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Expression of Major Surface Protein 2 Antigenic Variants during Acute *Anaplasma marginale* Rickettsemia

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Antigenic variants of *Anaplasma marginale* major surface protein 2 (MSP-2), a target of protective immune responses, have been detected by use of copy-specific monoclonal antibodies reactive with some, but not all, organisms during acute rickettsemia. The presence of polymorphic *msp-2* genes was confirmed by cloning and sequencing two gene copies, 11.2 and DF5, each of which encodes a full-length MSP-2 with a unique amino acid sequence. Transcription of *msp-2* genes during acute rickettsemia was analyzed by use of cDNA cloning of hybrid-selected *msp-2* mRNA. Sequencing of cDNA clones, designated AR1 to AR14, indicated that DF5 *msp-2* was transcribed during acute rickettsemia. Two classes of variant *msp-2* genes were also transcribed during acute rickettsemia. The first class of variant transcripts, typified by clones AR3, AR4, AR7, and AR14, each encoded a single or small number of amino acid substitutions relative to DF5. The second type, AR5, encoded a large region of amino acid polymorphism, including additions, deletions, and substitutions, as compared to DF5. Specific antibody directed against the AR5 polymorphic region bound a unique MSP-2 expressed on *A. marginale* that was not recognized by antibody generated against DF5. Similarly, anti-AR5 peptide antibody reacted with a different MSP-2 that was not bound by anti-DF5 antibody. This expression confirmed that variant *msp-2* transcripts encode structurally distinct MSP-2 molecules which bear unique B-cell epitopes. These results support the hypothesis that the large *msp-2* gene family, which constitutes a minimum of 1% of the genome, encodes antigenic variants critical to evasion of a protective immune response directed against surface MSP-2 epitopes.

Anaplasma marginale is an arthropod-borne rickettsial pathogen of cattle that invades and replicates in mature erythrocytes (7). Acute infection is characterized by high levels of rickettsemia (>10^9 infected erythrocytes per ml) and severe anemia which frequently results in abortion or death (7). Immunity against acute *A. marginale* rickettsemia is directed against outer membrane surface proteins (10, 15, 16), and cattle immunized with *A. marginale* outer membranes develop significantly lower levels of rickettsemia following challenge than adjuvant-immunized controls do (13, 15, 18). However, immunized animals do not completely clear *A. marginale* and remain persistently infected (3, 5). Development and maintenance of persistent infection, in which continuous cycles of low-level rickettsemia occur, have been hypothesized to reflect the emergence of antigenic variants (6).

The presence of *A. marginale* antigenic variants during acute rickettsemia is suggested by the detection of a small percentage of organisms that express epitopes on the major surface protein 2 (MSP-2) that are different from the epitopes expressed by the majority of organisms (9, 11). This variation in MSP-2 epitope expression has been observed consistently during acute rickettsemia regardless of the *A. marginale* strain used to initiate infection (9). The mechanism by which the variant MSP-2 epitopes are generated is unknown. Recently, we cloned and expressed a single complete *msp-2* gene copy, designated 11.2. Using the 11.2 *msp-2* as a probe, we identified a *msp-2* gene family that is represented extensively in the *A. marginale* genome and distributed widely throughout the chromosome (11). We hypothesize that these *msp-2* genes encode structurally unique polypeptides which result in the expression of variant MSP-2 epitopes. In this article, we report the testing of this hypothesis by examining the transcription and expression of *msp-2* genes during acute rickettsemia.

