Differential transcription of two highly divergent gut-expressed Bm86 antigen gene homologues in the tick *Rhipicephalus appendiculatus* (Acari: Ixodida)

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Abstract

The transcriptional control of gene expression is not well documented in the Arthropoda. We describe transcriptional analysis of two exceptionally divergent homologues (Ra86) of the Bm86 gut antigen from *Rhipicephalus appendiculatus*. Bm86 forms the basis of a commercial vaccine for the control of *Rhipicephalus (Boophilus) microplus*. The *R. appendiculatus* Ra86 proteins contain 654 and 693 amino acids, with only 80% amino acid sequence identity. Reverse-transcription PCR of gut cDNA showed transcription of only one genotype in individual female ticks. PCR amplification of 3′ untranslated sequences from genomic DNA indicated that both variants could be encoded within a single genome. When both variants were present, one of the two Ra86 genotypes was transcriptionally dominant.

Keywords: *Rhipicephalus appendiculatus*, tick, vaccine, gut antigen, Ra86, differential transcription.

Introduction

Transcriptional control of gene expression occurs at two different levels: regulation of expression of allelic variants at single copy loci in diploid organisms, or control of expression of individual genes located within multicopy gene families. The mechanisms underpinning these processes may, in some systems, overlap. Examples of the former have been described for a number of human genes (Ohlsson *et al.*, 1998; Lo *et al.*, 2003), while the latter group includes the generation of diversity in the antibody repertoires and odorant receptors in vertebrates, together with antigenic variation in eukaryotic pathogens, particularly *Plasmodium falciparum,* the aetiological agent of the most serious form of malaria in humans (Borst, 2002; Scherf *et al.*, 2008). In most members of the phylum Arthropoda, transcriptional regulation of genes has been relatively little documented. Ixodid tick genomics has recently been initiated and expressed sequence tag databases have been generated from several species including the salivary glands of *Rhipicephalus appendiculatus* (Nene *et al.*, 2004), normalized whole tissues of *Rhipicephalus (Boophilus) microplus* (Wang *et al.*, 2007), and *Ixodes scapularis*, a major vector of Lyme disease in humans, for which there will soon be 240 000 ESTs in the public databases (Van Zee *et al.*, 2007). These data have highlighted the complexity of the transcriptomes in these organisms (Jongejan *et al.*, 2007). Re-association kinetics and flow cytometry have demonstrated that tick genomes are comparable in size, or in some cases larger than those of most vertebrates (Ullman *et al.*, 2005; Geraci *et al.*, 2007). A 6X coverage draft assembly of the genome sequence is now available for *I. scapularis* (Van Zee *et al.*, 2007) and the genome is currently being annotated. Genomic DNA sequencing has confirmed that tick genomes appear more similar in their organisation to vertebrates than to that of the model arthropod *Drosophila*. There is a high frequency of repetitive DNA, including enigmatic short repeats comprising 5% of the genome of *I. scapularis,* SINE retroposons in *R. appendiculatus* and Type I and Type II transposable elements in *R. (B)*

microplus (Van Zee *et al.*, 2007; Sunter *et al.*, 2008; Guerrero *et al.*, 2010.; Meyer *et al.*, 2010).

Recombinant forms of a tick midgut-expressed glycoprotein, designated Bm86, form the basis of commercial vaccines TickGARD and Gavac which have been used for the control of *R. (B) microplus* and *Rhipicephalus annulatus* in Australia and Latin America (Willadsen, 2004, 2008; de la Fuente *et al.*, 2007). The major benefit of the vaccine has been a significant reduction in the application of acaricides in integrated tick control strategies, combining vaccination and acaricidal control (de la Fuente *et al.*, 2007). This vaccine elicits cross-protection against infestation with some heterologous ixodid tick species, including those in the genus *Hyalomma* (de Vos *et al.*, 2001), but not against other species including *R. appendiculatus*, the vector of the cattle disease East Coast fever caused by *Theileria parva* (de Vos *et al.*, 2001; Odongo *et al.*, 2007).

Sequence diversity within the Bm86 antigen gene between different isolates of *R. (B) microplus* has been shown to be relatively limited (Willadsen, 2008). Currently available data also indicates that the ability of the Bm86 antigen to induce cross-protection against heterologous tick species infestation does not necessarily appear to be closely related to the degree of sequence identity of the Bm86 homologue (Willadsen, 2008; Nijhof *et al.*, 2009). This suggests that other factors, such as levels of expression of the gene or the volume of host antibodies imbibed by the tick while feeding, may be more important in determining protection. Two Ra86 gene homologues that exhibit 95% identity and are probably allelic were recently isolated from an *R. apppendiculatus* tick stock from Mozambique (Nijhof *et al.*, 2009). Quantitative real-time PCR data, generated using gut cDNA derived from Bm86 and Ra86, indicated that differences in expression levels alone probably did not explain the lack of protection against *R. appendiculatus* induced by recombinant Bm86 (Nijhof *et al.*, 2009). In the present study we describe the isolation of two exceptionally divergent alleles of Ra86 from a Kenyan *R. appendiculatus* laboratory stock that are differentially transcribed by individual female ticks. The expression data is consistent with the hypothesis that the two Ra86 genotypes represent an example of allelic exclusion in an arthropod.

