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The *msp*1β Multigene Family of *Anaplasma marginale*: Nucleotide Sequence Analysis of an Expressed Copy

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A gene for the β subunit of the immunoprotective surface antigen MSP-1 of *Anaplasma marginale* was previously cloned and expressed in *Escherichia coli*. A nucleic acid probe based on this gene detects *A. marginale* infection in carrier cattle and in the tick vector. We report here the sequence and structural features of the cloned *msp*1β gene and expressed polypeptide. The gene codes for a polypeptide of 756 amino acids that contains domains of tandemly repeated sequence and glutamine-rich regions at the N and C termini. The cloned copy is a member of a multigene family with multiple restriction fragment length polymorphisms in isolates of this rickettsia from different geographical regions. The availability of the sequence will allow use of the polymerase chain reaction in diagnostic assays and the preparation and testing of different vaccine constructs in cattle.

Anaplasmosis is a hemoparasitic disease of cattle caused by the rickettsia *Anaplasma marginale*. The disease is devastating to the production, movement, and utilization of cattle world wide, including many areas of the United States (23). Although chemotherapy can control infections (18), it is too expensive for routine use in less-developed countries. Current methods of vaccination with attenuated or killed preparations are unreliable and associated with problems such as neonatal isoerythrolysis and the risk of transmission of other pathogens (7).

One approach toward vaccine development is to use a surface protein of the organism produced and possibly delivered by recombinant methods. The protein MSP-1 (for major surface protein), when prepared by affinity chromatography from infected erythrocytes, confers immunity on cattle against challenge with homologous (19) and heterologous (20) isolates of *A. marginale*. MSP-1 contains two noncovalently linked polypeptides that are products of separate genes and have similar apparent molecular weights (100,000 to 105,000) in a Florida isolate (3). The genes coding for each of these polypeptides, *msp*1α and *msp*1β (the *msp*1β gene product was previously termed Am105L [3]), have been cloned and expressed in *Escherichia coli*, and *msp*1α has been sequenced (1).

Current diagnostic methods have high rates of false-positives and -negatives, especially in carrier animals with low parasitemia (17). A diagnostic nucleic acid probe was developed, based on an *Sst*I fragment of the *msp*1β gene. This probe specifically detects *A. marginale* in infected ticks (12) and, due to cyclic variation in rickettsemia levels (15), detects some but not all *A. marginale* infections of long-term carrier cattle (9). To achieve adequate sensitivity to detect all carrier infections, it may be necessary to amplify target DNA with the polymerase chain reaction (24).

In this report we define the nucleotide sequence of an *msp*1β gene from a Florida isolate and compare the structure of this gene in different *A. marginale* isolates by restriction enzyme digestion and hybridization analysis. These data should allow the development and testing of different recombinant vaccine constructs and use of the *Sst*I probe and the polymerase chain reaction in diagnosis.

MATERIALS AND METHODS

Derivation of recombinant plasmids. Plasmid pAM97 contains a 3.9-kb *Bgl*II fragment of *A. marginale* DNA inserted in the *Bam*HI site of the plasmid vector pBR322, whereas pAM113 contains the same *Bgl*II fragment in the opposite orientation in pBR322 (3). *E. coli* strains carrying pAM97 and pAM113 express a recombinant *A. marginale* polypeptide of about 100,000 apparent molecular weight (determined by sodium dodecyl sulfate [SDS]-gel electrophoresis) (3). Subclones of pAM97 were used in sequencing experiments; these contained either a 2-kb or a 0.5-kb *Sst*I fragment or a 1.4-kb *Hin*cII-*Hin*dIII fragment inserted in the multiple cloning site of the plasmid pGEM3 (Promega).

