Antigenic Variation of Anaplasma Marginale: Major Surface Protein 2 Diversity during Cyclic Transmission between Ticks and Cattle

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The rickettsial pathogen Anaplasma marginale expresses a variable immunodominant outer membrane protein, major surface protein 2 (MSP2), involved in antigenic variation and long-term persistence of the organism in carrier animals. MSP2 contains a central hypervariable region of about 100 amino acids that encodes immunogenic B-cell epitopes that induce variant-specific antibodies during infection. Previously, we have shown that MSP2 is encoded on a polycistronic mRNA transcript in erythrocyte stages of A. marginale and defined the structure of the genomic expression site for this transcript. In this study, we show that the same expression site is utilized in stages of A. marginale infecting tick salivary glands. We also analyzed the variability of this genomic expression site in Oklahoma strain A. marginale transmitted from in vitro cultures to cattle and between cattle and ticks. The structure of the expression site and flanking regions was conserved except for sequence that encoded the MSP2 hypervariable region. At least three different MSP2 variants were encoded in each A. marginale population. The major sequence variants did not change on passage of A. marginale between culture, acute erythrocyte stage infections, and tick salivary glands but did change during persistent infections of cattle. The variant types found in tick salivary glands most closely resembled those present in bovine blood at the time of acquisition of infection, whether infection was acquired from an acute or from a persistent rickettsemia. These variations in structure of an expression site for a major, immunoprotective outer membrane protein have important implications for vaccine development and for obtaining an improved understanding of the mechanisms of persistence of ehrlichial infections in humans, domestic animals, and reservoir hosts.

Anaplasma marginale is an animal pathogen of major economic importance to livestock production throughout many areas of North and South America, Africa, Australia, and Asia (22). Anaplasmosis causes economic losses in the United States of approximately $300 million/year (cost in 1986 U.S. dollars) (20). A. marginale is classified as a genogroup II ehrlichial agent, closely related taxonomically to other animal and human ehrlichial pathogens (9). Genogroup I and II ehrlichial agents include Cowdria ruminantium, causative agent of heartwater disease in ruminants; Ehrlichia canis, a causative agent of canine ehrlichiosis; Ehrlichia chaffeensis, which causes human monocytic ehrlichiosis; and agents related or identical to Ehrlichia phagocytophila and Ehrlichia ewingii that cause animal and human granulocytic ehrlichioses. The human ehrlichioses are classified as emerging diseases, with >500 cases confirmed since 1985 and an estimated 5% fatality rate.

There are a number of common features to these ehrlichial infections. After an initial acute phase infections may persist for long periods, even with antibiotic treatment (2, 10, 11, 16, 17, 29). In persistent infections caused by A. marginale, use of DNA probes and quantitative PCR revealed recurrent cyclic peaks of rickettsemia that probably continue for the lifetime of an infected animal (13, 19). A. marginale organisms express an outer membrane protein, major surface protein 2 (MSP2), approximately 36 kDa in size, which is strongly recognized by B and T cells from infected animals and partially protects immunized animals against challenge (8, 12, 23, 24). MSP2 is significantly similar to the major outer membrane protein of other ehrlichial organisms in amino acid sequence and is also encoded by a multigene family (25). Like outer membrane proteins of an agent of human granulocytic ehrlichiosis, MSP2 contains a single hypervariable region in the central part of the molecule (14). In the recurrent peaks of a single infection caused by A. marginale there are at least three different genetic and antigenic variants of MSP2 expressed in each peak (15). We have demonstrated previously that the predominant msp2 mRNA transcript in erythrocyte stages of A. marginale is a polycistronic mRNA containing msp2 and three other genes. Also, we have demonstrated that msp2 variation proceeds through the formation of different sequence mosaics in the expression site for this polycistronic mRNA (4). The availability of the sequence of this msp2 expression site, together with the recent development of an in vitro culture system for A. marginale (21), permits analysis of the extent and limitation of msp2 variation as organisms cycle between their different stages in infected tick and mammalian cells.

