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The *Trypanosoma rangeli* histone H2A gene sequence serves as a differential marker for KP1 strains☆

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Abstract

Trypanosoma rangeli has recently been divided in two primary lineages denoted as KP1(+) and KP1(−) strains because of epidemiological and evolutionary interest in the molecular differentiation of these two groups. We report the molecular characterization of the genes encoding histone H2A protein from a *T. rangeli* KP1(+) strain (H14), its comparison to *T. rangeli* KP1(−) strain (C23) histone H2A coding genes [Puerta, C., Cuervo, P., Thomas, M.C., López, M.C., 2000. Molecular characterization of the histone H2A gene from the parasite *Trypanosoma rangeli*. Parasitol. Res. 86, 916–922], and its application in a low-stringency single specific primer polymerase chain reaction (LSSP-PCR) assay to differentiate these parasite groups. The results show that the locus encoding the H2A protein in the H14 strain is formed by at least 11 gene units measuring 799 nucleotides in length, organized in tandem, and located in two chromosomes of approximately 1.9 and 1.1 Mb in size. Remarkably, in KP1(−) strains these genes are on pairs of chromosomes of about 1.7 and 1.9 Mb. In addition, there is a hybridization signal in the compression region above 2.1 Mb in all *T. rangeli* strains. Therefore, the chromosomal location of these genes is a useful marker to distinguish between KP1(+) and KP1(−) *T. rangeli* strains. The alignment of the H2A nucleotide sequences from H14 and C23 strains showed an identity of 99.5% between the coding regions and an identity of 95% between the non-coding regions.

The deduced amino acid sequences proved to be identical. Based on 5% of the difference between the intergenic regions, we developed a LSSP-PCR assay which can differentiate between KP1(+) and KP1(−) strains.

Keywords: Histone H2A; *Trypanosoma rangeli* KP1(+); *Trypanosoma rangeli* KP1(−); KP1 minicircle; Low-stringency single specific primer polymerase chain reaction (LSSP-PCR)

1. Introduction

Trypanosoma rangeli is a parasite of man, domestic and wild animals in Central and South America. In contrast to the pathogenic *Trypanosoma cruzi*, it is harmless to the mammalian host. However, both parasites share the same hosts and are transmitted to humans by the same vectors in the endemic areas of the Chagas' diseases. For these reasons, epidemiological and biological analyses of *T. rangeli* have been carried out within the *T. cruzi* context (D'Alessandro, 1976; D'Alessandro and Saravia, 1992, 1999; Guhl and Vallejo, 2003).

Several studies using isoenzymes (Steindel et al., 1994), karyotypes (Henriksson et al., 1996; Toaldo et al., 2001), mini-exon PCR amplification (Grisard et al., 1999a,b), or randomly amplified polymorphic DNA (RAPD) (Steindel et al., 1994) as molecular markers showed that *T. rangeli* exhibited genetic polymorphism depending on geographic region (southern Brazil, Central America, or northern South America). Recently, two important epidemiological groups of *T. rangeli* have been described: KP1(−) strains, associated with the adaptive line of *Rhodnius*, represented by *R. colombiensis*, *R. pallescens*, and *R. ecuadoriensis*, and KP1(+) strains, associated with *R. prolixus* (Vallejo et al., 2002, 2003; Urrea et al., 2005). These two groups have been defined on the basis of independent mitochondrial (minicircle profile dimorphism obtained by kDNA PCR amplification) and nuclear (mini-exon PCR amplification) markers. Although both KP1(+) and KP1(−) strains can be

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found in the gut of any vector, each strain is only able to reach the haemolymph and to be transmitted to the vertebrate host by the saliva of the insect in a particular vector.

Besides these two *T. rangeli* primary lineages, which share the same geographic regions in Colombia (El Chaparral, Tolima) (Vallejo et al., 2002, 2003), two further groups composed of isolates from Brazilian Amazonia and Santa Catarina (southern Brazil) have been recently identified (Da Silva et al., 2004).

