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Transcription Analysis of the Major Antigenic Protein 1 Multigene Family of Three In Vitro-Cultured *Ehrlichia ruminantium* Isolates

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Ehrlichia ruminantium*, an obligate intracellular bacterium transmitted by ticks of the genus *Amblyomma*, causes heartwater disease in ruminants. The gene coding for the major antigenic protein MAP1 is part of a multigene family consisting of a cluster containing 16 paralogs. In the search for differentially regulated genes between *E. ruminantium* grown in endothelial and tick cell lines that could be used in vaccine development and to determine if differences in the *map1* gene cluster exist between different isolates of *E. ruminantium*, we analyzed the *map1* gene cluster of the Senegal and Gardel isolates of *E. ruminantium*. Both isolates contained the same number of genes, and the same organization as found in the genome sequence of the Welgevonden isolate (H. Van Heerden, N. E. Collins, K. A. Brayton, C. Rademeyer, and B. A. Allsopp, Gene 330:159–168, 2004). However, comparison of two subpopulations of the Gardel isolate maintained in different laboratories demonstrated that recombination between *map1-3* and *map1-2* had occurred in one subpopulation with deletion of one entire gene. Reverse transcription-PCR on *E. ruminantium* derived mRNA from infected cells using gene-specific primers revealed that all 16 *map1* paralogs were transcribed in endothelial cells. In one vector (*Amblyomma variegatum*) and several nonvector tick cell lines infected with *E. ruminantium*, transcripts were found for between 4 and 11 paralogs. In all these cases the transcript for the *map1-1* gene was detected and was predominant. Our results indicate that the *map1* gene cluster is relatively conserved but can be subject to recombination, and differences in the transcription of *map1* multigenes in host and vector cell environments exist.

Ehrlichia ruminantium (formerly *Cowdria ruminantium* [12]) is the causative agent of heartwater, a rickettsial disease transmitted by ticks of the genus *Amblyomma* which causes major economic losses in wild and domestic ruminants. The disease is endemic in sub-Saharan Africa and also is present on some Caribbean islands (33), where it poses a risk of spreading to the American mainland. Feeding ticks transmit *E. ruminantium* to vertebrate hosts in their saliva and/or by gut regurgitation (8, 19). Phylogenetic studies have revealed a close relationship between *E. ruminantium*, *Ehrlichia canis*, and *Ehrlichia chaffeensis* (30, 39).

In infections with these ehrlichial agents, the serological response is mainly directed against outer-membrane proteins of approximately 30 kDa. The genes coding for these proteins have been designated the major antigenic protein 1 (*map1*) in *E. ruminantium* (32, 40), the outer membrane protein p28 (*omp-1*) in *E. chaffeensis*, and the p30 outer membrane protein (*p30*) in *E. canis* (27, 28, 30, 35, 36, 42, 43). The OMP-1 and P30 protein families are each encoded by a multigene family consisting of 22 genes arranged in a cluster between a hypothetical transcriptional regulator (upstream) and the *secA* gene (downstream). The 5′ end of the cluster contains paralogs with short intergenic spaces, whereas the paralogs at the 3′ end are separated by larger intergenic spaces. Previously only four *E. ruminantium map1* paralogs had been identified, including one located downstream from *map1*, designated *map1+1*, which was located upstream from *secA* (37). Very recently the whole *map1* cluster was characterized for the Welgevonden isolate of *E. ruminantium* and shown to contain 16 genes located downstream from a hypothetical transcriptional regulator and the *secA* gene (38).

In vitro systems for the propagation and study of *E. ruminantium* using mammalian endothelial cells have been available for nearly 20 years, but systems for the propagation of *E. ruminantium* in tick cell lines have become available only recently (4–6). Using these in vitro systems, it was shown that there are clear differences in morphology between bacteria grown in endothelial cell and tick cell cultures (6) and also in their immunogenicity and pathogenicity for sheep (7). Together these data strongly suggest that differences may occur in gene transcription and protein expression by *E. ruminantium* grown in endothelial and tick cell cultures.

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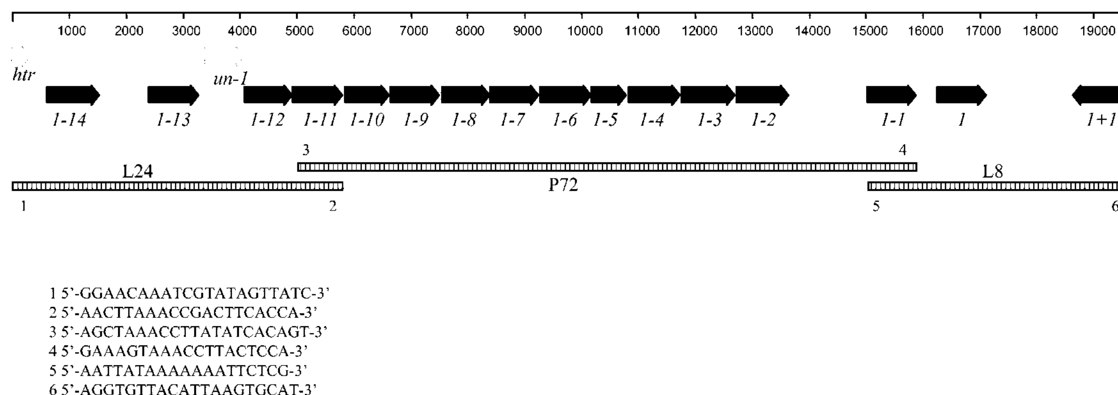

FIG. 1. Schematic representation of the *E. ruminantium* Senegal *map1* multigene family. The genes and their orientations are represented as arrows. The bars indicate the three clones that were constructed with long-template PCR. The *map1* paralog names are indicated below the solid arrows and the other gene names below the open arrows. *htr*, hypothetical transcriptional regulator; *un-1*, unknown gene.

Various studies investigating the transcription of ehrlichial multigene families have been conducted using both in vitro cultures and in vivo material collected from infected vertebrate hosts and ticks. All 22 paralogs in the *E. canis p30* multigene family and 16 paralogs in the *E. chaffeensis p28* multigene family were found to be transcribed when reverse transcription-PCR (RT-PCR) was used on infected monocyte cultures using gene-specific primers (23, 27). The *p30* paralogs with short intergenic regions were cotranscribed in monocyte cultures (27). In infected vertebrates, 16 out of 22 and 11 out of 14 paralogs studied were found to be transcribed for *E. chaffeensis* and *E. canis*, respectively, whereas only 1 paralog was found to be transcribed in various tick stages (13, 35, 36). We recently investigated the transcription of *map1-2*, *map1-1*, and *map1* of *E. ruminantium* grown in bovine endothelial cells, tick cell lines, and in *Amblyomma variegatum* ticks (2). In that study we found that *map1* was always transcribed and *map1-1* was transcribed in tick cells in vitro, ticks in vivo, and by attenuated organisms in endothelial cells, but we could not detect a transcript for *map1-2* in any of the studied samples. However, van Heerden et al. (38) showed that all 16 *map1* paralogs were transcribed in infected endothelial cell cultures.