Results

Ra86 sequence analysis

The sequences of 19 Ra86 cDNA clones isolated from the *R. appendiculatus* (Muguga laboratory stock) gut library were deposited in GenBank under the accession numbers FJ850975-FJ850978 and GU288578-GU288592 (Supporting Information Table S1). Two main groups of predicted amino acid sequences of the Ra86 clones were identified, designated Ra86-4 and Ra86-5, that could be differentiated by sequence and size. Ra86-4 was represented by five cDNA clones (three full-length and two partial sequences), and Ra86-5 was represented by 14 cDNA clones (six full-length and eight partial sequences). Figure 1 shows an amino acid alignment of representative examples of the full-length clones of Ra86-4 (clone 85A) and Ra86-5 (clone 64A). The full-length Ra86-4 and Ra86-5 proteins are predicted to contain 654 and 693 amino acids, respectively. The 39-amino-acid size difference between the Ra86 variants is attributable to a 40-amino-acid indel (an additional sequence in Ra86-5 located towards the N-terminus) combined with a single amino acid indel (position 225 in Ra86-4). In addition to the size differences, there are also numerous amino acid positions which differ between Ra86-4 and Ra86-5, which equates to 80% sequence identity overall between the groups. For example there are 129 amino acid positions that differ between cDNA clones 85A (Ra86-4) and 64A (Ra86-5). Within each of the Ra86-4 and Ra86-5 sequence groups there was ≥95% and ≥98% amino acid sequence identity, respectively. Therefore, it is unlikely that the two major types correspond to sampling variation within the dataset.

Ra86-4 and Ra86-5 sequences were compared with homologous sequences from other ixodid species that were available in GenBank (Table 1). For this analysis we selected available sequences of *R. appendiculatus* (Nijhof *et al.*, 2009), *R. (B) microplus* (Rand *et al.*, 1989; Andreotti *et al.*, 2008; Nijhof *et al.*, 2009), *Rhipicephalus decoloratus* (Odongo *et al.*, 2007), *R. annulatus* (Canales *et al.*, 2009), *Rhipicephalus sanguineus* (Q. Q. Fang, unpubl. data), *Hyalomma anatolicum* (de Vos *et al.*, 2001), *Haemaphysalis longicornis* (Liao *et al.*, 2007), and two recently-isolated Ra86 sequences from South Africa (Nijhof *et al.*, 2009).

The amino acid identity (80%) between the Ra86-4 and Ra86-5 homologues of the Kenyan *R. appendiculatus* is much less than between the two sequences from South African *R. appendiculatus* (95% amino acid identity between Ra86-1 and Ra86-2). The level of identity between Ra86-5 and the South African sequences Ra86-1 and Ra86-2 (78% and 79%) is comparable with that between the two Kenyan sequences. Conversely, Ra86-4 appears to be very similar in size and sequence to both Ra86-1 and Ra86-2 (97% and 96% sequence identity). There is less sequence identity between the *R. appendiculatus* Ra86 homologues that we describe (78–80%) than between homologues from *R. decoloratus* and *R. (B) microplus* (85–88%). Such extensive intraspecies sequence diversity is not observed within other species where more than one sequence is available. For example, *R. decoloratus* exhibits 93% amino acid identity between Bd86-1 and Bd86-2. In the case of *R. (B)*
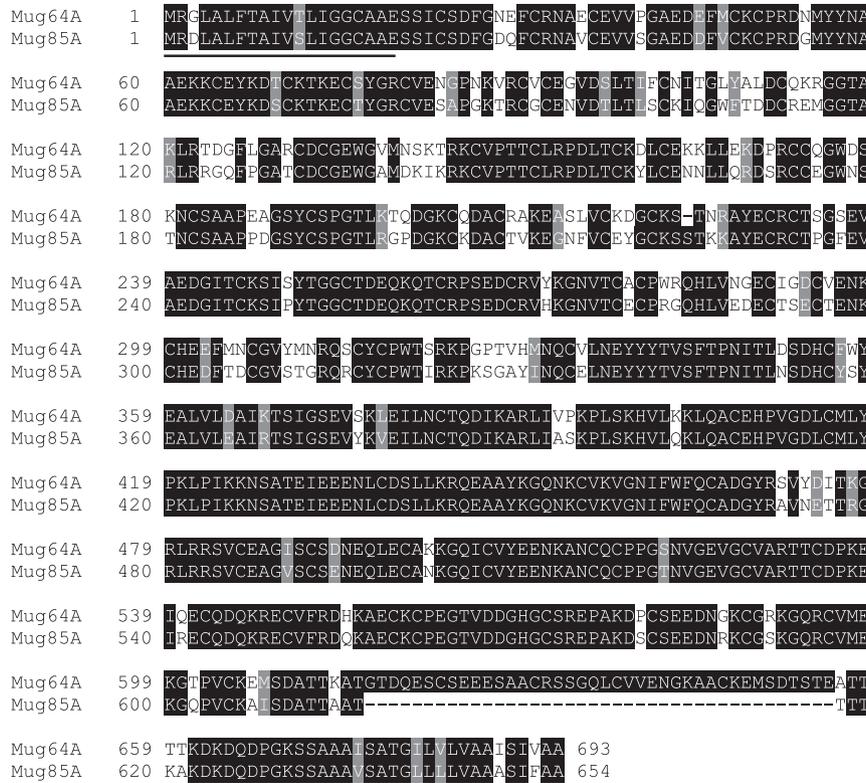
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Mug64A   1   MRGLALFTAIVLLIGGCAAESSICSDFGNEFCRNAECEVVPGAEDEFMCKCPRDNMYYNA
Mug85A   1   MRDLALFTAIVSLIGGCAAESSICSDFGDQFCRNAVCEVVSGAEDDFVCKCPRDGMYYNA

Mug64A  60   AEKKCEYKDICKTKECSYGRCVENGPNKVRGVCEGVDSLTIFCNITGLYALDCQKRGGTA
Mug85A  60   AEKKCEYKDSCKTKECTYGRCVESAPGKTRCGCENVDTLTLSCKIQGWTDDCREMGGTA

Mug64A 120   KLRTDGFLGARCDCGEWGVMNSKTRKCVPTTCLRPDLTCKDLCEKKLLEKDPRCCQGWDS
Mug85A 120   RLRRGQFPGATCDCGEWGAMDKIKRKCVPTTCLRPDLTCKYLCENNLLQRDSRCCEGWNS

Mug64A 180   KNCSAAPEAGSYCSPGTLKTQDGKCQDACRAKEASLVCKDGCKS-TNRAYECRCTSGSEV
Mug85A 180   TNCSAAPPDGSYCSPGTLRGPDGKCKDACTVKEGNFVCEYGCKSSTKKAYECRCTPGFEV

