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NOTES

Molecular Cloning, Sequence Analysis, and Expression of the Gene Encoding the Immunodominant 32-Kilodalton Protein of *Cowdria ruminantium*

ARNOUD H. M. VAN VLIET,[1]* FRANS JONGEJAN,[1,2] MIRINDA VAN KLEEF,[3] AND BERNARD A. M. VAN DER ZEIJST[1]

Departments of Bacteriology[1] and Parasitology,[2] Institute of Infectious Diseases and Immunology, School of Veterinary Medicine, University of Utrecht, 3508 TD Utrecht, The Netherlands, and Onderstepoort Veterinary Institute, Onderstepoort 0110, Republic of South Africa[3]

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***Cowdria ruminantium*, the causative agent of heartwater disease, expresses an immunodominant and conserved 32-kilodalton protein (MAP1; formerly called Cr32), which is currently in use for serodiagnosis of the disease. The gene encoding this protein, designated *map1*, was detected, cloned, and characterized. The gene is conserved between four different stocks of *C. ruminantium* originating from Senegal, Sudan, South Africa, and Zimbabwe. Homology searches revealed MAP1 to be homologous to the *Anaplasma marginale* surface protein MSP4, a potential protective antigen. The MAP1 protein, expressed in *Escherichia coli* fused with glutathione *S*-transferase, is specifically recognized by sera from animals infected with seven different stocks of *C. ruminantium*.**

Heartwater or cowdriosis is a rickettsial disease of wild and domestic ruminants. It is caused by *Cowdria ruminantium* and transmitted by ticks of the genus *Amblyomma*. The disease is endemic to sub-Saharan Africa (43) and has also been detected in the Caribbean region (36). The continuing spread of the African tick *Amblyomma variegatum* in the Caribbean and the presence on the American continent of yet uninfected *Amblyomma* species capable of spreading the disease pose a serious threat to livestock industries on the American mainland (3).

C. ruminantium has developmental stages resembling those of *Chlamydia* species (25). The deduced developmental cycle consists of an extracellular stage capable of infecting host cells (elementary body) and an intracellular stage capable of multiplication within the host cell (reticulate body). However, phylogenetic studies based on 16S rDNA sequence comparison revealed no significant phylogenetic relationship between *C. ruminantium* and *Chlamydia* spp. (9, 45) but a very close relationship among *C. ruminantium*, several *Ehrlichia* species (45), and *Anaplasma marginale* (9, 45).

Cloning and expression of major protein antigens of *C. ruminantium* would make it possible to test whether these antigens are protective, as has been described for *Anaplasma marginale* (1, 33, 34, 41) and *Ehrlichia risticii* (14, 39), or could facilitate the use of recombinant antigen in serological tests. Studies on immunodominant protein antigens of *C. ruminantium* identified a 32-kDa protein (20) and a 27-kDa protein (37). The 32-kDa protein, which was designated Cr32, is conserved in all isolates tested (17, 20, 22). It has also been reported to have a molecular weight of 31,000 (37). Actually, the Cr32 protein varies in molecular weight depending on the origin of the *C. ruminantium* stock (2). Therefore, definition by molecular weight is inappropriate, and we will stick to the newly coined name MAP1 (major antigenic protein) (2).

MAP1-specific monoclonal antibodies (MAbs) have been raised (22), and one of these MAbs has been used in a competitive enzyme-linked immunosorbent assay (ELISA) for the detection of *C. ruminantium*-specific antibodies in animal sera (22). Antigen for ELISA is derived from in vitro-cultured *Cowdria* organisms, a laborious and expensive method which also results in antigen batches of varying quality. Recombinant antigen would allow production of large batches of antigen of high quality and at lower costs.

Several attempts to clone the gene encoding the MAP1 protein by screening expression libraries with polyclonal antibodies and MAbs have not resulted in positive clones (46). This study describes the production of a MAP1-specific DNA probe which has been successfully used to detect the entire cloned gene encoding the MAP1 protein (*map1*). The gene has been characterized and expressed in *Escherichia coli*.