To study whether all 16 *E. ruminantium map1* paralogs were also transcribed in a tick environment, we studied the transcription in one vector and several nonvector tick cell lines. In order to be able to do this, the *map1* multigene sequence of two isolates was determined. The study led to the identification of recombination between two *map1* paralogs in one of the *E. ruminantium* isolates and demonstrated the occurrence of differential transcription of *E. ruminantium map1* paralogs. Analysis of differential transcription of *map1* multigenes in host and vector will aid in understanding the role these genes (and their products) may play in the pathogenesis and transmission of heartwater.

MATERIALS AND METHODS

Growth of *E. ruminantium* in bovine endothelial cells and tick cell lines. The Gardel (34), Senegal (18), and Welgevonden (11) isolates of *E. ruminantium* were cultured in bovine pulmonary artery (BPC), aorta endothelial (BAE), or umbilical cord (BUE) cells as described previously (17, 26). Two subpopulations of the Gardel isolate were used; both originated from CIRAD-EMVT, Guadeloupe. One culture, designated IBET Gardel, had been transferred to the Instituto de Biologia Experimental e Tecnologica, Oeiras, Portugal, in 2001, and thence to Utrecht University in 2002. The other culture, designated CTVM Gardel, was transferred to the Centre for Tropical Veterinary Medicine (CTVM), University of Edinburgh, Scotland, in 1993. For RNA extraction the following cultures were used: CTVM Gardel in BPC between passages 12 and 15, IBET Gardel in BAE between passages 45 and 66, Senegal in BAE between passages 4 and 14, and Welgevonden in BAE between passages 7 and 16. The CTVM Gardel and Welgevonden isolates of *E. ruminantium* were maintained in vitro in tick cell lines at temperatures between 28°C and 37°C as described previously (4–6). Both isolates were grown in the following cell lines: *Ixodes scapularis* embryo-derived IDE8 (25), *Amblyomma variegatum* larva-derived AVL/CTVM13 (6), and *Rhipicephalus appendiculatus* nymph-derived RAN/CTVM3 (2). CTVM Gardel was also grown in BDE/CTVM16, BME/CTVM2, and IRE/CTVM18 derived from embryos of *Boophilus decoloratus*, *Boophilus microplus*, and *Ixodes ricinus*, respectively (4), and Welgevonden was also grown in two other *R. appendiculatus* lines: embryo-derived RAE/CTVM1 (3) and neonate larva-derived RAE25 (20). Bacterial growth was monitored in Giemsa-stained cytocentrifuge smears.

PCR amplification, cloning, and sequencing of the *map1* multigene family. For the Senegal isolate, the *Ehrlichia canis p30* multigene family sequence (accession number AF078553) and the *E. chaffeensis p28* multigene family sequence (accession numbers U72291 and AF021338) were used to do BlastN searches in the *E. ruminantium* genome database of the Welgevonden isolate (Sanger Centre; www.sanger.ac.uk/Projects/Microbes/). Single-hit runs were aligned where possible, and several primers were designed to amplify the *E. ruminantium map1* locus. Using a Long and Accurate PCR (LA-PCR) kit (Takara Biomedical, Ohtsu, Japan), three PCR products (Fig. 1) were generated from genomic DNA extracted from *E. ruminantium* (Senegal) grown in bovine endothelial cells using the following PCR conditions: initial denaturation of 4 min at 94°C followed by 35 cycles of 20 s at 98°C, 10 s at 55°C, 15 min at 68°C, and a final extension of 10 min at 72°C. The PCR products were cloned into the pGEMT-easy vector (Promega Benelux, Leiden, The Netherlands) following the manufacturer's instructions and were transferred to *Escherichia coli* JM109 cells. Positive clones were selected by PCR amplification of part of the insert. Three overlapping clones were selected, and the sequence of the inserts was determined (Baseclear, Leiden, The Netherlands). Both primer walking and random shotgun cloning were used to determine the sequences.

DNA and RNA isolation. Total RNA from endothelial and tick cell cultures was extracted as described previously (2), except that DNase treatment was done after elution from the column. DNA of the various *E. ruminantium* isolates was extracted as described before (40) or using the tissue protocol provided with the QIAamp extraction kit (Westburg, Leusden, The Netherlands).

cDNA synthesis and paralog-specific PCR. Random hexamer primers were used to prepare cDNA using the SuperScript first-strand synthesis system (Invitrogen, Breda, The Netherlands). Between 5 and 10 μl of DNase-treated RNA was used to prepare 40 μl of cDNA using 100 ng of random hexamer primers according to the manufacturer's instructions. Control samples were prepared by omitting reverse transcriptase from the reaction. Two microliters of cDNA was subsequently used as template in a PCR containing 1× *Taq* PCR buffer (Promega, Leiden, The Netherlands), 3 mM $MgCl_2$, 1.25 U of *Taq* polymerase

TABLE 1. Primers used for the RT-PCR on the *map1* cluster of *E. ruminantium* Senegal and Gardel

