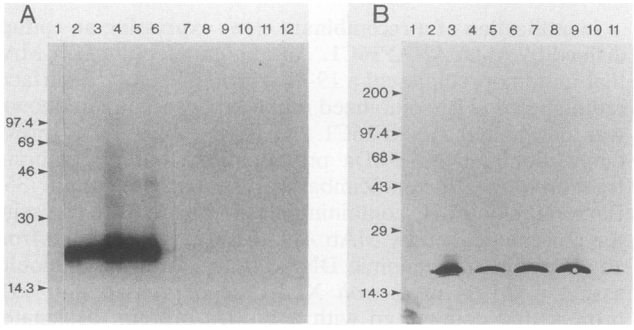

FIG. 5. (A) Immunoblot reacted with ANAF16C1 (lanes 1 through 6) and isotype control MAbs (lanes 7 through 12) demonstrating conservation of MSP-5 among *Anaplasma* strains. Lanes: 1 and 7, lysates of uninfected erythrocytes; 2 through 5 and 8 through 11, 2.5 × 10^9 initial bodies of the Mississippi, Washington-O, North Texas, and Missouri strains, respectively, per lane; 6 and 12, affinity purified native MSP-5 (Florida strain). (B) Immunoblot containing equivalent lysates of stabilates with parasitemia and reacted with MAb ANAF16C1. Lanes: 2, 4, 6, 8, and 10, no sample; 1, uninfected erythrocytes; 3, Florida strain of *A. marginale* (56% parasitemia); 5, Israeli *A. centrale* lysate (44% parasitemia); 7, nontailed Israeli *A. marginale* strain (63% parasitemia); 9, tailed Israeli *A. marginale* strain (57% parasitemia); 11, Idaho *A. ovis* strain (12% parasitemia).

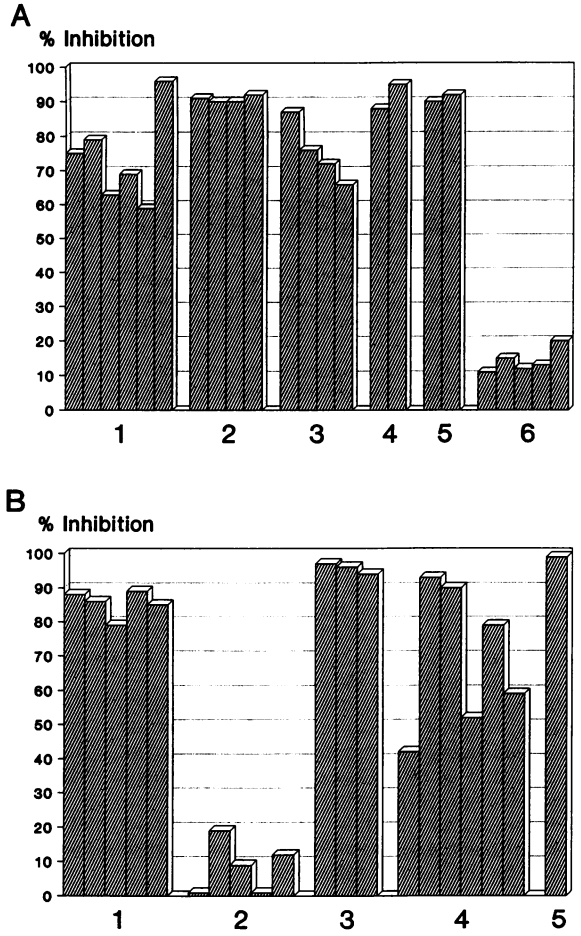

FIG. 6. Demonstration of a conserved epitope on recombinant MSP-5. (A) Percent inhibition of immune sera against U.S. *A. marginale* strains. Columns (strains): 1, Florida; 2, Virginia; 3, southern Idaho; 4, northern Texas; 5, Washington-O; 6, preinfection sera. (B) Percent inhibition of immune sera against the following: 1, Norton (Zimbabwe) *A. marginale* outer membrane fraction; 2, Norton inner membrane fraction; 3, Food and Agricultural Organization reference sera; 4, bovine *A. centrale* postinfection sera; 5, rabbit anti-*A. centrale* serum.