(Preliminary results of this research were presented at the Second Biennial Meeting of the American Society of Tropical Veterinary Medicine (ASTVM-93) in Guadeloupe, French West Indies, 2–6 February 1993, and have appeared in the proceedings of that conference [46]).

Eight stocks of *C. ruminantium* were used in this study: one each from Senegal (Senegal [23]), Sudan (Um Banein [19]), Zambia (Lutale [23]), and Zimbabwe (Crystal Springs [7]) and four stocks from South Africa (Ball 3 [16], Kümm [13], Kwanyanga [27], and Welgevonden [10]). Four stocks of *C. ruminantium* (Crystal Springs, Senegal, Um Banein, and Welgevonden) were cultivated in vitro in bovine umbilical endothelial cells (isolate BUE 9) under conditions described previously (17).

Genomic DNA was obtained from BUE 9 cell cultures infected with *C. ruminantium*. When the cultures reached an

* Corresponding author. Mailing address: Department of Bacteriology, Institute of Infectious Diseases and Immunology, P.O. Box 80.165, 3508 TD, Utrecht, The Netherlands. Phone: (31) 30-534992. Fax: (31) 30-540784. Electronic mail address (Internet): dddzeij@cc.ruu.nl.

infection score of 3+ for elementary bodies and reticulate bodies (17), infected endothelial cells were scraped from the bottom of the flask and mixed with the rickettsia-containing supernatant. This mixture was centrifuged for 10 min at 15,000 × *g* and 4°C and resuspended in 1 ml of sucrose-phosphate-glutamate buffer (6) per 162-cm^2 flask. Cells were kept at −80°C overnight, thawed, centrifuged for 10 min at 15,000 × *g*, and resuspended in 1 ml of TMN buffer (25 mM Tris [pH 8.0], 10 mM $MgCl_2$, 0.9% NaCl) supplemented with 10 μg of DNase I per ml to degrade the majority of contaminating bovine DNA. After 15 min of incubation at 37°C, DNase I was inhibited by the addition of EDTA to a final concentration of 0.05 M, and rickettsiae were pelleted by 10 min of centrifugation at 15,000 × *g* and 4°C. Genomic DNA of rickettsiae was purified by using repeated sodium dodecyl sulfate (SDS) and proteinase K incubations (45), phenol-chloroform-isoamyl alcohol extractions, and isopropanol precipitations (38); dissolved in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) to a concentration of 100 μg/ml; and stored at 4°C.

C. ruminantium organisms (Senegal stock) were purified on discontinuous Renografin density gradients (49). The proteins present in these purified organisms were separated in 15% (wt/vol) SDS-polyacrylamide gels (38) and electroblotted onto polyvinylidene difluoride membranes (Immobilon transfer membranes; Millipore, Bedford, Mass.). Blotted proteins were stained with Coomassie brilliant blue R250, and the 32-kDa protein band was excised. The N-terminal amino acid sequence of the MAP1 protein of the Senegal stock of *C. ruminantium* was determined with an Applied Biosystems model 470A Protein Sequencer, on-line, equipped with a model 120A PTH Analyzer at Eurosequence (PTH, Groningen, The Netherlands). The sequence is as follows: (N terminus) D V I Q E E N N P V G S V Y I S A K Y M P T . . . (C terminus). An oligonucleotide primer (32-1) derived from amino acids 3 to 11 of this sequence was synthesized and reads 5′-ATI CA(A/G) GA(A/G) GA(A/G) AA(C/T) AA(C/T) CCI GTI GG-3′. An internal amino acid sequence of the MAP1 protein of the Welgevonden stock was determined (44) and reads as follows: (N terminus) M P I A E D F G D T . . . (C terminus). The complete sequence was reverse translated, and its inverted and complemented sequence was used for the construction of oligonucleotide 32-2 [5′-GT(A/G) TCI CC(A/G) AA(A/G) TC(C/T) TCI GC(A/G/T) ATI GGC AT-3′].

