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Transcriptional analysis of the major antigenic protein 1 multigene family of *Cowdria ruminantium*

Cornelis P.J. Bekker[a,b], Lesley Bell-Sakyi[c], Edith A. Paxton[c], Dominique Martinez[d], Albert Bensaid[d], Frans Jongejan[b,e,*]

[a]*Division of Bacteriology, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Box 80165, 3508 TD Utrecht, The Netherlands*

[b]*Division of Parasitology and Tropical Veterinary Medicine, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Box 80165, 3508 TD Utrecht, The Netherlands*

[c]*Centre for Tropical Veterinary Medicine, University of Edinburgh, Easter Bush, Roslin, Midlothian, Scotland, UK*

[d]*CIRAD-EMVT, Pointe-à-Pitre, Guadeloupe, France*

[e]*Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa*

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Abstract

The major antigenic protein 1 (MAP1) of the tick-borne rickettsial pathogen *Cowdria ruminantium* is encoded by a multigene family containing conserved and variable genes. The part of a locus containing the *map1* multigene family that was characterized contained three homologous, but non-identical *map1* genes, designated *map1-2*, *map1-1*, and *map1*. Reverse transcriptase-polymerase chain reaction was used to study the transcriptional activity of these genes in isolates of *C. ruminantium* grown in bovine endothelial cells, in two different tick cell lines, and in *Amblyomma variegatum* ticks. The *map1* gene was always transcribed, whereas transcription of *map1-2* was not detected under any of the tested conditions. The *map1-1* gene transcript was detected in *A. variegatum* ticks, but was not found in virulent *C. ruminantium* Senegal grown in bovine endothelial cells at 30 or 37°C. Interestingly, transcripts of *map1-1* were also found in different passages of the in vitro attenuated Senegal isolate grown in bovine endothelial cells, as well as in the Gardel isolate grown in two tick cell lines. When transcribed, *map1-1* was present on a polycistronic messenger together with *map1*. © 2002 Published by Elsevier Science B.V.

Keywords: Differential transcription; *Amblyomma variegatum*; Ticks; Tick cell lines

1. Introduction

Cowdriosis (or heartwater) is a tick-borne disease caused by the rickettsia *Cowdria ruminantium* and transmitted by ticks of the genus *Amblyomma*. The disease affects both domestic and wild ruminants in sub-Saharan Africa and on certain Caribbean islands (Uilenberg, 1983). *C. ruminantium* and members of the related genera *Ehrlichia* and *Anaplasma* usually cause persistent infections in their natural hosts (Andrew and Norval, 1989; Gale et al., 1996; Breitschwerdt et al., 1998). Regulation of surface antigenicity may be an important mechanism for the establishment of such persistent infections in the host. Multigene families encoding for antigenic proteins are present in *C. ruminantium* (*map1*) (Sulsona et al., 1999), *Anaplasma marginale* (*msp-2*) (Palmer et al., 1994, 1998), *Ehrlichia canis* and *Ehrlichia chaffeensis* (*p28*) (Ohashi et al., 1998a; Reddy et al., 1998). The *map1* multigene family found in *C. ruminantium* is more closely related to the *p28* multigene family than to the *msp-2* multigene family, both in sequence similarity and in gene organization. The *map1* and *p28* genes are both located in a single cluster, whereas *msp-2* genes are dispersed over the genome. Recently, the complete *p28* multigene locus of *E. chaffeensis* containing 21 homologous *p28* genes was characterized (Yu et al., 2000), and the presence of a conserved, transcriptionally active *p28* multigene locus of *E. canis* (McBride et al., 2000) has been reported. In both studies only monocistronic transcripts were found even when two neighboring genes were transcribed simultaneously. However, in a study

Abbreviations: RT-PCR, reverse transcriptase-polymerase chain reaction; BEC, Bovine endothelial cells; MAP, major antigenic protein; DNase, deoxyribonuclease; RNase, ribonuclease; cDNA, DNA complementary to RNA

* Corresponding author. Tel.: +31-30-253-2568; fax: +31-30-254-0784.
E-mail address: f.jongejan@vet.uu.nl (F. Jongejan).

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wherein both gene clusters were compared it was found that all 22 paralogs of *E. canis* were transcriptionally active in monocyte cultures, and that paralogs with short intergenic spaces were co-transcribed (Ohashi et al., 2001).

Gene *p28-19* of the *E. chaffeensis* multigene family and *map1* of *C. ruminantium* encode surface-exposed proteins. The respective genes are largely conserved among different isolates except for three hypervariable regions (Reddy et al., 1996; Ohashi et al., 1998b). Studies using monoclonal antibodies have demonstrated diversity among *E. chaffeensis* isolates in the expressed P28 proteins (Yu et al., 1993) and this was shown to be related to diversity in the *p28* gene (Yu et al., 1999). However, complete conservation of a *p28* paralog in geographically different isolates of *E. canis* has also been reported (McBride et al., 1999). These findings show that both conserved and variable genes are present in the *p28* gene families of *E. canis* and *E. chaffeensis*, a situation that has also been reported for the *C. ruminantium map1* multigene family (Sulsona et al., 1999). Gaining more knowledge about these gene families is important as recombinant *E. chaffeensis* P28 and a DNA vaccine containing *map1* of *C. ruminantium* appeared to provide protection against an otherwise lethal challenge with the homologous isolate in mice (Nyika et al., 1998; Ohashi et al., 1998b). Furthermore, in a recent study it was shown that outer membrane protein-specific monoclonal antibodies protected SCID mice from fatal infection by *E. chaffeensis* (Li et al., 2001).