1, inhibition of 79 to 89% with sera against the outer membrane fraction of the Norton (Zimbabwe) *A. marginale* strain was obtained; however, sera against the inner membrane fraction, which did not recognize the 19-kDa protein (36), did not inhibit binding of MAb ANAF16C1 (Fig. 6B, lane 2). Sera from cattle immunized by infection with live *A. centrale* blood vaccine (29) were positive, with inhibition values of 42 to 93% (Fig. 6B, lane 4). Also, 99.5% inhibition was obtained with the rabbit anti-*A. centrale* serum (Fig. 6B, lane 5).

DISCUSSION

In contrast to what has been shown for the *A. marginale* proteins MSP-1 and MSP-2 (7, 23), the data presented here demonstrate molecular mass conservation of MSP-5 among all recognized *Anaplasma* species. MSP-1, a complex of two polypeptides, MSP-1a and MSP-1b (7), is characterized by MSP-1a size polymorphism of up to 50% (70 to 105 kDa) and more limited MSP-1b size variation of up to 3% (97 to 100 kDa) (23). MSP-2 varies up to 9% (33 to 36 kDa) among *A. marginale* strains (23).

Although the extent to which the size conservation of MSP-5 extends to genetic or antigenic conservation is not known, the data clearly show that MSP-5s from all recognized species of *Anaplasma* possess a common epitope defined by MAb ANAF16C1. Conservation of epitopes of MSP-1 and MSP-2 among *A. marginale* isolates from Kenya, Israel, and United States as well as *A. centrale* was previously shown (26). However, this is the first demonstration of a common epitope also found on *A. ovis*, which, unlike *A. marginale*, is primarily a pathogen of sheep.

Screening an *A. marginale* genomic DNA library for this common epitope was used to obtain a gene encoding MSP-5. Data demonstrating that the cloned *msp5* gene is expressed in *A. marginale* are (i) the shared epitope, defined by MAb ANAF16C1, of native and recombinant MSP-5; (ii) the ability of recombinant MSP-5 to deplete anti-native-MSP-5 antibody from bovine immune sera; (iii) the markedly similar molecular masses of native and recombinant MSP-5; (iv) Southern blot results, which indicate that the gene exists as a single copy within the *A. marginale* genome; (v) the identification of typical prokaryotic control elements encompassing the ORF; and (vi) the expression of the *msp5* gene in the opposite orientation relative to the *E. coli lacZ* promoter, indicating that the prokaryotic control elements identified are probably operative in the expression of recombinant MSP-5.

Gene structures with predicted promoter elements similar to those of *E. coli* genes were also identified in *msp1a* (2) and *msp1b* (6) of *A. marginale* and in genes from *Rickettsia prowazekii* and *Rickettsia tsutsugamushi* (9, 33). Also, comparison of the published amino acid sequences of a 17-kDa protein that is conserved among seven *Rickettsia* species (3, 4) showed 43.9% similarity and 17.8% identity with the deduced amino acid sequence of MSP-5.

Similar to the *msp5* gene, the *msp1a* gene is present as a single copy (2). However, the *msp1b* and *msp2* genes are present in multiple copies in the *A. marginale* genome (6, 19). Inducing the protective immunity with recombinant MSP-1b and MSP-2 proteins may require selecting the appropriate gene copy from the multigene family. The finding that the gene encoding MSP-5 is present as a single copy and the apparent uncomplicated molecular structure of MSP-5 simplify the testing of MSP-5 as a vaccine.

In summary, the size conservation, location in the initial body outer membrane, and presence of MSP-5 in all *Anaplasma* species tested, including *A. ovis*, suggest that MSP-5 is important in the *Anaplasma* life cycle. Also, the presence of antibody to the epitope defined by MAb ANAF16C1 in all postinfection sera tested indicates that this epitope is a potential diagnostic antigen for use in identifying persistently infected cattle.

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