With *Taq* DNA polymerase (Promega, Madison, Wis.) and primers 32-1 and 32-2, a fragment of 99 nucleotides was amplified from DNA derived from the Senegal stock of *C. ruminantium*, whereas this fragment was not detected when DNA derived from BUE 9 cells was used as template. By standard methods (38, 45) this fragment was cloned in pBluescript (Stratagene, La Jolla, Calif.), yielding pCRS16, and its nucleotide sequence was determined by using earlier-described procedures (5) and analyzed with the PC/Gene program (release 6.70; Genofit S.A., Geneva, Switzerland). This sequence contained an open reading frame (ORF) from which the first 11 amino acids were identical to amino acids 12 to 22 of the N-terminal amino acid sequence of the MAP1 protein. The fragment hybridized specifically with a 1.1-kb fragment in *Hin*dIII-digested DNA of *C. ruminantium*. This *Hin*dIII fragment was cloned in pBluescript, resulting in pCRS18, and was used for characterization of *map1*.

Analysis of the nucleotide sequence of the insert of clone pCRS18 revealed a single ORF lacking a stop codon. Using a 200-bp *Pst*I-*Hin*dIII fragment of pCRS18 (see Fig. 1) containing the C-terminal part of its ORF as probe allowed detection of a 1.2-kb *Pst*I-*Sau*3AI fragment which contained the remainder of the ORF. This fragment was cloned in pUC19 (50),

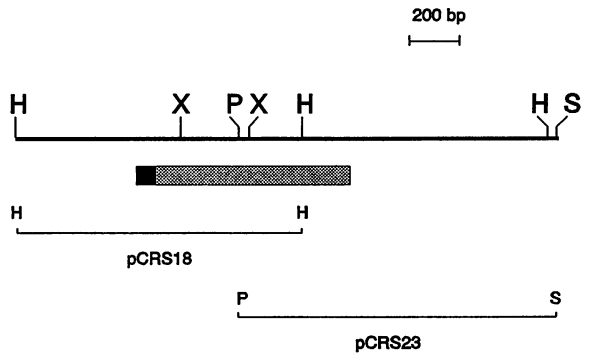

FIG. 1. Restriction map of the *map1* gene and the clones used to characterize it. (▨) ORF encoding the MAP1 protein; ■, signal sequence of MAP1. Restriction enzymes: H, *Hin*dIII; X, *Xba*I; P, *Pst*I; S, *Sau*3AI.

resulting in pCRS23, and the nucleotide sequence was determined up to 132 nucleotides downstream of the stop codon of *map1*. Clones pCRS18 and pCRS23 are indicated in Fig. 1, together with a restriction map of the genomic region containing *map1*.

The *map1* gene is 854 nucleotides long and encodes a protein with a calculated molecular weight of 27,919 after removal of the signal sequence. The nucleotide sequence has a high A+T content (70%), due to which numerous possible promoter sequences can be identified upstream of the *map1* gene. *E. coli* containing plasmid pCRS18 expresses low levels of a truncated MAP1 protein (data not shown), indicating the presence of promoter sequences active in *E. coli*. Figure 2 shows the complete nucleotide sequence of the *map1* gene and its flanking sequences together with the deduced amino acid sequence of *map1*. The experimentally determined N-terminal amino acid sequence is identical to the deduced amino acid sequence, but when it was compared with the internal amino acid sequence from the Welgevonden MAP1 amino acid sequence (44), only 6 of 10 amino acids matched. The N-terminal amino acid sequence is cleaved at a position predicted by the SigCleave program (47).

Homology searches in protein and nucleotide sequence data bases with the FASTA (35) programs, as supplied by the CAOS/CAMM Center (Nijmegen, The Netherlands), revealed the MAP1 protein to be homologous to the *Anaplasma marginale* major surface protein MSP4 (31). This 31-kDa outer membrane protein is a potential protective antigen in anaplasmosis (41), but nothing is known about its structure or function. An alignment of the MAP1 and MSP4 gene product sequences made with the Multalin program (8) is shown in Fig. 3.