In this study, we used RT-PCR to examine if there were differences in the transcriptional activity of three homologous, non-identical genes in the *map1* multigene family of *C. ruminantium* under different growth conditions. Conditions included in vitro in tick and bovine endothelial cells and in vivo in *Amblyomma variegatum* ticks. Finally, a comparison was made between transcription in different passages of virulent and attenuated *C. ruminantium* in endothelial cell cultures.

2. Materials and methods

2.1. Cowdria ruminantium isolates and endothelial cell culture conditions

The *C. ruminantium* isolates used in this study were: Senegal (virulent and attenuated) from Senegal, Welgevonden from South Africa, Sankat 430 from Ghana, and Gardel from Guadeloupe. Bovine endothelial cells (BEC) derived from umbilical cord were used to cultivate *C. ruminantium* isolates. To determine if the lower temperature used for the tick cell cultures (30°C instead of 37°C) influenced transcription of *map1* genes, infected BUE cultures were also incubated and passaged at 30°C. After three successful passages, total RNA was extracted from these cultures.

C. ruminantium-infected BEC cultures were regularly examined by preparation of Giemsa stained cytospin smears of culture supernate. When the cultures started showing signs of cell damage due to the infection, cells were scraped from the bottom of the culture flasks and pelleted by centrifugation for 10 min at $300 \times g$ and total RNA was extracted.

2.2. Infected tick cell cultures

The *Ixodes scapularis* cell line IDE8 was maintained at 30 ± 2°C in L-15B medium supplemented with 10% tryptose phosphate broth, 5% foetal calf serum (FCS), 0.1% bovine lipoprotein, 2 mM L-glutamine, and 100 units penicillin and 100 μg streptomycin per ml. A *Rhipicephalus appendiculatus* cell line designated RAN/CTVM3, comprising mainly haemocytes, fibroblast-like, and epithelial-like cells, was established from moulting nymphal ticks. RAN/CTVM3 cells were maintained at 28°C in H-Lac medium (Hanks BSS supplemented with 0.5% lactalbumin hydrolysate, 20% FCS, and L-glutamine and antibiotics as above).

IDE8 cells at passage 65–67 were infected with *C. ruminantium* (Gardel) and maintained as described previously (Bell Sakyi et al., 2000). Cell-free supernate from infected IDE8 cultures, obtained by centrifugation at $1000 \times g$ for 10 min, was used to infect RAN/CTVM3 cultures at passage 56–58, which were incubated thereafter at 30 ± 2°C with weekly medium changes.

C. ruminantium-infected tick cell cultures were examined by preparation of Giemsa stained cytospin smears of suspended cells. When at least 10% of the cells were infected, the cultures were harvested by pipetting off adherent cells and centrifuging the resultant cell suspension for 10 min at $300 \times g$. The cell pellets were thereafter used for total RNA extraction.

2.3. Infected Amblyomma variegatum ticks

Female sheep (Texelaar) between 6 and 12 months of age were inoculated intravenously using cryopreserved tissue culture (BEC) derived *C. ruminantium* stabilate (Senegal isolate). The sheep were treated with oxytetracycline (5 mg/kg) starting on the 3rd day of fever ($T > 40.0$°C) for three consecutive days to ensure recovery. Treatment with oxytetracycline does not clear the infection and animals become carriers. At 60 days post infection, approximately 100 uninfected *A. variegatum* nymphs were applied to the back of each sheep. The ticks originated from Burkina Faso and were maintained in a colony at Utrecht University. Engorged nymphs were collected and allowed to moult to the adult stage at 27°C and 95% relative humidity.

2.4. Cloning of map1 genes

C. ruminantium (Senegal) was purified from BEC cultures and genomic DNA was purified as previously described (van Vliet et al., 1992, 1994). A genomic library was constructed in pUC19 by ligating *Hin*dIII digested *C. ruminantium* DNA with *Hin*dIII digested, dephosphorylated pUC19. The ligation mix was used to transform *Escherichia*