MATERIALS AND METHODS

Derivation of A. marginale populations. An Oklahoma strain of A. marginale was propagated by in vitro culture, as described (6). Briefly, infected blood was

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collected in 1998 from a calf with clinical anaplasmosis from Wetumka, Okla., and was subinoculated into a susceptible, splenectomized calf. Blood collected at peak rickettsiaemia was frozen, thawed, and used as inoculum on confluent tick cell monolayers derived from *Ixodes scapularis* embryos. Colonies of *A. marginale* were grown on cell monolayers at 9 days postexposure, and infected tick cell monolayers reached 100% (terminal cultures) by 4 to 5 weeks postexposure. Cultures were passaged by placing terminal cultures onto fresh tick cell monolayers at a dilution of 1:5 to 1:10. By the third passage development of the cultured Oklahoma strain was similar to that of the Virginia strain described previously (21), and a 1.5 dilution resulted in 100% infection in 10 to 12 days.

After two serial passages were achieved, a 25-cm² flask with an infection of 90% was used to transmit a splenectomized calf (PA408). Cells were pipetted from the flask and disrupted with a ground-glass homogenizer. Cells were suspended in 1 ml of medium and inoculated intravenously. Calf PA408 was monitored daily for clinical signs and appearance of rickettsiae in Diff-Quik-stained blood films. Calf PA408 developed clinical anaplasmosis with a prepatent period of 20 days, a peak rickettsiaemia of 34%, and a minimum packed cell volume of 12%. When the *A. marginale* rickettsiaemia was approximately 30%, 200 male *Dermacentor variabilis* ticks were acquisition fed on calf PA408 for 7 days during the ascending rickettsiaemia. After the infected ticks were removed and held for 7 days in a humidity chamber, they were transmission fed on a second splenectomized calf (calf PA407). The *D. variabilis* ticks received a rickettsiaemia of 64%, with a prepatent period of 30 days, a peak rickettsiaemia of 45%, and a minimum packed cell volume of 12%. The *A. marginale* rickettsiaemia was approximately 30%, 200 male *Dermacentor variabilis* ticks were acquisition fed on calf PA408 for 7 days during the ascending rickettsiaemia. These ticks were transmission fed on a sheep for 7 days, to allow salivary gland stages of *A. marginale* to fully develop. Samples were taken from all *A. marginale* populations for DNA isolation and analysis of the *polycistronic msp2* expression site (see Fig. 1).

A second cyclic transmission was performed to investigate more closely the relationship between the msp2 expression site variants acquired by ticks from a persistently infected calf (calf PA147) and transmitted to a second calf (calf PA420). Calf PA147 was infected with the Oklahoma strain of *A. marginale* by transmission feeding with *D. variabilis* ticks. Calf PA147 experienced a rickettsiaemia peak of 64%, with a prepatent period of 22 days, a peak rickettsiaemia of 51% and a minimum packed cell volume of 13.5%. Calf PA407 recovered from acute anaplasmosis and remained a carrier of *A. marginale*, with recurrent cycles of erythrocytic rickettsiaemia approximately every 4 to 6 weeks, of generally decreasing amplitude with time of infection. Another group of *D. variabilis* ticks were acquisition fed on calf PA407 during the peak of persistent rickettsiaemia that occurred on 26 September 1998 and which represented the fifth microscopically detected peak of *A. marginale* in calf PA407. These ticks were transmission fed on a sheep for 7 days, to allow salivary gland stages of *A. marginale* to fully develop. Samples were taken from all *A. marginale* populations for DNA isolation and analysis of the *polycistronic msp2* expression site (see Fig. 1).

Southern blotting of *A. marginale* genomic DNA. Probes specific to msp2, orf3, or orf6 were prepared and used in Southern blotting of digested *A. marginale* genomic DNA as described previously (4). DNA probes were labeled with fluorescein-11-dUTP, washed under high-stringency conditions (60°C; 0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% SDS), and detected by chemiluminescence (ECL Plus: Amersham Pharmacia Biotech). Molecular size standards were illuminator nonradioactive markers (Stratagene).

