Molecular characterization of the kinetoplastid membrane protein-11 genes from the parasite *Trypanosoma rangeli*

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SUMMARY

Trypanosomatids are early divergent parasites which include several species of medical interest. *Trypanosoma rangeli* is not pathogenic for humans but shows a high immunological cross-reactivity with *Trypanosoma cruzi*, the causative agent of Chagas' disease that affects more than 17 million people throughout the world. Recent studies have suggested that *T. cruzi* KMP-11 antigen could be a good candidate for the induction of immunoprotective cytotoxic responses against *T. cruzi* natural infection. In the present paper the genes coding for the *T. rangeli* kinetoplastid membrane protein-11 have been characterized. The results show that the locus encoding this protein is formed by 4 gene units measuring 550 nucleotides in length, organized in tandem, and located in different chromosomes in KP1(+) and KP1(−) strains. The gene units are transcribed as a single mRNA of 530 nucleotides in length. Alignment of the *T. rangeli* KMP-11 deduced amino acid sequence with the homologous KMP-11 protein from *T. cruzi* revealed an identity of 97%. Interestingly, the T and B cell epitopes of the *T. cruzi* KMP-11 protein are conserved in the *T. rangeli* KMP-11 amino acid sequence.

Key words: *Trypanosoma rangeli*, *Trypanosoma cruzi*, kinetoplastid membrane protein-11, molecular characterization.

INTRODUCTION

Trypanosomatids are flagellated protozoan parasites which cause severe diseases in humans such as African sleeping sickness caused by *Trypanosoma brucei gambiense*, and *Trypanosoma brucei rhodesiense*, Chagas' disease caused by *Trypanosoma cruzi*, and leishmaniasis caused by a variety of species of *Leishmania*, including those from *Leishmania donovani* complex, *Leishmania mexicana complex*, *Leishmania tropica complex*, and *Leishmania braziliensis* complex. *Trypanosoma rangeli* is apparently not pathogenic to humans and animals but shows similar morphology, and high immunological cross-reactivity with *Trypanosoma cruzi* (Afchain *et al*. 1979; Basso *et al*. 1991, 2004; Saldana & Sousa, 1996; Palau *et al*. 2003), interfering with the Chagas' disease diagnosis (Guhl & Vallejo, 2003). Moreover, these parasites are sympatric in some regions of America, share the same hosts range producing mixed infections, and often have identical insect vectors such as *Rhodnius prolixus* and *Rhodnius colombiensis* (D'Alessandro, 1976; D'Alessandro & Saravia, 1992, 1999; Guhl & Vallejo, 2003). Recent studies have shown that immunization of mice with *T. rangeli* protects animals against *T. cruzi* infection (Basso *et al*. 1991, 2004; Palau *et al*. 2003).

Two important epidemiological groups of *T. rangeli* have recently been described, KP1(−), and KP1(+) strains, based on the KP1 mini-circle presence in the parasite kinetoplast and the association with different adaptive lines of vectors. *T. rangeli* KP1(+) strains are related with *Rhodnius* species from the prolixus group (*R. prolixus* and *Rhodnius neglectus*) (Vallejo *et al*. 2003) and, *T. rangeli* KP1(−) strains are associated with *Rhodnius* species from the pallescens group (*Rhodnius pallescens*, *R. colombiensis*, and *Rhodnius ecuadoriensis*) (Vallejo *et al*. 2003).

Chagas' disease affects more than 17 million people in 15 endemic countries in Central and South America (WHO, 2002), and it represents a serious health problem. There is no immunoprophylaxis available and the current treatment is rather toxic, not very effective, and only indicated for patients in the acute phase of the disease or for *T. cruzi*-infected asymptomatic young people. Many attempts have been made in order to characterize new parasite antigens that are capable of eliciting protective immune responses and that could be employed to provide an immunoprophylaxis therapy against this sickness.

The kinetoplastid membrane protein-11 (KMP-11) is a ubiquitous and abundant protein, mainly located in the flagellum and the flagellar pocket