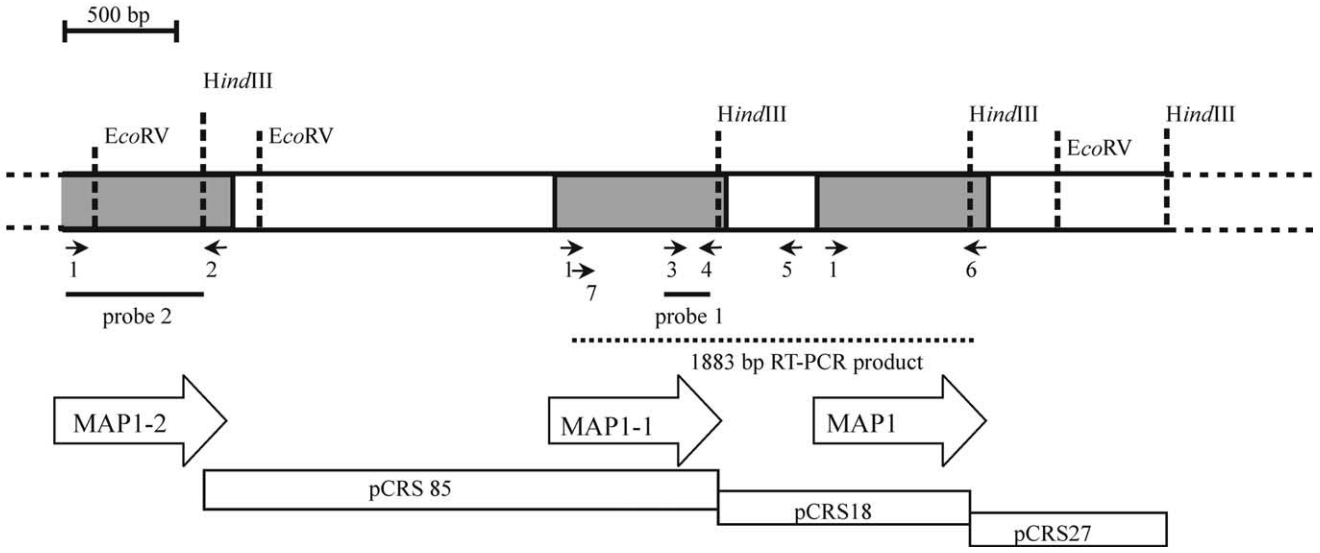

Fig. 1. Schematic representation of the *C. ruminantium* (Senegal isolate) *map1* gene locus (5128 bp) indicating the genomic orientation, primers and probes. The boxes indicated with pCRS represent the different clones obtained from the genomic *Hin*dIII library. The region amplified by RT-PCR to demonstrate the presence of a polycistronic transcript is indicated by the dotted line. The probes used to screen the genomic library are indicated with solid lines. Primers indicated and numbered were as follows; 1, map1g-for; 2, map1-2rev; 3, f5; 4, map1-1rev; 5, r-50; 6, map1-rev; and 7, map1-1for.

coli DH5α (Gibco) to ampicillin resistance. The cloning of *map1* from this library has been described previously (van Vliet et al., 1994). A primer specific for the upstream region of *map1*, designated r-50 (5′-AAA GCA AGC TAT AAT GTA AGT-3′), was used together with primer f5 (5′-GCG CAA AAT ACA TGC CAA CTG CAT C-3′) to amplify the region upstream of *map1*. The resulting PCR product was digested with *Hin*dIII and the 5′ part was used as a probe to screen the genomic library (Fig. 1, probe 1). One of the clones, designated pCRS85, was sequenced and shown to contain the 5′ part of *map1-1* and the 3′ part of the *map1-2* gene (Fig. 1). The partial *map1-2* gene was further characterized by sequencing a PCR product obtained after amplification of genomic DNA with primer map1-2rev, specific for the *map1-2* gene, and a general *map1* primer designated map1g-for (Table 1).

2.5. DNA sequencing

DNA was sequenced on an ABI Prism 310 (Perkin Elmer Applied Biosystems, Foster City, CA) or sent for sequencing to BaseClear (Leiden, The Netherlands)

2.6. Total RNA isolation from infected cells

Total RNA was isolated from *C. ruminantium*-infected BEC grown at either 30 or 37°C and from infected tick cells using a RNeasy mini kit (Qiagen). Total RNA was treated before elution with RNase-free DNase I (10 U). Following elution, the RNA was quantified in a spectrophotometer at A_{260}, while purity was confirmed by a spectrophotometric A_{260}/A_{280} ratio of over 1.8.

Table 1
Primers for cloning and gene-specific RT-PCR amplification

Name	Sequence of (f) forward and reverse (r) primers	Nucleotides[a]
Cloning		
f5	(f) GCG CAA AAT ACA TGC CAA CTG GAT C	602–626
r-50	(r) AAA GCA AGC TAT AAT GTA AGT	409–429
RT-PCR		
map1g-for	(f) TAA T(A/G)T CAT TA(A/G) TGT CAT TTT TAC C	515–539
map1g-rev	(r) A(A/T)(A/C/G/T) (C/T)AA A(C/T)C TT(A/C) (C/T)TC CAA (G/T)TT C	79–100, 1309–1330
map1-rev	(r) TGG ACT AAC AGC ACT ACT GGC	1246–1266
map1-1for	(f) CCA AGC ATA CCA CAT TTC AGA	This study
map1-1rev	(r) TGA AGC GGA AGT GCT TTG AGG	28–48
map1-2rev	(r) TAT TGC AGA TGT TAC TAA TGG GGA T	This study

[a] Numbers based on accession number X74250 (van Vliet et al., 1992).

2.7. Total RNA isolation from infected ticks and determination of infection status

Total RNA was isolated from unfed adult *A. variegatum* ticks, several weeks after moulting, using a total RNA isolation kit (totally RNA™, Ambion). Ticks were cut in half using sterile scalpel blades and one half was incubated in 1 ml of denaturation buffer until all internal organs were completely denatured. Total RNA was subsequently digested for 30 min at 37°C with RQ1 RNase-free DNase (10 U) (Promega). After the addition of 20 mM ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), DNase was inactivated by incubation for 10 min at 65°C. RNA was used without any further treatment in RT-PCR reactions.

DNA was extracted from the other half of each tick by boiling it for 10 min in PBS; subsequently SDS was added (1% final concentration) and the sample was spun for 5 min at full speed. DNA was phenol-extracted from the supernatant and subsequently ethanol-precipitated and resuspended in TE. Extracted DNA was used in a PCR using primers EHR-F (5′-ggaattcAGAGTTGGATCMTGGYTCAG-3′) and primer EHR-R (5′-cgggatccCGAGTTTGCCGGGACTTYTTCT-3′) to demonstrate the presence of *C. ruminantium*.