To map the gene required for expression of the *A. marginale* protein, a subclone of pAM97 was prepared in the plasmid vector pUC19. pAM97 was digested with *Xma*III and *Hpa*I, and the 2.5-kb fragment was isolated from an agarose gel. This fragment contains the majority of the open reading frame (ORF) in Fig. 1 except for DNA coding for the N-terminal 20 amino acids. The 2.5-kb *Xma*III-*Hpa*I fragment was then filled in with deoxynucleotide triphosphates, ligated to *Hin*cII-digested pUC19 DNA, and used to transform competent *E. coli* (DH5α; Bethesda Research Laboratories) to ampicillin resistance. Clones containing the desired 2.5-kb fragment in each orientation were identified by restriction enzyme digestion, and the validity of the constructs was confirmed by DNA sequencing of the junction regions between vector and insert DNAs.

Determination of nucleotide sequences. DNA sequences were determined by the dideoxynucleotide chain-termination method (26) with either Sequenase (United States Biochemical) on denatured double-stranded DNA (16) or reverse transcriptase on in vitro-synthesized (Promega protocol) RNA transcripts. Sequence ambiguities were resolved with 7-deaza-2′-deoxyguanosine-5′-triphosphate and 2′-deoxyinosine-5′-triphosphate (4). SP6 and T7 promoter-specific primers were used to sequence the termini of inserts, and then new oligonucleotide primers were synthesized as

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required to extend the sequence. Sequences were determined completely for both DNA strands. To directly sequence *A. marginale* RNA transcripts, total RNA was isolated from infected erythrocytes and sequenced by the method of Inoue and Cech (14), using synthetic oligonucleotide primers complementary to different regions of pAM97 DNA.

Computer analyses of sequence data. Sequences were analyzed by using the Sequence Analysis Software (International Biotechnologies), GCG (Genetics computer group, University of Wisconsin), MIT (Whitehead Institute), and PCGENE (Intelligenetics) sequence analysis programs.

Analysis of recombinant proteins. Recombinant proteins were analyzed either by immunoprecipitation (27) of [^{35}S] methionine labeled products from plasmid-directed in vitro transcription and translation reactions (Amersham) or by immunoblotting (11). For immunoblots, *E. coli* isolates containing recombinant plasmids were pelleted from 3 ml of an overnight culture and suspended in 300 μl of SDS-gel sample buffer. Then 40 μl of solubilized bacteria was separated per gel lane, transferred to nitrocellulose, and probed with rabbit antisera to MSP-1 (R874 at a 1:4,000 dilution) and ^{125}I-labeled protein A. The preparation and specificity of the anti-MSP-1 serum designated R874 were described previously (3). Plasmid pFL10 was used as a control in immunoblots; this contains an *msp*1α gene from a Florida isolate and expresses a recombinant protein of about 105,000 apparent molecular weight recognized by anti-MSP-1 sera (1). Proteins were separated by electrophoresis on 7.5 to 17.5% (wt/vol) SDS–polyacrylamide gradient gels. ^{14}C-labeled protein molecular weight standards (phosphorylase b, 92,500; bovine serum albumin, 69,000; ovalbumin, 46,000; carbonic anhydrase, 30,000; lysozyme, 14,300) were obtained from Amersham.

Southern blotting. *A. marginale* genomic DNA was isolated from washed, infected erythrocytes at about 50% parasitemia after passage through a CF11 cellulose column (2). DNA (2 μg) was digested with restriction enzyme; the fragments were separated by agarose gel electrophoresis, transferred to nitrocellulose or nylon membranes, and hybridized as described previously (25). Blots were hybridized with either an RNA probe labeled with ^{32}P by in vitro transcription from the 2-kb *Sst*I fragment of pAM97 inserted in the plasmid vector pGEM3 (Promega protocol) and linearized with *Pvu*II or with fragments of pAM97 DNA purified from agarose gels and ^{32}P labeled by random priming (25). Except for the hybridization experiment described below, in which wash conditions of varied stringency were used, the final wash of filters was performed at 65°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% (wt/vol) SDS. To determine the extent of homology, blots were hybridized and then washed under conditions of varied stringency. Filters were washed either five times at 65°C in 5× SSPE (1× SSPE is 180 mM NaCl–10 mM NaH_2PO_4–1 mM EDTA [pH 7.4]) containing 0.25% *N*-lauroylsarcosine or three times in 1× SSPE containing 0.1% *N*-lauroylsarcosine and then two more times in 0.1× SSPE containing 0.0033% *N*-lauroylsarcosine, also at 65°C. A *Hin*dIII digest of bacteriophage λ DNA was used as a molecular weight standard on agarose gels (fragments shown in figures are 23.1, 9.4, 6.6, 4.4, 2.3, and 2.0 kb).