Gene[a]	Senegal (5′-3′)	Gardel (5′-3′)
map1-14(f)	TGTTGACTTTTCCAATGAGAGTGA	TGTTGACTTTTCCAATGAGAGCGA
(r)	TTGGTGAAAGTAAAAACCCCATAC	TTGGTGAAAGTAAAAACCCTACAC
map1-13(f)	AGATCTTAAAGAGGATGGGTACAA	AGATCTTAAAGAGGATGGATACAA
(r)	GGTAATAACCTTCTGCAACAGCTA	CTAAAATAAACCTTACTCCTATAC
un-1(f)	ATTAACAGCACTCCCAATCCATTA	ATTAGCAGCACTCCTAATCCAGTA
(r)	AATTTGTACAGCTGCTTGGAAAAA	GGAAAACAACACTTTTTGTGGGTG
map1-12(f)	AATACAAGCCAAGCATTTCGTACT	AATACAAGCCAAGCATTTCGTATT
(r)	TAACATTGAATTTTGCTGATGATG	TAACGTTGAATTTTGCTGATGATG
map1-11(f)	TATCAAACTTTCGAAACTTCCACA	TATCAAACTTTCGAAACTTTCACA
(r)	CACTGATCAGGAGATTTGTTCTTG	CACTGATCAAGAGATTTGTTCTTG
map1-10(f)	ATTACCACCCAATTTAAGCCTACT	ATTACCAGCCAATTTAAGCCTACT
(r)	AATCCTTAACCCGACTTCGCTACC	AATCCTTAACCCGACTTCGCTACC
map1-9(f)	GTAACGTACCAAATACTGAAGATG	GTAACGTACCAAATACTGAAGATG
(r)	TATTAAGTTTGGCTATTGCTGAA	TATTAAGTTTGGCTATTGCTGAAG
map1-8(f)	TTTAACATTCTTATTAGCGTTTTC	TTTAACATTCTTATTAGCGTTCTC
(r)	CTGATTTTAGGAGATACGCGATAA	CTGATTTTAGGAGATACACGATAA
map1-7(f)	TGGACAATATAAGCCAGGAGTTCC	TGGACAATATAAGCCAGGAGTTCC
(r)	ACTGCACTAGTAATTTTGGGTTCA	GTTGCACTAGTAATTTTGGGTTCA
map1-6(f)	TTTTTACCTCACCAAGCACTTTC	TTTTTACCTCACCAAGTACTTTC
(r)	AGCTAATGTTTAACGTTGATGTTG	AGCTAATGTTTAATGTTGATGTTG
map1-5(f)	CTTTTGCCTTCATTCATACATTTA	ATACAAAACTAGAAAAAGAGAATA
(r)	TACTTACTTTGACATTATATGCTA	TACTTACTTTGACATTATATGCTA
map1-4(f)	GTTTCAGTGGCACTATAGGACAGT	GTTTTAGTGGCACTATTGGACAGT
(r)	TTGGGATTATTTGCAAGCATACGA	TTGGGATTATTTGCAAGCATACGA
map1-3(f)	CAATTTTCGTAACTTCTCAGTCAA	CAATTTTCGTAATTTCTCAGTTAA
(r)	TTTAAATTTATTGCCTGCTACTTT	TTTAAATTTATTACCTACTACTTT
map1-2(f)	AATAAACTCATTGCAACAGGTATA	AACAAACTCATTGCAACAGGTATA
(r)	CTAATGGGATGTTATAGAGATCAG	CTAATGGGGTGTTATTCAGATCAG
map1-1(f)	CCAAGCATACCACATTTCAGA	CCAAGCATACCACACTTCAGA
(r)	TGAAGCGGAAGTGCTTTGAGG	TGAAGCGGAAGTGCTTTGAGG
map1(f)	TAATATCATTAGTGTCATTTTTACC	TAATATCATTAGTGTCATTTTTACC
(r)	TGGACTAACAGCACTACTGGC	GTGTTGCTGATGCAAAACCTGG
map1+1(f)	CTTAGTTAAACCAGGGATTG	ATAATCAATATCTAAATTAGCAGA
(r)	AGGTGTTACATTAAGTGCAT	GGTGTTACATTAAGTGCATTGTTG

[a] f, forward; r, reverse.

(Promega), 400 μM of each deoxynucleoside triphosphate, and 10 pmol of each primer set (Table 1) in a 25-μl reaction. Reactions were carried out on an iCycler (Bio-Rad Laboratories b.v., Veenendaal, The Netherlands) using the following program: 2 min at 94°C followed by 40 cycles of 30 s at 94°C, 30 s at 55°C, 1 min at 72°C, and a final elongation step of 7 min at 72°C. Positive controls included genomic DNA instead of cDNA. PCR products were visualized by running the samples on ethidium-bromide-stained agarose gels.

Southern blot with *map1-2*, *map1-3*, and recombination site-specific probes. DNA from *E. ruminantium* Gardel (IBET or CTVM)-infected BUE cells was obtained by scraping cells from the bottom of culture flasks. The cell suspension was passaged 10 times through a 25-gauge needle and subsequently spun at 2,000 × *g* for 5 min at 4°C. The supernatant containing bacteria was then spun for 15 min at 15,000 × *g* at 4°C. The pellet was resuspended in phosphate-buffered saline (PBS) and treated with DNase (Promega Benelux, Leiden, The Netherlands) for 30 min at 37°C to digest the host cell DNA. Bacteria were washed three times with PBS, and DNA was extracted using the QIAamp kit (Westburg, Leusden, The Netherlands). DNA from uninfected cells was directly extracted with the same kit. Genomic DNA was digested with HindIII and separated in a 0.7% agarose gel. DNA was transferred to a Nylon membrane (Zeta-probe; Bio-Rad, Veenendaal, The Netherlands), and blots were prehybridized at 50°C in prehybridization solution (6×SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA {pH 7.7}], 0.5% sodium dodecyl sulfate [SDS], 100 μg/ml denatured salmon sperm DNA, and 5×Denhardt's solution [1× Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin]) for 2 h and hybridized at 50°C in prehybridization solution containing biotin-labeled probe (Isogene, Maarssen, The Netherlands). Blots were then washed twice for 10 min in 2× SSPE, 0.1% SDS at 50°C. Subsequently the membrane was incubated in 20 ml of 1:4,000-diluted peroxidase-labeled streptavidin (Boehringer, Mannheim, Germany) in 2× SSPE–0.1% SDS for 1 h at 50°C. The membrane was washed twice in 2× SSPE–0.1% SDS for 10 min at 50°C with shaking and once in 2× SSPE for 10 min at room temperature. The membrane was thereafter incubated for 1 min in 5 ml of ECL detection fluid and exposed to an ECL hyperfilm (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). The membrane was stripped by two washes in 1% SDS–0.1× SSPE for 15 min at 95°C and one at 20°C. For reuse the membrane was placed between sheets of Whatman paper and sealed in a plastic bag and stored at 4°C. The 5′ biotin-labeled probes were GAAATCCAAATCCTGGACCT for *map1-3*, AAC AAACTCATTGCAACAGGTATA for *map1-2*, and ACATCTGCAATAGC(G/T)ACACTT for the recombination site.

Nucleotide sequence accession numbers. The obtained sequence for the *E. ruminantium* Senegal isolate *map1* multigene family was deposited as GenBank accession number AF319940. For the IBET Gardel isolate the sequence of the *map1* cluster (accession number AY652746) was taken from the whole-genome sequence that was obtained by random shotgun cloning (IGH-CIRAD *Ehrlichia ruminantium* genome project). The *map1* cluster sequence of the Welgevonden isolate was obtained from the whole-genome sequence of this isolate (accession number NC_005295).

