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The Immunoprotective *Anaplasma marginale* Major Surface Protein 2 Is Encoded by a Polymorphic Multigene Family

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An *Anaplasma marginale* Florida *msp-2* gene was cloned and expressed in *Escherichia coli*. Pulsed-field gel electrophoresis and Southern blot analysis revealed the presence of multiple *msp-2* gene copies that were widely distributed throughout the chromosomes of all three strains examined. Genomic polymorphism among copies was greatest in the 5′ end of *msp-2* but also occurred in 3′ regions. The presence of gene-copy-specific epitopes was indicated by the reactivity of the cloned *msp-2* copy with some, but not all, monoclonal antibodies that bound native MSP-2. Multiple antigenically distinct MSP-2 molecules were expressed within strains and were coexpressed by individual *A. marginale* organisms. These results suggest that expression of polymorphic *msp-2* gene copies is responsible for the significant percentages of *A. marginale* organisms within strains that do not react with individual anti-MSP-2 monoclonal antibodies. Sequence analysis revealed highly significant MSP-2 homology with two rickettsial surface proteins, *A. marginale* MSP-4 and *Cowdria ruminantium* MAP-1. Immunization with MSP-4 has been shown to induce protective immunity in a manner similar to that of immunization with MSP-2. These findings support the hypothesis that *A. marginale* surface proteins are targets of protective immune responses but are antigenically polymorphic.

Anaplasma marginale is an arthropod-borne rickettsial pathogen that induces severe anemia, abortion, and death in cattle following invasion and replication within mature erythrocytes (32). Protective immunity is conferred following either natural infection or deliberate infection with strains of reduced virulence (16). Currently, inoculation of cattle with infected blood is the most effective and common method of immunization worldwide (16). However, the sustainability of this method has been constrained by technical problems concerning such matters as standardization, storage, and delivery (26). In addition, the risk of direct blood-borne transmission of both known and newly emergent pathogens limits continued reliance on this method of immunoprophylaxis (15, 26, 33). Consequently, there is interest in developing improved vaccines based on defined antigens that can be produced and standardized in vitro.

Immunization of cattle with *A. marginale* organisms or purified outer membranes induces protection against severe rickettsemia and anemia (21, 35). Significantly, protection against homologous challenge correlates with antibody titer against outer membrane polypeptides, including an approximately 36-kDa protein designated major surface protein 2 (MSP-2) (35). Immunization with native immunoaffinity-purified MSP-2 induces partial protection against challenge with both homologous and heterologous *A. marginale* strains (31). MSP-2 is conserved between *A. marginale* and *Anaplasma centrale*, a less virulent species that induces protection against *A. marginale* (28, 34), and is present in morphologically distinct *A. marginale* stages in the infected tick vector *Dermacentor andersonii* (29). Consistent with its conservation in both the intraerythrocytic and tick vector stages is the recent observation that MSP-2 may function as an adhesin for erythrocyte binding (18).

In this paper, we report the cloning and expression of an *msp-2* genomic copy from the Florida strain and the identification of multiple related gene copies distributed throughout the chromosome. By using the cloned *msp-2*, genomic and antigenic polymorphism was identified in this complex multigene family. Importantly, sequence analysis revealed significant homology to a second immunoprotective *A. marginale* surface protein, MSP-4. These findings support the hypothesis that *A. marginale* surface proteins are targets of protective immune responses but are antigenically polymorphic.

MATERIALS AND METHODS

***A. marginale* organisms.** The Florida, South Idaho, and Virginia strains of *A. marginale* were maintained as liquid nitrogen-cryopreserved stabilates of infected bovine erythrocytes in dimethyl sulfoxide–phosphate-buffered saline (PBS) (17). *A. marginale* organisms were isolated from thawed, infected bovine erythrocytes by sonication and differential centrifugation as previously described (30). For preservation of intact *A. marginale* DNA to prepare large chromosomal fragments, infected erythrocytes were isolated from freshly collected blood and embedded in agarose (2). This procedure is described in detail below. Blood smears from cattle experimentally infected with the Florida strain were prepared as previously described (20) and stored at −70°C prior to use.

Antibodies. A panel of eight anti-MSP-2 monoclonal antibodies (MAbs) was produced by immunization of mice with *A. marginale* and generation of cloned hybridomas as previously described (8). MAbs used (the *A. marginale* strains used to immunize mice are in parentheses) were as follows: (i) ANAO45A2, ANAO50A2, ANAO58A2, ANAO66A2, and ANAO70A2 (Washington-O); (ii) ANAF19E2 (Florida); and (iii) ANAR20A and ANAR79A (Virginia). The reactivities of

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the MAbs in groups i and ii with all examined strains of *A. marginale*, including the Florida strain, have been previously described (20, 28). The reactivity of group iii MAbs with MSP-2 was identified by using binding to the 36-kDa Florida strain surface polypeptide in immunoblot and immunoprecipitation assays (data not shown), as previously described (29, 31). MAb ANA15D2, which is directed against MSP-1a (27) and reacts with 100% of the *A. marginale* organisms, regardless of the strain (20, 28), was used as a positive control to detect all organisms present. MAb Tryp1E1, reactive with *Trypanosoma brucei* VSG, and MAb 14/16.1.7, reactive with *Babesia bigemina* RAP-1, were used as negative control antibodies. Polyclonal rabbit anti-MSP-2 serum (R883) was produced by immunization of rabbits with 100 μg of isolated native MSP-2 in complete Freund's adjuvant followed by three booster immunizations of MSP-2 in incomplete Freund's adjuvant at 3-week intervals. Normal rabbit serum was used as a negative control.