Nucleotide sequence accession number. The sequence reported in this paper has been deposited in the GenBank data base with accession number M59845.

RESULTS

Nucleotide sequence of an in vivo-expressed *msp*1β gene. Recombinant plasmids pAM97 and pAM113, derived previously (3), contain the same 3.9-kb *Bgl*II fragment of *A. marginale* DNA in opposite orientations with respect to the vector. Bacteria containing these plasmids express a recombinant polypeptide of about 100,000 molecular weight which is recognized by anti-MSP-1 sera and which is structurally homologous to one component of MSP-1. In preliminary experiments we attempted to locate the region of the insert DNA which was required for expression. First, pAM97 DNA was digested with different restriction enzymes selected from the map described previously (3). The digested DNA fragments were then transcribed and translated into protein and immunoprecipitated with anti-MSP-1 serum (R874). From this experiment it appeared that the central 3.0-kb region of the inserted DNA, encompassed approximately by the right *Sst*I and left *Hpa*I sites (3), was necessary for protein expression (data not shown). Accordingly, this region of DNA was sequenced and shown to contain a single long ORF of 2,268 bases (Fig. 1).

To prove that this reading frame was responsible for expression of an *A. marginale* protein recognized by anti-MSP-1 serum, we inserted the 2.5-kb *Xma*III-*Hpa*I fragment in frame into the expression vector pUC19. Recombinant pUC19 plasmids were obtained that contained the *Xma*III-*Hpa*I fragment in both orientations. DNA sequencing confirmed that in one orientation the *Xma*III-*Hpa*I fragment of *A. marginale* DNA had been placed in frame with the N-terminal sequence of the pUC19 *lacZ* gene. This should result in expression of a fusion protein in which the N-terminal 19 amino acids of the *A. marginale* ORF have been replaced by the N-terminal 11 amino acids and regulatory elements from pUC19 (the Ser residue at the junction is reconstituted). In the opposite orientation there should be no expression, since there is no ORF and any *A. marginale* promoter elements present in pAM97 have been removed. Recombinant bacteria containing plasmids in either possible orientation were analyzed for reaction with R874 in immunoblots (Fig. 2). Expression of a polypeptide that was reactive with R874 was obtained from pUC19 plasmids containing the *Xma*III-*Hpa*I fragment in the correct orientation (plasmids 7, 8, 12, 13, 14, 18, 19, and 20) but not in the reverse orientation (plasmid 16). In contrast, recombinant bacteria containing the complete 3.9-kb *Bgl*II fragment expressed full-sized, immunoreactive protein in either orientation (pAM97 and pAM113), indicative of a functioning *A. marginale* promoter. As expected for an 8-amino-acid difference, no molecular weight change in the expressed recombinant protein was detected between the pUC19 constructs and pAM97 or pAM113. This observation does suggest, however, that the methionine residue at amino acid position 1 (Fig. 1) is a more probable N terminus than the next methionine 51 amino acid residues downstream. Expression from the downstream methionine would have resulted in a polypeptide 4 to 5 kDa smaller from pAM97 and pAM113 than from the pUC19 constructs.