Mug64A 239   AEDGITCKSISYTGGCTDEQKQTCRPSEDCRVYKGNVTCACPWRQHLVNGECIGDCVENK
Mug85A 240   AEDGITCKSIPYTGGCTDEQKQTCRPSEDCRVHKGNVTCECPRGQHLVEDECTSPCTENK

Mug64A 299   CHEEFMNCGVYMNRQSCYCPWTSRKPGPTVHMNQCVLNEYYYTVSFTPNITLDSDHCFWY
Mug85A 300   CHEDFTDCGVSTGRQRCYCPWTIRKPKSGAYINQCELNEYYYTVSFTPNITLNSDHCYSY

Mug64A 359   EALVLDAIKTSIGSEVSKLEILNCTQDIKARLIVPKPLSKHVLKKLQACEHPVGDLCMLY
Mug85A 360   EALVLEAIRTSIGSEVYKVEILNCTQDIKARLIASKPLSKHVLQKLQACEHPVGDLCMLY

Mug64A 419   PKLPIKKNSATEIEEENLCDSLLKRQEAAYKGQNKCVKVGNIFWFQCADGYRSVYDITKG
Mug85A 420   PKLPIKKNSATEIEEENLCDSLLKRQEAAYKGQNKCVKVGNIFWFQCADGYRAVNETTRG

Mug64A 479   RLRRSVCEAGISCSDNEQLECAKKGQICVYEENKANCQCPPGSNVGEVGCVARTTCDPKE
Mug85A 480   RLRRSVCEAGVSCSENEQLECANKGQICVYEENKANCQCPPGTNVGEVGCVARTTCDPKE

Mug64A 539   IQECQDQKRECVFRDHKAECKCPEGTVDDGHGCSREPAKDPCSEEDNGKCGRKGQRCVME
Mug85A 540   IRECQDQKRECVFRDQKAECKCPEGTVDDGHGCSREPAKDSCSEEDNRKCGSKGQRCVME

Mug64A 599   KGTPVCKEMSDATTKATGTDQESCSEEESAACRSSGQLCVVENGKAACKEMSDTSTEATT
Mug85A 600   KGQPVCKAISDATTAAT------------------------------------------TTT

Mug64A 659   TTKDKDQDPGKSSAAAISATGILVLVAAISIVAA 693
Mug85A 620   KAKDKDQDPGKSSAAAVSATGLLLLVAAASIFAA 654
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Figure 1. An alignment of the predicted amino acid sequences of Ra86-5 and Ra86-4, represented by full-length cDNA clones 64A and 85A, respectively. Positions of identical residues are indicted by black shading and non-identical, but functionally similar, residues are highlighted in grey. The sequences highlighted by the solid and broken underscores indicate the positions of a predicted signal peptide sequence and a transmembrane helix, respectively.

microplus, intraspecies diversity is limited over a wide geographical range, e.g. there is 96–97% amino acid identity between Bm86 Yeerongpilly, the type specimen from Australia, and Bm86 sequences from Brazil and Mozambique (Table 1). Based on extensive sequencing studies of Bm86, the lowest identity so far observed within the *R. (B) microplus* complex is 94% (Sossai *et al.*, 2005; Willadsen, 2008).

Table 1. Percentage identity between predicted amino acid sequences of *R. appendiculatus* Ra86-4, Ra86-5 and homologous tick proteins

Species/ homologue type	Ra86-5	Ra86-1	Ra86-2	Bd86-1	Bd86-2	Bm86-Y	Bm86-M	Bm86-B	Ba86	Rs86	Ha98	Hl86
Ra86-4	80	97	96	75	75	74	74	75	74	75	65	39
Ra86-5	–	78	79	75	75	74	75	76	76	75	62	41
Ra86-1		–	95	74	74	72	73	74	74	74	65	39
Ra86-2			–	74	75	73	74	75	75	75	66	39
Bd86-1				–	93	86	86	87	88	71	67	39
Bd86-2					–	85	85	87	88	71	67	39
Bm86-Y						–	96	96	93	71	64	37
Bm86-M							–	97	93	71	66	36
Bm86-B								–	94	72	66	36
Ba86									–	72	67	38
Rs86										–	68	42
Ha98											–	41

R. appendiculatus: Ra86-4 (clone 85A) & Ra86-5 (clone 64A) (Muguga-Kenya, accession numbers FJ850978 & FJ85097); *R. appendiculatus*: Ra86-1 & Ra86-2 (South Africa, FJ809944 & FJ809945; Nijhof *et al.*, 2009); *R. decoloratus*: Bd86-1 & Bd86-2 (Kenya, DQ630523 & DQ630524; Odongo *et al.*, 2007); *R. microplus*: Bm86-Y (Yeerongpilly-Australia, M29321; Rand *et al.*, 1989), Bm86-M (Mozambique, ACZ55133; Nijhof *et al.*, 2009), Bm86-B (Brazil, ACA57829; Andreotti *et al.*, 2008); *R. annulatus*: Ba86 (USA, ACR19242; Canales *et al.*, 2009); *R. sanguineus*: Rs86 (EF222203, Fang, Q. Q., unpubl. data); *Hyalomma anatolicum*: Ha98 (India, AF347079; de Vos *et al.*, 2001); *Haemaphysalis longicornis*: Hl86 (Okayama, AB251858; Liao *et al.*, 2007).
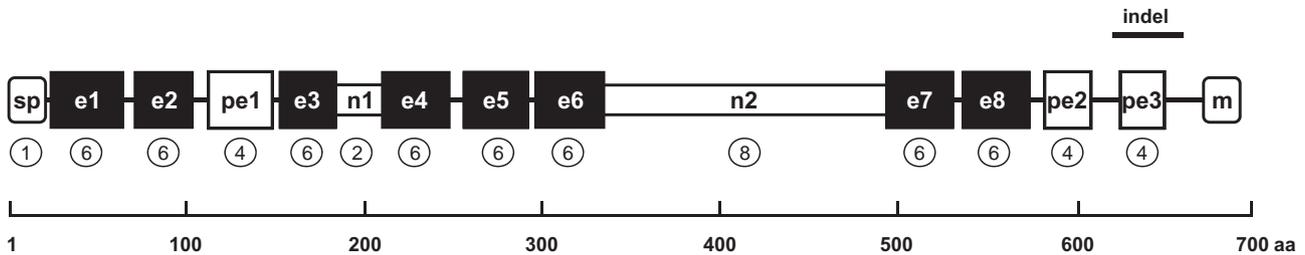
indel