***msp-2* cloning and sequencing.** Genomic DNA was isolated from *A. marginale* Florida as previously described (4, 6) and used to construct two libraries. The first library was made from *Pst*I-digested *A. marginale* DNA ligated into *Pst*I-digested and dephosphorylated pKK233-2 (Pharmacia). *Escherichia coli* XL-1 Blue (Stratagene) was transformed with recombinant pKK233-2, and ampicillin-resistant colonies were screened for *msp-2* expression by using MAb ANAR20A as previously described (6). A recombinant colony bound by MAb ANAR20A was identified and designated pCKR5.2. Both DNA strands of the pCKR5.2 insert were sequenced by using sequentially derived primers initiating dideoxynucleotide chain reactions (Sequenase 2.0; U.S. Biochemical). Oligonucleotide primers were synthesized with an Applied Biosystems 381 DNA synthesizer. The sequence of pCKR5.2 revealed an open reading frame truncated at the 5′ end of the insert. A second library was composed of mechanically sheared *A. marginale* DNA ligated with *Eco*RI adaptors and inserted into lambda ZapII. Phage-transfected *E. coli* Y1090 organisms were immunoscreened by using MAb ANAF19E2, and a reactive colony, pCKR1.8, was identified. The sequence of a PCR-derived subclone of pCKR1.8 revealed an open reading frame truncated at the 3′ end. On the basis of the derived sequences of pCKR5.2 and the pCKR1.8 subclone, which contained a 66-bp overlap, *msp2* was amplified from genomic *A. marginale* DNA by using *Taq* DNA polymerase. The 5′ primer, 5′-AGGAGT TAGCCATGGTGAGTGCTGTAAGT, derived from the PCR subclone of the pCKR1.8 sequence, incorporated an *Nco*I site (underlined) by substitution into the sequence bridging the start of the open reading frame. The incorporation of the *Nco*I site into the primer was predicted to alter the initial MSP-2 amino acid sequence from M-S-A-V-S-N to M-V-S-A-V-S-N. Insertion into the *Nco*I site in pKK233-2 was selected to optimally place the initiation codon downstream of the vector *trc* promoter and the *lacZ* ribosome binding site (3). The 3′ primer, 5′-CCTAAGCCATGGATCTAGAAGGAACTA GAAGGC, derived from the pCKR5.2 sequence downstream of the termination of the open reading frame, also incorporated an *Nco*I site (underlined). Following amplification, the 1.3-kb PCR product was ligated into *Nco*I-digested, dephosphorylated pKK233-2 and used to transform *E. coli* XL-1 Blue. Colonies reactive with MAbs ANAF19E2 and ANAR20A were selected by immunoscreening. A single clone, pCKR11.2, was used in all further experiments. Plasmid DNA was extracted from pCKR11.2 for restriction enzyme digestion and Southern blotting and for double-stranded DNA sequencing. Sequence analysis using the Genetics Computer Group package from the University of Wisconsin, version 7.3, was performed on a Vax 11/785 computer.

Representation of pCKR11.2 *msp-2* in the *A. marginale* genome. To verify that the PCR-amplified and cloned *msp-2* in pCKR11.2 accurately represented genomic *msp-2*, multiple restriction sites in plasmid and genomic DNA were compared. *A. marginale* genomic DNA and pCKR11.2 DNA were digested with restriction enzymes selected to yield fragments of defined sizes (see Fig. 4). Southern blotting was performed under prehybridization and hybridization conditions as previously described (24), and blots were probed with biotin-labeled pCKR11.2 *msp-2*. The molecular sizes of comigrating plasmid and genomic fragments were determined by comparison to 0.56-, 2.0-, 2.3-, 4.4-, 6.6-, 9.4-, and 23.1-kb size standards.

Native MSP-2 isolation and N-terminal sequencing. Native MSP-2 was isolated from *A. marginale* Florida by immunoaffinity chromatography (29). Column fractions were analyzed for the presence of MSP-2 by using immunoblots probed with MAb ANAF19E2 and for purity by silver staining following electrophoresis in sodium dodecyl sulfate (SDS)-containing polyacrylamide gels (data not shown), as previously described (29, 31). Fractions highly enriched with MSP-2 were dissolved in sample buffer and heated at 55°C for 10 min (12). MSP-2 was electrophoresed in SDS-containing polyacrylamide gels, modified as described previously (12) to minimize destruction of amino acid side chains and to prevent amino-terminal blockage by gel contaminants. MSP-2 was then electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore), stained with 0.1% Coomassie blue, and destained with 45% methanol–10% acetic acid. The portion of the membrane containing bound MSP-2 was excised and used to derive the amino-terminal sequence by previously described methods (7) using an Applied Biosystems 475A amino acid sequencer. Sequences were derived from three analyses of two different chromatographic isolations of MSP-2.

Immunoblotting of recombinant MSP-2. The molecular size of the pCKR11.2-expressed product was determined by immunoblotting. *E. coli* cells transformed with pCKR11.2 or control *E. coli* cells transformed with nonrecombinant pKK233-2 were grown overnight in Luria-Bertani broth containing 50 μg of ampicillin per ml and lysed 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% Nonidet P-40, 0.1% SDS). Bacterial lysates or similarly prepared lysates of *A. marginale* Florida were electrophoresed on SDS-containing polyacrylamide gels and transferred to nitrocellulose (13, 25). Membranes were reacted with 2 μg of either MAb ANAF19E2, MAb ANAR20A, or the negative control MAb TRYP1E1 per ml and then with peroxidase-labeled goat anti-mouse immunoglobulin G (IgG) with enhanced chemiluminescent detection.

Presence of multiple *msp-2* gene copies. *A. marginale* genomic DNA extracted from either the Florida strain or the South Idaho strain was digested with *Apa*I, *Bgl*I, *Hin*dIII, *Kpn*I, or *Nco*I. Calf thymus DNA was digested identically as a control. The electrophoretically separated and blotted fragments were hybridized with one of five *msp-2* probes prepared by PCR amplification: whole *msp-2* (1,216 bp; amplified between nucleotides [nt] 10 and 1226); the 5′ half of *msp-2* (512 bp; amplified between nt 151 and 663); the 5′ end of *msp-2* (325 bp; amplified between nt 10 and 335); the 3′ half of *msp-2* (560 bp; amplified between nt 666 and 1226); and the 3′ end of *msp-2* (348 bp; amplified between nt 878 and 1226). A probe representing the single-copy *A. marginale msp-4* gene was used as a control (24). Prehybridization and hybridization were done as previously described (24). All probes were labeled with

digoxigenin, and hybridization was detected by chemiluminescence.

Distribution of *msp-2* copies in the *A. marginale* chromosome. The locations of multiple *msp-2* copies in the chromosome were determined by Southern blotting of large *A. marginale* genomic fragments separated by clamped homogeneous electric field electrophoresis (CHEF). Briefly, erythrocytes infected with either the Florida strain, the South Idaho strain, or the Virginia strain of *A. marginale* were separated from leukocytes by using an α-cellulose–microcrystalline cellulose column, and washed intact erythrocytes were embedded in 0.7% agarose (2). Agarose plugs were incubated in a solution containing 0.5 M EDTA (pH 9.5), 1% *N*-lauroylsarcosine, and 2 mg of proteinase K per ml for 48 h at 37°C and then stored at 4°C in fresh proteinase K solution. Plugs containing intact *A. marginale* were digested with *Sfi*I as previously described (2) and electrophoresed in 1% agarose in TBE buffer (45 mM Tris, 45 mM borate, 1 mM EDTA) at 14°C by using a CHEF-DRII apparatus (Bio-Rad). Electrophoresis was done for 18 h at 14°C by using 180 V and an 8-s switch rate. Lambda DNA-*Hin*dIII fragments and Promega Delta 39 markers were included as size standards. The gel was stained with ethidium bromide for 20 min and photographed. The same gel was depurinated with 250 mM HCl for 15 min, washed twice for 15 min in 0.4 M NaOH–0.6 M NaCl, and then washed twice more for 15 min in 1.5 M NaCl–0.5 M Tris-HCl (pH 7.5) (2). Chromosomal digests were transferred to nylon membranes in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), washed for 1 min in 0.4 M NaOH followed by 0.2 M Tris-HCl (pH 7.5)–2× SSC, and then bound to the membrane by using UV light. The membrane was prehybridized for 2 h at 65°C and then hybridized with the digoxigenin-labeled whole *msp-2* probe, and hybridization was detected by chemiluminescence as previously described.