2.8. Reverse transcriptase PCR (RT-PCR)

RNA (1 μg) from in vitro cultured *C. ruminantium*-infected cells was used to generate cDNA using a first strand cDNA synthesis system (SUPERSCRIPT™, Life Technologies) and random hexamer primers, according to the manufacturer's instructions. Primer pairs (0.4 μM), one general (g-for) and one specific for each *map1* gene were used to amplify each *map1* gene in a PCR reaction using the same cDNA batch as template (Table 1). PCR parameters were as follows: amplification for 30 cycles at 94°C for 30 s, 50°C for 1 min, and 72°C for 1 min; and a final elongation step at 72°C for 5 min. RNA from *C. ruminantium*-infected ticks was amplified using an Access RT-PCR system (Promega) using gene specific primers for first strand cDNA synthesis. The thermal cycling profile consisted of reverse transcription at 42°C for 50 min followed by 10 min at 94°C. The subsequent PCR step was as described above. A negative control that included all reagents except the reverse transcriptase was included in every test to confirm that genomic DNA was not present in the RNA preparations. Amplified (RT-)PCR products were sequenced to verify the specificity of the primers.

2.9. Nucleotide sequence accession number

The complete sequence of the pCRS85 clone has been submitted to the DDBJ/EMBL/GenBank database under accession number AF319940.

3. Results

3.1. Cloning and sequencing of map1 genes of C. ruminantium (Senegal)

A study by van Vliet et al. (1994) identified an incomplete open reading frame (ORF) adjacent to the *map1* gene of the Senegal isolate that showed homology with 32% similarity to the 3′ end of the *map1* gene. Previous attempts to identify *map1* homologous genes using part of the *map1* gene as a probe had failed and it was originally thought that *map1* was a single copy gene (Reddy et al., 1996). In an attempt to clone the entire gene, reverse primer r-50 specific for the intergenic upstream region of *map1* and forward primers that target regions within the *map1* gene, were used for PCR amplification. Whereas all other primer combinations failed we obtained a PCR product with primer f5. Apparently primer f5 was also able to hybridize to a region upstream of *map1*. The 5′ *Hin*dIII part of the resulting PCR product was used as a probe to screen a *C. ruminantium* (Senegal) *Hin*dIII library (Fig. 1, probe 1). Several positive clones were lifted from the library and digested with *Hin*dIII to determine the insert size. Clone pCRS85, which contained an insert of approximately 2.4 kb, was selected and sequenced. The clone contained the unidentified part of the gene which was named *map1-1*. Upstream of this *map1-1* gene an additional partial *map1* homologue was identified (designated *map1-2*) separated from *map1-1* by a 1385 bp intergenic region (Fig. 1) In order to clone the missing 5′ part of this gene, primer map1-2rev specific for the newly identified gene, and primer map1-gfor, which targets a conserved region at the 5′ end of known *map1* paralogs, were used for PCR amplification. A 0.8 kb PCR product was amplified and sequenced and shown to contain one continuous open reading frame. Use of the 5′ *Hin*dIII fragment of this PCR product as a probe (Fig. 1, probe 2) to screen the *C. ruminantium Hin*dIII library did not reveal any positive clones.

3.2. Amino acid homology

The deduced amino acid sequences of MAP1 and of MAP1-1 and the partial MAP1-2 proteins of the Senegal isolate were aligned using the ClustalV method (Fig. 2). The amino acid homology ranged from 32.8 to 46.7% among the *C. ruminantium* proteins when the entire ORFs of *map1* and *map1-1* and the partial ORF of *map1-2* were compared (Table 2). Higher amino acid homologies were observed between *C. ruminantium* MAP1 and homologous proteins from *E. canis* and *E. chaffeensis* (Table 2); the same was observed for MAP1-1 and MAP1-2. Clear conserved and hypervariable domains such as those found in MAP1 were not detected in MAP1-1 or MAP1-2 (Fig. 3). When MAP1 of the Senegal isolate was aligned with MAP1-1 and MAP1-2 of the same isolate neither clear conserved nor hypervariable domains could be detected (Fig. 2). There-