RESULTS

Sequence analysis and cluster comparison. Sequence analysis of the *map1* cluster of both the Senegal (Fig. 1) and IBET Gardel isolates revealed the same number of *map1* paralogs in these isolates as described for the Welgevonden isolate (38). Furthermore, the position of the cluster in the genome (between a hypothetical transcriptional regulator gene and the *secA* gene) and the arrangement of the cluster were conserved among the three different isolates. All genes within the cluster contained both start and stop codons, and only the genes *un-1*

TABLE 2. Properties of three *E. ruminantium map1* multigene families[c]

Gene	ORF (bp)[a]	Intergenic space (bp)[a]	Amino acid no.[a]	Region for RT-PCR[b] (nt position and size [bp])
map1-14	939/924/930	887/876/883	312/307/309	383–1038 (655) 374–1044 (670)
map1-13	885	88	294	2234–2817 (583) 2251–2978 (727)
un-1	711	−4	236	3247–3582 (335) 3264–3498 (234)
map1-12	828	15	275	3886–4554 (668) 3903–4571 (668)
map1-11	882	27	293	4744–5325 (581) 4761–5342 (581)
map1-10	774/795/774	16	257/264/257	5649–6296 (647) 5666–6292 (626)
map1-9	870	24/24/25	289	6536–6559 (620) 6532–6555 (620)
map1-8	849	10	282	7260–7907 (647) 7256–7279 (647)
map1-7	852	19	283	8215–8871 (656) 8211–8867 (656)
map1-6	900/897/888	8	299/298/295	9012–9813 (801) 9008–9812 (804)
map1-5	618/624/618	39	205/207/205	9902–10328 (426) 9998–10321 (323)
map1-4	894	20	297	10842–11343 (501) 10835–11336 (501)
map1-3	948	23/22/23	315	11583–12261 (678) 11576–12254 (678)
map1-2	873/921/921	1386/1384/1392	290/306/306	12413–13250 (837) 12407–13196 (789)
map1-1	849	372/376/372	282	14859–15503 (644) 14807–15451 (644)
map1	873/855/873	1620/1532/1606	290/284/290	15971–16722 (751) 15915–16703 (788)
map1+1	852/ND[c]/858		283/ND/285	18972–19140 (168) 18410–19189 (779)

[a] Where size differences occur, the order in which the isolates are indicated is Gardel, Senegal, Welgevonden.
[b] Data are presented for the Senegal (top) and Gardel (bottom) isolate. nt, nucleotide.
[c] ND, not determined.

and *map1-12* did not contain an intergenic region and instead had a 4-nucleotide overlap (Table 2). Fifteen of the paralogs were orientated in a head-to-tail direction, with small intergenic regions (8 to 39 nucleotides) near the 3′ end region (paralogs *map1-12* to *map1-2*) and large intergenic regions (372 to 1,621 nucleotides) in the 5′ region (paralogs *map1-2* to *map1+1*) of the cluster. Comparison of individual paralogs using ClustalV analysis on the amino acid sequence showed highly conserved paralogs (>95% similarity; e.g., *map1-9*, *map1-8*, and *map1-1*) and less conserved (variable) paralogs (<86% similarity; e.g., *map1-5*, *map1-2*, and *map1*). The sizes of the intergenic spaces were highly conserved for the paralogs *map1-13* to *map1-2* and showed the greatest variation near the ends of the cluster.

Detection of recombination in CTVM Gardel. When testing the gene-specific primers, no product was detected for *map1-2* when genomic DNA from the CTVM Gardel isolate was used. However, when genomic DNA from the IBET Gardel isolate was used the expected product was found. To investigate this further a new primer (map1-2R2) was designed in the intergenic region between *map1-2* and *map1-1*. No product was amplified from CTVM Gardel when this primer was used in combination with primer map1-2F. However, a product was amplified when the primer was used in combination with primer map1-3F. The resulting product was approximately 1 kb, whereas the same primer combination yielded a product of approximately 1.8 kb (as expected from the determined sequence) from IBET Gardel (data not shown). Sequencing of the 1-kb PCR product showed that the first part (bp 1 to 713) of the sequence was 99.5% identical to *map1-3* while the second part (bp 700 to 837) was identical to *map1-2* and the intergenic region between *map1-2* and *map1-1* (with the exception of 1 nucleotide), suggesting that a recombination between *map1-3* and *map1-2* had occurred (Fig. 2). A sequence of 14 bp was detected which was present in both the sequences of IBET Gardel *map1-2* and *map1-3*. The resulting "hybrid" gene had a continuous open reading frame (ORF) which was the same size as the *map1-3* gene in IBET Gardel. To confirm that the observed deletion in the Gardel CTVM isolate was not due to artifacts introduced by the PCR, Southern blots using *map1-2*- and *map1-3*-specific probes and a probe specific for the recombination site were conducted. As expected, the *map1-2*-specific probe only reacted with a HindIII fragment (2,277 bp) in the Gardel IBET lane, the *map1-3* specific-probe reacted with different HindIII fragments in the Gardel IBET (2,277 bp) and Gardel CTVM (4,085 bp) lanes, and the re-

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map1-3 AGC GGA CAA TAT AAA CCA AGT GTT TCC AAT TTT CGT AAT TTC TCA GTT AAA GAA ACT AAC
CTVM                                                                          ACT AAC
map1-2 AGT GGA CAA TAT AAA CCA AGT GTT CCT CAT TTT AGT AAC TTT TCA ATT AAT GAA ACT AAT

map1-3 ACT TAT ACA AAA AAT CTA ATA GGT GTT AAA AAG GAC ATT ACA TCT TTA GAA GTA CAT ACA
CTVM   ACT TAT ACA AAA AAC CTA ATA GGT GTT AAA AAG GAC ATT ACA TCT TTA GAA GTA CAT ACA
map1-2 ACT GAT ATA CAA AAT TTA GTA GCT GCC AAA CAA AAT ATT TCA TCA TTA GAT ATT GAT ACA

map1-3 AAT AAT AAC AAA CAC ATT GTA AGT AGA AGA AAT CCA AAT CCT GGA CCT ACT ATT AAA GCT
CTVM   AAT AAT AAC AAA CAC ATT GTA AGT AGA AGA AAT CCA AAT CCT GGA CCT ACT ATT AAA GCT
map1-2 GTC CTT --- AAA --- --- GAA --- --- --- --- --- --- --- --- --- --- --- --- ---