RNA isolation and RT-PCR. Total RNA was isolated from infected tick salivary glands preserved in RNA later (Ambion, Austin, Tex.) using the RNAqueous kit (Ambion). Isolated RNA was digested with DNase I (DNA-free; Ambion) before use in reverse transcription (RT)-PCR. mRNA transcripts of the *msp2* gene were reverse transcribed into DNA using the RETRO script kit (Ambion) and primer AB198 (5′AGGGCAAACTCAACCCCACTACCA CCAS′), which anneals to the conserved 3′ end of the coding region of the *msp2* gene. Primary RT-PCRs were used to amplify the *A. marginale* genomic *msp2* expression site. Polycistronic *msp2* PCR products were also cloned into the pCR-XL-TOPO vector (Invitrogen), and plasmid DNA was sequenced to verify the structure of amplified DNA. Sequence accessions. The sequence reported here have been assigned GenBank accession numbers AF317720 to AF317776.

**RESULTS**

**Structural conservation of the polycistronic msp2 expression site in *A. marginale* from culture, cattle, and ticks.** Figure 1 shows the derivation of *A. marginale* organisms used for analysis of the *msp2* expression site. Genomic DNA was extracted from each of the seven different populations of *A. marginale*, and the ~3.9-kbp *msp2* expression site was amplified by PCR and sequenced. The structure and sequence were similar to that described previously for the *msp2* polycistronic expression site from Florida and Idaho strains of *A. marginale* (4). There were three open reading frames upstream of the *msp2* gene, encoding polypeptides predicted by the PSORT algorithm (http://psort.nibb.ac.jp) to be outer membrane proteins. As in Florida and Idaho strains (4), orf3 and orf4 encoded polypeptides significantly similar to the outer membrane protein OMP1b of *E. chaffeensis*. When the different Oklahoma strain DNA sequences were aligned there were very few
changes observed in orf2, orf3, or orf4 between the different life cycle stages and populations of the Oklahoma strain A. marginale (a total of 5 amino acid changes between all seven populations in polypeptides encoded by orf2, orf3, and orf4). In contrast, many differences were present in the central msp2 hypervariable region including substitutions, insertions and deletions (Fig. 2a). There was more variability, particularly in orf4 when the expression site sequences of acute erythrocyte stages from Oklahoma, Florida, and Idaho strains were aligned (Fig. 2b). No changes were present between Oklahoma (all stages), Florida, and Idaho strains in the 5'-flanking region of orf4, which was previously shown to contain the +1 site for transcription and a predicted prokaryotic promoter (4).
MSP2 is encoded on a polycistronic transcript in *A. marginale* from tick salivary glands. To demonstrate whether or not this genomic site encoded an *msp2*-containing mRNA transcript in tick salivary gland stages of *A. marginale*, RT-PCR was used to amplify a fragment that contained linked *msp2*, *orf2*, *orf3*, and *orf4* genes. The expected fragment of 2.1 kbp was amplified from total RNA prepared from salivary glands of both *D. variabilis* and *D. andersoni* infected with *A. marginale* (Fig. 3). No PCR products were detected in control reactions without reverse transcriptase. Sequencing of the cloned 2.1-kbp RT-PCR product (Fig. 3) revealed that it contained the expected linked regions containing *msp2*, *orf2*, *orf3*, and *orf4* genes. Hence, this genomic site appears to be transcribed into *msp2* mRNA in *A. marginale* isolated from infected tick salivary glands, as has been shown previously for bloodstream stages (4). As in Florida and Idaho strains of *A. marginale*, there were multiple copies of the *msp2* gene in Oklahoma strain genomic DNA. Only a single band was detected, however, when using DNA probes containing *orf2*, *orf3*, and *orf4* (Fig. 4). The multiple *msp2* copies were polymorphic between the different strains, and only the *msp2* copy derived from the expression site also contained contiguous coding sequence for *orf2*, *orf3*, and *orf4* (Fig. 4). Therefore, the other *msp2* copies could not be expressed as a polycistronic mRNA containing all 4 open reading frames without recombination.