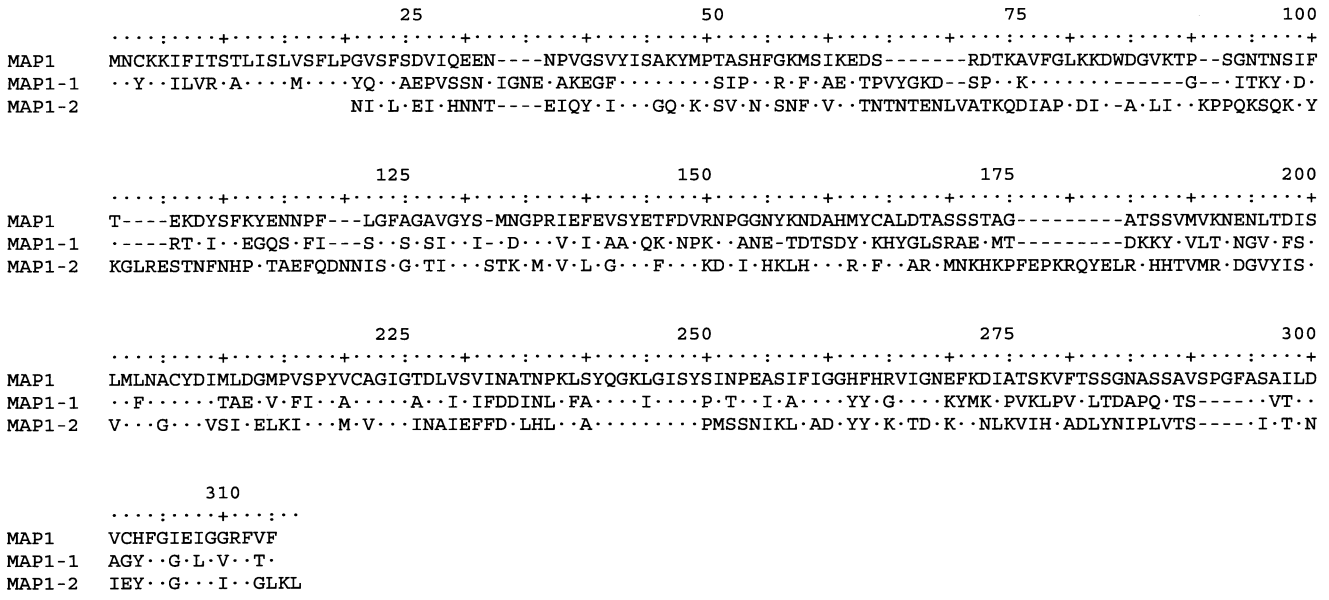

Fig. 2. Alignment of *C. ruminantium* MAP1, MAP1-1, and MAP1-2 of the Senegal isolates. The *map1* gene and its two paralogs *map1-1* and *map1-2* were translated and aligned using the ClustalV method (Lasergene 5.0 package DNAstar Inc.). The complete amino-acid sequence of MAP1 is presented. Dots indicate identity with MAP1 and dashes were introduced to obtain the best alignment.

fore, ambiguous bases had to be incorporated in the map1-gfor and map1-grev primers (Table 1). All primers were tested on genomic DNA from the isolates used and shown to amplify the expected genes, except for the *map1-2* primer which did not give a product when the Gardel isolate was used (Fig. 5A).

3.3. Transcriptional analysis of map1 paralogs of C. ruminantium isolates grown in bovine endothelial cells

To study the transcriptional activity total RNA was extracted from *C. ruminantium* grown in bovine endothelial cells and cDNA was prepared using random hexamer primers. Amplification of the three gene transcripts from cDNA prepared from *C. ruminantium* (Senegal, Welgevonden, Sankat 430)-infected endothelial cells grown at 37°C was performed using gene specific reverse primers and primer map1g-for (Table 1). RNA transcripts were detected only for the *map1* gene in the three *C. ruminantium* isolates (Fig. 4A). To determine if the lower temperature in ticks compared to mammals could have an effect on transcription, cultures infected with the Senegal, Welgevonden, or Sankat 430 isolate were incubated at 30°C and total RNA was extracted after three passages (23 days). Again RNA transcripts were detected only for the *map1* gene (Fig. 4A). All RT-PCR products were sequenced and confirmed to be *map1* gene sequences, indicating specificity of the primer pairs for *map1*.

In vitro attenuated *C. ruminantium* Senegal isolate (passage 32 or 37) grown in endothelial cells at 30°C or 37°C was also tested using RT-PCR to determine if the same transcription pattern was present as in virulent passages, from which the attenuated culture was derived. As in a virulent passage a RNA transcript was found for *map1* and no transcript was found for *map1-2* (Fig. 4B). However, in contrast to the virulent passage an additional

Table 2
Percentage identity (upper triangle) and divergence (lower triangle) of amino acid sequences of *C. ruminantium* MAP1 and *E. canis* (Eca) and *E. chaffeensis* (Ech) P28 proteins calculated using the MegAlign option of the Lasergene 5.0 package (DNAstar Inc.)

	1	2	3	4	5	6	7	8	9
1 MAP1		46.7	32.8	39.4	46.4	65.5	38.6	47.0	67.2
2 MAP1-1	88.5		35.1	40.3	79.7	49.6	39.4	78.0	47.8
3 MAP1-2	135.5	115.2		57.4	35.7	35.4	56.5	34.5	33.3
4 Eca P28-1	126.7	110.3	56.9		40.9	40.6	69.3	40.0	38.0
5 Eca P28-2	88.5	28.5	110.9	106.4		47.5	39.7	83.2	48.7
6 Eca P28-8	50.7	78.3	119.6	118.4	86.2		40.0	48.7	73.9
7 Ech P28-11	125.6	109.5	56.6	44.9	107.0	118.0		39.7	38.6
8 Ech P28-14	87.9	32.9	116.9	110.3	22.8	81.9	106.7		49.3
9 Ech P28-19	45.9	80.9	124.7	126.4	78.3	35.6	119.5	76.3	

RNA transcript was detected for *map1-1* in the attenuated passage (Fig. 4B). Transcripts from the genes were analyzed for the presence of polycistronic messages with RT-PCR using the same cDNA and the gene-specific *map1-1* forward primer (map1-1for) and the gene specific *map1* reverse primer (map1-rev) (Table 1, Fig. 1). An approximately 1900 bp amplicon was detected in the attenuated passage demonstrating the presence of a polycistronic mRNA (Fig. 4B).