The 1.2-kb *Hin*dIII insert of clone pCRS18 containing two-thirds of the *map1* gene was labelled with [α-^{32}P]dATP (Amersham, Buckinghamshire, England) by use of a random primer labelling kit (Boehringer, Mannheim, Germany) and was used as probe on a Southern blot (38) of *Hin*dIII-digested DNA of *C. ruminantium* stocks originating from different geographical regions within Africa: Senegal (Western Africa), Um Banein (Sudan, Eastern Africa), and Crystal Springs and Welgevonden (Zimbabwe and South Africa, Southern Africa). As all DNA samples still contained bovine DNA originating from the endothelial cells used to cultivate *C. ruminantium*, *Hin*dIII-digested bovine DNA was also included on the blot. The filter was prehybridized in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, 1 mM EDTA [pH 7.7])–5× Denhardt's

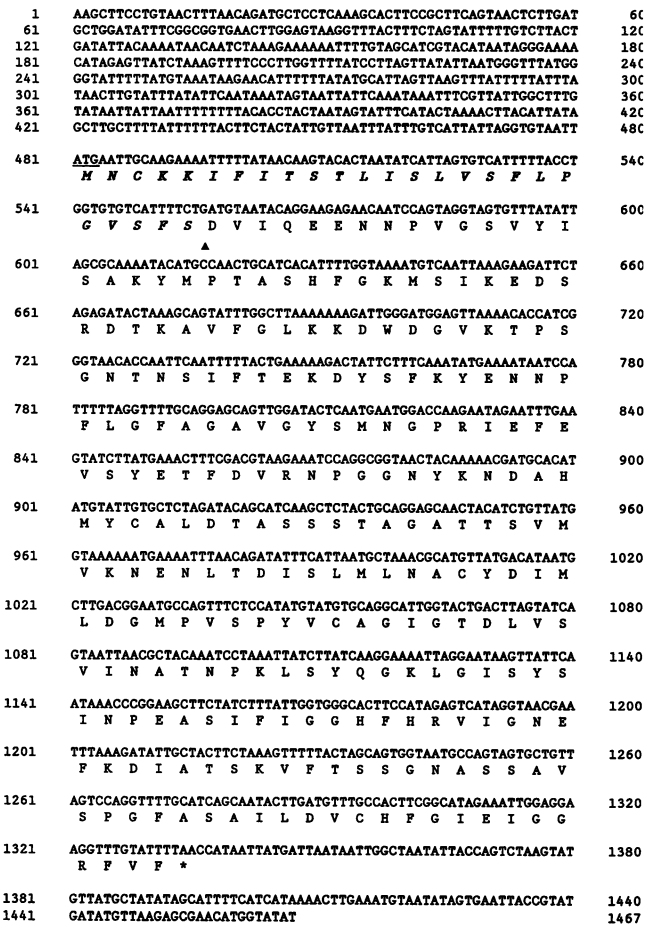

FIG. 2. Nucleotide sequence and translation of the *map1* gene. The start of the *map1* gene is underlined, the signal sequence is indicated in boldface, the cleavage site between the signal peptide and the N-terminal amino acid sequence is indicated by ▲, and the translational stop is indicated by an asterisk.

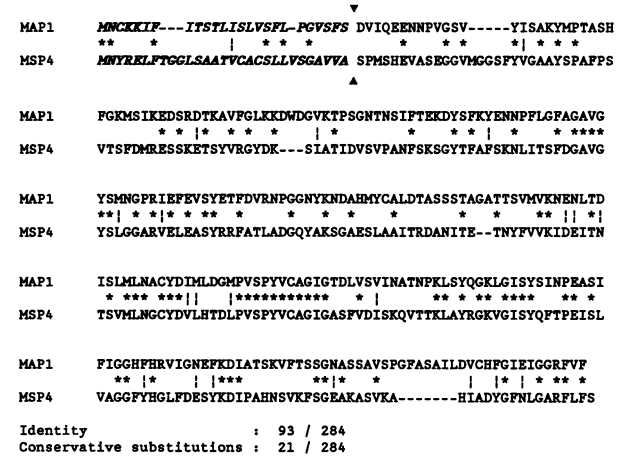

FIG. 3. Alignment of protein sequences of the *C. ruminantium* MAP1 protein and the *Anaplasma marginale* MSP4 protein. Amino acid identity is indicated by an asterisk; conservative substitutions are indicated by | . The cleavage site of the signal peptide is indicated by ▲ or ▼.