**Polymorphism in the msp2 hypervariable region in cyclically transmitted rickettsiae.** Three to five different variants were found in each *A. marginale* population. Some of these variants were shared between different populations; therefore, a total of 24 different variants of this *msp2* expression site were identified in the 49 clones examined. The amino acid sequences of the different MSP2 hypervariable regions found in each life cycle stage of *A. marginale* are shown aligned in Fig. 5. They differ from one another by multiple insertions, deletions, and substitutions, with some sequences appearing to be “mosaics” of others, e.g., Ok407per2VarC is identical to Ok407acVarB in the first part of the MSP2 hypervariable region but identical to Ok407per2VarB in the last part. These differences among the variants suggest that templated intragenic recombination may be occurring between the multiple genomic *msp2* copies and the polycistronic expression site.

We examined where identical variant sequences were found...
in the polycistronic expression site in different *A. marginale* populations (Fig. 5). A predominant variant found in *A. marginale* was OkculVarA. The identical MSP2 variant was also present in the first (acute) bloodstream rickettsia of the animal infected with these cultures (Ok408acVarA), the ticks that acquisition fed during this acute rickettsiaemia (OksgacVarA), and also the first bloodstream rickettsiaemia of the animal infected by these ticks (Ok407acVarA). This was the predominant, but not the only variant type in each of these *A. marginale* populations. Minor variants were also conserved in the transmission cycle: acute bloodstream rickettsiaemia to ticks. RT-PCR analysis of isolated RNA from infected salivary glands with AB198 as the RT primer, AB765 and AB766 as primary PCR primers, and AB192 and AB783 as secondary (nested) PCR primers. A 2.1-kbp product was specifically amplified in reactions containing reverse transcriptase enzyme (+) but was not present in control reactions without reverse transcriptase (−). This 2.1-kbp band hybridized to an *orf2* probe (arrow). Cloning and sequencing of the 2.1-kbp product demonstrated that it contained sequence from *msp2, orf2, orf3,* and *orf4.* Low-molecular-weight hybridizing bands are also present in RNA (lanes labeled +), which may represent amplified products from partially degraded *A. marginale* RNA.

FIG. 3. A polycistronic RNA transcript containing the *msp2* gene is present in *A. marginale*-infected salivary glands from *D. variabilis* and *D. andersoni* ticks. RT-PCR analysis of isolated RNA from infected salivary glands with AB198 as the RT primer, AB765 and AB766 as primary PCR primers, and AB192 and AB783 as secondary (nested) PCR primers. A 2.1-kbp product was specifically amplified in reactions containing reverse transcriptase enzyme (+) but was not present in control reactions without reverse transcriptase (−). This 2.1-kbp band hybridized to an *orf2* probe (arrow). Cloning and sequencing of the 2.1-kbp product demonstrated that it contained sequence from *msp2, orf2, orf3,* and *orf4.* Low-molecular-weight hybridizing bands are also present in RNA (lanes labeled +), which may represent amplified products from partially degraded *A. marginale* RNA.

found were different, both from those observed in the acute rickettsemia and from those in cultured *A. marginale* or in the ticks that initiated the bovine infections (Fig. 5). This increase in diversity of the expression site parallels the increase in diversity of *msp2* mRNA observed previously in persistent infections (15). Interestingly, when ticks acquired infection from a persistent rather than from an acute stage of a bovine infection, the variants found in the tick salivary glands (Oksgper variants [Fig. 5]) also appeared similar to the erythrocyte stage variants circulating at the time of tick feeding. One of the tick stage variants (OksgperVarB) was identical to an erythrocyte stage variant circulating during tick acquisition feeding (Ok407per1VarB).