Structure of the *msp*1β gene. To define the start of transcription of the *msp*1β gene, total RNA was isolated from *A. marginale*-infected erythrocytes and was sequenced with the oligonucleotide primers 5′ CGCTTCCAGTGACTTCATGT GGGG 3′ and 5′ CTTGTCGTCTTCTGTCATCGCACC 3′, which are complementary to bases 314 through 337 and 164 through 187, respectively (Fig. 1). The start of transcription was identified by both primers as the same base which we

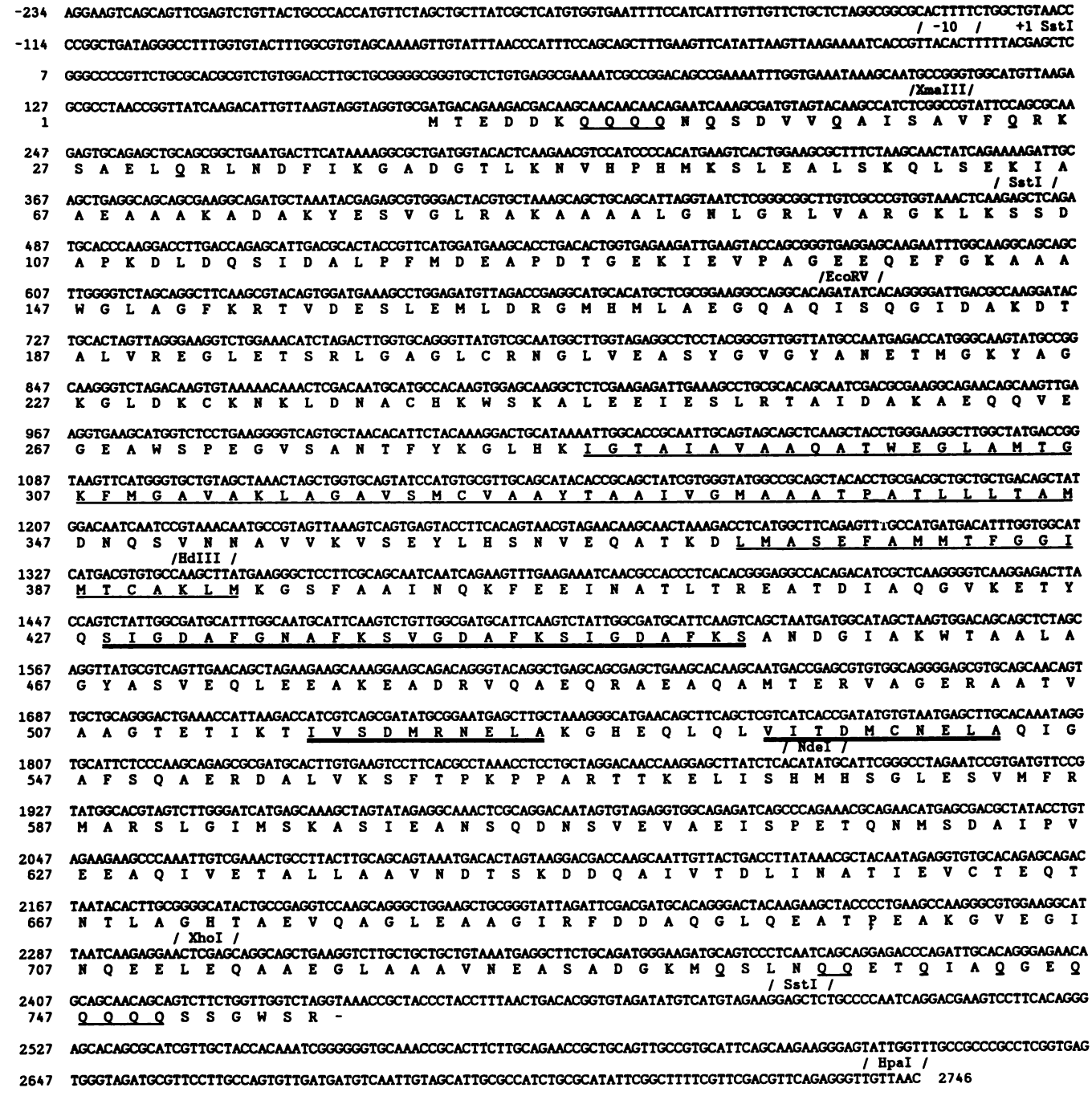

FIG. 1. Nucleotide and predicted amino acid sequence of the cloned *msp*1β gene. The start of transcription, −10 promoter sequence, and selected restriction enzyme sites are indicated above the sequence. Glutamine-rich regions at the N and C termini and potential transmembrane helices are indicated by underlining; domains containing repeated sequences are double underlined.

define as no. 1 and which occurs within an *Sst*I site. An upstream sequence (TACACT) was found which agrees with the *E. coli* consensus −10 promoter region (TAtAaT [13]) and which is correctly spaced with respect to the transcription start. A −35 promoter sequence was not precisely identified in this study. However, three overlapping sequences (TTAAGTT, TTCATAT, and TTGAAGT) are present which have reasonable agreement with the *E. coli* −35 consensus sequence (TTGACat) and which are separated from the −10 region by 14 to 26 bases. The transcription start site is followed by an untranslated region of 169 bases with termination codons in