map1-3 ACA GGA ATT AGC AAT CCT AGT AAT TTT AAT ATT CCT TAT AAT CCA GAA TTT CAA GAT AAT
CTVM   ACA GGA ATT AGC AAT CCT AGT AAT TTT AAT ATT CCT TAT AAT CCA GAA TTT CAA GAT AAT
map1-2 --- --- --- --- --- CCT AGC AAC TTT AAC CAT CCA TAT ACT ACA GAA TTT CAA GAT AAC

map1-3 ATA ATA AAT TTT AGT GGA ACA ATC GGT TAT CAA TTT TCA AAA AGT AAA AGA ATA GAA ATA
CTVM   ATA ATA AAT TTT AGT GGA ACA ATC GGT TAT CAA TTT TCA AAA AGT AAA AGA ATA GAA ATA
map1-2 AAC ATA AGC TTT GGT GGA GCT ATT GGT TAC TAC TCC ACT GAA GGC ACA AGA ATT GAA TTA

map1-3 GAA GGA TCT TAT AAA ATA TTT GAT GTA AAG GAT CCT GGT GGA TAT ATG CTT TAT GAT GCA
CTVM   GAA GGA TCT TAT AAA ATA TTT GAT GTA AAG GAT CCT GGT GGA TAT ATG CTT TAT GAT GCA
map1-2 GAA GGA TCT TAC GAA TTC TTT GAT GTA AAA GAC CCT AGC GGT TAT AAA CTA CAT GAT GCA

map1-3 TAT CGA TAC TTT GCA TTA GCA CGT GAA ATG AAT GAT ACA AAA TTT GAG CCA AAA CCA TAT
CTVM   TAT CGA TAC TTT GCA TTA GCA CGT GAA ATG AAT GAT ACA AAA TTT GAG CCA AAA CCA TAT
map1-2 TAT AGG TAT TTT GCA TTA GCA CGT GAT ATG AAT AGA AAA AAA TCT --- TTT GAA CCA AAA

map1-3 CAA CTA GAC AAT TTT TTC AAC AAC TTT TAT CAT ACA GTT ATG AAA AAT ACA GGT CTG TCA
CTVM   CAA CTA GAC AAT TTT TTC AAC AAC TTT TAT CAT ACA GTT ATG AAA AAT ACA GGT CTG TCA
map1-2 AAA CAG ATT GGA TTA AGA ACA --- --- AAT TAT ACA GTC ATG CGA AAT AAT GGA TTA TTT

map1-3 ATC ATA TCT GTT ATG ATT AAT GGA TGT CAT GAT TTT CAT GTA AAT GAA TTA AAA ATA TCA
CTVM   ATC ATA TCT GTT ATG ATT AAT GGA TGT CAT GAT TTT CAT GTA AAT GAA TTA AAA ATA TCA
map1-2 ATT TCA TCT GTT ATA CTT AAT GGG TGT TAT GAT TTT TCT ATA AAT GAA TTG AAA ATA TCA

map1-3 CCT TAT ATA TGC GCA GGC GTT GGA ATA AAT ACT ATA GAA TTT TTT GAT ACC TCA CAT ATA
CTVM   CCT TAT ATA TGC GCA GGC GTT GGA ATA AAT ACT ATA GAA TTT TTT GAT ACC TCA CAT ATA
map1-2 CCT TAT ATG TGT GTA GGT ATT GGT ATA AAT GCT ATA GAA TTT TTC GAT GCA TTA CAC CTA

map1-3 AAG TTT GCT TAT CAA GGC AAA ATT GGC ATT AGT TAT CCA CTA TCT AAC AAC ATT AAA GTA
CTVM   AAG TTT GCT TAT CAA GGC AAA ATT GGC ATT AGT TAT CCA CTA TCT AAC AAC ATT AAA GTA
map1-2 AAA CTT GCC TAT CAA GGT AAA TTT GGT ATA AGC TAT CCC ATA TCT AAC AAT ATT AAA TTA

map1-3 TTT TCA AAT GGG TAT TAT CAT AAA GTA GTA GGT AAT AAA TTT AAA AAC TTA GAA GTT ATT
CTVM   TTT TCA AAT GGG TAT TAT CAT AAA GTA GTA GGT AAT AAA TTT AAA AAA CTA GAA GTT ATT
map1-2 TTC GCA GAT GGA TAT TAC TAC AAA GTA ACA GAC AAT AAA TTC AAA AAT CTC AAA GTT ATT

map1-3 CAT GTT GCC AAT TTA CAC AAT GCA CCA TGG TAT ACA TCT GCA ATA GCG ACA CTT AAT ATA
CTVM   CAT GTT GCC AAT TTA CAC AAT GCA CCA TGG TAT ACA TCT GCA ATA GCT ACA CTT AAT GTT
map1-2 TAT GTT GCT GAT CTG AAT AAC ACC CCA TTA GTA ACA TCT GCA ATA GCT ACA CTT AAT GTT

map1-3 GGA TAT TTC GGA GCC GAA GTT GGA ATA AGA TTA GGC TTA AAA TTA TAA
CTVM   GAA TAT TTT GGT GGA GAA ATT GGA ATA AGA TTT GGA TTA AAG TTA TAA TAT TAT ATA AAA
map1-2 GAA TAT TTT GGT GGA GAA ATT GGA ATA AGA TTT GGA TTA AAG TTA TAA TAT TAT ATA AAA

CTVM   AAT AAG TTT TTC TCT GGT AAA CAA ATT TAG CTT AAT GGA TAT CAT ATA AGT
map1-2 AAT AAG TTT TTC TCT GGT AAA TAA ATT TAG CTT AAT GGA TAT CAT ATA AGT TTC TTA TTA