| sp | e1 | e2 | pe1 | e3 | n1 | e4 | e5 | e6 | n2 | e7 | e8 | pe2 | pe3 | m |

| ① | ⑥ | ⑥ | ④ | ⑥ | ② | ⑥ | ⑥ | ⑥ | ⑧ | ⑥ | ⑥ | ④ | ④ |

1   100   200   300   400   500   600   700 aa

Figure 2. Schematic representation of Ra86-5 structural organization. Epidermal growth factor (EGF)-like and partial EGF-like modules are represented by shaded and unshaded rectangles, and marked e1 to e8 and pe1 to pe3, respectively. Non-EGF-like regions that contain cysteine residues are indicated as n1 and n2. The number of cysteine residues in each region are shown within the circles. Also shown are the predicted signal peptide (sp.) and the predicted membrane anchor (m). Ra86-4 has a similar structure except it is missing a 39-amino-acid sequence towards the C-terminus (indicated by 'indel').

Predicted functional features of Ra86

Analysis of the amino acid sequences of Ra86 indicated a predicted N-terminal signal peptide and a C-terminal 23-amino-acid *trans*-membrane helix (Fig. 1). The inter-vening sequence had features associated with hydrophilic and surface-exposed proteins. This general structure is similar to homologous proteins of *R. (B) microplus* (Bm86; Rand *et al.*, 1989), *H. anatolicum* (Ha98; de Vos *et al.*, 2001), *R. decoloratus* (Bd86; Odongo *et al.*, 2007) and *Hae. longicornis* (Hl86; Liao *et al.*, 2007).

Epidermal growth factor-like modules in Ra86

As described for *R. (B) microplus* Bm86 (Rand *et al.*, 1989) and other ixodid species (Nijhof *et al.*, 2009), the predicted amino acid sequences of Ra86-4 and Ra86-5 also comprise a series of epidermal growth factor (EGF)-like module structures, containing repeated cysteine resi-dues (Fig. 2). Eight EGF-like domains, each containing six cysteine residues, and two or three 'partial' EGF-like modules, each containing four cysteine residues, were identified in Ra86-5. The EGF-like and partial EGF-like modules are generally conserved in length in the Bm86 homologue of all species, including *R. decoloratus, H. anatolicum* and *R. appendiculatus*. EGF molecules nor-mally contain approximately 40–60 amino acids per EGF module, which contains six cysteine residues separated by a pattern of other amino acid residues, as shown by computer-based characterization of EGF precursor (Tellam *et al.*, 1992). The major size difference between Ra-86-4 and Ra86-5 is attributable to indel corresponding to a partial EFG-like module towards the C-terminus. Figure 3 summarizes the organization and spacing of the EGF-like and partial EGF-like module within Ra86-5. The consensus amino acid sequence for the EGF modules is $C(i)-X_{4-8}-C(ii)-X_{3-6}-C(iii)-X_{8-18}-C(iv)-X_{0-1}-C(v)-X_{7-15}-C(vi)$. Partial EGF-like module pe1 comprises cysteines C(iii)-C(vi), while pe2 and pe3 comprise cysteines C(i)-C(iv). Although a pattern of repeated cysteine residues is clearly evident, there is no experimental evidence that they create disulphide bridges in Ra86. However, most cys-teine residues in Bm86 have been shown to be involved in disulphide bond formation (Rand *et al.*, 1989).

Ra86 transcription in individual Rhipicephalus appendiculatus *female ticks*

Reverse transcription (RT)-PCR, using primers flanking the indel that distinguishes the Ra86-4/Ra86-5 genes, was undertaken to investigate transcription of the Ra86-4 and Ra86-5 at the individual tick level. RT-PCR was performed on total RNA extracted from the midguts of 20 adult females and 20 adult males of *R. appendiculatus*. Individual female ticks express either the Ra86-4 gene or the Ra86-5 gene, but not both (Fig. S1). The specificity of the assay was confirmed using control cDNA clones, representing Ra86-4 and Ra86-5 (Fig. S1). To further confirm these results, an

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EGF-e1        C SDFGNEF  C RNAE    C EVVPGAEDEFM           C K C  PRDNMYYNAAEKK    C
EGF-e2        C KTKE     C SYGR    C VENGPNKVR             C V C  EGVDSLTIF        C
Part EGF-Pe1                       C QKRGGTAKLRTDGFLGAR    C D C  GEWGVMNSKTRK     C
EGF-e3        C LRPDLT   C KDL     C EKKLLEKDPR            C   C  QGWDSKN          C
EGF-e4        C RAKEASLV C KDG     C KSTNRAYE              C R C  TSGSEVAEDGIT     C
EGF-e5        C TDEQKQT  C RPSED   C RVYKGNVT              C A C  PWRQHLVNGE       C
EGF-e6        C VENK     C HEEFMN  C GVYMNRQS              C Y C  PWTSRKPGPTVHMNQ  C

EGF-e7        C SDNEQLE  C AKKGQI  C VYEENKAN              C Q C  PPGSNVGEVG       C
EGF-e8        C DPKEIQE  C QDQKRE  C VFRDHKAE              C K C  PEGTVDDGHG       C

Part EGF-Pe2  C SEEDNGK  C GRKGQR  C VMEKGTPV              C
Part EGF-Pe3  C SEEESAA  C RSSGQL  C VVENGKAA              C
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Figure 3. Amino acid sequences of epidermal growth factor (EGF)-like (e) and partial EGF-like (pe) modules in Ra86-5 (clone 64A), showing alignments of the cysteine residues (bold).