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of different species of kinetoplastids including *Trypanosoma*, *Leishmania*, *Crithidia*, *Leptomonas* and *Phytomonas* (Jardim *et al*. 1995; Stebeck *et al*. 1995, 1996; Berberich *et al*. 1997; Bridge *et al*. 1998; Ramirez *et al*. 1998; Thomas *et al*. 2000). Moreover, in *T. cruzi*, it has been demonstrated that 50% of the protein is associated with the cytoskeleton of the parasite (Thomas *et al*. 2000). KMP-11 protein is expressed during all parasite stages exhibiting higher levels in the insect stages (Stebeck *et al*. 1995). KMP-11 is well conserved among kinetoplastids and its predicted secondary structure consists of two α-helices separated by a random-coil segment. One third of each helical side is formed by hydrophobic residues, which are thought to interact with the lipidic bilayer in the kinetoplastid cell membrane (Stebeck *et al*. 1996). Interestingly, this protein presents a significant homology to the apolipoprotein B, as well as to the cytoskeleton-associated protein CIP1 from *Arabidopsis thaliana* (Thomas *et al*. 2000), and to calcium-binding proteins like LAV1-2, an EF-hand protein of 40 kDa from *Physarium polycephalum* (Fuertes *et al*. 2001). Based on these facts, it has been suggested that this protein may function in part to increase bilayer pressure, stabilizing molecules such as lipophosphoglycan within the parasite pellicular membrane (Jardim *et al*. 1995) as well as being implicated in the parasite mobility, and its attachment to the host cell (Thomas *et al*. 2000). On the other hand, it has been reported that KMP-11 protein is a potent inducer of humoral and cellular immune responses (Tolson *et al*. 1994) in infected animals and in leishmaniasis and chagasic patients (Berberich *et al*. 1997, 2003; Jensen *et al*. 1998; Ramirez *et al*. 1998, 2001; Mukhopadhyay *et al*. 1999; Trujillo *et al*. 1999; Marañon *et al*. 2001; Planelles *et al*. 2001, 2002; Thomas *et al*. 2001; de Carvalho *et al*. 2003). Moreover, it has recently been demonstrated that *T. cruzi* KMP-11 when fused to the heat shock protein HSP70 from this parasite elicits a specific cytotoxic and humoral immune response against the antigen and leads to protection in an experimental murine model (Planelles *et al*. 2001). In this context, herein we report the isolation and molecular characterization of the gene coding for KMP-11 protein from *T. rangeli* and its comparison to the homologous gene in *T. cruzi*.

MATERIALS AND METHODS

Parasites

Epimastigotes from KP1 (+) *T. rangeli* strains: H14 from Honduras (MHOM/Hond/H14, Acosta *et al*. 1991), Choachi from Colombia (IRHO/CO/82/Choachi), and Colombian KP1 (−) Tre strain (Morales *et al*. 2002) were grown at 28 °C in modified REI medium supplemented with 2% (v/v) heat-inactivated fetal bovine serum. *T. cruzi* epimastigotes, Munanta and Shubacbarina strains (Rodriguez *et al*. 1998) were grown at 28 °C in liver infusion triptone (LIT) medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum.

Southern blot analysis

Genomic DNA (1–4 μg) from *T. rangeli* was digested with different restriction enzymes and also partially with *Hinf*I enzyme for increasing periods of time, resolved on 0·8% agarose gels and transferred to nylon membranes (Bio-Rad) by standard procedures (Sambrook, Russell & Irwin, 2000). Full length KMP-11 coding sequence from *T. cruzi* and *T. rangeli* (KMP11Tr) were PCR amplified using, respectively, *T. cruzi* and *T. rangeli* genomic DNA and two primers KMP11F (5′-ATGGCCACCACT CTTGAG-3′) and KMP11R (5′-TTACTTTCCT GGGAACTG-3′) that map at the *T. cruzi* KMP-11 5′ end (including start codon) and at the *T. cruzi* KMP-11 3′ end (Thomas *et al*. 2000). Probes were labelled by the random primer method using [α-^{32}P] dCTP (Feinberg & Vogelstein, 1983). Hybridizations were carried out using the methodology previously described by Puerta *et al*. (1994). Briefly, hybridization was performed overnight at 37 °C (heterologous probe) or 42 °C (homologous probe) in 50% formamide/5 × SSC/0·1% SDS/5 × Denhart's/0·05 M Na_2HPO_4/NaH_2PO_4 buffer/0·25 mg/ml heat-denatured herring sperm DNA. Post-hybridization washes were performed 4 times in 2 × SSC/0·1% SDS at room temperature and once in 0·1 × SSC/0·1% SDS at 65 °C for 1 h and exposed to Curix RP2 medical X-Ray film (Kodak).

Chromosomal blot analysis

For pulsed field gel electrophoresis (PFGE) analysis, agarose blocks containing about 5×10^7 parasites were prepared as described by Clark *et al*. (1990) and stored at 4 °C in 0·5 M EDTA, pH 9·5. Then 1/5 of each block was electrophoresed in an LKB 2015 Pulsaphor System apparatus (Pharmacia LKB, Sweden), using 1% agarose gels and 0·5 × TBE buffer (40 mM Tris, 45 mM boric acid, 1 mM EDTA pH 8·3) at 13 °C with pulse times of 250, 500, 750 and 1000 s at 84 V for 80 h. Resolved chromosomes were transferred to a nylon filter and hybridized with the KMP11Tr probe as described above.