MAb reactivity with recombinant MSP-2. The reactivity of recombinant MSP-2, expressed by *E. coli* transformed with pCKR11.2, with a panel of MAbs against native *A. marginale* MSP-2 was determined by using indirect immunofluorescence. Briefly, *E. coli* cells transformed with either pCKR11.2 or control pKK233-2 were grown overnight, washed in PBS, resuspended in PBS-BSA buffer (PBS containing 1.5% bovine serum albumin [BSA]), and used to prepare acetone-fixed cytospins. Acetone-fixed smears of *A. marginale* Florida-infected erythrocytes were used as positive controls. Approximately 0.5 μg of MAb or a 1:50 dilution of polyclonal rabbit antibody in PBS-BSA buffer was added, and the cells were incubated for 30 min at 4°C. Slides were washed three times in PBS-BSA buffer, incubated for 90 min at 4°C with either goat anti-mouse IgG or goat anti-rabbit IgG fluorescein-labeled antisera, and washed an additional three times in PBS-BSA. Slides were examined at a magnification of ×400, and an excitation wavelength of 450 to 490 nm was used to detect fluorescein-labeled organisms.

The expression of MSP-2 on the surfaces of *E. coli* cells transformed with pCKR11.2 was tested by using a live immunofluorescent assay modified from those of previous descriptions (19). Briefly, mid-logarithmic-phase bacteria were collected, washed once in PBS-BSA buffer, and resuspended in the same buffer. Bacteria were incubated with either MAb or polyclonal rabbit antibody, washed, and detected by using fluorescein-labeled antisera as described above except that all incubations, washes, and microscopic examinations were done in the fluid phase.

Coexpression of unique *msp-2* copies. To detect simultaneous expression of different *msp-2* copies, pairs of anti-MSP-2 MAbs of different isotypes were reacted with acetone-fixed smears of *A. marginale* Florida and the binding of each MAb was detected by using fluorescein-labeled goat anti-mouse IgG1 and phycoerythrin-labeled goat anti-mouse IgG2a. Washing and incubation were done as described above. Slides were examined at a magnification of ×1,000, and excitation wavelengths of 450 to 490 nm were used to detect fluorescein-labeled organisms and a wavelength of 546 nm was used to detect phycoerythrin-labeled organisms.

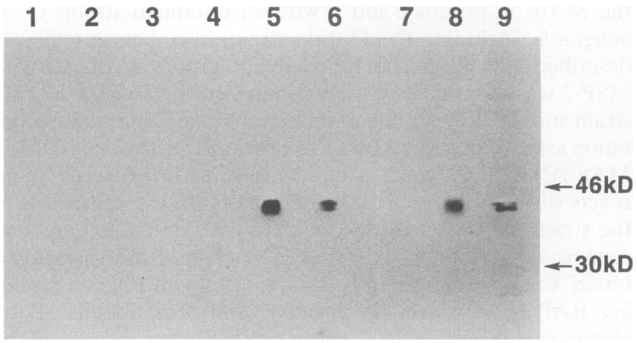

FIG. 1. Expression of recombinant MSP-2. Lanes 1, 4, and 7 contain pKK233-2-transformed *E. coli* XL-1 Blue; lanes 2, 5, and 8 contain pCKR11.2-transformed *E. coli* XL-1 Blue; and lanes 3, 6, and 9 contain *A. marginale* Florida. Lanes 1 through 3 were reacted with MAb TRYP1E1; lanes 4 through 6 were reacted with MAb ANAF19E2; and lanes 7 through 9 were reacted with MAb ANAR20A. The positions of the 46- and 30-kDa molecular size markers are indicated at the right.

Nucleotide sequence accession number. The nucleotide sequence of the pCKR11.2 *msp-2* gene has been assigned GenBank accession number UO7862.

RESULTS

***msp-2* cloning, expression, and sequencing.** *E. coli* transformed with pCKR11.2 expressed a recombinant protein that comigrated with native *A. marginale* Florida MSP-2 (Fig. 1). Both the pCKR11.2 recombinant MSP-2 and native *A. marginale* MSP-2 bound MAbs ANAF19E2 and ANAR20A but not the negative control MAb TRYP1E1 (Fig. 1). *E. coli* transformed with nonrecombinant pKK233-2 was unreactive with all three MAbs.

The nucleotide sequences of both strands of the pCKR11.2 insert were determined. The insert open reading frame (409 amino acids) at the first methionine included the predicted M-V-S-A-V-S-N alteration of the true *msp-2* copy sequence M-S-A-V-S-N (the nucleotide and deduced amino acid sequences shown in Fig. 2 represent the *A. marginale msp-2* copy, not the PCR-modified pCKR11.2. sequences). The N-terminal 60-amino-acid region is hydrophobic, and the signal cleavage site with the highest score predicted by using the SigCleave algorithim is between amino acids 30 and 31. The actual sequence of the amino terminus of native immunoaffinity-purified MSP-2 was identical to the sequence deduced from the nucleotide sequence of pCKR11.2 *msp-2* beginning at amino acid 31. The amino-terminal sequence APAAGAGA GGEGLFSGA (amino acids 31 through 47) appeared in all three protein sequencing assays with the exception of amino acid 40 (underlined), which was identified as G in two runs and as S in a single run. The predicted molecular mass of the entire MSP-2 was 42,365 Da, and that of the mature protein without the signal peptide was 39,411 Da.