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MATERIALS AND METHODS

Cloning and sequencing of genomic copies of *msp-2*. The original 11.2 *msp-2* was obtained by immunoscreening a *msp-2* library generated from *A. marginale* Florida genomic DNA with PCR amplification. The selection of the 5′ and 3′ primers, immunoscreening with monoclonal antibody AnaF19E2, expression, and sequence of the 11.2 *msp-2* have been described previously (11). A second genomic copy, designated DF5, was isolated from the PCR-generated *msp-2* genomic library by screening with a 32-base oligonucleotide (Fig. 1A). This oligonucleotide was derived from *msp-2* clone pCKR5.2 selected from a library of *Pst*I-digested genomic *A. marginale* DNA inserted into pKK233-2 by immunoscreening transformed *Escherichia coli* XL-1 Blue with anti-MSP-2 monoclonal antibody ANAR20A (data not shown). The sequence of pCKR5.2, truncated at its 5′ end, was identical to that of 11.2 except for a central region with 70% identity over 119 nucleotides. The 32-base oligonucleotide was selected from a region of pCKR5.2 sequence that was 50% identical with that of the 11.2 *msp-2*. The PCR-generated genomic library was screened by colony hybridization with the 32-base oligonucleotide, which was labeled with digoxigenin 11-ddUTP by using terminal transferase (17). A single clone, DF5, was selected and plasmid DNA was extracted for double-stranded sequencing with sequentially derived primers initiating dideoxynucleotide chain reactions. Sequence analysis with the Genetics Computer Group package from the University of Wisconsin, version 7.3, was performed on a Vax11/785 computer.

Isolation of *msp-2* mRNA expressed during acute rickettsemia. A seronegative Holstein calf (no. 198) was inoculated with 10^{10} erythrocytes infected with the Florida strain of *A. marginale*. Peripheral blood was collected daily and examined microscopically for infected erythrocytes by use of Giemsa-stained smears. At a rickettsemia level of 70% infected erythrocytes, total RNA was isolated and extracted from whole blood by use of lithium chloride and urea (1, 19). To confirm that the total RNA preparation from rickettsemic blood contained *msp-2* RNA, the total RNA was Northern (RNA) blotted and probed with labeled 11.2

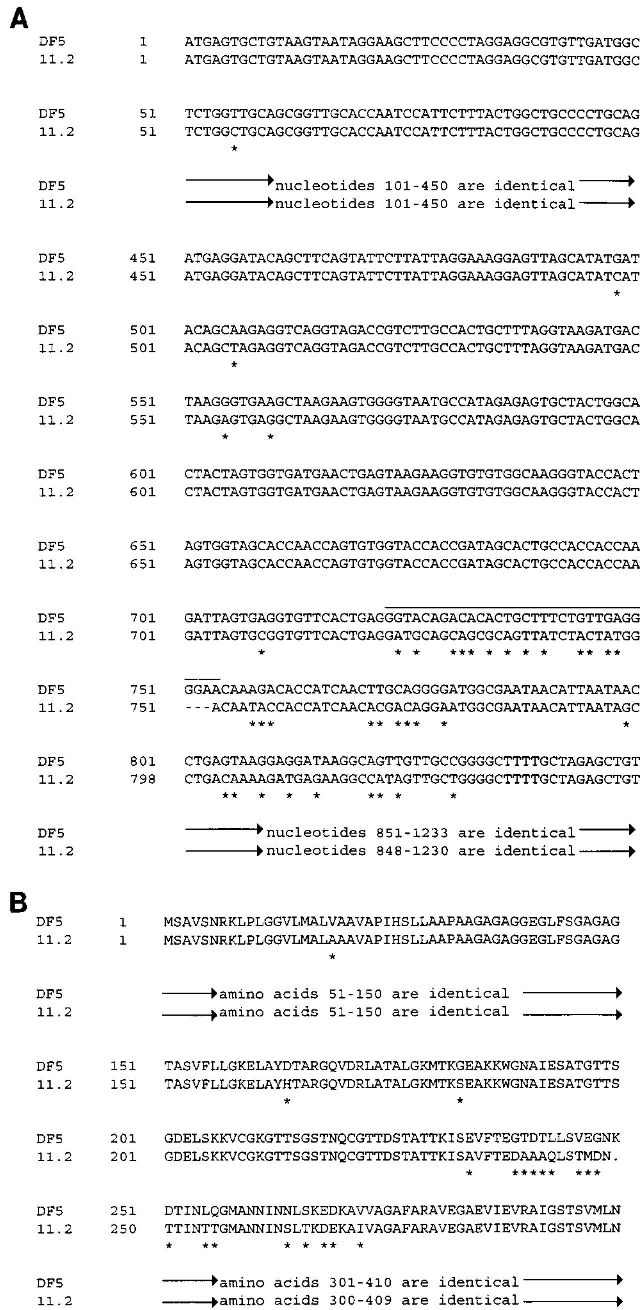

FIG. 1. (A) Nucleotide sequence comparison between 11.2 and DF5 *msp-2* gene copies. The asterisks designate nucleotide substitutions, and the dashes designate deletions. The overline from DF5 nt 723 to 754 indicates the oligonucleotide used to screen the genomic library for DF5 *msp-2*. (B) Amino acid sequence comparison between 11.2 and DF5 MSP-2. The asterisks designate amino acid substitutions, and the dot designates a deletion.