map1-2 TTT TGA TTT TAT ATA TAA TGT ATA TCA GTA T
```

FIG. 2. Sequence alignment of the relevant part of *E. ruminantium* Gardel *map1-3* (top), *map1-2* (bottom), and the sequence obtained from CTVM Gardel with primers MAP1-3F and MAP1-2R2 (underlined). A black background indicates nucleotides that are different between the two sequences. The putative recombination site is boxed in and boldface.

combination site-specific probe reacted with two fragments (2,277 and 2,704 bp) in the Gardel IBET lane and only one fragment (4,085 bp) in the Gardel CTVM lane (Fig. 3). None of the probes reacted with HindIII-digested genomic DNA from uninfected endothelial cells. To confirm that both samples were indeed the Gardel isolate, the *map1* genes from the two samples were amplified and sequenced. Both sequences were identical to the Gardel *map1* sequence (accession number U50832) published in GenBank (data not shown).

Transcriptional analysis of the *map1* gene cluster in bovine endothelial cell cultures. To assess possible differences in transcription of members of the *map1* gene cluster of different isolates during growth in endothelial cells, cDNA was prepared from endothelial cell cultures infected with the Gardel (IBET

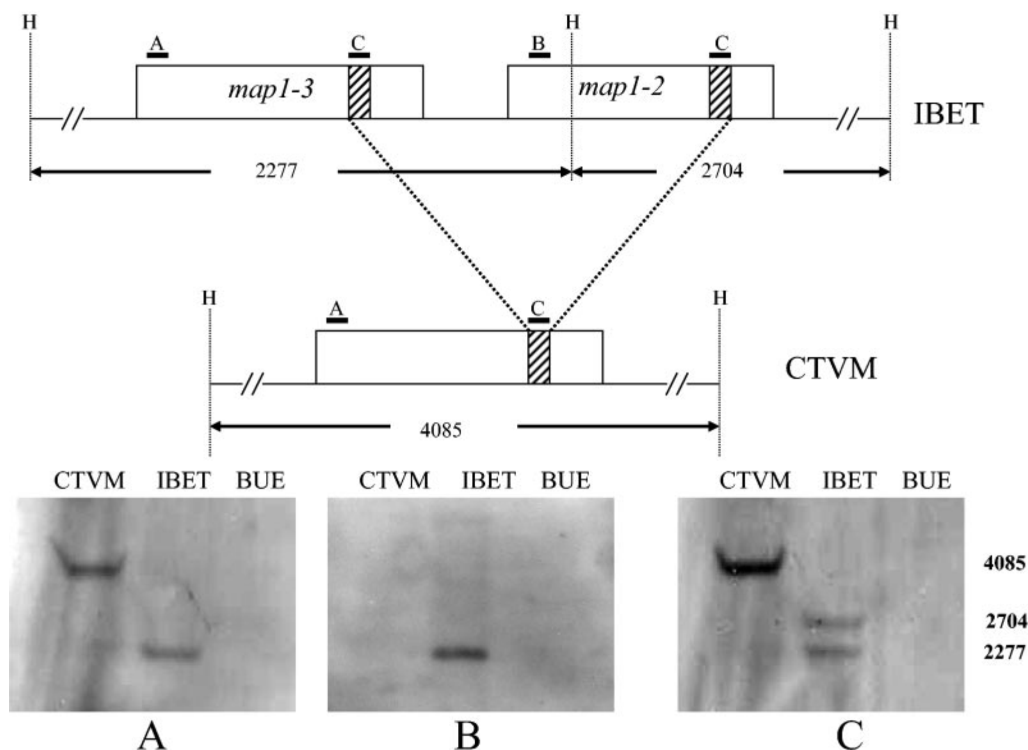

FIG. 3. Southern blot on HindIII-digested genomic DNA. The top shows a schematic representation of the genomic region containing *map1-3* and *map1-2* with the position of the HindIII restriction sites (H) and the location of the different probes (A to C). The bottom shows the genomic Southern blot with probe MAP1-3F (A), MAP1-2F (B), and the recombination site (C). BUE indicates uninfected endothelial cells.

and CTVM), Senegal, and Welgevonden isolates of *E. ruminantium* and analyzed by PCR using *map1* paralog- and in some cases isolate-specific primers. Independent of the source of endothelial cells (pulmonary artery or umbilical cord) or the passage number of the isolate, all *map1* paralogs were transcribed in the three studied isolates.

Transcriptional analysis of the *map1* gene cluster in tick cell cultures. Gene transcription after growth of the CTVM Gardel or Welgevonden isolates in different tick cell lines was determined with *map1* paralog-specific primers. For all tick cell lines tested, a transcript for *map1-1* was present and was the predominant transcript (Table 3). Transcripts for *map1-3* and *map1-11* were never observed in any of the infected tick cell lines. Some transcripts were only detected in nonvector tick cell lines, namely *map1-12*, *map1-10*, *map1-6*, and *map1-5* (Table 3). Figure 4 shows the results obtained from the AVL/CTVM13 and RAN/CTVM3 cell lines infected with the Welgevonden isolate of *E. ruminantium* and illustrates the variation in the amount of gene product amplified for the individual paralogs.

DISCUSSION

We report here the observation that the *E. ruminantium map1* gene cluster is relatively conserved among different isolates but can be subject to recombination leading to removal of entire genes from the cluster. Furthermore, the transcriptional analysis of the genes within the locus in vitro in host endothelial and vector and nonvector tick cell lines is reported. All genes were found to be transcribed in endothelial cell culture as described recently (38), independent of the source of the endothelial cells. In tick cell lines however, transcription of some paralogs could not be detected, and variation in the amount of amplified product was observed with *map1-1* always being the most prominent transcript.

One of our most remarkable results was that two subpopulations of the Gardel isolate exhibited a different gene composition, despite the apparent conservation in gene organization of the *map1* gene cluster among *E. ruminantium* isolates. The deletion of the *map1-2* gene in the CTVM Gardel subpopulation indicates that recombination can occur which may influence the phenotype of the bacteria. At the site of the deletion a repeat motif was detected of 14 bp which occurred only in *map1-2* and *map1-3*. The absence of the *map1-2* gene in the CTVM Gardel *map1* gene cluster can explain the inability to amplify this gene in previous experiments (2). The observation that the Southern blot with the *map1-2*-specific probe did not show any fragment in the Gardel CTVM lane suggests that the gene is not present elsewhere in the genome. Unfortunately it was not possible to completely trace the history of the CTVM Gardel isolate, but following arrival at CTVM the isolate was used to infect goats, and cultures were established from their blood. As the oldest identifiable cultures derived from these goats at CTVM already showed the absence of *map1-2* by passage 2, we assume the recombination event occurred in the initial infections at CTVM or had already occurred in the material obtained from Guadeloupe.

Reddy et al. (29) described recombinase motifs and a high

TABLE 3. Transcription of the *E. ruminantium map1* multigene family in infected tick cell lines

Gene	Cell line, cell line passage no., isolate, and isolate passage no.[a]											
	AVL13		IDE8		IRE18	RAN3			RAE1	RAE25	BME2	BDE16
	18 G 0 (5)[b]	18 W 0 (2)	82 G 5	83 W 6	23 G 5	56 G 14 (7)	61 W 2 (1)	56 W 5 (1)	85 W 1 (1)	147 W 1 (1)	78 G 5 (4)	26 G 8 (4)