To more closely examine this relationship between the circulating variants in persistent infection and those acquired and transmitted by the tick vector, we performed a second series of cyclic transmissions. Since acquisition feeding occurs over 7 days, we sampled the circulating expression site variants in persistently infected calf PA417 before, during, and after tick feeding, as well as in tick salivary glands and the acute transmitted rickettsemia in calf PA420. The relationship between the *msp2* hypervariable region sequences obtained is shown in the dendrogram in Fig. 7, with brackets to the right of the figure indicating identical variants found in the different rickettsial populations. The same circulating variant was observed prior to, during, and after tick feeding (ok417-9-13VarA is identical to ok417-9-20VarG and ok417-9-27VarA) as well as in the salivary glands of infected ticks (oksg-VarA) and in the acute rickettsemia transmitted to calf PA420 (ok420-VarA). Five other variant types were also shared between some, but not all, *A. marginale* populations. These included variant types shared in the three samplings of the persistent infection in PA417 and also present in tick salivary glands (ok417-9-13VarC, ok417-9-20VarC, ok417-9-27VarA, and oksg-VarG), other variants shared between circulating bloodstream variants and ticks (ok417-9-20VarB and oksg-VarD; ok417-9-27VarG and oksg-VarH), and an identical circulating variant in calf PA417 and in the acute rickettsemia of calf PA420 (ok417-9-20VarA and ok420-VarD).

It is necessary to evaluate the artifactual contribution to sequence diversity in the above data that could result from PCR and sequencing errors. Previously, it was demonstrated that major and minor *msp2* hypervariable region sequences observed in genomic clones of the polycistronic expression site corresponded to those found in *msp2* mRNA in the same sample (4). However, some sequence changes could result from PCR-derived mutations. To assess this possibility we analyzed *msp2* sequence in the conserved region of the polycistronic expression site upstream of the hypervariable region. In 15 independent clones of the expression site from different *A. marginale* populations, comparing 239 bp per clone of upstream sequence, there were base changes at four positions. At two of these positions there was an identical base substitution in 7 of 15 clones; therefore, this change probably represents a true sequence polymorphism. At the other two positions there was a base substitution unique to 1 of 15 clones; therefore, these may represent artifactual changes. This gives a potential error rate of 2 of 3,585 bp or potentially a 1-bp change for every four or five hypervariable region sequences. This error rate cannot explain the extensive base substitutions, insertions,
and deletions observed in the hypervariable region of the msp2 expression site in the different rickettsial populations (Fig. 5).

Similar variants are present in salivary glands of different tick species acquisition fed on the same bloodstream rickettsemia. D. andersoni and D. variabilis ticks were allowed to acquire A. marginale by feeding at the same time on an acute rickettsemia in calf PA411. The sequence of the msp2 hyper-variable region was determined in seven independent clones of the polycistronic msp2 expression site from salivary gland DNA isolated from both D. andersoni and D. variabilis. Of the seven expression site clones from D. andersoni, five encoded the same MSP2 hypervariable region sequence and this was identical to a sequence found in two expression site clones from D. variabilis DNA. A second variant sequence was present in three expression site clones from D. andersoni, five encoded the same MSP2 hypervariable region sequence and this was identical to a sequence found in two expression site clones from D. variabilis DNA. A second variant sequence was present in three expression site clones from D. variabilis DNA and also in one clone from D. andersoni DNA. Two other variant types were unique to D. variabilis, and one was unique to D. andersoni. These data do not support any substantial differences in elaboration of msp2 expression site variants in these different tick species.

**DISCUSSION**

The sequence data reveal conservation in overall structure of a polycistronic expression site for the msp2 gene in different strains and life cycle stages of A. marginale. In infections of both tick cells and mammalian erythrocytes the expression site contains three genes 5’ to msp2. DNA containing these three genes and msp2 is transcribed into a polycistronic RNA in tick salivary gland and erythrocyte stages and in all strains of A. marginale examined. msp2 and the three upstream genes are predicted to encode outer membrane proteins. Unlike msp2, multiple hybridizing copies of the three upstream genes are not found in A. marginale genomic DNA. The sequence of the three upstream genes and 5’ and 3’ flanking regions are conserved between different strains and stages, with the exception of some amino acid substitutions between strains, particularly in the polypeptide encoded by orf4. Greater polymorphism is found within the msp2 coding region itself, both between strains and between different stages in the life cycle of a single strain. This polymorphism is largely confined to a central hypervariable region of the msp2 gene in the polycistronic site encoding about 100 amino acids. Many variant forms of this hypervariable region exist in single populations of A. marginale, whether derived from culture, infected tick salivary glands, or infected bovine erythrocytes. The variant forms differ from one another by multiple insertions, deletions, and substitutions. Analysis of variant forms present in populations of A. marginale derived by cyclical transmission between culture, cattle, and ticks reveals most diversity in this expression site during persistent infections in the bovine host.