Searching of the GenBank (release 81), SwissProtein (re-

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   1 ATGAGTGCTGTAAGTAATAGGAAGCTTCCCCTAGGAGGCGTGTTGATGGCTCTGGCTGCA  60
     M  S  A  V  S  N  R  K  L  P  L  G  G  V  L  M  A  L  A  A

  61 GCGGTTGCACCAATCCATTCTTTACTGGCTGCCCCTGCAGCTGGTGCTGGCGCAGGCGGC 120
     A  V  A  P  I  H  S  L  L  A▲A  P  A  A  G  A  G  A  G  G

 121 GAGGGTCTATTTTCAGGCGCCGGAGCCGGGAGTTTTTACATAGGACTGGACTACAGCCCA 180
     E  G  L  F  S  G  A  G  A  G  S  F  Y  I  G  L  D  Y  S  P

 181 GCATTCGGCAGCATCAAGGACTTTAAAGTCCAAGAGGCTGGCGGCACTACTAGAGGTGTG 240
     A  F  G  S  I  K  D  F  K  V  Q  E  A  G  G  T  T  R  G  V

 241 TTCCCGTACAAGCGAGATGCTGCAGGTCGGGTGGATTTCAAGGTCCACAACTTCGACTGG 300
     F  P  Y  K  R  D  A  A  G  R  V  D  F  K  V  H  N  F  D  W

 301 AGTGCTCCAGAACCCAAGATTAGCTTCAAGGATAGCATGCTCACAGCCCTAGAGGGGAGC 360
     S  A  P  E  P  K  I  S  F  K  D  S  M  L  T  A  L  E  G  S

 361 ATTGGGTATAGTATTGGAGGAGCAAGGGTTGAAGTAGAAGTAGGGTATGAAAGGTTTGTC 420
     I  G  Y  S  I  G  G  A  R  V  E  V  E  V  G  Y  E  R  F  V

 421 ATTAAGGGAGGTAAGAAGTCTAATGAGGATACAGCTTCAGTATTCTTATTAGGAAAGGAG 480
     I  K  G  G  K  K  S  N  E  D  T  A  S  V  F  L  L  G  K  E

 481 TTAGCATATCATACAGCTAGAGGTCAGGTAGACCGTCTTGCCACTGCTTTAGGTAAGATG 540
     L  A  Y  H  T  A  R  G  Q  V  D  R  L  A  T  A  L  G  K  M

 541 ACTAAGAGTGAGGCTAAGAAGTGGGGTAATGCCATAGAGAGTGCTACTGGCACTACTAGT 600
     T  K  S  E  A  K  K  W  G  N  A  I  E  S  A  T  G  T  T  S

 601 GGTGATGAACTGAGTAAGAAGGTGTGTGGCAAGGGTACCACTAGTGGTAGCACCAACCAG 660
     G  D  E  L  S  K  K  V  C  G  K  G  T  T  S  G  S  T  N  Q

 661 TGTGGTACCACCGATAGCACTGCCACCACCAAGATTAGTGCGGTGTTCACTGAGGATGCA 720
     C  G  T  T  D  S  T  A  T  T  K  I  S  A  V  F  T  E  D  A

 721 GCAGCGCAGTTATCTACTATGGACAATACCACCATCAACACGACAGGAATGGCGAATAAC 780
     A  A  Q  L  S  T  M  D  N  T  T  I  N  T  T  G  M  A  N  N

 781 ATTAATAGCCTGACAAAAGATGAGAAGGCCATAGTTGCTGGGGCTTTTGCTAGAGCTGTT 840
     I  N  S  L  T  K  D  E  K  A  I  V  A  G  A  F  A  R  A  V

 841 GAAGGTGCGGAGGTGATAGAGGTCAGGGCTATAGGGTCTACTTCTGTAATGCTCAATGCT 900
     E  G  A  E  V  I  E  V  R  A  I  G  S  T  S  V  M  L  N  A

 901 TGTTATGACCTTCTGACTGATGGTATTGGGGTTGTTCCTTATGCTTGTGCTGGTATCGGT 960
     C  Y  D  L  L  T  D  G  I  G  V  V  P  Y  A  C  A  G  I  G

 961 GGTAACTTTGTCAGTGTTGTTGATGGTCACATTAATCCTAAGTTTGCTTACAGAGTGAAG 1020
     G  N  F  V  S  V  V  D  G  H  I  N  P  K  F  A  Y  R  V  K

1021 GCTGGTCTGAGTTATGCTCTGACTCCTGAAATTTCTGCCTTTGCTGGGGCTTTCTACCAC 1080
     A  G  L  S  Y  A  L  T  P  E  I  S  A  F  A  G  A  F  Y  H

1081 AAGGTGTTAGGTGATGGTGATTATGATGAGCTACCTTTGAGTCATATTTCTGATTATACT 1140
     K  V  L  G  D  G  D  Y  D  E  L  P  L  S  H  I  S  D  Y  T

1141 GGTACTGCTGGTAAGAATAAGGATACTGGTATTGCTTCATTTAACTTCGCTTACTTTGGT 1200
     G  T  A  G  K  N  K  D  T  G  I  A  S  F  N  F  A  Y  F  G

1201 GGTGAGTTGGGTGTTAGGTTTGCCTTCTAG 1230
     G  E  L  G  V  R  F  A  F  *
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FIG. 2. Nucleotide sequence and translation of *msp-2* (pCKR11.2-cloned copy). The signal peptide cleavage site is indicated by the arrowhead between amino acids 30 and 31. The termination codon is indicated by the asterisk.

lease 27.0), Protein (release 38.0), and EMBL (release 7.3) databases revealed significant amino acid sequence similarity to two rickettsial surface proteins, *A. marginale* MSP-4 (23) and *Cowdria ruminantium* MAP-1 (36). The homology between MSP-2 and MSP-4 (60% similarity; 39% identity) was sequence specific and not only a reflection of content, as indicated by a BestFit quality score of 21 standard deviations above the mean quality score following ten random shuffles of MSP-4. BestFit alignment of MSP-2 and MSP-4 indicated that multiple oligopeptide sequences were conserved throughout the proteins (Fig. 3). The BestFit quality score for the homology between MSP-2 and the 32-kDa MAP-1 *C. ruminantium* surface protein (59% similarity; 33% identity) was 16 standard deviations above the mean for 10 randomly shuffled MAP-1 sequences. Multiple oligopeptide sequence homology between MSP-2 and MAP-1 also occurred throughout the proteins (data not shown).