all three reading frames. Immediately following the last set of termination codons is an ATG which begins the ORF. Ten residues upstream of the ATG is the sequence AGTAG, which could mediate binding to the conserved 5′ CUCCU 3′ segment of 16S rRNA (28). After the end of the ORF at base 2438 are termination codons in all three reading frames. A predicted (5) prokaryotic terminator site is present between bases 2616 and 2666.

Analysis of protein structure. The ORF codes for a protein

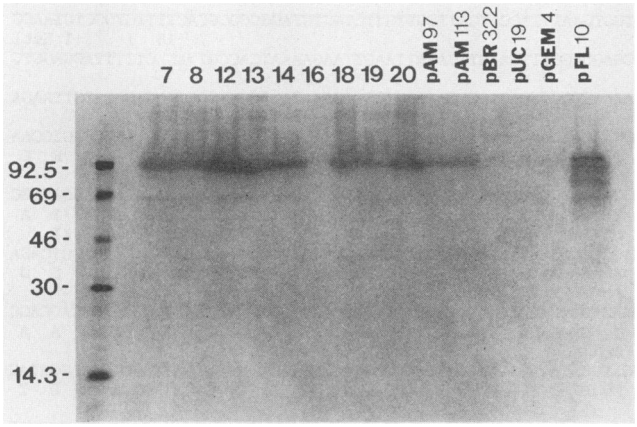

FIG. 2. Expression of the cloned *msp*1β gene in *E. coli*. *E. coli* isolates containing different recombinant plasmids were analyzed for expression of the *msp*1β polypeptide by SDS-gel electrophoresis and immunoblotting with anti-MSP-1 serum. Plasmids 7, 8, 12, 13, 14, 18, 19, and 20 contain the 2.5-kb *Xma*III-*Hpa*I fragment of pAM97 (Fig. 1) inserted in frame, and in the correct orientation for expression of the ORF, into the *Hin*cII site of pUC19. Plasmid 16 contains the same 2.5-kb fragment in the *Hin*cII site of pUC19 but in the opposite orientation. pAM97 and pAM113 contain the *msp*1β gene on a 3.9-kb *Bgl*II fragment of *A. marginale* genomic DNA in opposite orientations in the *Bam*HI site of pBR322. pFL10 contains the *A. marginale msp*1α gene (from a Florida isolate) inserted into the *Sma*I site of pGEM4. pBR322, pUC19, and pGEM4 are control plasmids containing no insert DNA. ^{14}C-labeled molecular weight standards (in thousands) are shown on the left.