T. rangeli can lead to protection against *T. cruzi* infection in animal models (Basso et al., 2004; Palau et al., 2003). Therefore, besides its evolutionary and epidemiological interest, it is important to establish the molecular characteristics of the major groups of *T. rangeli* in order to distinguish them and determine if they have the same effect on *T. cruzi* infection. In an effort to contribute to the molecular characterization and differentiation of *T. rangeli* major groups, herein we report the molecular characterization of the genes encoding histone H2A protein from a *T. rangeli* KP1(+) strain (H14), its comparison to *T. rangeli* KP1(−) strain (C23) histone H2A coding genes (Puerta et al., 2000), and its application in a low-stringency single specific primer polymerase chain reaction (LSSP-PCR) assay to differentiate these parasite groups.

2. Materials and methods

2.1. Parasites

Epimastigotes from the *T. rangeli* KP1(+) strains: MHOM/Hond/H14 (Acosta et al., 1991), I/PRX/CO/84/P19 (Vallejo et al., 2002), and IRHO/CO/82/Ch (Schottelius, 1987), and *T. rangeli* KP1(−) strains: MHOM/CO/99/5048 (Morales et al., 2002), Tre (Morales et al., 2002), MAOT/CO/82/C23 (Zuñiga et al., 1997), and Trs (Coyaima, Tolima), were used in this study. They were characterized by using S35/S36/KP1L PCR (Vallejo et al., 2002).

Parasites were grown at 28 °C in modified REI medium supplemented with 2% (v/v) heat-inactivated fetal bovine serum. The *T. rangeli* H14 and Tre strains were grown at 28 °C in Tobie biphasic medium modified by M.C. Ruiz-Andreu (IPBLN-CSIC, Spain): liquid phase, DMEM medium supplemented with 10% (v/v) of heat-inactivated fetal bovine serum (SBFI) and solid phase 1.5 g bacto beef, 2.5 g bacto peptone, 4 g KCl, 7.5 g bacto agar, 5 g K_2HPO_4 in 500 ml of H_2O. *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.

2.2. PCR amplification, cloning, and nucleotide sequence

PCRs were performed using the Trangeli1 (5′-ATGG-CAACCCCGAAGCAGG-3′) and Trangeli2 (5′-CCCGACG-CACCGACACGAC-3′) primers which, based on the C23 strain H2A coding sequence, amplify exclusively both groups of *T. rangeli* in a final volume of 25 μl containing 100 ng of purified genomic DNA, 1× reaction buffer (10 mM Tris–HCl pH 9.0, 50 mM KCl, and 0.1% Triton X-100), 1.25 units of *Taq* DNA polymerase (Promega), 1.5 mM of $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, and 20 pmol of each primer. Reactions were carried out in a MJ Research PTC-100 DNA thermal cycler, using the following profile: 94 °C/5 min, 30 cycles of 95 °C/30 s, 62 °C/1 min and 72 °C/30 s, and a final incubation of 72 °C for 5 min. The amplified fragments were purified from agarose gel using GFX Gel Band Purification kit (Amersham Biosciences) and cloned into the pGEM®-T Easy plasmid (Promega).

Both strands of the cloned fragments were sequenced by the Sanger method (Sanger et al., 1977) in a 373 Automatic DNA sequencer (Pharmacia LKB), using universal and specific primers. Homology searches were performed in the GenBank and EMBL databases using the FASTA program (Pearson, 1990) and sequences were aligned using MULTALIGN (Corpet, 1988) and LALIGN programs (Pearson, 1990).

2.3. Southern analyses

A 1–4 μg of total DNA was digested with *Sph*I, *Bgl*I, and *Pvu*II restriction enzymes, resolved in 0.8% agarose gels and transferred to a nylon membrane (Biorad) by standard procedures (Sambrook and Russell, 2001). In order to detect the copy number of the H2A gene genomic DNA was also partially digested with the *Sph*I and *Bgl*I enzymes. The hybridization conditions were carried out using the methodology previously described by Puerta et al. (1994).

The probes were labeled by the random primer method using [α-^{32}P] dCTP (Feinberg and Vogelstein, 1983). The last wash of the filters was performed in 0.1× SSC/0.1% SDS at 65 °C for 1 h and the membrane was exposed to Curix RP2 medical X-ray film (AGFA).

2.4. Chromosomal blot analysis

For pulsed-field gel electrophoresis (PFGE) analysis, agarose blocks containing about 5×10^7 parasites were prepared as previously described (Puerta et al., 2000) and stored at 4 °C in 0.5 M EDTA, pH 9.5.