reagent–0.5% SDS–100 μg of denatured herring sperm DNA per ml for 3 h at 60°C. Labelled, denatured probe was added, and hybridization was performed for 18 h at 60°C. Washing conditions were as follows: two times for 15 min each with 2× SSPE–0.1% SDS at 42°C. Filters were autoradiographed with intensifying screens at −80°C and with Fuji XR films (Fuji Photo Film Co., Ltd., Tokyo, Japan). Figure 4 shows this hybridization. No hybridization signal was detected with bovine DNA, whereas all *C. ruminantium* stocks hybridize with the *map1*-specific probe. The three bands reacting in DNA from the Crystal Springs stock are probably caused by incomplete digestion of the DNA.

The use of recombinant antigen in heartwater diagnostics requires that sera from various animal species which have been infected with different *C. ruminantium* stocks recognize the MAP1 protein but do not cross-react with a carrier used for high-level expression. The *map1* gene without the region encoding the signal peptide was PCR amplified and expressed, fused with glutathione *S*-transferase (GST), using expression vector pGEX-2T (40). MAP1-GST fusion product (molecular weight, 57,000) and GST (molecular weight, 27,000) were expressed in *E. coli* and purified (15, 40). Complete purification of MAP1-GST, as described for soluble GST-fusion products (15, 40), was not possible because the MAP1-GST protein was insoluble in extraction and elution buffers. Partial purification was achieved by repeated sonication followed by centrifugation to pellet the insoluble fusion protein. Antisera were raised in experimental animals to seven of the eight *C. ruminantium* stocks mentioned (no antiserum was raised against the Um Banein stock). Immunization against heartwater was carried out by an infection and treatment method, which has been described elsewhere in detail (21). Three goats (Saanen breed) were immunized with Lutale (goat 89046), Kümm (goat 8334), and Senegal (goat 8910) stocks. Three sheep (Dutch Texel breed) were immunized with Welgevonden (sheep 8834), Ball 3 (sheep 8614), and Senegal (sheep 8531) stocks. Three experimental calves (Friesian) were immunized with Lutale (calf 57), Crystal Springs (calf 70), and Senegal (calf 130) stocks. Finally, three mouse-pathogenic *C. ruminantium* stocks (Kümm, Kwanyanga, and Welgevonden) were used to infect and subsequently treat three groups of mice.

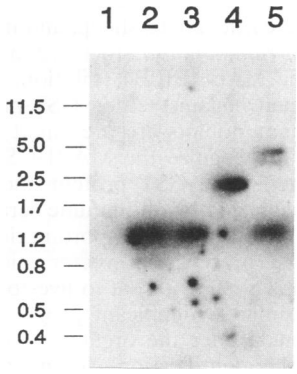

FIG. 4. Southern hybridization of *Hin*dIII-digested genomic DNA derived from bovine cells and several stocks of *C. ruminantium* with a *map1*-specific probe. The size of lambda marker fragments in kilobases is indicated on the left. Lanes: 1, bovine DNA; 2, Senegal stock; 3, Welgevonden stock; 4, Um Banein stock; 5, Crystal Springs stock.