3.4. Transcriptional analysis of map1 paralogs of C. ruminantium grown in tick cells

To investigate possible differences in transcription of genes between tick and mammalian stages, total RNA was extracted from *C. ruminantium*-infected tick cell cultures grown at 30 ± 2°C. Amplification of gene transcripts from *C. ruminantium* (Gardel) infected IDE8 or RAN/CTVM3 cells was performed. RNA transcripts were detected for

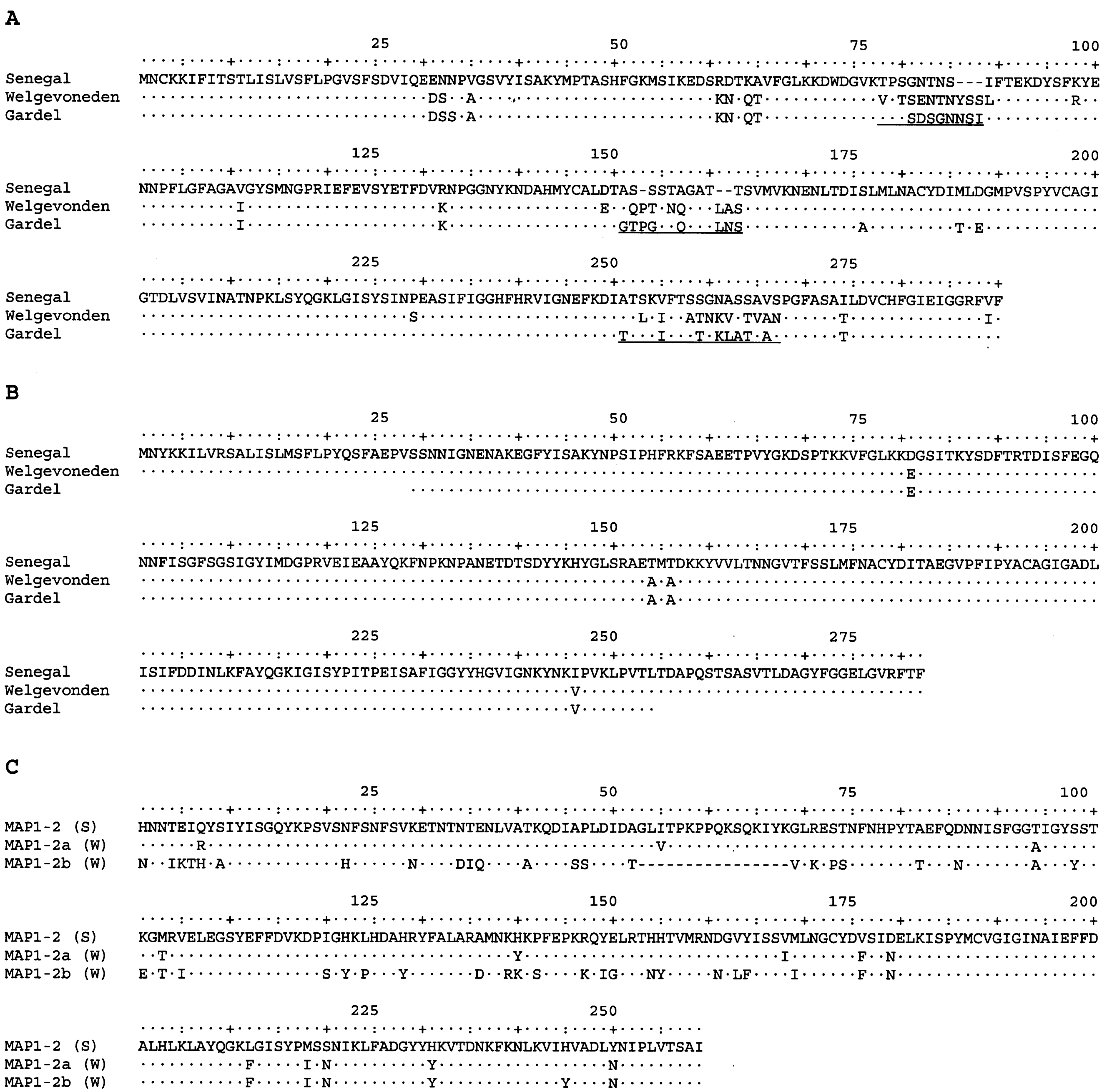

Fig. 3. Alignment of *C. ruminantium* MAP1 (A); MAP1-1 (B); and MAP1-2 (C); from the Senegal (S), Welgevonden (W) and Gardel isolates. The *map1* gene and its two paralogs *map1-1* and *map1-2* were translated and aligned using the ClustalV method (Lasergene 5.0 package DNAstar Inc.). The complete amino-acid sequence MAP1, MAP1-1 and MAP1-2 of the Senegal isolate is presented. Dots indicate identity with MAP1 and dashes were introduced to obtain the best alignment. The hypervariable regions in MAP1 are underlined.