Table 2. Ra86 genotypes and expression in 29 individual adult female
R. appendiculatus ticks

Tick no.	Ra86-4 or Ra86-5 expression*	Ra86-4 3′ UTR PCR‡	Ra86-5 3′ UTR PCR§	3′ UTR genotype†
2	4	+	+	4,5
3	5	−	+	5,5
4	4	+	+	4,5
5	4	+	+	4,5
6	5	−	+	5,5
7	5	−	+	5,5
8	4	+	−	4,4
9	4	+	+	4,5
10	4	+	+	4,5
11	5	−	+	5,5
12	4	+	−	4,4
13	4	+	−	4,4
14	5	−	+	5,5
15	4	+	−	4,4
16	5	−	+	5,5
17	5	−	+	5,5
18	5	−	+	5,5
19	4	+	+	4,5
20	5	−	+	5,5
21	5	−	+	5,5
22	4	+	+	4,5
23	4	+	+	4,5
24	4	+	−	4,4
25	5	−	+	5,5
26	4	+	+	4,4
27	5	−	+	5,5
28	5	−	+	5,5
29	5	−	+	5,5
30	4	+	+	4,5
Controls				
Pooled egg DNA	ND	+	+	4,5
Plasmid clone 85A	4	+	−	4
Plasmid clone 92A	5	−	+	5

* Expression of Ra86-4 or Ra86-5 in the tick gut was determined by reverse-transcription PCR using primers that flank an indel in the coding regions of Ra86-4 and Ra86-5.
† Ra86 genotype was determined by Ra86-4‡ or Ra86-5§ 3′UTR-specific PCR.
"+": positive PCR; "−": negative PCR; ND: Not done.

additional 30 gut cDNAs from individual female ticks were analysed, and amplicons were generated from 29 of these corresponding to the size expected for either Ra86-4 or Ra86-5 (Table 2). RT-PCR of individual male ticks was unsuccessful (data not shown), which may have been a consequence of the small size of the males relative to females and a concomitant low yield of RNA.

Sequence variation between the 3′ untranslated regions of genes encoding Ra86 proteins of different sizes

We attempted to amplify the corresponding C-terminal coding region of the Ra86 gene using genomic DNA from the same ticks from which the second batch of gut cDNA was generated. However, we were unable to amplify this region from genomic DNA, despite experimenting with a variety of conditions. We therefore investigated whether

there was sufficient variation in associated genomic sequences to differentiate the genomic DNA of Ra86-4 and Ra86-5. An analysis of an alignment of the 250 bp 3′ untranslated region (UTR) of the 19 cloned cDNA sequences isolated from the Ra86 cDNA, revealed 28 nucleotide substitutions between Ra86-4 and Ra86-5 (a consensus of 88% identity overall) in paired comparisons of the 3′ UTR of Ra86-4 and Ra86-5 sequences. Within the Ra86-4 genotype, the 250 bp 3′ UTRs were 100% identical except for base 51 which differed by one nucleotide substitution. Within Ra86-5, the 3′UTR paired sequence comparisons exhibited 98–100% identity. The maximum number of nucleotides substituted between different Ra86-5 sequences was five. The alignment of the 3′ UTR sequences is shown in Supporting Information Fig. S2.

Analysis of Ra86 transcripts and genotypes in individual Rhipicephalus appendiculatus *female ticks*

Following the identification of two distinct genotypes within the 3′ UTR and the association of these with the two different Ra86 coding sequences, the set of 29 individual *R. appendiculatus* adult females was analysed by PCR using both gut cDNA and genomic DNA templates. This experiment used Ra86 primers that were designed to be specific for (1) coding the region flanking the indel; and (2) the 3′UTRs of Ra86-4 and Ra86-5, as described above. The specificity of the primers associated with Ra86-4 and Ra86-5 was confirmed using cDNA clones 85A (representing Ra86-4) and 92A (representing Ra86-5) (Fig. 4B, bottom right panel).

When gut cDNA was used as the template, as in the first experiment, individual ticks expressed either a Ra86-4 or Ra86-5 transcript, but not both. Representative results for ticks 21–25 are shown in Fig. 4A (left panel), together with plasmid controls encoding full-length cDNA (Fig. 4A, right panel). The 3′UTR PCR assays performed on genomic DNA using the Ra86-4- or Ra86-5-specific primers revealed that ticks expressing Ra86-4 transcripts were always associated with a Ra86-4 3′ UTR in genomic DNA, while ticks expressing a Ra86-5 transcript were always associated with a Ra86-5 3′ UTR in genomic DNA. Some individual ticks generated genomic DNA amplicons from both Ra86-4 and Ra86-5 UTRs. These ticks invariably expressed a Ra86-4-type transcript as assessed by PCR using gut cDNA. The combined data for genomic DNA- 3′ UTR PCR and gut cDNA PCR for the 29 individual ticks is summarized in Table 2.