Representation of pCKR11.2 *msp-2* in the *A. marginale* genome. To verify that the PCR-amplified and cloned pCKR11.2 *msp-2* faithfully represented the genomic copy, pCKR11.2 DNA and the Florida strain genomic DNA were digested with pairs of restriction enzymes predicted to yield defined *msp-2* fragments. The enzymes used, the predicted restriction sites, and the resulting comigrating pCKR11.2 *msp-2* and genomic fragments detected by using Southern blots probed with whole pCKR11.2 *msp-2* are shown in Fig. 4. All

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  4 VSNRKLPLGGVLMALAAAVAPIHSLLAAPAAGAGAGGEGLFSGAG..AGSFYIGLDYSPA 61
    :. |.|  ||    |.||...  |||...|..|:: :.:: |::|  :||||:| .||||
  1 MNYRELFTGG....LSAATVCACSLLVSGAVVASPMSHEVASEGGVMGGSFYVGAAYSPA 56

 62 FGSIKDFKVQEAGGTTRGVFPYKRDAAGRVDFKVHNFDWSAPEPKISFKDSMLTALEGSI 121
    |.|:..|.:.|.: .|. | .|.:. |. :|..|.. ::| .: .:.|...::|.::|.:
 57 FPSVTSFDMRESSKETSYVRGYDKSIAT.IDVSVPA.NFSKSGYTFAFSKNLITSFDGAV 114

122 GYSIGGARVEVEVGYERFVIKGGKKSNEDTASVFLLGRELAYHTARGQVDRLATALGKMT 181
    |||:||||||:|.:| ||.                                . | |. .
115 GYSLGGARVELEASYRRFA.....................................TLADGQYA 141

182 KSEAKKWGNAIESATGTTSGDELSKKVCGKGTTSGSTNQCGTTDSTATTKISAVFTEDAA 241
    ||:|..:: ||......|..: :
142 KSGAESLA.AITRDANITETNYF..................................... 163

242 AQLSTMDNTTINTTGMANNINSLTKDEKAIVAGAFARAVEGAEVIEVRAIGSTSVMLNAC 301
                                                |:.: .|.-||||||:|
164 .....................................VVKIDEITNTSVMLNGC 180

302 YDLLTDGIGVVPYACAGIGGNFVSVVDGHINPKFAYRVKAGLSYALTPEISAFAGAFYHK 361
    ||:| .::.| ||.||||:.||.: . ::..|:|||.|.|:||.:||||| .||:|||
181 YDVLHTDLPVSPYVCAGIGASFVDISK.QVTTKLAYRGKVGISYQFTPEISLVAGGFYHG 239

362 VLGDGDYDELPLSHISDYTGTAGKNKDTGIASFNFAYFGGELGVRFAF 409
    ::::: |.::|  :  .:.|.|  . .. ||.:.|     :||.|| |
240 LFDES.YKDIPAHNSVKFSGEAKASVKAHIADYGF.....NLGARFLF 281
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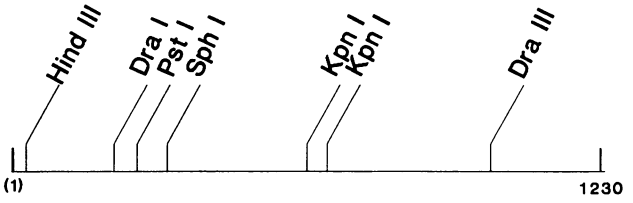
FIG. 3. BestFit alignment of MSP-2 (top row) and MSP-4 (bottom row) protein sequences. Identical amino acids are indicated by a vertical line. Conservative substitutions are indicated by one dot (.) where the residue comparison values are ≥0.1 and by two dots (:) where the residue comparison values are ≥0.5 (9). The symbol ..... denotes a gap used to achieve optimal alignment between the sequences.

digests yielded pCKR11.2 fragments of the predicted size that comigrated with a fragment in the genomic DNA. Colinearity between the plasmid and the genome was observed in six restriction enzyme fragments extending from the 5′ end (the *Hin*dIII site at nt 22) through the 3′ end (the *Dra*III site at nt 1084).



Restriction enzyme	Cleavage sites in pCKR11.2 *msp-2*	Size of co-migrating plasmid and genomic fragments
*Hin*dIII/*Sph*I	22/339	317
*Hin*dIII/*Dra*III	22/1084	1062
*Dra*I/*Dra*III	204/1084	880
*Pst*I/*Dra*III	264/1084	820
*Sph*I/*Dra*III	339/1084	745
*Kpn*I/*Dra*III	638;668 /1084	30;416

FIG. 4. Representation of pCKR11.2 *msp-2* in the *A. marginale* genome. Diagram of pCKR11.2 *msp-2* indicating the double restriction enzyme digests and resulting fragments used to map pCKR11.2 *msp-2* to the genome. Nucleotide positions of cleavage sites in pCKR11.2 *msp-2* are indicated. Sizes of comigrating plasmid and genomic fragments are those of comigrating bands detected in Southern blots of plasmid and genomic DNA by using the whole pCKR11.2 *msp-2* probe. *Kpn*I cleaves at nt 638 and 668; the 30-bp fragment for *Kpn*I-*Dra*III was not detected in Southern blots.