One-fifth of each block was electrophoresed in a Gene Navigator Pulsed Field System apparatus (Amersham Pharmacia Biotech), using 1% agarose gels and 0.5× TBE buffer (40 mM Tris, 45 mM boric acid, 1 mM EDTA pH 8.3) at 84 V for 96 h at 12 °C. Pulses of 250, 500, 750, and 1000 s were applied for a period of 24 h. The resolved chromosomes were transferred to a nylon filter by standard techniques (Sambrook and Russell, 2001) and hybridized with the insert of clone C1 labeled by the random primer method using [α-^{32}P] dCTP (Feinberg and Vogelstein, 1983) as a probe. The profile of the hybridization signals was analyzed using the Quantity One program, version 4.5 (Biorad). The resolved chromosomes were transferred to a nylon filter and hybridized with the insert of clone C1 as a probe. The profile of the hybridization signals was analyzed using the Quantity One program, version 4.5 (Biorad).

2.5. LSSP-PCR

For the histone H2A intergenic region amplification, 125 ng of genomic DNA were subjected to a specific PCR using the H14INTF (5′-TAGATACACCTGGGGGAACG-3′) and H14INTR (5′-TTGCTTGTTGCAGCTGTGAT-3′) primers. The reaction was performed in a final volume of 25 μl containing 1× reaction buffer (10 mM Tris–HCl pH 9.0, 50 mM KCl, and 0.1% Triton X-100), 1.25 units of *Taq* DNA polymerase (Promega), 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, and 20 pmol of each primer. The following profile was used: 95 °C/5min, 35 cycles of 95 °C/30 s, 65 °C/40 s and 72 °C/40 s, and a final extension at 72 °C for 5 min.

The PCR product was run on a 2.5% agarose gel and the gel stained with ethidium bromide. The amplified band was excised from the gel and purified using GFX Gel Band Purification kit (Amersham Biosciences). Gene signatures were produced by LSSP-PCR following the protocol described by Pena et al. (1994) with some modifications; 120 pmol of H14INTR primer were used per reaction in a final volume of 50 μl containing 200 μM of each dNTPs, 4.0 units of *Taq* polymerase (Promega), 10 mM Tris–HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.001% gelatin, and 2.5 μl of the first reaction product. The PCR profile was as follows: 94 °C/3 min, 35 cycles of 94 °C/45 s, 30 °C/45 s and 72 °C/45 s, and additional extension at 72 °C for 10 min. A 10 μl of the amplified products was electrophoresed in 10% polyacrylamide gels and stained with silver nitrate (Sambrook and Russell, 2001). The profiles of *T. rangeli* strains were analyzed using the Quantity One program, version 4.5 (Biorad).

3. Results

3.1. Amplification and cloning

In order to isolate the histone H2A coding genes from KP1(+) *T. rangeli* H14 strain, PCR was performed using genomic DNA of the mentioned parasite as template and Trangeli1/2 primers. Genomic DNA from IRHO/CO/82/Ch (Ch) strain was used as a KP1(+) control and genomic DNA from *T. cruzi* Munanta strain or a reaction without DNA template were used as negative controls. As it was expected, two fragments of approximately 147 and 935 nt were amplified in H14 and Ch strains (data not shown).

The H14 935 bp fragment was cloned into the pGEM®-T Easy plasmid, named C1 clone, and sequenced. Analysis of the clone C1 sequence (Accession no. AY147905) showed that it contains a complete ORF followed by an intergenic region of 391 bp, and by the 5′ end of a truncated ORF in frame (146 nt) (Fig. 1A). The sequences of both ORFs showed to be identical and to share an identity of 88% with *T. rangeli* C23 strain H2A gene (Puerta et al., 2000), confirming that a H14 histone H2A partial cluster was cloned. Thus, the H14 histone H2A gene is 799 nt in length, and is composed of a 5′ non-coding region of 148 bp, a 408 bp coding region, and a 3′ non-coding region of 243 bp. In the intergenic region, upstream from the start codon, there is a polypyrimidine track followed by five putative splicing acceptor AG dinucleotides. In addition, a potential hairpin was detected in the 3′ non-coding region (Fig. 1B) using the Palindrome application from EMBOSS. The G/C content of the coding region is 68%, and 47% for the non-coding region.