Northern blot analysis

The *T. rangeli* cytoplasmic RNA was isolated using standard procedures (Marañon *et al*. 2000). RNA (5 μg) was size-fractionated on 1% agarose/formaldehyde gels, transferred to nylon membrane using a 40 mM NaOH solution and hybridized with the radio-isotope labelled KMP11Tr probe as described above.

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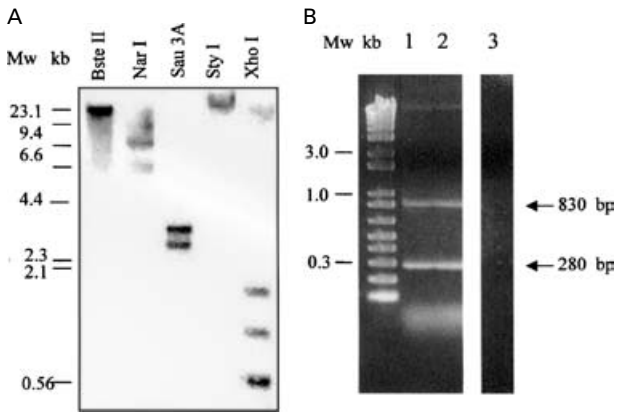

Fig. 1. (A) Southern blot of *Trypanosoma rangeli* genomic DNA. (A) Samples of 4 μg from epimastigotes of the *T. rangeli* Tre strain were digested with *Bste*II, *Nar*I, *Sau*3A, *Sty*I and *Xho*I restriction endonucleases and hybridized to the *T. cruzi* KMP-11 coding sequence. *Hind*III digested λ Phage DNA was used as molecular weight marker. (B) Ethidium bromide-stained 1% agarose gel showing the PCR amplified products using *Taq*I DNA polymerase, KMP11F/R primers and *T. rangeli* Tre strain genomic DNA (lanes 1 and 2) or no DNA (lane 3). A 100 bp ladder (Promega) was used as molecular weight marker.

PCR amplification and cloning

The *T. rangeli* KMP-11 coding region was PCR-amplified employing KMP11F and KMP11R primers and *T. rangeli* genomic DNA as template. Two different polymerases, standard *Taq* DNA polymerase (Promega) and a proof-reading *Tli* DNA polymerase (Promega) were used. Following ethidium bromide staining the product amplified by the *Tli* DNA polymerase and the 2 fragments produced by *Taq* DNA polymerase were gel extracted, purified using GFX Gel Band Purification kit (Amersham Biosciences) and cloned into the pGEM®-T Easy plasmid (Promega).

Both strands of the cloned inserts were sequenced by the Sanger method (Sanger *et al*. 1977) in a 373 Automatic DNA sequencer (Pharmacia LKB), using the universal and KMP11F and KMP11R primers.

Sequence analyses

Homology searches were performed using the GenBank and EMBL databases and FASTA program (Pearson, 1990). Sequence alignments were performed using MULTALIN (Corpet, 1988) and LALIGN programs (Pearson, 1990).

RESULTS

Isolation and characterization of the gene coding for Trypanosoma rangeli *KMP-11 protein*

In order to verify the presence of the KMP-11 gene in *T. rangeli*, we took advantage of the close phylogenetic relationship between *T. rangeli* and *T. cruzi*. Thus, *T. rangeli* genomic DNA was digested with different restriction enzymes and hybridized with the sequence coding for KMP-11 protein from *T. cruzi* (Fig. 1A). Several hybridization bands were observed in Southern blot analysis indicating the existence of homologous KMP-11 genes in *T. rangeli*. As a first approach for isolating the homologous genes in *T. rangeli*, primers KMP11F and KMP11R with map, respectively, at the 5′ and 3′ ends of *T. cruzi* KMP-11 coding gene were used in a PCR reaction using *T. rangeli* genomic DNA as template and two different DNA polymerases, *Tli* and *Taq*. When *Tli* polymerase was employed, a single band of approximately 280 bp was observed (data not shown). When *Taq* polymerase was used, 2 bands of approximately 830 bp and 280 bp were amplified (Fig. 1B). All the amplified fragments were cloned into pGEM®-T Easy plasmid (Promega) and sequenced by use of the Sanger method.