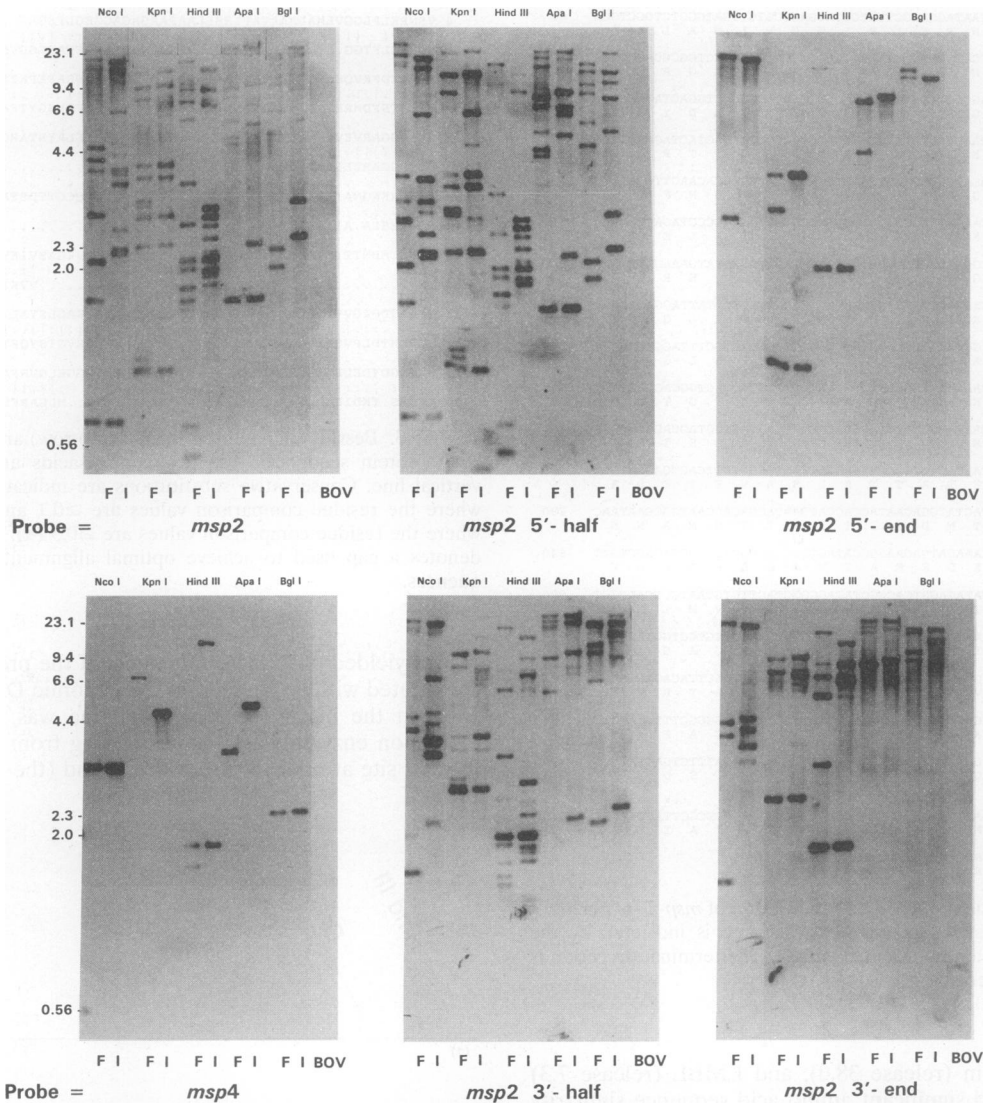


FIG. 5. Presence of multiple *msp-2* copies in the *A. marginale* genome. *A. marginale* DNA from either the Florida strain (F) or the South Idaho (I) strain was digested with the restriction enzyme indicated at the top of each lane and Southern blotted. Undigested bovine DNA was used as a negative control (BOV). Blots were hybridized with either whole *msp-2* (1,216 bp; nt 10 through 1226); the 5′ half of *msp-2* (512 bp; nt 151 through 663); the 5′ end of *msp-2* (325 bp; nt 10 through 335); whole *msp-4*; the 3′ half of *msp-2* (560 bp; nt 666 through 1226); or the 3′ end of *msp-2* (348 bp; nt 878 through 1226). Molecular size markers in kilobases are indicated at the left.

Presence of multiple *msp-2* gene copies. *A. marginale* genomic DNA extracted from either the Florida strain or the South Idaho strain was digested with restriction enzymes, Southern blotted, and probed with either whole *msp-2* or PCR-generated probes representing 5′ or 3′ *msp-2* regions. Hybridization with whole pCKR11.2 *msp-2* identified multiple partially homologous sequences in all five restriction enzyme digests of both the homologous Florida strain and the South Idaho strain (Fig. 5). *Apa*I, *Bgl*I, and *Nco*I do not cut within *msp-2* (on the basis of the pCKR11.2 sequence; the *Nco*I site in pCKR11.2 was created by PCR cloning and was not in the genomic copy) and therefore should generate a single band if only one *msp-2* genomic copy is present. There is a single *Hin*dIII site (at nt 22), which should yield two bands for a single-copy gene. There are two *Kpn*I sites (at nt 638 and 668), which should also result in two observable bands for a single-copy gene, since the small 30-bp fragment would not be seen. Therefore, the multiple bands that hybridized with pCKR11.2 *msp-2* represent partially homologous *msp-2* copies. It is unlikely that these bands represent 5′ or 3′ sequences conserved among otherwise unrelated *A. marginale* genes, as the nonoverlapping 5′-half and 3′-half probes hybridized to the multiple bands identified with the whole *msp-2* probe (Fig. 5). The exact number of *msp-2* copies cannot be determined, since restriction site polymorphisms in other *msp-2* copies may result in the production of several bands from a single copy. Hybridization of the digested genomic DNA with *msp-4*, a single-copy gene (23, 24), revealed the expected number of bands and provided a control for multiple bands due to incomplete genomic DNA digestion. *Apa*I, *Bgl*I, *Kpn*I, and *Nco*I do not cut within *msp-4* (23) and therefore generated a single band; *Hin*dIII cleaves *msp-4* once (23, 24) and yielded the expected two bands (Fig. 5).

To determine if defined *msp-2* regions were conserved

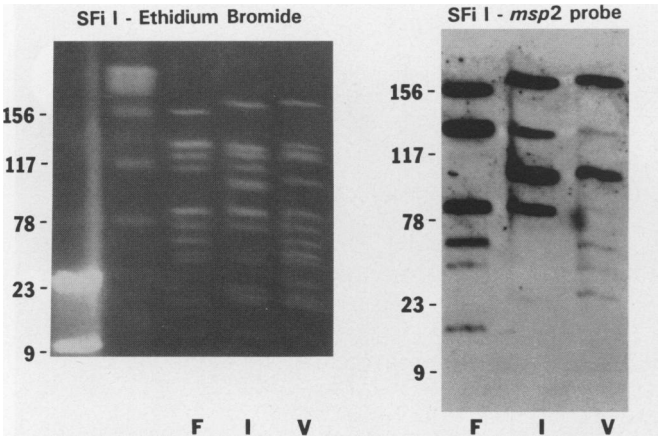


FIG. 6. Distribution of *msp-2* copies in the *A. marginale* chromosome. *Sfi*I-digested *A. marginale* genomic DNA (F, Florida strain; I, South Idaho strain; V, Virginia strain) separated by clamped homogeneous electric field gel electrophoresis and stained with ethidium bromide. The first two lanes contain lambda DNA-*Hin*dIII fragments and Promega Delta 39 markers, respectively, as size markers. The fragments were Southern blotted and hybridized with whole pCKR11.2 *msp-2*. Molecular size markers in kilobases are indicated at the left of each panel.

among multiple copies, the digested genomic DNA was probed with 5′-end, 5′-half, 3′-half, and 3′-end probes. Comparison of the results from hybridization of the region-specific probes with the whole *msp-2* probe indicated that the largest number of copies was detected by using the 5′-half probe. This suggested that the 5′ region (nt 151 through 663) was largely conserved among the different copies. In contrast, the 5′-end probe bound relatively few of the multiple copies, consistent with a more highly polymorphic region extending from nt 10 through 335. The 3′-half and 3′-end probes bound fewer copies than the 5′-half probe but more than the 5′-end probe; this is indicative of some polymorphism in these 3′ regions.