The above data and other published data on variation of MSP2 epitopes during persistent infection (14, 15) are consistent with the following hypothetical model for antigenic variation of A. marginale. The complete and incomplete genes encoding MSP2 (4, 23) may be silent until recombined into the polycistronic expression site containing the three upstream genes and promoter region. The recombination events intro-

**FIG. 4.** Structure of msp2 and orf2 to orf4 in genomic DNA of Florida, South Idaho, and Oklahoma strains of A. marginale. Southern blots of Florida (F), South Idaho (I), Oklahoma acute erythrocyte stage (Oe), or culture stage (Oc) genomic DNA digested with the restriction enzyme FspI and hybridized with probes specific for either msp2, orf2, orf3, or orf4 (probe is shown at bottom of figure). FspI cleaves 41 nucleotides 5’ to orf4 and 268 nucleotides 3’ to msp2 to release a fragment of 3.76 kbp that contains the complete polycistronic msp2 expression site sequence (see Fig. 2) from all genomic DNAs. Molecular size standards (Std) are shown in the left lane of each blot. Multiple msp2-related sequences are present in genomic DNA of all strains; only msp2 sequences located in the expression site are contiguous with orf2, orf3, and orf4.
FIG. 5. Multiple different msp2 variants are present in the polycistronic expression site in each population of A. marginale. The major variant type is conserved during passage of A. marginale between culture, acute erythrocyte stage infection, and tick salivary glands but is not conserved in persistent cattle infections. The expression site was amplified by PCR using primers which annealed 288 bp 3' to the termination codon of msp2 (AB752) and to the intercistronic sequence between orf3 and orf4 (AB750) to generate a product of 2.9 kbp from A. marginale genomic DNA that contained msp2, orf2, and orf3. The PCR product was cloned in pCR-XL-TOPO vector (Invitrogen), and independent colonies containing a 2.9-kbp insert were selected for sequencing of cloned plasmid DNA. The hypervariable region of the msp2 gene was sequenced on both strands in seven independent clones derived by PCR amplification from genomic DNA of each of the A. marginale populations described in Fig. 1. DNA sequences were translated to amino acids, and the different variant sequences were aligned with PILEUP. The proportion of each sequence variant in that population is indicated in brackets; e.g., the major sequence variant detected in cultured A. marginale was variant A, which was found in three of seven independent clones of the expression site. Identical amino acids shared between all variants are indicated by a dash and are shown on the bottom row of the alignment. Variant types present in different A. marginale populations are indicated by identical symbols to the left of the sequence alignments.
duce gene segments encoding the MSP2 hypervariable region into the expression site, probably via gene conversion employing flanking conserved sequences. This generates complex mosaics of sequence in the expression site which encode epitopes that are exposed on the surface of *A. marginale*. These epitopes are targeted by T and B cells (8) which results in the elimination of some variant types, the selection of other variants, and the sequential peaks of rickettsemia that are observed in persistent cattle infections (19). Immune selection based on MSP2 does not operate in ticks or in naive cattle prior to the first peak of acute rickettsemia. If there is a constant rate of msp2 recombination affecting a minority of the *A. marginale* population at any time, one may not detect substantial changes in MSP2 variants until there is immune selection.

Features of this model have similarities to antigenic variation in other organisms. In African trypanosomes, gene conversion of a polycistronic expression site by pseudogenes encoding a surface glycoprotein generates new antigenic variants in chronic infections (3, 26). In the family *Picomaviridae*, a virus population may consist of a swarm of slightly different individual genomes, and distinct repertoires of antigenic variants are observed in the presence and absence of immune selection (7). Similar molecular evasion mechanisms may have evolved in different organisms to allow persistent infections and onward transmission.