Cytochrome oxidase gene sequences of individual Rhipicephalus appendiculatus *ticks that express different Ra86 variants in their gut transcriptome*

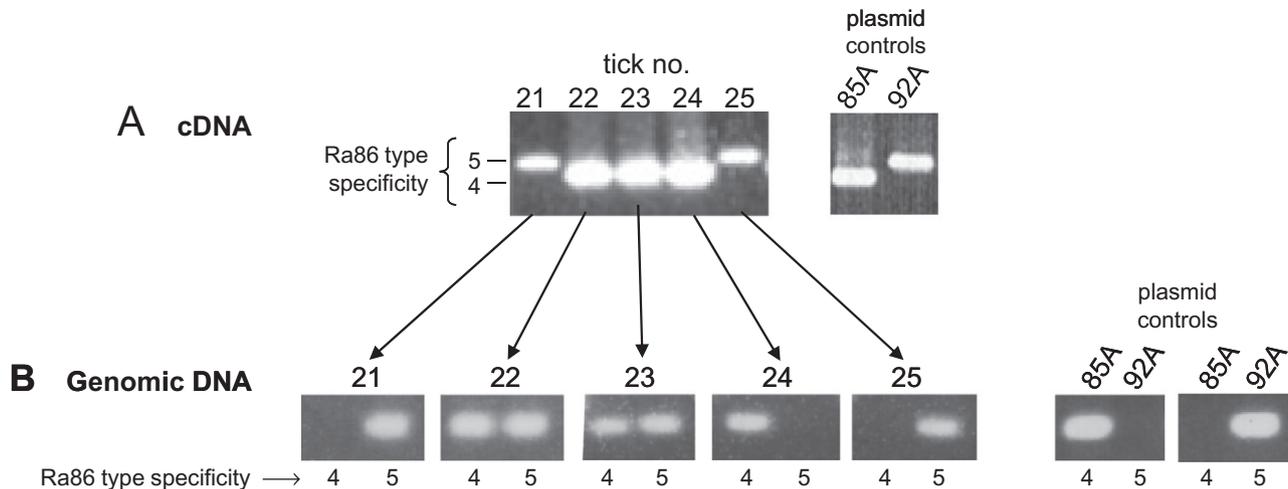Cytochrome oxidase (CO) 1 gene sequences were PCR amplified from mitochondrial DNA of individual *R. appen-*

Figure 4. Expression and genomic organisation of Ra86 in five individual ticks. Panel A (left): Reverse transcription-PCR products generated from gut RNA of five individual ticks (tick numbers 21–25) using conserved primers that flank an indel in the Ra86 coding sequence. The Ra86-4 and Ra86-5 RT-PCR products have predicted sizes of 904 and 1024 bp, respectively. Panel A, right: cDNA PCR specificity was confirmed using plasmids containing cDNA from Ra86-4 (clone 85A) and Ra86-5 (clone 92A) Panel B, left: Genomic DNA isolated from the corresponding ticks (21–25) were analysed by PCR with primers specific for Ra86-4 and Ra86-5 3′ untranslated region (UTR) sequences. The predicted size for both Ra86-4 and Ra86-5 3′ UTR PCR products is 136 bp. Panel B, right: Ra86 3′ UTR PCR specificity was confirmed using plasmids containing cDNA from Ra86-4 (clone 85A) and Ra86-5 (clone 92A).

diculatus ticks expressing either Ra86-4 or Ra86-5 in their gut cDNA. Individual CO1 sequences from ticks expressing Ra86-4 and Ra86-5 exhibited 99–100% identity between one another. The consensus CO1 sequence from ticks expressing Ra-86-4 was identical to that from ticks expressing Ra86-5. BLAST analysis of the CO1 consensus sequence against the GenBank nr database revealed ≥96% identity with all *R. appendiculatus* CO1 sequences (Table S2). These matches were significantly higher than with CO1 sequences from other ixodid tick species. The results are consistent with both Ra86-4 and Ra86-5 being derived from *R. appendiculatus* rather than a mixture of two tick species in the stock, despite the significant sequence differences between Ra86-4 and Ra86-5. However, mitochondrial sequences have recently been demonstrated to be intra-specifically variable, therefore, additional genetic analyses using higher resolution markers, such as microsatellites or single nucleotide polymorphisms (SNPs), will be required in the future to confirm this provisional conclusion.

Discussion

With a level of 80% identity between amino acid sequences, the variation between Ra86-4 and Ra86-5 exceeds that typically observed at alleles located at a single diploid locus. However, recent theoretical analyses suggest that in contrast to traditional models involving gene duplication and subsequent divergence, allelic divergence associated with altered protein functionality can precede gene duplication (Proulx & Phillips, 2006). A

well studied example is the lens crystallins of vertebrates, which may also be bi-functional (Piatigorsky & Wistow, 1991). One example of two extremely divergent alleles, which encode proteins with <80% amino acid sequence identity, is the *Plasmodium falciparum* surface antigen MSP1 gene (Roy *et al.*, 2008). Although *P. falciparum* is haploid for most of its life cycle, it does have a transient diploid phase and an obligate sexual cycle, suggesting that suppression of recombination could be occurring, which would explain the maintenance of these divergent genotypes (Roy *et al.*, 2008). It is also noteworthy that the diploid genome of James Watson contains 356 indels of varying size between alleles of potentially protein-encoding open reading frames (Wheeler *et al.*, 2008). Therefore, despite the high sequence divergence and presence of indels, by analogy with a range of other species, it seems quite possible that Ra86-4 and Ra86-5 represent different allelic types at a single locus. The genomic organisation of the genes encoding Bm86-like proteins is not yet fully clarified, although Bm86-like homologues have been identified across all major phylogenetic divisions within the suborder Ixodida. Southern blot data for Bm86 is ambiguous because of the existence of cross-hybridizing domains (J. de la Fuente, pers. comm.), and no tick genome is yet sufficiently complete to confirm the copy number of any Bm86-like homologues. The *I. scapularis* draft genome, which is currently derived from an assembly corresponding to 6X coverage of the estimated 2.1 gigabase genome (Van Zee *et al.*, 2007), only contains a single contig with a full-length Is86 gene (R. Bishop and

S. Patel, unpubl. obs.). However, the present data from the genomic 3′UTR PCR amplification indicates that both Ra86-4 and Ra86-5 variants can be present within a single *R. appendiculatus* genome. In the case of East African Bd86 (Odongo *et al.*, 2007) and Ra86 from South Africa (Nijhof *et al.*, 2009), isolation of cloned sequences comprising two distinct variants of the gene exhibiting approximately 95% and 93% amino acid identity, respectively, is also consistent with allelic divergence at a single locus. If the Ra86 gene represents a single copy locus then expression of only type-4 (Ra86-4) in the heterozygous individuals constitutes an example of allelic exclusion, which to our knowledge has not yet been described among invertebrates.