When the H14 histone H2A coding region was compared with the 0.76 and 1.2 kb units of *T. cruzi*, an identity of approximately 90% was found (Fig. 2). However, the *T. rangeli* H14 and *T. brucei* H2A coding region only showed 80% identity (Fig. 2). A similar analysis was carried out with the histone H2A intergenic region from H14 and C23 revealing an identity of 95% at nucleotide level (Fig. 3). Further comparisons between intergenic regions of the *T. rangeli* H14 and the 0.76 and 1.2 kb *T. cruzi* histone H2A units showed an identity of 58% and 69%, respectively (Fig. 3).

Sequence alignment of the H2A deduced amino acid sequence from H14 and C23 *T. rangeli* strains shows a 99.5% of identity at amino acid level. The deduced amino acid sequence of the genes contained in clone C1 corresponds to a protein of 135 amino acids that has a theoretical isoelectric point of 12.2 and a theoretical molecular weight equivalent of 14.4 kDa.

The histone H2A characteristic motifs, such as AGLXFPV, RSAK, and the histidine at position 123 (Puerta et al., 2000) are present. Moreover, the deduced amino acid sequences of H14 and C23 are identical. Nevertheless, differences were found in the codon usage among the *T. rangeli* groups, and *T. rangeli* and *T. cruzi*. For instance, the GTA triplet coding for valine was used only in the H14 sequence and the GAT (aspartic acid), CTT (leucine), and TAT (tyrosine) triplets were used in *T. cruzi* but not in *T. rangeli*. On the contrary, the CGC (arginine), TCT, and AGT (serine) codons were used in *T. rangeli* but not in *T. cruzi.*

3.2. Genomic organization

The presence of a hybridization band of similar size obtained after digestion with *Sph*I and *Bgl*I restriction endonucleases which cut once inside histone genes suggests the existence of several copies of the histone H2A gene organized in tandem arrays in *T. rangeli* H14 strain (Fig. 4A). Moreover, the hybridization pattern generated after digestion with *Pvu*II that cuts twice into H2A gene supports this genomic organization.

For estimating the copy number of histone H2A genes, the parasite' genomic DNA was partially digested with *Sph*I and *Bgl*I restriction endonucleases and Southern blot carried out using radiolabeled C1 clone as a probe.