It has been proposed that, no matter which bloodstream variants of MSP2 are ingested by the tick, there is reversion to expression of a small number of specific “tick stage” sequence variants of MSP2 on transmission (27). These tick stage variants were the major variants present in tick salivary glands and in the first rickettsemia peak of cattle infected with a South Idaho strain of *A. marginale* (27). We did not obtain evidence for reversion in the present study. In contrast, our data are most consistent with the presence of a large number of different variants in persistent infections and transmission of the circulating bloodstream variants through ticks to naive cattle. That more shared variants were not observed in both PA417 and PA420 erythrocyte stage infections as well as in infected tick salivary glands is probably due to the sample size, i.e., the sequencing of only 10 independent clones of the msp2 expression site from each population. Possible explanations for differences from the results of Rurangirwa et al. (27) are the use of splenectomized calves in the present study, expression of tick stage MSP2 variants from a different expression site, or strain differences in msp2 expression. The infection of splenectomized calves with the Oklahoma strain results in microscopically visible relapsing peaks of rickettsemia which are easily monitored as a source of organisms and DNA. Although the type and extent of MSP2 expression site diversity between different bloodstream populations was similar in this study to that observed previously with spleen-intact calves (4), it is possible that the diversity of bloodstream variants observed can be influenced by splenectomy. Arguing against a different locus for MSP2 expression in the tick are, firstly, RT-PCR data showing that a polycistronic RNA encoding MSP2 is transcribed from the same genomic locus in *A. marginale* from tick salivary glands as erythrocyte stages. Secondly, the tick stage variants SGV1 and SGV2, identified in South Idaho strain *A. marginale* (27), utilized the same polycistronic expression site as described in this study (4). This suggests a similar mechanism of expression of tick stage SGV1 and SGV2 variants to other bloodstream variants. In contrast to their first results,
substantial diversity in tick stage antigenic types was found by Rurangirwa et al. using two other strains of *A. marginale* (28). They suggested that there may be strain-specific selection for certain MSP2 variants in ticks. It is possible that we did not find restriction of MSP2 variants because the progenitor population of *A. marginale* (okcul) was already selected by growth in tick cell culture.

It is unlikely that a vaccine could be developed based on MSP2 variants present in tick salivary glands and conserved in the first rickettsemia peak as was initially proposed (27). Our analysis of salivary gland stages and acute bloodstream rickettsemias within a single strain of *A. marginale* identified numerous sequence variants in this polycistronic expression site. There is some basis for development of “region-specific” vaccines, as variants of one strain tended to group together in multiple alignment profiles (data not shown). This perhaps relates to observations made with different anaplasmosis vaccines that have been tested against field challenge, with less protection generally afforded to animals by immunization with geographically heterologous strains of *A. marginale* (22). Any optimism in this regard must be counterbalanced by consideration of the >20 *msp2* expression site variants found (Fig. 5) in a few transmissions with one strain. A greater possibility for vaccine development may be to identify exposed T- and B-cell epitopes on other outer membrane proteins. Those epitopes encoded by the more conserved orf234 represent potential vaccine targets.

In conclusion, analysis of a polycistronic *msp2* expression site in *A. marginale* from culture, tick salivary glands, and acutely or persistently infected cattle reveals sequence conservation between these stages of the Oklahoma strain throughout most of this expression site, including 5’ and 3’ flanking regions. The exception is in the expression site region encoding the central hypervariable region of MSP2. This region of the expression site is very polymorphic within individual populations of *A. marginale*. Although polymorphic, the major sequence variants present did not change on passage of *A. marginale* between culture, acute-stage erythrocyte infections, and tick salivary glands but did change during persistent infections of the bovine host. The sequence variants found in tick salivary glands most closely resembled those present in the blood at the time of acquisition of infection, whether infection was acquired from an animal with an acute or a persistent rickettsemia. These variations in structure of an expression site for a major, immunoprotective outer membrane protein have important implications for vaccine development against *A. marginale* and related ehrlichial pathogens. The data on *msp2* variation suggest an unusual flexibility in the small 1.2-Mb genome (1) that may be employed in adaptation to and persistence in different host environments.

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