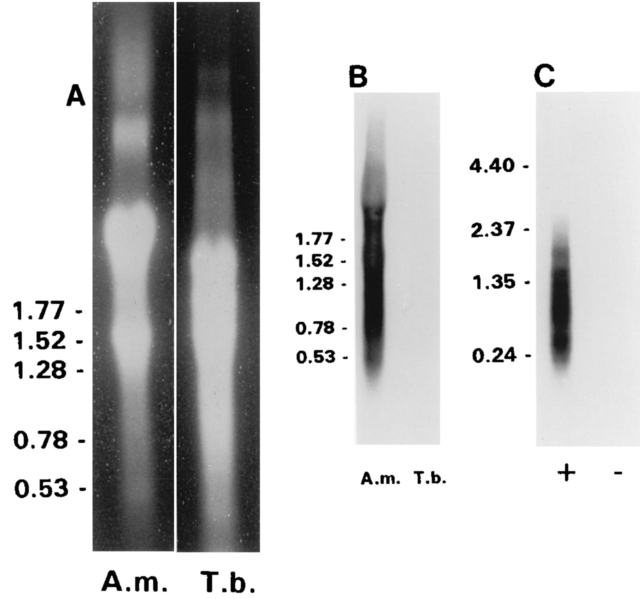

FIG. 2. (A) Ethidium bromide stain of electrophoretically separated total RNA isolated from *A. marginale* rickettsemic blood (A.m.) or *T. brucei* parasitemic blood (T.b.). (B) Hybridization of digoxigenin-labeled 11.2 *msp-2* with total RNA isolated from *A. marginale* rickettsemic blood (A.m.) or *T. brucei* parasitemic blood (T.b.). (C) Hybridization of digoxigenin-labeled 11.2 *msp-2* with *A. marginale* RNA hybrid selected on either nitrocellulose filters containing 11.2 *msp-2* DNA (+) or filters containing bovine DNA (−).

msp-2 DNA. Total RNA from *A. marginale* rickettsemic blood was electrophoresed in a denaturing gel, transferred in a 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer to a nylon membrane, and fixed by UV light cross-linking (17). Digoxigenin labeling of 11.2 *msp-2*, prehybridization, hybridization, and detection of bound probe by use of chemiluminescence have been described previously (11). Total RNA obtained from *Trypanosoma brucei* parasitized rat blood was processed identically and included as a negative control. *A. marginale msp-2* RNA was specifically isolated from total RNA by a hybrid select procedure (8). Twenty micrograms of 11.2 *msp-2* DNA or, as a negative control, 20 μg of bovine thymus DNA was cross-linked to 3-mm^2 nitrocellulose filters with UV light. The filters were hybridized with 50 μg of total RNA isolated from *A. marginale* rickettsemic blood in a hybridization solution of 20 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); pH 6.4], 65% (vol/vol) formamide, 0.25% (wt/vol) sodium dodecyl sulfate (SDS), and 0.4 M NaCl. Following hybridization at 50°C for 3 h, the filters were washed 10 times in a 65°C solution of 10 mM Tris (pH 7.6), 0.15 M NaCl, 1 mM EDTA, and 0.5% SDS. The filters were washed an additional two times in the wash solution without SDS, and then bound RNA was eluted with boiling water. The eluted mRNA was extracted with phenol and chloroform, precipitated with ethanol, and then treated with RNase-free DNase to eliminate any possible *msp-2* DNA freed from the nitrocellulose. The eluted *msp-2* mRNA was Northern blotted and probed with the digoxigenin-labeled 11.2 *msp-2* as already described. As a control, *A. marginale* RNA eluted from the bovine DNA-containing nitrocellulose filters was also Northern blotted and probed with 11.2 *msp-2*.