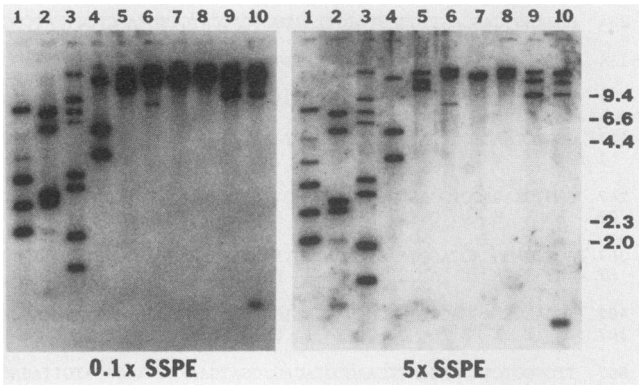

FIG. 3. Multiple copies of the *msp*1β gene in genomic DNA from a Florida isolate of *A. marginale*. DNA was digested with different restriction enzymes and analyzed by Southern blotting with a ^{32}P-labeled RNA probe complementary to bases 483 through 2312 of Fig. 1 with wash conditions of high or low stringency. DNAs in lanes 1 through 10 were digested with *Sst*I, *Bgl*I, *Hin*dIII, *Bgl*II, *Mlu*I, *Bam*HI, *Eco*RI, *Kpn*I, *Sma*I, and *Apa*I, respectively. The migration positions of molecular weight standards (in kilobase pairs) are shown on the right.

of 756 amino acids with a calculated molecular weight of 80,350. Repeated regions (double underlined in Fig. 1) are found in this gene, as in *msp*1α (1), but are not as extensive. The first repeat domain indicated in Fig. 1 contains a 7-amino-acid peptide, SVGDAFK, degenerate versions of which occur four times in tandem (SIGDAF, GNAFK, SVGDAFK, and SIGDAFKS; amino acids 428 through 453). The sequence IVSDMRNELA (amino acids 516 through 525) is repeated later as VITDMCNELA (amino acids 534 through 543). An unusual feature of the polypeptide is the presence of a glutamine-rich segment at both the N and C termini, including a run of 4 or 5 glutamines 7 residues in from both termini. Interestingly, glutamine-rich domains have been observed in other proteins, where they are thought to form a structural framework for the formation of multimeric complexes (6, 21). The *A. marginale* polypeptide is predicted (10) to be primarily α-helical in structure, with two potential transmembrane segments (amino acid residues 287 through 346 and 373 through 393 [22]). Since the *msp*1β gene product lacks an obvious N-terminal signal sequence and yet appears to be located on the surface of *A. marginale* initial bodies (3), one of the predicted transmembrane segments could be an uncleaved internal signal sequence (29) for localization of the polypeptide to the outer membrane.

Comparison of *msp*1β genes of different *A. marginale* isolates. The cloned *msp*1β gene is one member of a multigene family, the members of which all appear to be highly homologous (3) (Fig. 3). The use of the *msp*1β gene in diagnostic analyses with polymerase chain reaction amplification requires that constant sequences can be found in different *A. marginale* isolates. If the *msp*1β gene product is to be used for vaccination, it must also contain constant immunoprotective epitopes. To begin an examination of the variability of this gene, we compared restriction enzyme fragment patterns generated from the *msp*1β genes of geographically diverse *A. marginale* isolates.