Sequence analysis of the small amplified band (Accession number AY147904) showed correspondence with the *T. rangeli* KMP-11 coding sequence (Fig. 2A) which has an identity of 88% with the *T. cruzi* KMP-11 coding region. The G/C content of this ORF is slightly lower than the A/T content (50·2%). In addition, a strong preference for codons ending in G or C for charged amino acids was observed. For example, 4/5 aspartic acid residues, 13/15 glutamic acid residues, 5/5 histidine residues, 14/15 lysine residues, 3/3 asparagine residues, and 4/5 glutamine residues present in the *T. rangeli* KMP-11 protein are encoded by GAC, GAG, CAC, AAG, AAC, and CAG codons, respectively.

Sequence analysis of the large band (Accession number AY325812) revealed 2 copies of the KMP-11 coding gene separated by an intergenic region of 270 bp (Fig. 2B). In this intergenic region there is a polypyrimidine track followed by 4 putative splicing acceptor sites (AG dinucleotides). Alignment of the sequences of the two ORFs within the 829 bp band showed an identity at nucleotide level of 90·3% whereas the comparison among the *Tli* amplified band and these two ORFs showed that they shared a higher percentage of similarity with the first ORF (98%) than with the second one (91%). Interestingly, the percentage of identity among the KMP-11 coding gene copies is higher between *T. rangeli* and *T. cruzi* (Accession numbers AJ000077, AF167435, and AF167434) than between *T. rangeli* and *T. brucei* (Accession number AF028726) genes. Comparison between the *T. rangeli* and *T. cruzi* intergenic regions revealed an identity of 58% suggesting that higher homology is focused on coding sequences.

Genomic organization of T. rangeli *KMP11 coding gene*

The genomic organization of the *T. rangeli* KMP-11 genes was studied by Southern blot analysis of

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A

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   1 atggccaccactcttgaggagttttccgccaaacttgaccggttggatgccgagtttgca
   1 M  A  T  T  L  E  E  F  S  A  K  L  D  R  L  D  A  E  F  A
             ●  ●              ♦
  61 aagaagatggaggagcagaacaagaagttctttgcggacaagccggacgaaagcaccctg
  21 K  K  M  E  E  Q  N  K  K  F  F  A  D  K  P  D  E  S  T  L
                                                          ■
 121 tctcctgaaatgaaggagcactacgagaagttcgagaggatgattcaggagcacaccgac
  41 S  P  E  M  K  E  H  Y  E  K  F  E  R  M  I  Q  E  H  T  D
                                                          ♦
 181 aagttcaacaagaagatgcacgagcactctgagcactttaagtcgaagtttgctgagctg
  61 K  F  N  K  K  M  H  E  H  S  E  H  F  K  S  K  F  A  E  L

 241 ctcgagcaacagaagaacgctcagttcccaggaaagtaa 279
  81 L  E  Q  Q  K  N  A  Q  F  P  G  K  *  92
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B

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   1 atggccaccactcttgaggagttttccgccaaacttgaccggttggatgccgagtttgca
   1 M  A  T  T  L  E  E  F  S  A  K  L  D  R  L  D  A  E  F  A

  61 aagaagatggaggagcagaccaagaagttctttgcggacaagccggacgaaagcaccctg
  21 K  K  M  E  E  Q  T  K  K  F  F  A  D  K  P  D  E  S  T  L

 121 tctcctgaaatgaaggagcactacgagaagttcgagaggatgattcaggagcacaccgac
  41 S  P  E  M  K  E  H  Y  E  K  F  E  R  M  I  Q  E  H  T  D

 181 aagttcaacaagaagatgcacgagcactctgagcactttaagtcgaagtttgctgagctg
  61 K  F  N  K  K  M  H  E  H  S  E  H  F  K  S  K  F  A  E  L

 241 ctcgagcaacagaagaacgctcagttcccgggcaagtaggcagagaggagagaatggatg
  81 L  E  Q  Q  K  N  A  Q  F  P  G  K  *

 301 tgactatggtttgtggtacgcgcgccattgtctccatttttcgatgttttaatagaatag

 361 gggtttttttggttatgtttctggacttatcagacggcagctaccgggtttgcgtggcc

 421 accaatgttgcattctttttttttttttattttgctttcaggtgggtgatgccgccgccat
                                          ♦
 481 ttcttcctgtgttctttgctgcttttctctcgcagtttgattgcgcccaagctatatctt
                                  ♦               ♦
 541 taagtgaaacatggccaccacccttgagaagttttccgccaaacttgaccggttggatgc
   1   ♦       M  A  T  T  L  E  K  F  S  A  K  L  D  R  L  D  A

 601 cgagtttgcaaagaagatggaggagcagaacaagaagttcttcgcggacaaacccgacga
  18  E  F  A  K  K  M  E  E  Q  N  K  K  F  F  A  D  K  P  D  E