Synthesis, cloning, selection, and sequencing of *msp-2* cDNA. The hybrid-selected *msp-2* mRNA was used as a template for first-strand cDNA synthesis with random hexamer primers and SuperScript RNase H$^-$ reverse transcriptase (Gibco). Following second-strand synthesis and addition of *Eco*RI adapters (17), cDNA inserts were ligated into *Eco*RI-digested and dephosphorylated pGEM-7Zf(+). Competent *E. coli* XL-1 Blue was transformed with ligated vector and screened with digoxigenin-labeled 11.2 *msp-2* and chemiluminescence. To identify clones with unique *msp-2* inserts, plasmid DNA was digested with *Eco*RI and Southern blotted with 11.2 *msp-2* as a probe, and the 5′ and 3′ ends of each insert were sequenced. Plasmid DNA from unique clones was double strand sequenced, and sequences were analyzed as described above. With the prefix AR for acute rickettsemia, cDNA clones were designated AR1 through AR14.

Generation of antibody against polymorphic MSP-2 peptides and identification of variant MSP-2 expressed during acute rickettsemia. Peptides were selected from the polymorphic regions of the derived amino acid sequences of DF5 and AR5 *msp-2* transcripts. A 15-residue DF5 peptide (amino acids 221 to 235) and a 17-residue AR5 peptide (amino acids 217 to 233) were synthesized and coupled to maleimide-activated keyhole limpet hemocyanin (Pierce). Mice were immunized subcutaneously with peptide-keyhole limpet hemocyanin (50 μg of total protein) emulsified in complete Freund's adjuvant. Following three subsequent immunizations in incomplete Freund's adjuvant, sera were obtained and tested for reactivity with MSP-2 expressed during acute rickettsemia. Lysates were prepared from *A. marginale*-infected erythrocytes collected from animal 198 during acute rickettsemia and electrophoresed on SDS-containing polyacrylamide gels as described previously (11, 14). Separated proteins were transferred electrophoretically to nitrocellulose filters. Membranes were reacted with either a 1:500 dilution of one of the anti-MSP-2 peptide sera or a negative control anti-bovine interleukin 4 peptide serum. Bound antibody was detected with peroxidase-labeled goat anti-mouse immunoglobulin G with enhanced chemiluminescent detection (11).

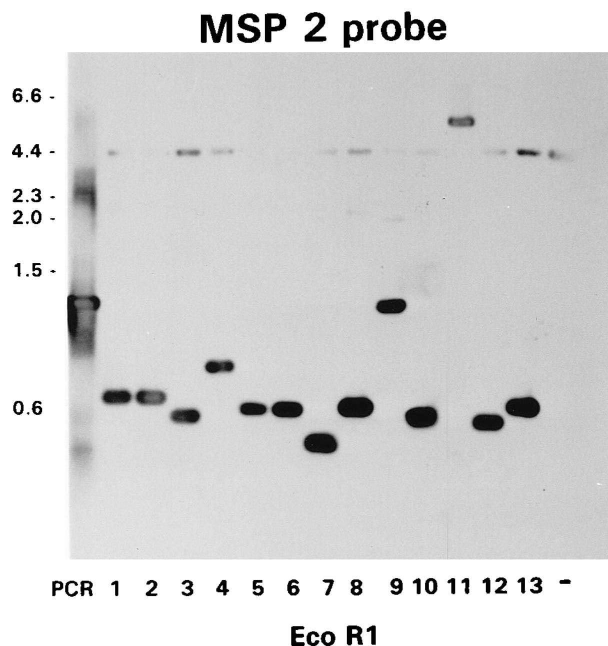

FIG. 3. Determination of *msp-2* cDNA insert sizes by *Eco*RI digestion of plasmid DNA followed by electrophoresis and hybridization with digoxigenin-labeled 11.2 *msp-2*. Lanes: 1 to 13, excised inserts from cDNA clones AR1 to AR13, respectively; PCR, positive control containing PCR-amplified 11.2 *msp-2* DNA; −, negative control containing pGEM-7Zf(+) DNA without an insert. *Eco*RI digestion of plasmid DNA from AR11 linearized the plasmid but did not excise the *msp-2* insert (lane 11). AR14 had an *msp-2* insert size of <0.2 kb (data not shown).