The restriction fragment profile of genomic DNA probed with *msp*1β gene sequences was generally consistent with the presence of three or four different gene copies in the Florida isolate. The number of related fragments observed was not increased by reducing the stringency of hybridization from 0.1× to 5× SSPE (Fig. 3). Considerable restriction enzyme fragment length polymorphism was observed among these copies in different isolates (Fig. 4a and b). For example, the 2-kb *Sst*I fragment, which contains most of the coding sequence described above in the Florida isolate, was found in a Virginia isolate but not in Texas, Idaho, or St. Croix isolates (Fig. 4a). Two other *Sst*I fragments were observed in all five isolates. From the sequence presented in Fig. 1, one may select enzymes which yield a defined fragment from the pAM97 ORF and use that fragment as a probe on different genomic DNAs, digested with the enzymes used to produce the probe. Such an experiment will reveal changes within the coding sequence of the genomic copy of the cloned *msp*1β gene among the *A. marginale* isolates. Digestion with *Eco*RV-*Hin*dIII should release a fragment of 641 bp from the center of the ORF in Florida DNA; this fragment was found in all three isolates examined (Fig. 4c). In contrast, a *Hin*dIII-*Nde*I fragment of 553 bases, encompassing the repeat region, was found in DNA from Florida and Virginia isolates but not in DNA from a Texas isolate.

DISCUSSION

The nucleotide sequence and structure of the cloned *msp*1β gene described here may be compared with that of *msp*1α (1), currently the only other *A. marginale* gene sequence available. Both genes are expressed in *E. coli* under the control of *A. marginale* promoters, which resemble the *E. coli* consensus sequence. Both *A. marginale* genes contain domains of tandemly repeated sequence, although these are not so extensive in the *msp*1β gene as in the *msp*1α gene. Interestingly, three sequences in the *msp*1β gene have