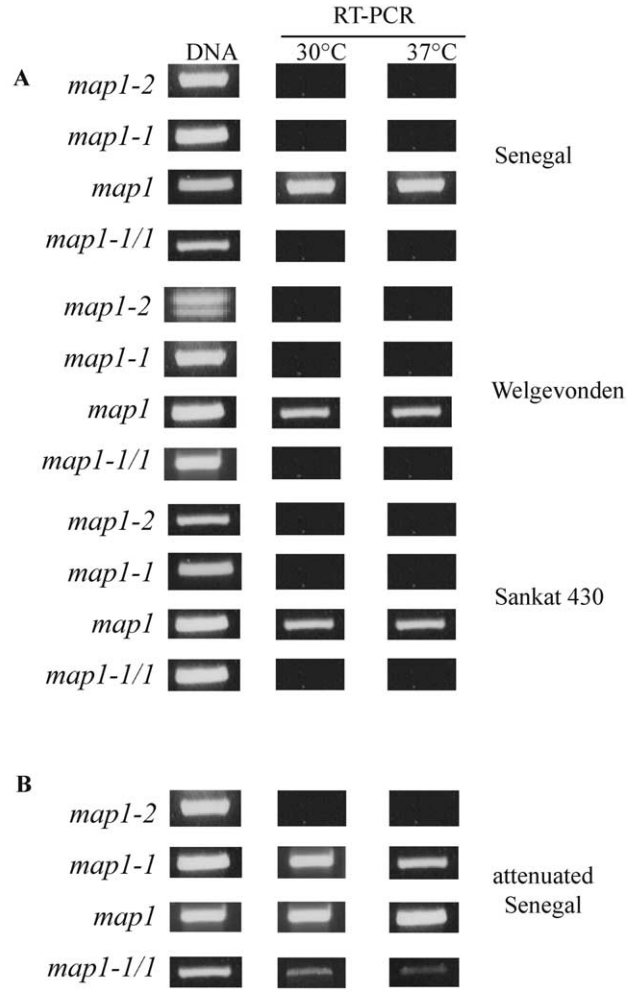

Fig. 4. Transcriptional analysis of the *map1* gene locus of *C. ruminantium* isolates grown in bovine endothelial cells at 30 and 37°C using RT-PCR. Three virulent isolates (A); and one attenuated isolate (B). Reverse primers specific for each gene (Table 1) were used to amplify *map1-2*, *map1-1* and *map1*, a forward primer specific for *map1-1* was used in combination with map1-rev to show the presence of a polycistronic messenger (map1-1/1 row). A PCR control on genomic DNA (first column) is included to show proper primer function.

the *map1* and *map1-1* gene in both cultures by RT-PCR (Fig. 5A). A *map1-1/map1* polycistronic mRNA was detected in both *C. ruminantium*/tick cell line combinations. The same results were observed for attenuated Gardel in both IDE8 and RAN/CTVM3 and for Sankat 430 in RAN/CTVM3 (data not shown).

3.5. Transcriptional analysis of map1 genes of C. ruminantium from infected ticks

In order to determine if the in vitro tick cell culture results were indicative of the in vivo situation, infected *A. variegatum* adults, which were obtained by feeding nymphs on *C. ruminantium* (Senegal) infected sheep, were tested. RNA transcripts were detected for *map1* and *map1-1* in these ticks, and a *map1-1/map1* polycistronic messenger was also detected (Fig. 5B) as was demonstrated in vitro for the Gardel isolate in two tick cell cultures.

4. Discussion

We report here the differential transcription of *C. ruminantium map1* paralogs under different in vitro conditions. It was found that in BEC cultures only the *map1* gene of several virulent *C. ruminantium* isolates was transcribed both at 37 and 30°C. However, both *map1* and *map1-1* were transcribed as a polycistronic messenger in attenuated passages of the Senegal isolate grown in endothelial cells, and in passages of the Gardel isolate grown in two different tick cell lines. Furthermore, the transcription of both *map1* and *map1-1* was also found in the virulent Senegal isolate in vivo in *A. variegatum* ticks.

The basis for our findings was that the Senegal isolate contained at least three *map1* paralogs. These paralogs were found to be located in a head-to-tail orientation within a single cluster. Findings suggest a similar organization in the Welgevonden isolate of *C. ruminantium* (Sulsona et al., 1999). Using a *map1-2* PCR amplified product as a probe (Fig. 1, probe 2) we were not able to detect any *Hin*dIII clone containing the 5′ part of the *map1-2* gene in our library. The genomic *Hin*dIII fragment containing the 5′ part of the *map1-2* gene may have been too large for the pUC19 vector. It is also possible that *C. ruminantium* DNA

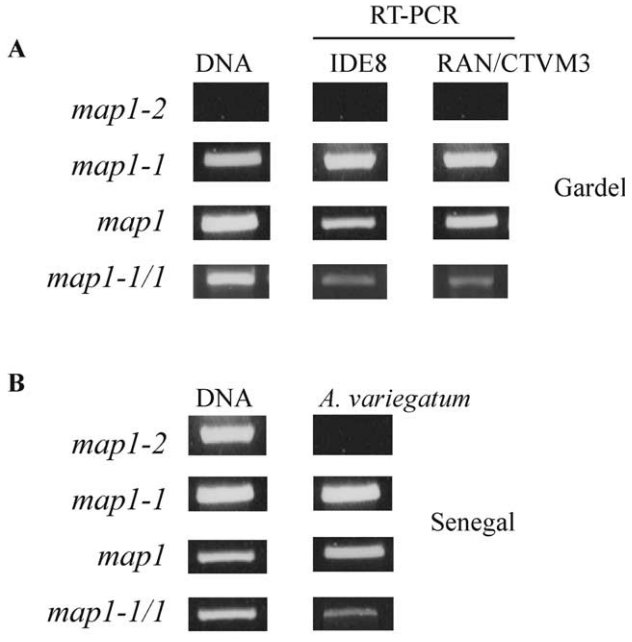

Fig. 5. Transcriptional analysis of the *map1* gene locus of *C. ruminantium* (Gardel isolate) grown in vitro in IDE8 and RAN/CTVM3 tick cells (A); or *C. ruminantium* (Senegal isolate) grown in vivo in *A. variegatum* ticks (B). Reverse primers specific for each gene (Table 1) were used to amplify *map1-2*, *map1-1* and *map1*, a forward primer specific for *map1-1* was used in combination with map1-rev to show the presence of a polycistronic messenger (map1-1/1 row). A PCR control on genomic DNA (first column) is included to show proper primer function.