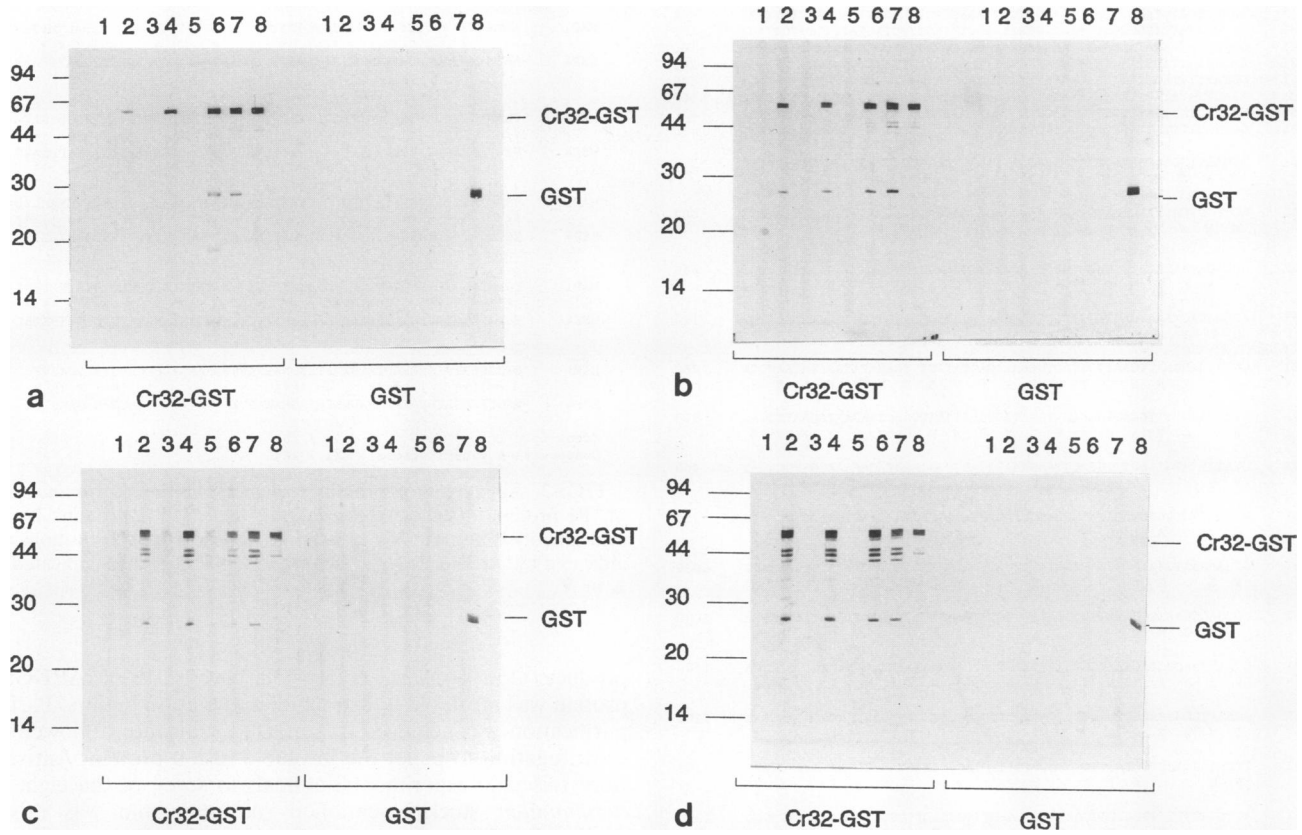

FIG. 5. Western blots (immunoblots) of MAP1-GST and GST proteins incubated with preimmune and immune antisera of four different animal species infected with *C. ruminantium* stocks. Marker sizes in kilodaltons are indicated on the left; MAP1-GST and GST are shown on the right. Controls on each blot are included in lanes 7 and 8: lane 7, incubation with the MAP1-specific MAb 4F10B4; lane 8, incubation with a rabbit antiserum directed against the GST protein. (a) Goat antisera. Lanes 1 and 2, incubation with preimmune and immune serum of goat of 89046 (Lutale); lanes 3 and 4, incubation with preimmune and immune serum of goat 8334 (Kümm); lanes 5 and 6, incubation with preimmune and immune serum of goat 8910 (Senegal). (b) Bovine antisera. Lanes 1 and 2, incubation with preimmune and immune serum of calf 130 (Senegal); lanes 3 and 4, incubation with preimmune and immune serum of calf 70 (Crystal Springs); lanes 5 and 6, incubation with preimmune and immune serum of calf 57 (Lutale). (c) Sheep antisera. Lanes 1 and 2, incubation with preimmune and immune serum of sheep 8531 (Senegal); lanes 3 and 4, incubation with preimmune and immune serum of sheep 8614 (Ball 3); lanes 5 and 6, incubation with preimmune and immune serum of sheep 8834 (Welgevonden). (d) Mouse antisera. Lanes 1 and 2, incubation with preimmune and immune serum of a mouse infected with the Welgevonden stock; lanes 3 and 4, incubation with preimmune and immune serum of a mouse infected with the Kwanyanga stock; lanes 5 and 6, incubation with preimmune and immune serum of a mouse infected with the Kümm stock.