map1-14	+	+		+							+	+
map1-13	+	+	+	+			+	+		+		+
un-1	+	+		+			+	+				
map1-12											+	
map1-11												
map1-10					+						+	+
map1-9	+	+	+	+		+	+	+				+
map1-8	+	+	+	+		+	+	+				
map1-7	+	+	+	+		+	+	+	+		+	+
map1-6				+	+		+				+	+
map1-5											+	+
map1-4	+	+	+	+			+		+		+	+
map1-3												
map1-2		+		+			+	+	+			
map1-1	+	+	+	+	+	+	+	+	+	+	+	+
map1	+	+	+	+			+	+	+	+		+
map1+1	+	+	+	+	+	+	+	+	+	+	+	+

[a] Infected cell lines IDE8, RAN3, RAE1, RAE25, BME2, and BDE16 were incubated at 31°C, IRE18 at 28°C, and AVL13 at 37°C. "/CTVM" has been omitted from cell line names for simplicity. G, CTVM Gardel lacking *map1-2*; W, Welgevonden.

[b] The first number indicates the *E. ruminantium* passage level in the cell line, the number in parentheses is the passage level in the IDE8 culture used to infect the cell line. IDE8 and IRE18 were infected directly from bovine endothelial cells (4).

frequency of repeat elements in the *p28* multigene locus of *E. chaffeensis* suggesting that recombination could occur but found no detectable rearrangements over a period of 2.5 years in an in vitro culture of the Arkansas isolate (29). Collins et al. (10) recently reported that the genome of *E. ruminantium* is very rich in tandemly repeated and duplicated sequences and that these repeats have mediated numerous translocation and inversion events that have resulted in the duplication and truncation of some genes and have also given rise to new genes (10). As the *map1-2* gene is present in the Welgevonden and Senegal isolate and could also be amplified from other isolates (data not shown), we assume that the gene is deleted from CTVM Gardel. Cheng et al. (9) showed that molecular heterogeneity of *E. chaffeensis* isolates occurred in the α-repetitive region of the cluster, giving rise to three genetic groups containing either 22 (group I and III) or 21 genes (group II). Two locations were identified where insertions and/or deletions occurred (9). The recombination described in the present study appeared however in the β-repetitive region and was observed in what was considered to be a single isolate. As all *E. ruminantium* isolates for which the *map1* gene sequence is known are unique, and we obtained identical *map1* sequences from both Gardel sources, we assumed we were dealing with only one isolate. However, it was shown for another isolate (Kümm) of *E. ruminantium* that it in fact consisted of two genetically different "strains" which were separated and identified through their different host cell tropism in vitro (44), so it is possible that the original material received at CTVM contained two "strains," of which one was subsequently established in culture and the other was not.

We found all the *map1* paralogs and the gene of unknown function (*un-1*) to be transcriptionally active when the Senegal, Gardel, and Welgevonden isolates were cultured in endothelial cells, confirming the recent report of van Heerden et al. (38). We did not investigate whether or not paralogs were cotranscribed. Cultures were tested at different passage numbers and in different endothelial cell lines, but in all cases the results were the same. For unknown reasons transcription could not be detected in all cases when samples were tested the first time but were detected when samples were retested. These results differ from the data in endothelial cells previously reported by us, in which we only found *map1* transcribed and no transcripts for *map1-1* or *map1-2* (2). This may be explained by the fact that in the present study we used two specific primers for each paralog and prepared cDNA using random hexamer primers, whereas previously we used *map1* cluster-specific primers to generate the cDNA. Discrepancies in gene transcription have also been reported from studies in monocyte cultures of the *p28* and *p30* multigene families in *E. chaffeensis* and *E. canis* (23, 24, 30, 42). The same gene families were also studied in vivo in dogs and in tick vectors. In monocytes of infected dogs, 11 out of 14 *E. canis p30* genes and 16 out of 22 *E. chaffeensis p28* genes were transcribed, whereas transcription of only 1 gene (*p30-10* or *omp-1B*) was detected in *Rhipicephalus sanguineus* or *Amblyomma americanum* ticks, respectively (13, 35, 36).

We found that the *E. ruminantium* ortholog of *p30-10* and *omp-1B*, *map1-1*, was transcribed in all the *E. ruminantium*-infected tick cell lines and was the most dominant transcript present. However, we also found other *map1* paralogs transcribed in tick cell lines indicating that, in vitro at least, several paralogs can be transcribed in tick cells. We previously reported that in *A. variegatum* ticks both *map1-1* and *map1* of the Senegal isolate are transcribed (2). In the vector cell line AVL/CTVM13 we found 10 out of 16 *map1* paralogs transcribed for the Welgevonden isolate and 9 for the CTVM Gardel isolate,

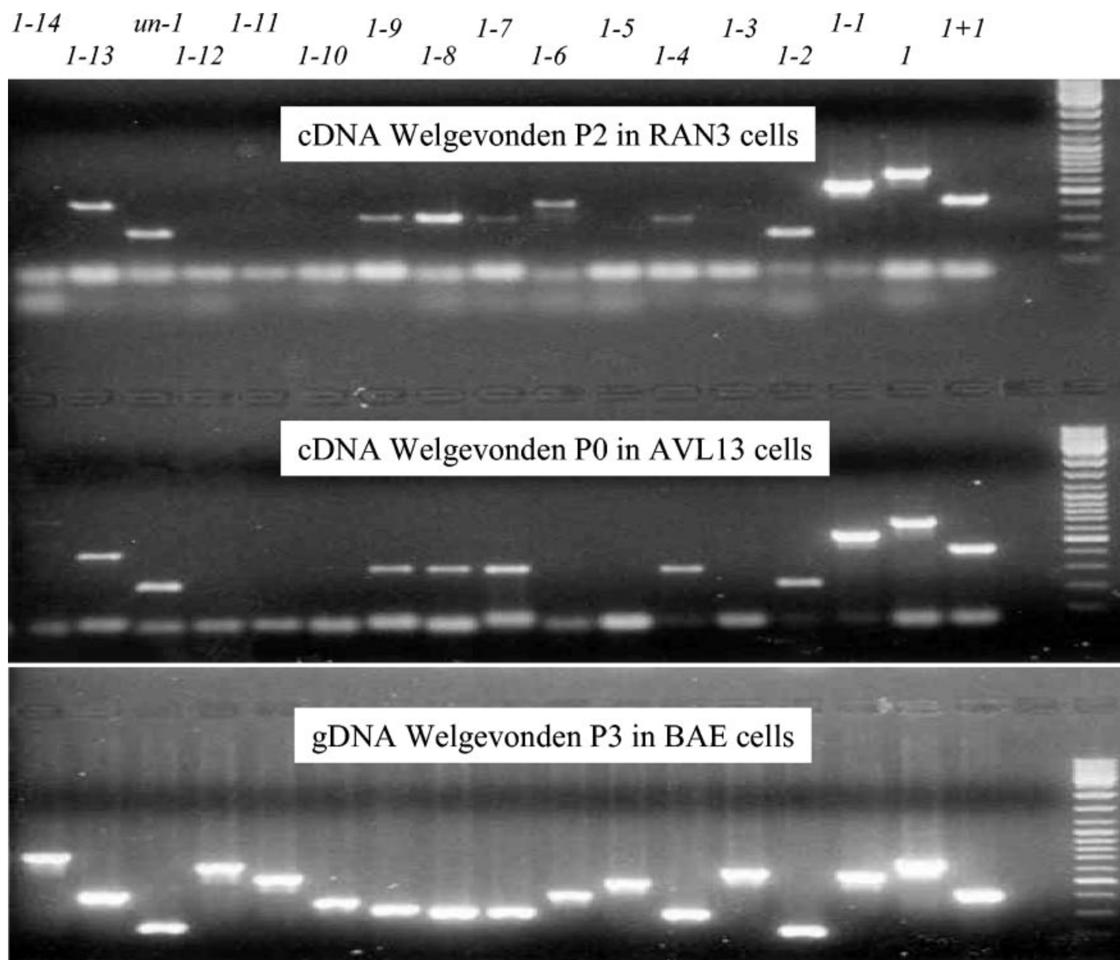

FIG. 4. RT-PCR of the *map1* multigene family of *E. ruminantium* Welgevonden in the *A. variegatum* cell line AVL/CTVM13 (middle) and an *R. appendiculatus* cell line RAN/CTVM3 (top). The bottom panel shows a control PCR with the same primer set using genomic DNA (gDNA) as template. Lanes 1 to 17 contain PCR products for *map1* paralogs as shown, lane 18 contains the PCR negative control, and lane 19 contains molecular weight markers.