 661 gagcacgctctcccctgaaatgaaggagcactatgaaaagtttgaaaaaatgattcagga
  38  S  T  L  S  P  E  M  K  E  H  Y  E  K  F  E  K  M  I  Q  E

 721 gcacaccgacaaattcaacaaaaagatgcacgagcactctgagcacttcaaggccaaatt
  58  H  T  D  K  F  N  K  K  M  H  E  H  S  E  H  F  K  A  K  F

 781 tgctgagttgcttgagcagcagaagaacgctcagttcccaggaaagtaa 829
  78  A  E  L  L  E  Q  Q  K  N  A  Q  F  P  G  K  *
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Fig. 2. Nucleotide sequence and deduced amino acid sequence from the *Trypanosoma rangeli* KMP-11 coding genes. (A) Sequence of the DNA fragment amplified using *Tli* enzyme. Numbers to the left of the sequence indicate the nucleotide and amino acid position. The stop codon is marked by an asterisk. The *Xho*I restriction site is in bold. ● indicates the two protein kinase C phosphorylation sites, ◆ marks the casein kinase II phosphorylation sites, and ■ denotes the *O*-glycosylation site. Amino acids that comprise the two theoretical α-helices are underlined. (B) Sequence from the 829 bp amplified fragment. Numbers to the left of the sequence indicate the nucleotide and amino acid positions. Stop codons are marked by an asterisk. The *Xho*I restriction site is in bold and, *Xmn*I restriction site is both in bold and in italic. The polypyrimidine track is underlined inside the intergenic region. ◆ indicates the putative spliced leader acceptor sites.

genomic DNA using the *T. rangeli* KMP-11 coding region as probe. The presence of a highly intense hybridization band of 550 bp in length in lane *Xho*I (Fig. 1A) and lanes *Age*I and *Hinf*I (Fig. 3A, fragment I), restriction endonucleases which cut once within the KMP-11 unit, is consistent with the existence of

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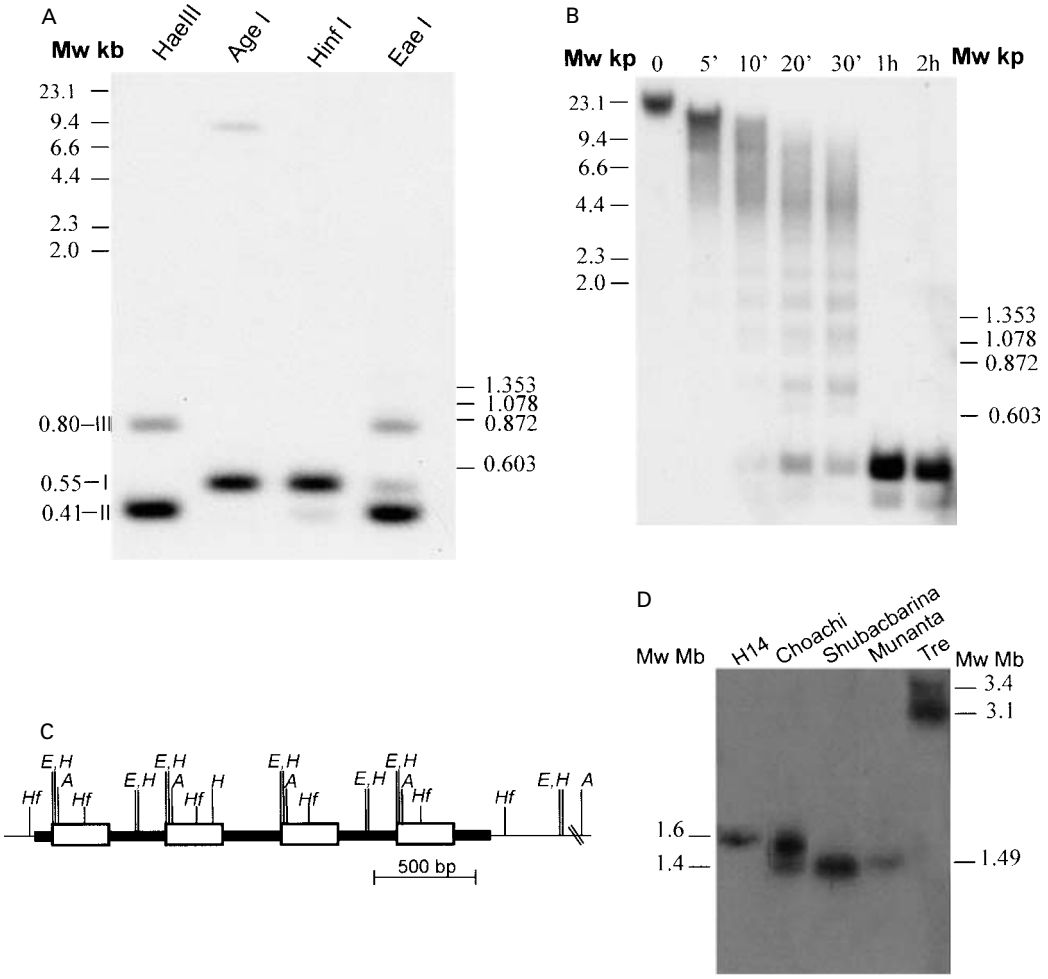