Nucleotide sequence accession number. The nucleotide sequences of the *msp-2* genes have been assigned the following GenBank accession numbers: 11.2 *msp-2*, UO7862; DF5 *msp-2*, U36193.

RESULTS

Cloning and sequencing of genomic copies of *msp-2*. Hybridization with the 32-base oligonucleotide identified a genomic *msp-2* clone, designated DF5, with a nucleotide sequence different from that of the initial 11.2 *msp-2* genomic clone (Fig. 1A). The nucleotide sequences encoding the MSP-2 open reading frames were 97% identical (Fig. 1A). Nucleotide substitutions and the addition of 3 bases in the DF5 gene were clustered (Fig. 1A), resulting in a region of 55% identity (11.2, nucleotides [nt] 724 to 776; DF5, nt 724 to 779). As a result of the addition of a codon at nucleotide 751 in the DF5 sequence, the open reading frame for DF5 was 410 amino acids, as compared with 409 amino acids for 11.2 (Fig. 1A). The nucleotide substitutions and additions resulted in an overall amino acid identity of 95% (Fig. 1B). The amino acid differences between DF5 and 11.2 were clustered primarily between DF5 amino acids 234 to 272 (11.2, amino acids 234 to 271), resulting in sequence identity of 54% in this region (Fig. 1B). The signal peptide cleavage site (following the alanine at position 30) and the stop codon (11) were conserved between the two *msp-2* genomic clones.

Isolation of *msp-2* mRNA expressed during acute rickettsemia. Total RNA was isolated during acute *A. marginale* rickettsemia or, as a negative control, from acute *T. brucei* parasitemia (Fig. 2A). Specific hybridization with 11.2 *msp-2* as a probe indicated that *msp-2* mRNA had been obtained (Fig. 2B). There was no 11.2 *msp-2* hybridization to *T. brucei* RNA (Fig. 2B). The *msp-2* mRNA was hybrid selected with 11.2

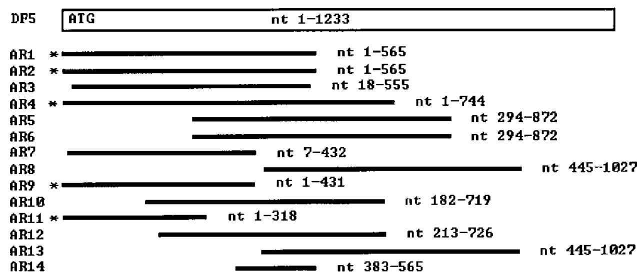

FIG. 4. Alignment of cDNA clones AR1 to AR14 with the DF5 *msp-2* gene based on nucleotide sequence comparison. The asterisk indicates clones in which the sequence extended 5′ to the MSP-2 open reading frame.

msp-2 DNA-loaded nitrocellulose filters or, as a negative control, bovine thymus DNA-loaded nitrocellulose filters. Following elution, RNA was treated with RNase-free DNase. RNA eluted from the *msp-2* DNA filters hybridized with an 11.2 *msp-2* probe in Northern blots, indicating that *msp-2* mRNA had been selected (Fig. 2C, "+" lane). There was no hybridization of the 11.2 *msp-2* probe to RNA eluted by an identical protocol from bovine thymus DNA-loaded filters (Fig. 2C, "−" lane).