was unstable as shown before in other vector systems (Brayton et al., 1999). Using the map1-2rev primer in combination with the general forward primer we found different results depending on the isolate used. The Senegal and Sankat 430 isolate each yielded one product, whereas the Welgevonden isolate showed two products, and no products were observed with the Gardel isolate (Figs. 4 and 5). These results indicate that the sequences of the MAP1 paralogs show variation between different isolates of *C. ruminantium.* While MAP1 from different isolates shows sequence variation particularly located in three hypervariable domains, the sequence variation between MAP1-1 proteins of different isolates was quite limited or non-existent (Fig. 3B) as has been reported before (Sulsona et al., 1999). The sequence variation between MAP1-2 of the Senegal isolate and MAP1-2a of the Welgevonden isolate (Fig. 3C) was also quite limited. Additional sequence data from other isolates is required to determine the extent of variation between different MAP1-2 proteins. The results also show that the *map1-2* specific primer needs further improvement for it to be useful for all isolates. The fact that no transcripts of *map1-2* were found in any of the tested systems could be due to improper function of this primer in RT-PCR.

The protein sequence identities of MAP1, MAP1-1, and MAP1-2 of the Senegal isolate were lower (32.8–46.7%) when compared to each other than to orthologs in *E. canis* and *E. chaffeensis* (Table 2). The highest percentage of identity of MAP1-2 is found with *E. canis* P28-1 (57.4%) and *E. chaffeensis* P28-11 (56.5%), MAP1-1 with *E. canis* P28-2 (79.7%) and *E. chaffeensis* P28-14 (78.0%), and MAP1 with *E. canis* P28-8 (56.5%) and *E. chaffeenis* P28-19 (67.2%). Furthermore, it has been shown that the *E. chaffeensis p28-19* gene is divergent among different isolates (Yu et al., 1999), as is the case for *map1* of *C. ruminantium* (Reddy et al., 1996). The limited homology between the different MAP1 paralogs within a single isolate (Fig. 2) provided the scaffolding to analyze the transcription of the corresponding genes under various conditions using specific primers.

To our knowledge our data provide the first evidence of differential transcription of *map1* paralogs between bovine endothelial and tick cell lines. The finding that several genes can be transcribed simultaneously could explain the occurrence of multiple bands in the 28–30 kDa range observed in immunoblots for *Cowdria* (Rossouw et al., 1990; Jongejan, 1991) and for *E. canis* and *E. chaffeensis* (Yu et al., 1993, 1999; Rikihisa et al., 1994). Transcription analysis of the *p28* multigene families in *E. canis* and *E. chaffensis* has been described for in vitro cultures in the canine macrophage cell line DH82 (Reddy et al., 1998; McBride et al., 2000; Yu et al., 2000; Ohashi et al., 2001). It was shown by Reddy et al. (1998) that only one *E. chaffeensis p28* gene was transcribed and four other identified genes were silent. However, when the complete p28 multigene family in *E. chaffeensis* became known, it was shown that six out of 10 *p28* genes were transcribed (Yu et al., 2000), including two which were studied by Reddy et al. (1998). This discrepancy could be explained by the use of different primers as the authors mention, or by differences in the culture techniques used. Transcriptional analysis of *p28* genes in *E. canis* showed that all five genes studied were transcribed (McBride et al., 2000). In all three studies monocistronic transcripts were reported. However, Ohashi et al. (2001) showed that in the *omp* cluster of *E. canis*, paralogs with short intergenic spaces (5′-end half of cluster) were co-transcribed, whereas transcripts for the adjacent genes connected by long intergenic spaces in the 3′-end half of the cluster were undetectable or detectable only at low levels relative to the DNA control. For *C. ruminantium* we found a polycistronic messenger for two adjacent genes (*map1-1* and *map1*) connected by a long intergenic space.

The detection of a polycistronic messenger in tick cells and in ticks may be due to the use of a different promoter under those circumstances. The same promoter could be active in the attenuated Senegal culture thus explaining the detection of *map1-1* transcripts in that culture. Since nothing is known yet about promoter sequences in *C. ruminantium*, more research is required to confirm the presence of different promoters and their specific activity. Active transcription of genes in the tick vector has been described for *Anaplasma marginale* in *Dermacentor* ticks (Rurangirwa et al., 1999), in which a restricted repertoire of major surface protein 2 (MSP2) variants of *A. marginale* were expressed in the salivary gland of infected male *D. andersoni* ticks.

As only a limited number of passages were tested and a non-quantitative detection method was used, low levels of *map1-1* messenger could have gone undetected in virulent passages. Results obtained with the attenuated Gardel isolate grown in endothelial cells, although using different primers and RT-PCR conditions, showed that a polycistronic messenger (*map1-1* and *map1*) seemed to be present in lower quantities in passage 53 (virulent) than in passage 224 (attenuated) (Bensaid and Martinez, unpublished data). Whether the in vitro transcription of *map1-1* in attenuated cultures has any connection with attenuation per se needs to be further investigated. If so, this could be used as a marker for in vitro attenuation of *C. ruminantium.*

Further studies, especially on in vivo transcription in the ruminant host and tick vector, are essential to select valuable vaccine candidate genes which will become available with the ongoing *C. ruminantium* genome sequencing project (www.sanger.ac.uk). Furthermore, clarification of the regulation of transcription and expression of genes in the *C. ruminantium map1* gene family will aid our understanding of the role of these genes in host-parasite interactions and possible immune evasion.

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