Fig. 3. (A) Samples of 4 μg from the *Trypanosoma rangeli* Tre strain genomic DNA were digested with different restriction endonucleases and hybridized with the *T. rangeli* KMP-11 coding sequence (KMP11Tr). Molecular weight markers, *Hind*III digested λ Phage DNA and *Hae*III digested Φ X174 RF DNA were used. (B) Samples of 1 μg of *T. rangeli* Tre strain genomic DNA were partially digested with the *Hinf*I restriction endonuclease for 5 min, 10 min, 20 min, 30 min, 1 h and 2 h at 37 °C. *Hind*III digested λ Phage DNA and *Hae*III digested Φ X174 RF DNA were used as molecular weight markers. The approximately 630 bp hybridization band observed after 20 and 30 min of enzyme digestion corresponds to the fragment formed by 1 gene unit plus the *Hinf*I short fragment located at the locus 5′ end. (C) Schematic representation of the KMP-11 locus. The coding regions (open boxes), the intergenic regions, the 5′ and 3′ non-coding regions of the cluster (thick black lines) and restriction endonuclease cleaving sites, *Hae*III (H), *Age*I (A), *Hinf*I (Hf) and *Eae*I (E) are indicated. (D) Pulsed-field gel electrophoresis of chromosomes from *T. rangeli* H14, Choachí and Tre, *T. cruzi* Shubacbarina and Munanta strains. The size of the hybridization bands is indicated.

several copies of the KMP-11 gene separated by their intergenic regions of similar size in the *T. rangeli* genome. Digestion of genomic DNA with *Hae*III and *Eae*I enzymes, which cut twice inside most KMP-11 units produces, as expected, an intense hybridization band of approximately 415 bp (fragment II in Fig. 3A). A slightly hybridization band of 800 bp was also generated (fragment III in Fig. 3A). This fragment corresponds to the KMP-11 unit located at the 3′ end of the cluster and part of its flanking region. In addition, nucleotide polymorphism in the second copy of the KMP-11 locus creates a new *Hae*III restriction site at nucleotide 219 (as seen by DNA sequencing of the larger amplified band, shown in Fig. 2B). This polymorphism produces an additional slight hybridization fragment of approximately 520 bp which originated due to the absence of the *Eae*I/*Hae*III restriction site located at the intergenic region comprised between the second and third copy of the KMP-11 locus.

To determine the copy number of *T. rangeli* KMP-11 gene Southern blotting and hybridization with the KMP11Tr coding region employing genomic DNA partially digested with *Hinf*I was carried out. The obtained results, shown in Fig. 3B, indicate that there are 4 copies of KMP-11 genes in *T. rangeli*. Copy number estimation was confirmed by densitometric analysis of the *Hinf*I-hybridized bands. The 550 bp fragment obtained after 30 min of digestion with *Hinf*I was used as reference in order to compare with the same fragment obtained after 1 and 2 h of digestion. A map representing the genomic

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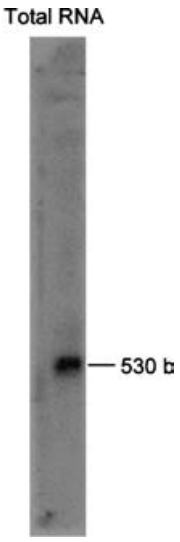

Fig. 4. Northern blot analysis of total RNA of epimastigotes from *Trypanosoma rangeli* Tre strain in the logarithmic phase of growth. The filter was hybridized with the radio-isotope labelled KMP11Tr probe.

organization of the KMP-11 cluster is shown in Fig. 3C. To identify the chromosome that contains the KMP-11 cluster, pulsed-field gel electrophoresis was carried out with different *T. rangeli* strains (Fig. 3D). Analysis of the hybridization bands revealed the existence of divergence in the cluster location among strains. Thus, the KMP-11 cluster is located in two chromosomes of 3·1 and 3·4 Mb in the KP1(−) Tre strain while in the KP1(+) Choachi strain it is located in two chromosomes of 1·6 and 1·4 Mb. In KP1(+) H14 strain the KMP-11 cluster is contained in a single chromosome of approximately 1·6 Mb. In the Shubacbarina and Munanta strains, which belong to group I of *T. cruzi*, the KMP-11 genes are positioned on a chromosomal band of 1·49 Mb.