***msp-2* cDNA clones.** The cDNA clones derived from *msp-2* hybrid-selected mRNA were screened by colony hybridization with 11.2 *msp-2* DNA as a probe. Fourteen positive clones were identified and designated AR1 to AR14. Digestion of plasmid DNA with *Eco*RI and hybridization with 11.2 *msp-2* DNA revealed a range of *msp-2* insert sizes. Inserts from clones AR1 to AR13 are shown in Fig. 3 and vary from approximately 0.4 to 1.2 kb. *Eco*RI digestion of plasmid DNA from AR11 linearized the plasmid but did not excise the *msp-2* insert (Fig. 3), indicating that one of the restriction sites had been lost and a possible artifact had been introduced. AR14 had an *msp-2* insert size of <0.2 kb (not shown). The 5′ and 3′ ends of AR1 to AR14 were sequenced by primer extension from the SP6 and T7 vector sequences and aligned relative to the published sequence of 11.2 *msp-2* (Fig. 4). The regions corresponding to the *msp-2* open reading frame were sequenced, with both strands of DNA, for clones AR1, -3 to -5, -7 to -12, and -14. For pairs of clones that had the same-size insert with identical 5′ and 3′ sequences (AR1 and AR2, AR5 and AR6, and AR8 and AR13), only one clone was sequenced. The sequences of clones AR1 and AR7 to AR12 were identical to the sequence of the DF5 genomic copy. The identical cDNA sequences extended from nt 1 to nt 1027 of the 1,233-bp DF5 *msp-2* sequence. No AR cDNA clone represented the region from nt 1028 to 1233 (Fig. 4). Clones AR3, -4, and -14 each had a single (AR4) or several (AR3 and AR14) base substitutions relative to the sequence of DF5 (Table 1). Three of these nucleotide substitutions resulted in amino acid changes, and

TABLE 1. Nucleotide and amino acid changes between DF5 and clones AR3, -4, and -14

Nucleotide substitution	Clone no.	Amino acid change
nt 164 G→T	AR4	G→V (residue 55)
nt 490 G→C	AR3, AR14	D→H (residue 164)
nt 498 A→T	AR3, AR14	None (residue 166)
nt 547 G→A	AR3	G→S (residue 183)
nt 552 A→G	AR3	None (residue 184)
nt 555 T→C	AR14	None (residue 185)

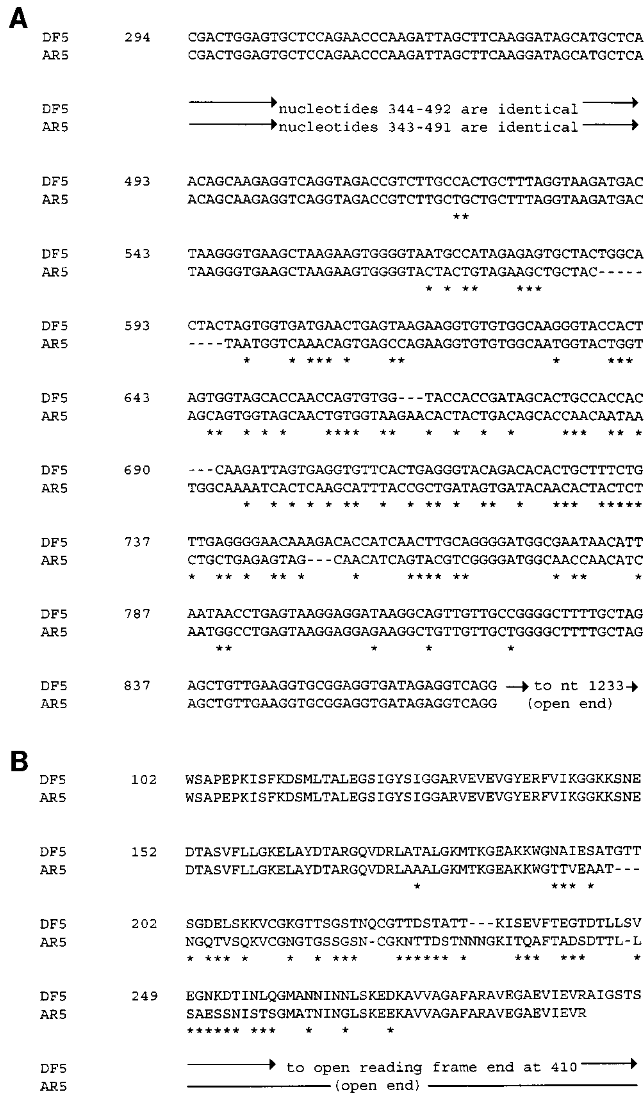

FIG. 5. (A) Nucleotide sequence comparison between AR5 cDNA and DF5 *msp-2*. The asterisks designate nucleotide substitutions, and the dashes designate deletions. (B) Amino acid sequence comparison between AR5 and DF5 MSP-2. The asterisks designate amino acid substitutions, and the dashes designate deletions.