MAP1-GST and GST proteins were immobilized on nitrocellulose membrane (42) and incubated with preimmune and immune sera from cattle, goats, sheep, and mice infected with seven different *C. ruminantium* stocks (dilution, 1:200), with the MAP1-specific MAb 4F10B4 (dilution, 1:1,000), or with antibodies directed against the GST-protein (dilution, 1:4,000). These immunoblots are presented in Fig. 5. Preimmune sera did not recognize the MAP1-GST and GST proteins, whereas the MAP1-GST protein and its degradation products were recognized by all immune sera.

Heartwater is one of the most important tick-borne diseases in Africa, and the presence of vector and disease in the Caribbean region is a major threat to livestock production on the American mainland. Significant progress in research on heartwater was made once the organism could be cultivated (4), allowing for the identification, cloning, and expression of major protein antigens.

The first cloning experiments with *C. ruminantium* focussed on the development of specific DNA probes for use in hybridization and PCR amplification (30, 48). In this study, we describe the cloning of a *C. ruminantium* gene, encoding an immunodominant and conserved 32-kDa outer membrane protein. Because conventional methods such as screening of genomic expression libraries with MAbs and polyclonal antibodies did not yield MAP1-positive clones (46), amino acid sequences were used to construct a MAP1-specific DNA probe. Since *C. ruminantium* can only be cultivated in the cytoplasm of eucaryotic cells, almost all DNA samples and DNA libraries contained bovine DNA. This requires a specific probe to detect cloned *C. ruminantium* genes. The method used to obtain a specific probe may also be suitable for detection of other genes encoding *C. ruminantium* proteins. The nucleotide sequence of the *map1* gene was determined, and the gene was shown to be conserved between several isolates of *C. ruminantium*. The gene was expressed, and epitopes on recombinant MAP1 were specifically recognized by antisera from four different animal species immunized with *C. ruminantium*. This indicated that recombinant MAP1 antigen could be useful for serodiagnostic purposes.

Recently, research on the specificity of serological tests for detection of heartwater has shown that cross-reactions between *C. ruminantium* and antibodies to *Ehrlichia* spp. exist

(11, 12, 18, 24, 26, 29). Whether specific epitopes on the MAP1 protein can be identified and used to circumvent these problems in serodiagnosis of heartwater is an approach currently under evaluation.

Furthermore, the potential of recombinant MAP1 as a component of a subunit vaccine against heartwater can now also be evaluated. In this respect, the homology with the *Anaplasma marginale* MSP4 protein is very interesting, since that is one of the proteins recognized by sera from animals that are protected against anaplasmosis (41) and one of the proteins to be tested as part of a recombinant subunit vaccine (32). No other significant homologies with other proteins were found; therefore, no function of the MAP1 protein can be postulated. It is most probably not involved in virulence, since it is also present in attenuated organisms of the Senegal stock of *C. ruminantium* prepared by repeated in vitro culture (17). The MAP1 protein could be involved, however, in the induction of a protective immune response against *C. ruminantium*, because goats and sheep vaccinated with in vitro-attenuated *C. ruminantium* (Senegal) demonstrated an immunodominant recognition of this antigen (17). Although animals immunized with MAP1 extracted from polyacrylamide gels were not protected against challenge with virulent organisms (44), these experiments have to be repeated with recombinant antigen. A recently cloned immunogenic 21-kDa *C. ruminantium* antigen (28) should also be taken into account in these studies. Finally, more studies are required to determine the appropriate presentation of these recombinant antigens in animals susceptible to heartwater.

Nucleotide sequence accession number. The nucleotide sequence of the *C. ruminantium map1* gene has been assigned accession number X74250.

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