The reverse-transcription PCR data from female ticks, combined with 3′UTR genomic PCR, strongly suggests transcriptional control of expression at the Ra86 loci. Control at the transcriptional level is reminiscent of members of multicopy gene families such as the olfactory receptor genes in mammals and the Pfemp1 (var) genes of *P. falciparum* (Borst, 2002). However, it is interesting that in *Drosophila*, in contrast with mammalian systems, expression of variant members of odorant receptor multicopy gene families do not appear to be subject to transcriptional control (Elmore & Smith, 2001), perhaps suggesting that this mechanism is less frequent in arthropod multicopy gene families.

It is notable that in all instances where both Ra86-4 and Ra86-5 3′UTRs were detected in genomic DNA from a single tick the transcript was of the Ra86-4 type. This suggests that whatever the mechanism of transcriptional control, Ra86-4 was 'dominant'. We were unable to generate Ra86 amplicons from male tick gut cDNA. This contrasts with the results obtained in a recent study using a real-time PCR assay with primers derived from Ra86 or Bm86 genes using pooled tick gut samples as a template (Nijhof *et al.*, 2009). One explanation is that there was insufficient Ra86 RNA present in individual male ticks to generate a detectable product using a standard PCR assay.

Using sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis, it was previously observed that both unfed and fed individual ticks exhibit distinct protein expression profiles in their salivary glands (Wang *et al.*, 1999, 2001). Assuming that both Ra86 variants are expressed at the protein level, this variation in the expressed protein repertoire therefore extends to the gut as well as salivary gland. Gut proteins have been considered 'hidden antigens', since they are not directly exposed to the host antibody response in natural tick infestations (Kemp *et al.,* 1989; Willadsen, 2004). It can, however, be argued that the immune system may act indirectly by affecting the quality and quantity of the blood meal that reaches the tick gut. In support of this it has recently been

shown that in the feeding females of the ixodid tick *Amblyomma hebraeum* a critical weight, which is dependent on the host response, is a prerequisite for vitellogenesis, which also occurs in a 'hidden' tissue (Friesen & Kaufman, 2009). The selective pressure driving diversification of the putative variant *R. appendiculatus* proteins therefore remains unclear.

The 80% level of protein identity observed between the two major variants of Ra86, which were isolated from a single laboratory stock of *R. appendiculatus* (Muguga), is remarkable given that the most distinct homologues of Bm86 in *R. (B) microplus* ticks isolated from different continents exhibit >94% identity (Garcia-Garcia *et al.*, 1999; de la Fuente *et al.*, 2000; Sossai *et al.*, 2005; Willadsen, 2008). By contrast, two Bm86 homologues from a single tick stock *R. decoloratus* (Bd86) exhibited 93% identity (Odongo *et al.*, 2007). The polymorphism identified within the Bm86 protein is based on extensive sequencing of the gene in tick isolates from Australia and South America, where the TickGARD and GAVAC commercial vaccines have been extensively deployed. In the case of *R. appendiculatus* the variation observed is within a single tick stock that has been maintained in captivity for over 50 years. One explanation for the limited diversity observed within *R. (B) microplus* is that the full range of genetic diversity was not sampled in these studies, since the species may have evolved in South-East Asia and only spread to Australia, South America and Sub-Saharan Africa relatively recently (Jongejan & Uilenberg, 2004). In addition to sequence variation, length variation as a result of indels is apparent both within Ra86 and between homologues in other tick species, with the sizes of the predicted protein ranging from 615–634 amino acids. However, the 20% divergence in amino acid identity between Ra86-4 and Ra86-5 represents, by a wide margin, the largest difference yet observed between Bm86-homologue variants shown to be encoded within a single tick genome.

Despite the fact that the two major variants of Ra86 within the Muguga tick stock have only 80% amino acid identity, this does not necessarily imply that Ra86-4 and Ra86-5 will not induce immunity to challenge with *R. appendiculatus* ticks expressing the alternative Ra86 variant protein. For example, TickGARD has been shown to induce a significant degree of cross-immunity against *H. anatolicum* challenge (de Vos *et al.*, 2001), although the level of amino acid sequence identity of the *H. anatolicum* homologue, Ha98, with Bm86 is only approximately 65% (Azhahianambi *et al.*, 2009). Recent comparative analysis of the relative amount of transcription between Bm86 and Ra86, using a real-time PCR assay, indicates that although expression levels are more variable in *R. appendiculatus*, they are probably not sufficient to explain the lack of cross-protection (Nijhof *et al.*,

2009). The evidence that factors in addition to levels of sequence identity determine the efficacy of Bm86 against heterologous challenge has been reviewed recently (Willadsen, 2004, 2008).

The Ra86 molecule is organized in repeated EGF-like and partial EGF-like cysteine-rich structural domains, which has also been observed for Bm86 (Rand *et al.*, 1989) and homologues from other species including *H. anatolicum* (Ha98) (Azhahianambi *et al.*, 2009) and *Hae. longicornis* (Hl86) (Liao *et al.*, 2007). Virtually all cysteines in Bm86 have been demonstrated to be involved in disulphide bond formation (Rand *et al.*, 1989). The clustering of the cysteine residues is a general characteristic of proteins with extensive intramolecular disulphide bonds as revealed by computer simulation (Tellam *et al.*, 1992). However, as reported for Bm86, the EGF-like peptides are not processed and released as functional peptides in contrast to the EGF precursor molecule. The function of Bm86 and the homologues in other tick species remains unknown, although the similarity in structural organization to EGF-precursors and localization to the surface of the microvilli of gut cells is consistent with a role in the regulation of cell growth of gut digest cells (Tellam *et al.*, 1992; Gough & Kemp, 1993; Liao *et al.*, 2007). In future it will be interesting, although technically difficult, to investigate whether the sequence divergence between Ra86-4 and Ra86-5 has implications for the structure or function of the two predicted variant proteins.