Distribution of *msp-2* copies in the *A. marginale* chromosome. *Sfi*I digestion of *A. marginale* genomic DNA resulted in large fragments separated by clamped homogeneous electric field electrophoresis (Fig. 6). These fragments have been previously shown to be nonoverlapping and to represent the entire 1,250-kb *A. marginale* genome (2). The fragments were blotted onto a nylon membrane and probed with whole pCKR11.2 *msp-2*. Partially homologous *msp-2* copies were detected in six large chromosomal fragments from the Florida (159, 129, 97, 57, 41, and 17.5 kb) and Virginia (185, 135, 112, 61, 41, and 31 kb) strains and in five chromosomal fragments from the South Idaho strain (176, 132, 110, 97, and 31 kb).

MAb reactivity with recombinant MSP-2. MAbs ANAF 19E2 and ANAR20A and anti-MSP-2 rabbit serum R883 bound recombinant *E. coli* expressing pCKR11.2 *msp-2* as detected by immunofluorescence (Table 1). In addition, these three antibodies bound the surface of recombinant *E. coli* expressing *msp-2* as detected by immunofluorescence assay of live bacteria. Anti-MSP-2 MAbs ANAO45A2, ANAO50A2, ANAO58A2, ANAO66A2, ANAO70A2, and ANAR79A were unreactive with pCKR11.2 *msp-2* (Table 1). The negative control MAbs TRYP1E1 and 14/16.1.7 and the normal rabbit serum did not bind either acetone-fixed or live recombinant *msp-2*-expressing *E. coli*. All anti-MSP-2 MAbs reacted with *A. marginale* Florida but bound <75% of the organisms present, as previously described (20, 28). As a positive control, MAb ANA15D2, directed against MSP-1a (27), bound 100% of the *A. marginale* organisms present, as previously described (20, 28). None of the MAbs or sera tested reacted with control *E. coli* transformed with the nonrecombinant pKK233-2 or with uninfected bovine erythrocytes.

TABLE 1. Reactivity of anti-MSP-2 antibodies with *A. marginale* Florida and *E. coli* expressing pCKR11.2 *msp-2*

Monoclonal antibody	Reactivity with *A. marginale* as determined by fixed IFA[a]	Reactivity with pCKR11.2 MSP-2 as determined by:	
		Fixed IFA[a]	Live IFA[b]
R883[c]	+	+	+
NRS[d]	−	−	−
ANAR20A	+	+	+
ANAF19E2	+	+	+
ANAR79A	+	−	−
ANAO45A2	+	−	−
ANAO50A2	+	−	−
ANAO58A2	+	−	−
ANAO66A2	+	−	−
ANAO70A2	+	−	−
14/16.1.7	−	−	−
TRYP1E1	−	−	−

[a] Fixed IFA, immunofluorescence on acetone-fixed organisms.
[b] Live IFA, immunofluorescence on live bacteria.
[c] R883, polyclonal rabbit antibody against native MSP-2.
[d] NRS, normal rabbit serum.

Coexpression of unique *msp-2* copies. MAb ANAF19E2, which binds an epitope expressed by the cloned pCKR11.2 *msp-2* copy, and ANAO50A2, which is unreactive with the pCKR11.2-expressed copy, bound MSP-2 primarily on distinct subpopulations of *A. marginale* Florida (Fig. 7A and B). However, individual *A. marginale* organisms that expressed both MSP-2 epitopes were identified (Fig. 7A and B). A similar pattern of reactivity was observed when ANAF19E2 was tested with either ANAR79A, ANAO66A2, or ANAO70A2 (data not shown).

DISCUSSION

The evidence that the pCKR11.2 clone accurately represents a genomic *msp-2* copy includes (i) the expression of a protein that comigrates with native MSP-2 and is bound by anti-MSP-2 MAbs ANAF19E2 and ANAR20A, (ii) the immunoreactivity of the expressed protein with rabbit polyclonal antibody produced by immunization with native MSP-2, (iii) the identity of the deduced amino acid sequence following the predicted signal peptide cleavage site with the native MSP-2 N-terminal amino acid sequence, and (iv) the alignment of six restriction enzyme fragments from the clone, extending from nt 22 to 1084, with the genome.

Southern blots probed with the pCKR11.2 *msp-2* copy identified multiple partially homologous *msp-2* copies in each of two strains, Florida and South Idaho. In addition, *msp-2* hybridization to several large nonoverlapping chromosomal fragments indicated that the multiple *msp-2* copies also occur in the Virginia strain. The number of *msp-2* copies present cannot be conclusively determined because the restriction sites selected for cutting external to or once within the pCKR11.2 *msp-2* sequence may be polymorphic in other copies and would therefore misrepresent the copy number. However, if 7 to 10 copies are present, each with an average size of 1.2 kb, the size needed to encode MSP-2 (all described MSP-2 molecules migrate at 33 to 41 kDa) (1, 21, 22, 31), ≥1% of the 1,250-kb *A. marginale* genome may be attributed to *msp-2* (2). Unlike