Expression product of the T. rangeli *KMP-11 gene*

The expression product of the *T. rangeli* KMP-11 genes was analysed by Northern blotting using total RNA from *T. rangeli* hybridized to the *T. rangeli* KMP-11 coding region. As shown in Fig. 4, a single hybridization band of 530 nt in length was detected in the epimastigote form of the parasite.

The *T. rangeli* KMP-11 deduced amino acid sequence corresponds to a protein of 92 amino acids (Fig. 2A) with a molecular weight of 11 kDa and a theoretical isoelectric point of 5·96. Prediction of post-translational modifications shows the existence in KMP-11 protein, of a single *O*-glycosylation site at threonine 39 as well as 4 phosphorylation sites, 2 of them depending on protein kinase C and 2 on casein kinase II (Fig. 2A). In addition, theoretical analysis of the secondary structure reveals the presence of 2 α-helices separated by a random-coil segment, like that found in other KMP-11 proteins (Stebeck *et al.* 1995; Thomas *et al.* 2000). All of these characteristics are conserved among the deduced amino acid sequence of the 3 sequenced KMP-11 ORFs with the exception of the isoelectric point of the second KMP-11 copy found in the 829 bp fragment which is 6·52. Alignment of the *T. rangeli* KMP-11 deduced amino acid sequences revealed that the ORF amplified by *Tli* has an identity of 98% with the first ORF contained in the 829 bp fragment and 96% with the second (Fig. 5). Moreover, comparison of the *Tli* amplified band with *T. cruzi*, *T. brucei* and *Leishmania panamensis* KMP-11 sequences showed an identity of 97%, 92·4% and 88%, respectively. The first ORF of the 829 bp clone revealed an identity of 96% with *T. cruzi*, 91% with *T. brucei* and 86% with *L. panamensis* KMP-11 sequences, whereas the second ORF showed an identity of 98%, 93%, and 85% with *T. cruzi*, *T. brucei* and *L. panamensis* KMP-11 proteins, respectively. Besides, it is important to note that the homology shared between *T. cruzi* KMP-11 and the cytoskeleton associated protein CIP1 from *Arabidopsis thaliana* (Thomas *et al.* 2000) is also present in all *T. rangeli* KMP-11 deduced sequences. In the same way, the homology between KMP-11 proteins and calcium-binding proteins is also kept in the *T. rangeli* KMP-11 protein (Fuertes *et al.* 2001). Finally, the B and T cell epitopes of *T. cruzi* KMP-11 are conserved in the *T. rangeli* KMP-11 protein.

DISCUSSION

Isolation and characterization of the *Trypanosoma rangeli* KMP-11 gene has allowed us to compare it with the sequence of the homologous protein in other trypanosomes. Comparison of genomic organization between KMP-11 from *T. rangeli* and *T. cruzi* demonstrated a similar organization of 4 tandemly repeated copies separated by intergenic regions of 270 bp in length. In contrast, *Leishmania* KMP-11 ORFs are separated by longer intergenic regions which exhibit different sizes. KMP-11 transcripts in both trypanosomes have approximately 500 nt in length, whereas in *Leishmania* the KMP-11 messenger is longer, with a length of 1300 nt. Thus, all of these KMP-11 genes transcribe a high portion of their intergenic regions. These differences can be the result of different regulatory mechanisms operating in these trypanosomatids. In fact, whereas in *T. cruzi* the level of the KMP-11 transcripts is the same through the different life-stages (Thomas *et al.* 2000), in *Leishmania infantum* the KMP-11 transcripts are upregulated in promastigotes (Berberich *et al.* 1998). Thus, it is possible that intergenic regions could include specific regulatory sequences responsible for operating at different levels, post-transcriptional and translational.