three were silent (Table 1). Clone AR5 contained a large region of variant nucleotide sequence, including additions, deletions, and substitutions, in comparison with the DF5 *msp-2* sequence (Fig. 5A). The variant region extended from DF5 nt 522 to DF5 nt 825 and was flanked by regions of identical sequence. These nucleotide changes resulted in five amino acid deletions and three additions as well as numerous substitutions in AR5, as compared with the sequence of DF5 (Fig. 5B). Within the variant region (DF5 amino acids 177 to 271), the identity between AR5 and DF5 MSP-2 was 47%. Assuming that the AR5 flanking regions continue to be invariant to the 5′ and 3′ ends of the open reading frame, AR5 *msp-2* encoded two fewer amino acids, with a predicted MSP-2 molecular size of 42,092 Da, as compared with a size of 42,548 Da for DF5 MSP-2. Without the signal peptide, the predicted molecular sizes are 39,102 Da for AR5 MSP-2 and 39,566 Da for DF5 MSP-2.

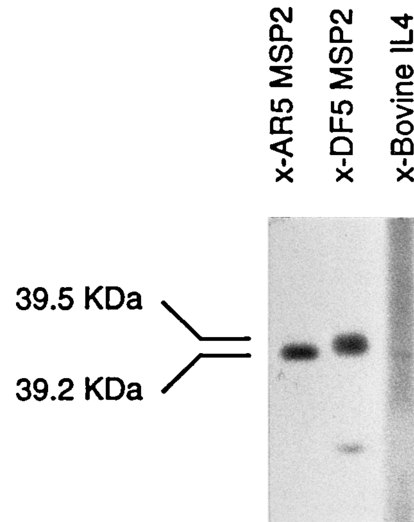

FIG. 6. Binding of antibody directed against MSP-2 polymorphic regions with distinct MSP-2 molecules expressed during acute rickettsemia. *A. marginale* was isolated during acute rickettsemia, and lysates were separated electrophoretically in SDS-containing polyacrylamide gels, transferred to nitrocellulose, and reacted with anti-AR5 peptide antibody (lane 1), anti-DF5 antibody (lane 2), or, as a negative control, anti-bovine interleukin-4 peptide antibody (lane 3). The apparent molecular sizes of the bound MSP-2 molecules are indicated in the left margin.

Identification of antigenically variant MSP-2 during acute rickettsemia. Antibody raised against the 15-residue DF5 peptide (amino acids 221 to 234) or the 17-residue AR5 peptide (amino acids 217 to 233) bound MSP-2 polypeptides in *A. marginale*-infected erythrocytes collected from animal 198 during acute rickettsemia. The anti-DF5 antibody reacted with a MSP-2 polypeptide with an apparent molecular size of 39.5 kDa, as compared with the apparent molecular size of 39.2 kDa for the polypeptide bound by the anti-AR5 antibody (Fig. 6). The anti-DF5 antibody did not react with the 39.2-kDa MSP-2, nor did anti-AR5 antibody react with the larger 39.5-kDa MSP-2 (Fig. 6).

DISCUSSION

The hybridization of 11.2 *msp-2* with numerous sequences present throughout the *A. marginale* chromosome suggested the presence of a large, multigene family capable of encoding polymorphic MSP-2 molecules (11). Whether these sequences, accounting for at least 1% of the genome, included full-length genes and were capable of being transcribed and translated in vivo was unknown. Genomic cloning and sequencing of the DF5 *msp-2* indicated that the chromosome does indeed contain additional polymorphic full-length *msp-2* genes. The polymorphism between the 11.2 and DF5 genomic clones is relatively limited but results in amino acid sequence variation. This limited polymorphism probably does not reflect the range of variation that occurs at the genomic level because DF5 was selected from an *msp-2* library generated by amplification of chromosomal DNA with primers based on the 11.2 sequence.