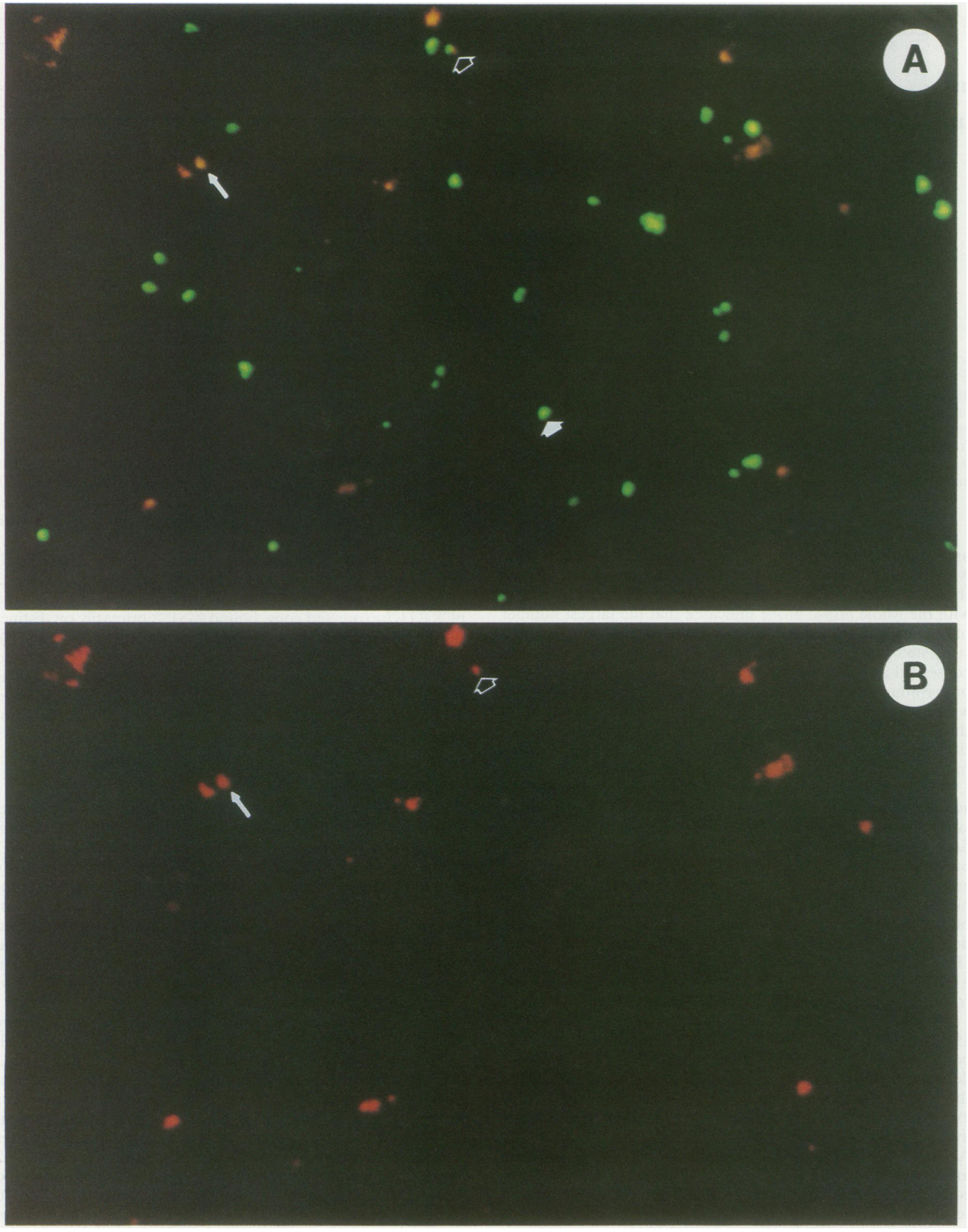

A
B

FIG. 7. Reactivity of MSP-2-copy-specific MAbs with *A. marginale* Florida. Reactivity of MAb ANAF19E2 (observed in panel A by using fluorescein-labeled goat anti-murine IgG1) and MAb ANAO50A2 (observed in panel B by using phycoerythrin-labeled goat anti-murine IgG2a; also observed as faint orange fluorescence in panel A) with *A. marginale* in the same microscopic field is shown. *A. marginale* organisms reactive with only ANAF19E2 (solid arrowhead), only ANAO50A2 (solid arrow), and both ANAF19E2 and ANAO50A2 (open arrowhead) were identified.

the members of many multigene families that arise by gene duplication and are present in tandem, the *msp-2* copies are widely distributed throughout the chromosome. This is also consistent with an unusually large representation of *msp-2* in the genome. The wide dispersion of *msp-2* copies throughout the chromosome suggests that any coordinated control of *msp-2* expression would involve *trans*-regulation.

Determination of the exact genetic and antigenic polymorphism of MSP-2 will require cloning and sequencing of additional *msp-2* copies. The hybridization data obtained by using 5′- and 3′-specific probes suggest that there is substantial nucleotide sequence variation among *msp-2* copies. In particular, probes based on the 5′ end bound only 1 to 3 of the partially homologous *msp-2* copies identified by using the whole *msp-2* probe. The relevance of *msp-2* nucleotide sequence polymorphism as a mechanism for epitope variation is indicated by the identification of copy-specific anti-MSP-2 MAbs. The pCKR11.2 MSP-2 reacted with only a subset (ANAF19E2 and ANAR20A) of the anti-MSP-2 MAbs reactive with the parent Florida strain. Two-color analysis of MAb binding to individual *A. marginale*-infected erythrocytes, each of which represents infection with a single organism, revealed that organisms may express an MSP-2 bearing epitopes represented on pCKR11.2 MSP-2 (bound by MAb ANAR20A or ANAF19E2) or an antigenically distinct MSP-2 bearing epitopes not represented on pCKR11.2 MSP-2. The ability of individual organisms to coexpress different *msp-2* copies is suggested by the binding of ANAF19E2 and ANAO50A2 to the same organism and the binding of both MAbs to discrete populations of organisms. The presence of multiple polymorphic *msp-2* copies expressed by *A. marginale* explains, at least in part, the observation that not all organisms belonging to a strain are reactive with a given anti-MSP-2 MAb (20). The present data suggest, but do not prove, that individual organisms each express at least one *msp-2* but that the degree of polymorphism is sufficiently great that no one MAb recognizes all expressed copies.

Whether this MSP-2 antigenic polymorphism plays a significant role in the recurrent rickettsemia that occurs throughout persistent infection is unclear (10, 11). During cyclic rickettsemia, *A. marginale* levels increase from $<10^4$ to $>10^6$ infected erythrocytes per ml (11, 14). The rickettsemia increases over a 10- to 14-day period and then rapidly diminishes, presumably under the control of a primary immune response to an antigenic variant (14). Identification of unique *msp-2* copy-specific sequences and epitopes will allow determination of whether antigenically variant MSP-2 copies arise during the rickettsial cycles.

The availability of cloned pCKR11.2 *msp-2* will allow testing of a recombinant MSP-2 vaccine for protection against acute disease. The expression of pCKR11.2 *msp-2* on the surface of recombinant *E. coli* and the surface exposure of the B-cell epitopes bound by MAbs ANAR20A and ANAF19E2 suggest that the recombinant MSP-2 is correctly inserted in the outer membrane. In addition, sera from cattle immunized with native affinity-purified MSP-2, shown to be protected following experimental *A. marginale* challenge (31), bound the ANAF 19E2-defined B-cell epitope on the recombinant *E. coli* surface (data not shown). If a single-copy-based MSP-2 immunogen can protect against challenge, this will suggest that conserved B- or T-cell epitopes are present among different MSP-2 proteins and that immunized cattle can respond to these epitopes. Analysis of sequences from additional *msp-2* gene copies will facilitate the mapping of these regions. The conservation of oligopeptide motifs between MSP-2 and MSP-4 may also be used to identify relevant epitopes. Recombinant MSP-4 has recently been shown to induce immunity against Florida strain challenge (5). The question of whether the regions conserved between MSP-4, encoded by a single-copy gene, and pCKR11.2 MSP-2 represent B- or T-cell epitopes and whether these regions are also conserved among the other MSP-2 copies is currently under investigation.

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