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Tr2tliA   MATTLEEFSAKLDRLDAEFAKKMEEQNKKFFADKPDESTLSPEMKEHYEKFERMIQEHTD 50
Tr1       ........................T.............................
Tr2       ......K.....................................................K.......
Tc        .....................................................K.......
Tb        ....Y...A..................R........A..............K.......
Lp        ....Y...A.......E..N...Q...A.........................K...E

Tr2tliA   KFNKKMHEHSEHFKSKFAELLEQQKNAQFPGK 92
Tr1       ................................
Tr2       ..............A.................
Tc        ..............A.................
Tb        ......R.......A.................
Lp        ..............H..........A..Y...
```

Fig. 5. Alignment of the deduced amino acid sequences from *Trypanosoma rangeli* KMP-11 coding sequences (Tr2tliA: Accession number Q8IS88, Tr1: Accession number AAP88967, and Tr2: Accession number AAP88968), *T. cruzi* (Tc: Accession number Q9U6Z1), *T. brucei* (Tb: Accession number Q26773), and *L. panamensis* (Lp: Accession number Q9NHU4). Dots represent identical amino acids.

Two important epidemiological groups of *T. rangeli* have recently been described, KP1(−) strains associated with *R. pallescens* group, and KP1(+) strains associated with *R. prolixus* group (Vallejo *et al.* 2002, 2003). Therefore, the differences observed in the KMP-11 locus location between KP1(+) and KP1(−) strains could be the result of an evolution process that facilitated an increase in the distance between these two lineages of *T. rangeli*. Indeed, there are several lines of evidence that indicate that these two groups of *T. rangeli* differ not only by using molecular markers such as KP1 mini-circle and PCR mini-exon amplifications, but also in their biological behaviour (Vallejo *et al.* 2002, 2003). Thus, the chromosomal location of KMP-11 genes constitutes another molecular marker that can differentiate these *T. rangeli* subpopulations. It is interesting to note that KMP-11 location in *T. cruzi* is also different among strains belonging to groups I and II. Thomas *et al.* (2000) reported the location of the KMP-11 gene in a chromosome of 1900 kb in the Y strain, which belongs to group II whereas in Munanta and Shubacbarina group I strains these genes are located in a chromosome of 1490 kb. Since the KMP-11 locus is located at different chromosomes depending on species and strains, it could be employed as a tool for diagnostic purposes. On the other hand, since KMP-11 genes are organized in a single locus, the presence of two chromosomal hybridization bands in *T. rangeli* Choachi and Tre strains can be the result of differences in size between homologous chromosomes. These polymorphisms have been also described in other trypanosomatids (Henriksson *et al.* 1995; Toaldo *et al.* 2001).

Sequence analysis also showed the existence of polymorphism at nucleotide level inside the *T. rangeli* KMP-11 coding sequences. For instance, the second unit of the 829 bp fragment lacks an *Xho*I restriction site and, however, contains a new *Xmn*I restriction site. This polymorphism was also detected by PCR-RFLP analysis of Tre and other KP1(−) strains (laboratory data). In addition, the *Hae*III and *Eae*I restriction enzymes, besides cutting at the beginning of the coding region of all genes, they also cut only inside the intergenic regions located between the first and second copies and the third and fourth copies. This finding could be indicative of a duplication event that has produced 4 copies from the originally existent 2 copies.

Polymorphism also affects the protein amino acid composition. Indeed, there are 4 changes among the known deduced amino acid sequences, 3 of them being conservative and 1 being non-conservative. These results contrast with the absence of polymorphism in the *T. brucei* KMP-11 proteins (Bridge *et al.* 1998). In *T. cruzi* KMP-11 gene units only the absence of the lysine residue located just before the protein stop codon in the deduced amino acid sequence of the first copy of the *T. cruzi* KMP-11 cluster is observed (Thomas *et al.* 2000).

Comparative sequence analysis showed that the intergenic region of *T. rangeli* KMP-11 genes has a greater identity with *T. cruzi* (58% in 269 nts) than with *T. brucei* (65% in 77 nts) region. The polypyrimidine tract, located upstream from the 4 putative splicing acceptor sites, is not preceded by the 9 GT dinucleotide repetitions observed in *T. cruzi* and *T. brucei* KMP-11 genes.

All the above-mentioned analyses demonstrated that the *T. rangeli* KMP-11 protein is phylogenetically closer to the *T. cruzi* homologous protein than to the *T. brucei* homologous protein. In fact, one copy from *T. rangeli* KMP-11 shares a higher homology with the *T. cruzi* than with its other own copies. This high degree of similarity between *T. cruzi* and *T. rangeli* should be corroborated with other molecular markers. Interestingly, Stevens *et al.* (1999), using the ssrRNA sequences, found a close evolutionary relationship between *T. rangeli* and *T. cruzi*, both of which are placed in the same clade.

Finally, it is important to remark that *T. rangeli* KMP-11 protein as *T. cruzi* protein conserves the predicted secondary structure as well as the motifs implicated in the hypothetical functions assigned to these proteins, including those for phosphorylation and calcium binding. The high degree of

conservation of this protein and its presence in all trypanosomatids render KMP-11 an important protein for these parasites.

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