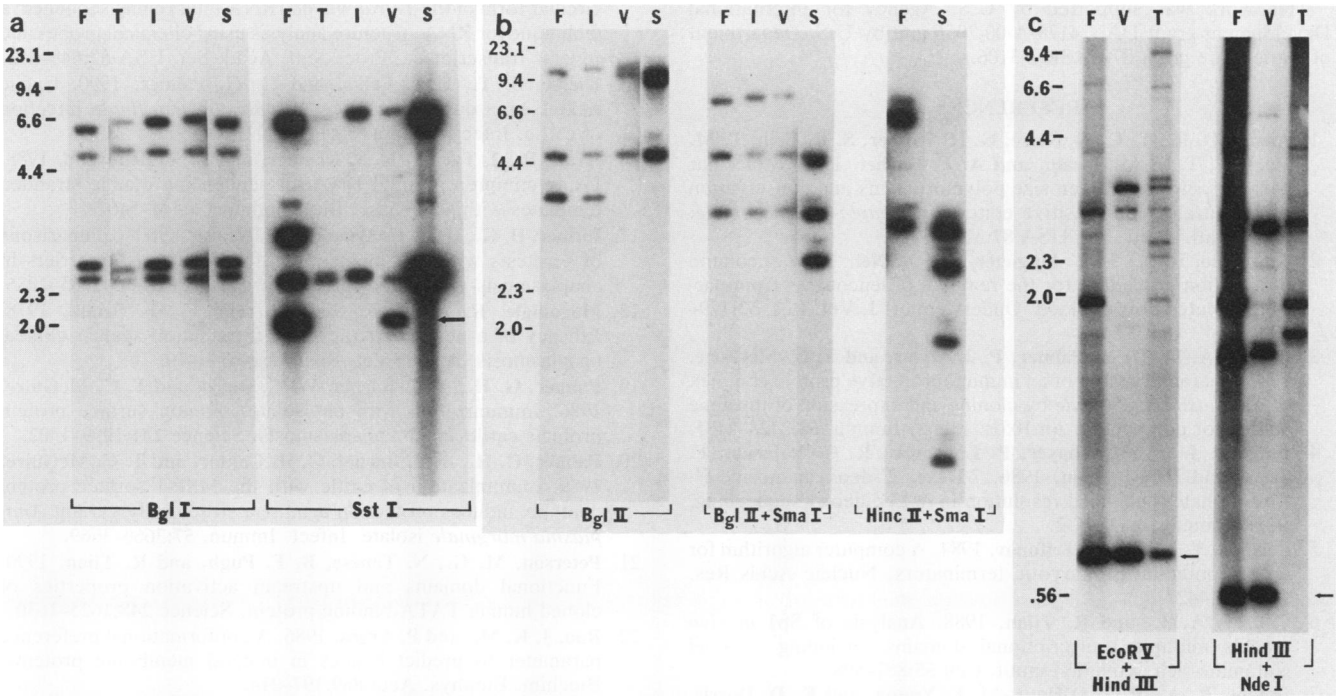

FIG. 4. Comparison of *msp*1β gene structure in genomic DNAs from different isolates of *A. marginale*. Genomic DNA was digested with restriction enzymes as indicated and analyzed by Southern blotting. The probe used in panels a and b was the same as that used in Fig. 3. In panel c, the probes were the 641-bp *Eco*RV-*Hin*dIII fragment (bases 700 through 1340 in Fig. 1) and the 553-bp *Hin*dIII-*Nde*I fragment (bases 1341 through 1893) of the cloned, Florida isolate *msp*1β gene. Lanes F, T, I, V, and S contained genomic DNA from Florida, Texas, Idaho, Virginia, and St. Croix isolates of *A. marginale*, respectively. Arrows on the right of the lanes in panels a and c indicate the migration positions of the expected genomic fragment corresponding to the probe. The positions of molecular weight standards (in kilobase pairs) are shown on the left.

similarity with the tandem repeats present in the *msp*1α gene: bases 575 through 588, 2002 through 2015, and 2398 through 2416 (86, 86, and 79% identity, respectively). Moreover, two of these three regions of *msp*1β overlap the same sequence of *msp*1α found not only in the *msp*1α tandem repeats but also in other regions within and outside the *msp*1α coding sequence. It was suggested that this sequence could be derived from ancestral invasion of the *A. marginale* chromosome by a mobile element (1). Otherwise, no significant homology was detected between the *msp*1α and *msp*1β genes. Similarly, a search of the GenBank data base did not reveal substantial similarities of *msp*1β with other sequences.

Unlike *msp*1α, the cloned *msp*1β gene is a member of a polymorphic multigene family. Evidence suggesting that the sequenced gene copy presented here is expressed in *A. marginale* is as follows: (i) the similarity between peptide maps of native and recombinant polypeptides (3); (ii) the presence in the DNA of typical prokaryotic control elements surrounding an ORF, which enables expression in *E. coli*; (iii) the *A. marginale* mRNA sequence determined directly by using synthetic oligonucleotide primers agreed with the DNA sequence presented here. We were unable, however, to obtain a totally unambiguous sequence for every mRNA base. It could be argued that expression from another, very similar gene copy would not have been distinguishable.

It is not clear whether all *msp*1β gene copies are capable of being expressed or whether some represent pseudogenes. The polymorphism in these copies between isolates gives cause for concern if the genes or their products are to be used for diagnosis or vaccination, especially since changes were found within the coding sequence (Fig. 4c). All copies were sufficiently homologous to be detected under high-stringency hybridization conditions, which suggests that the variability is not as great as in, for example, *Trypanosoma brucei* surface glycoprotein genes (8). To use the *msp*1β DNA sequence as the basis for synthesis of polymerase chain reaction primers will require that each primer be tested for amplification of target DNA from multiple isolates of *A. marginale* or that a strictly conserved region be identified.

The availability of the nucleotide sequence of an *msp*1β gene will allow testing of the above possibilities and further development of a sensitive diagnostic assay for *A. marginale* infection. This is especially important in anaplasmosis, in which the disease is persistent and diagnosis by light microscopy is difficult due to the lack of distinguishing morphologic features of the rickettsia. Also, novel constructs of the *msp*1β gene can be prepared, expressed, and tested as vaccines, perhaps in combination with the *msp*1α gene product to reconstitute the native *A. marginale* MSP-1 protein structure.

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