Future investigation of the mechanisms regulating differential expression of the two Ra86 variants will provide insights into arthropod functional genomics. By analogy with other eukaryotic organisms it is likely that this is based on epigenetic control, such as imprinting by methylation or sequestration of actively transcribed genes in specific sub-nuclear locations (Tykco & Morrison, 2002; Scherf *et al.*, 2008).

Experimental procedures

Tick material

Rhipicephalus appendiculatus (Muguga stock) was originally isolated from the Kiambu region of the central highlands of Kenya in the 1950s (T. Dolan, pers. comm.) and was maintained in the East African Veterinary Research Organisation, Muguga, Kenya, and subsequently in the International Livestock Research Institute (ILRI), Nairobi, Kenya. The stock has been passaged on cattle and rabbits according to methods described by Bailey (1960). For the current study, adult ticks were allowed to feed on rabbits for 4 days, after which the midguts were dissected from approximately 400 female ticks. Total RNA was isolated according to Xie & Rothblum (1991) and was used to construct a cDNA library in the Uni-Zap vector. Library construction was outsourced and performed by a commercial company (Stratagene, La Jolla, CA, USA).

Isolation and sequencing of Ra86 cDNA clones

Probes for screening the *R. appendiculatus* gut cDNA Uni-Zap library were prepared by RT-PCR of *R. appendiculatus* gut RNA. Briefly, primers were designed from the Bm86 sequence (accession number M29321; Rand *et al.*, 1989) and a partial Ra86 sequence (Cobon *et al.*, 1996). Primer pair 1 (ILO 8172 forward 5′-GTAGAGGGCACAGCAGAATCATCC-3′ and ILO 8021 reverse 5′-TTCTGACCCTTGTAGGCAGCTTCC-3′) was used to amplify a 1315 bp product from the 5′-end of the Ra86 gene. Primer pair 2 (ILO 8392 forwards 5′-ACAACAGCTGCGACT ACAACAACG-3′ and ILO 8329 reverse 5′-CCAAGCTAGGTGT TCGATGTAAGC-3′) was used to amplify a 211 bp product from the 3′-end of the Ra86 gene. The identity of the purified PCR products was verified by DNA sequencing using BigDye Terminator Cycle sequencing (Applied Biosystems, Foster City, CA, USA). A mixture of the two probes was radio-labeled with ^{32}P and used to screen the library. A secondary screen was performed to isolate clonal plaques. Ra86 clones were processed with ExAssist helper phage to release pBluescript phagemids (Stratagene), which were then sequenced. Sequences were analysed using BLAST (Basic Local Alignment Search Tool, http://www.ncbi.nlm.nih.gov/BLAST/) and ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/). Signal peptides and trans-membrane helix sequences were predicted with SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/; Bendtsen *et al.*, 2004) and TMHMM Server vs. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/), respectively.

Reverse transcription PCR of expression of Ra86 in the gut RNA of individual ticks

Guts were dissected from 50 adult females and 50 adult males of *R. appendiculatus* that had fed on rabbits for 4 days. Each individual gut was dispensed into a separate tube containing 0.5 ml chilled L15 buffer supplemented with 15% FCS. Total RNA was then rapidly isolated according to Xie & Rothblum (1991). RNA from each tick was used for RT-PCR of Ra86. PCR primers were designed to flank an indel that differentiates the Ra86-4 and Ra86-5 variants of the gene. The primer sequences were: ILO-8023 forwards 5′-TGAACTGCACGCAGGATATTAAGG-3′ and ILO-8329 reverse 5′-CCAAGCTAGGTGTTCGATGTAAGC-3′.

Specific PCR amplification of 3′ untranslated regions from cDNA

An alignment of 3′UTR sequences of Ra86-4 and Ra86-5 homologues revealed SNPs that enabled the design of sets of primers for specific amplification 3′UTR sequences of each type. The primers were Ra86-4 forward 5′-GTGAGGA/GAGATT/GTCAA GCTTCAAC-3′, Ra86-4 reverse 5′-AGCATCCGTGTAGTCGTG AGTACC-3′, Ra86-5 forward 5′-GTGAGGG/AAGATG/TTCAA GCTTCAGT-3′ and Ra86-5 reverse 5′-CACATCCGTGT/CAGT CGTGAGTAAC-3′. The 3′UTR PCRs yielded a product of 130 bp, and the primer specificities were confirmed on plasmid cDNA clones of Ra86-4 (clone 85A) and Ra86-5 (clone 92A).

Mitochondrial cytochrome oxidase 1 analysis of individual Rhipicephalus appendiculatus *ticks expressing different Ra86 homologues*

Cytochrome oxidase 1 sequences were obtained by PCR amplification from the DNA of 30 individual *R. appendiculatus* ticks

from the Muguga laboratory stock. Four sequences from each tick were generated and aligned with ClustalW2 to create a consensus sequence, which was then used to search the National Centre for Biotechnology Information nr database by blastn.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/j.1365-2583.2010.01043.x

Figure S1. PCR amplification of Ra86 from gut RNA from 20 individual female *R. appendiculatus* ticks. Controls: PCR amplification of cDNA plasmids representing Ra86-4 (clone 85A) and Ra86-5 (clone 92A), respectively. The predicted sizes of the Ra86-4 and Ra86-5 PCR products are 890 bp and 1010 bp, respectively. Molecular markers for sizes indicated (lane M).

Figure S2. Alignment of Ra86 3′ untranslated region sequences.

Table S1. GenBank accession numbers of 19 Ra86 homologues of the Bm86 antigen isolated from *Rhipicephalus appendiculatus*.

Table S2. Summary of sequence similarity of mitochondrial cytochrome oxidase 1 sequences representing a consensus sequence derived from individual ticks expressing Ra86-4 and Ra86-5 in gut cDNA. These were searched against the GenBank nr sequence database using blastn.

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