Importantly, the generation of cDNA clones with sequences identical to that of the DF5 genomic clone strongly suggests that the DF5 genomic copy is transcribed in vivo. The *msp-2*

mRNA used to generate the cDNA clones was hybrid selected on 11.2 *msp-2* DNA. However, despite this possible bias, none of the cDNA clones obtained during acute rickettsemia were identical to the 11.2 *msp-2* genomic clone. The 11.2 *msp-2* gene may not have been transcribed at the time blood was collected during acute rickettsemia, or alternatively, transcription occurred at a very low level or in very few organisms and was not highly represented among the cDNA library.

In addition to transcription of DF5 *msp-2*, two classes of variant transcripts were isolated during acute rickettsemia. The first class of variant transcripts, typified by clones AR3, AR4, AR7, and AR14, each encoded a single or small number of amino acid substitutions relative to the sequence of DF5. None of the substitutions altered the open reading frame or introduced a stop codon. However, the possibility that the substitutions were artifactually generated during the single round of reverse transcription prior to cDNA cloning has not been excluded. In addition, whether these transcripts result in expression of antigenically unique MSP-2 molecules is unknown. The second class is represented by clone AR5, which has a substantial region of nucleotide polymorphism, including substitutions, additions, and deletions, as compared with DF5 *msp-2*. Significantly, this nucleotide polymorphism alters the MSP-2 protein sequence, resulting in a 94-amino-acid variant region with only 47% identity between AR5 MSP-2 and DF5 MSP-2 and expression of polypeptides with slightly different molecular sizes. The expression of structurally variant MSP-2 polypeptides has been shown previously by comparison of *A. marginale* strains and can be detected within a strain by two-dimensional electrophoresis of whole organisms (2, 20) or SDS-polyacrylamide gel electrophoretic separation and silver staining of purified MSP-2 (12). From the present study, it is clear that at least part of this structural polymorphism can be attributed to transcription of different *msp-2* genes. The differences between DF5 and the variant transcripts may represent a minimal estimate of *msp-2* polymorphism. All transcripts were hybrid selected on 11.2 DNA, which has an overall identity of 95% with DF5. Consequently, markedly different *msp-2* transcripts may not have been efficiently selected by use of the described hybridization conditions. Nonetheless, it is evident that the *msp-2* gene family is polymorphic and is capable of encoding structurally unique polypeptides.

The ability of antibodies specific for either DF5 or clone AR5 to bind distinct MSP-2 polypeptides in *A. marginale* from acute rickettsemia indicates that variant transcripts are expressed in vivo and encode unique MSP-2 polypeptides. The structural polymorphism results in expression of at least one unique B-cell epitope on clone AR5, as compared with DF5, as well as the loss of at least one epitope. Whether the DF5 and AR5 variant polypeptides are coexpressed on individual organisms or expressed on nonoverlapping subpopulations of organisms has not been tested. However, expression of antigenically unique MSP-2 variants by individual organisms during acute rickettsemia, identified with variant-specific monoclonal antibodies (11), demonstrates that the different surface polypeptides are not simply coexpressed on all organisms. MSP-2 is a target of protective immune responses during acute rickettsemia (14), and immunity against homologous, but not heterologous, strain challenge has been shown to correlate with antibody titer against MSP-2 (13, 18). The presence of organisms with antigenically distinct surface polypeptides suggests that effective control of acute rickettsemia requires immune responses to multiple MSP-2 variants.

These different transcripts and antigenically variant surface polypeptides may reflect distinct stable subpopulations that occur within an *A. marginale* strain and are proportionally expanded during acute rickettsemia. If so, this is a common feature of natural *A. marginale* acute rickettsemia since the MSP-2 antigenic variants are present regardless of the strain used to initiate infection (9). Alternatively, MSP-2 variants may arise relatively frequently during *A. marginale* replication and therefore reflect a mechanism for continuous antigenic variation. Although which of these scenarios occurs is still unknown, either one may limit the effectiveness of vaccines incorporating one or few MSP-2 variants in controlling acute rickettsemia. However, if, as we hypothesize, MSP-2 antigenic variants evade the immune response and are responsible for persistent cyclic rickettsemia, then the multiple polymorphic *msp-2* gene copies, polymorphic transcripts, and antigenically variant surface polypeptides must reflect a mechanism for rapid generation of variants within infected cattle. This hypothesis will be tested in future studies.

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