including *map1-1* and *map1*. The transcription pattern for the two isolates was thus identical considering that *map1-2* is missing from the CTVM Gardel isolate used in these cultures. Although *Amblyomma* species are the only known vectors for *E. ruminantium* (41), several cell lines established from other tick genera have been shown to support growth of *E. ruminantium* (4, 5, 7). The number of transcripts detected in these nonvector cell lines ranged from 4 (RAE25) to 11 (IDE8). For the Welgevonden isolate all the transcripts found in the vector cell line AVL/CTVM13 were also found in the nonvector line IDE8. Despite the differences observed in transcription patterns between the various tick cell lines, transcripts of two paralogs (*map1-11* and *map1-3*) were never detected and two paralogs (*map1-1* and *map1+1*) were always found to be transcribed. A surprising observation was the transcription of four paralogs (*map1-12*, *map1-10*, *map1-6*, and *map 1-5*) in nonvector tick cell lines which were not detected in the vector cell line AVL/CTVM13 but which were transcribed in endothelial cells. Studying the transcription of the *map1* multigene family in infected ticks may show if the same paralogs are transcribed in vivo as in vitro in tick cell lines. At this point it should be noted that our RT-PCR results should be interpreted with caution, as finding a transcript does not necessarily imply that the mRNA is translated into a protein. In *E. chaffeensis*, despite the fact that multiple transcripts for *p28* (*omp-1*) were detected, the product of only one gene was found based on N-terminal amino acid sequencing of expressed proteins from cultured organisms (23, 28). Using a proteomics approach, Singu et al. (31) showed for the same organism the expression of the products of two *omp* genes (p28-Omp19 and p28-Omp20) in macrophages and one (p28-Omp-14) in tick cells and that these proteins are posttranslationally modified by phosphorylation and glycosylation to generate multiple expressed forms (31). Whether a similar discrepancy between transcription and translation and/or expression exist for *E. ruminantium* remains to be determined.

As there have not been any studies reported on *E. canis* and *E. chaffeensis* gene transcription in vitro in tick cell lines, it is difficult to evaluate our results. However, some work has been done on two other rickettsial pathogens in *I. scapularis* cell lines, namely *Anaplasma marginale* and *Anaplasma phagocytophilum*. The *A. marginale* major surface protein 2 (MSP2) is

encoded by a multigene family, and two MSP2 operon-associated proteins were expressed by *A. marginale* in IDE8 cells and in the bovine host during acute rickettsaemia, while only one was expressed in *Dermacentor* ticks during transmission feeding (22). Garcia-Garcia et al. (15) studied expression of *A. marginale* MSP1a, MSP1b, and MSP5 in infected bovine erythrocytes, IDE8 cells, and *Dermacentor* salivary glands. While the levels of MSP5 and MSP1b were similar in extracts of erythrocytes and IDE8 cells, MSP1a levels were much lower in IDE8 cells than in erythrocytes and were undetectable in sections of salivary glands. Transcriptional analysis indicated that the expression of MSP1a was probably regulated at the transcriptional level, since the amount of *msp1*α transcripts in infected IDE8 cells was lower than that in erythrocytes and undetectable in infected salivary glands, while transcripts for *msp1*β, *msp4*, and 16S rRNA were present in all three tissues (15). The immunodominant surface protein P44 of *A. phagocytophilum* is also encoded by a multigene family and is expressed in vitro in the human promyelocytic cell line HL-60. When the pathogen was transferred to the *I. scapularis* cell line ISE6 growing at 34°C, downregulation of a specific P44 antigen was observed in Western blots using a monoclonal antibody. This effect was not temperature dependent, since it was also seen in tick cells maintained at 37°C, but upregulation occurred after the pathogen was returned to HL-60 cells (16). Later it was shown that the *p44* gene in the expression site is polymorphic in vitro (HL-60 cells and ISE6 tick cells [1]) and in vivo (mice and *I. scapularis* ticks [14] and humans [1, 21]). Temperature does not seem to play a major role in the transcription of the *E. ruminantium map1* multigene family in vitro either, as the same paralogs transcribed at 37°C in AVL/CTVM13 cells were also detected at 31°C in IDE8 cells, and no differences were seen in transcription pattern between incubation temperatures of 30°C and 37°C for *E. ruminantium* in endothelial cells (2, 38).

Together, these data indicate that the *E. ruminantium map1* multigene family is regulated differently in the host and vector cell environments in vitro and can be subject to recombination leading to an altered gene arrangement. Studying the transcriptional activity using quantitative assays of the *E. ruminantium map1* multigene family in its natural environment in the host and vector in vivo combined with a proteomics approach should provide more insight into the regulation and function of this gene family.

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