Since 11 hybridization bands were observed, we can conclude that there are at least 11 copies of the histone H2A gene per haploid genome in *T. rangeli* H14 strain (Fig. 4B). Remarkably, pulsed-field gel electrophoresis (PFGE) analysis revealed that in the KP1(+) IRHO/CO/82/Ch and H14 strains the H2A genes are located on two chromosomes of 1.1 and 1.9 Mb, respectively, while in the Tre and 5048 KP1(−) strains are located in couple chromosomes of about 1.7 and 1.9 Mb (Fig. 4C). An additional hybridization band was observed at the compression region in all *T. rangeli* strains analyzed with the

```
     M   A   T   P   K   Q   V   S   K   K   A   S   R   K   R   S   G
1    ATG GCA ACC CCG AAG CAG GTA TCG AAG AAG GCG TCC AGG AAG CGC AGC GGC

     G   R   S   A   K   A   G   L   I   F   P   V   G   R   V   G   S
52   TCT GGC CGC GCG AAG GCC GGC TTG ATC TTC CCC GTG GGC CGC GTC GGC TCG

     L   L   R   R   G   Q   Y   A   R   R   V   G   A   S   G   A   V
103  CTG CTG CGC CGT GGC CAG TAC GCC CGT CGT GTC GGT GCG TCT GGC GCC GTG

     Y   M   A   A   V   L   E   Y   L   T   A   E   L   L   E   L   S
154  TAC ATG GCG GCG GTG CTG GAG TAC CTG ACA GCT GAG CTG CTG GAG CTC TCC

     V   K   A   A   S   Q   Q   A   K   R   P   K   R   L   T   P   R
205  GTG AAG GCG GCG AGC CAG CAG GCC AAG AGG CCC AAG CGC CTG ACA CCC CGC

     T   V   T   L   A   V   R   H   D   D   D   L   G   T   L   L   R
256  ACG GTG ACG CTC GCT GTG CGC CAC GAC GAC GAC CTC GGC ACC CTG CTG CGG

     N   V   T   L   S   R   G   G   V   M   P   S   L   N   K   A   L
307  AAC GTG ACC CTG TCG CGC GGT GGC GTG ATG CCG AGT CTG AAC AAG GCG CTG

     A   K   K   H   K   S   S   K   K   A   R   A   T   P   S   A   *
358  GCG AAG AAG CAC AAG AGC AGC AAG AAG GCG CGG GCG ACG CCC AGC GCT TAG

409  aggggccatg aagcccagcg cagagaggaa cgcgactcgc tagatacacc tgggggaacg

469  ccattgccca gccacggtga ctcacgtgtg gccccataga atagtgtgtt tgtttttatt

529  tatttatttg tgttggcgtg gcttctccca atttctcctc tcgcctcttc cacgggaatg

589  cgcaacgtcc ttgtttttttt tttggatttt tgaaacattt tcgccggcac ctggcggcgg

649  cgtgggtgct gcgtctgctt tctgtggctg catgcgtgct ttctcctttt ttctttttttt

709  tgtttccttc ttgtattgtt ttctttgttt ttcaagatca cagctgcaac aagcaaacaa

                                      M   A   T   P   K   Q   V   S   K
769  gccacttttt tcagtttttg catttcaaca ATG GCA ACC CCG AAG CAG GTG TCG AAG

     K   A   S   R   K   R   S   G   G   R   S   A   K   A   G   L   I
826  AAG GCG TCC AGG AAG CGC AGC GGC GGC CGC TCT GCG AAG GCC GGC TTG ATC

     F   P   V   G   R   V   G   S   L   L   R   R   G   Q   Y   A   R
877  TTC CCC GTG GGC CGC GTC GGC TCG CTG CTG CGC CGT GGC CAG TAC GCC CGT

     R   V   G   A   S
928  CGT GTC GGT GCG TCG GG
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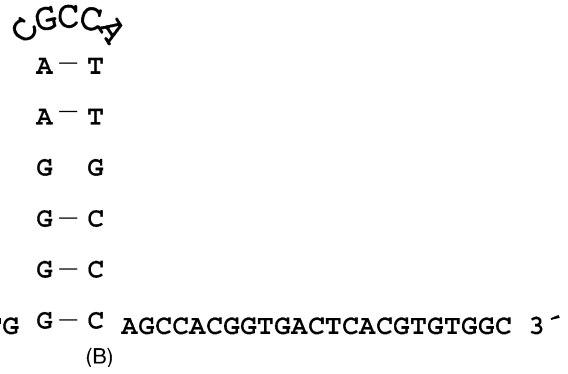
(A)



(B)

Fig. 1. (A) Nucleotide sequence and deduced amino acid sequence of the *Trypanosoma rangeli* C1 clone. Numbers to the left of the sequence indicate the nucleotide and amino acid position. The nucleotides corresponding to the coding regions are in capital letters. The ATG codons are boxed, and the asterisk indicates the TAG stop codon. Rhombi indicate the putative mini-exon addition sites, and the polypyrimidine track is underlined. The position of the putative stem loop is double underlined. (B). Secondary structure of the potential hairpin loop detected in the 3′ non-coding region of the histone H2A gene from *T. rangeli*.

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H14      ATGGCAACCCCGAAGCAGGTATCGAAGAAGGCGTCCAGGAAGCGCAGCGGCGGCCGCTCTG
C23      ATGGCAACCCCGAAGCAGGTGTCGAAGAAGGCGTCCAGGAAGCGCAGCGGCGGCCGCTCTG
Tc1200   ATGGCAACCCCGAAGCAGGCAGCGAAGAAGGCGTCCAAGAAGCGCAGCGGCGGCCGCTCCG
Tc760    ATGGCAACCCCGAAGCAGGCAGCGAAGAAGGCGTCCAAGAAGCGCAGCGGCGGCCGCTCCG
Tb       ATGGCAACACCCAAGCAGGCAGTGAAGAAGGCATC---GAAGGGCGGGAGCAGCCGCTCTG
         ******** ** *******    ********* **   **** ** *  ** ******* *

H14      CGAAGGCCGGCTTGATCTTCCCCGTGGGCCGCGTCGGCTCGCTGCTGCGCCGTGGCCAGTA
C23      CGAAGGCCGGCTTGATCTTCCCCGTGGGCCGCGTCGGCTCGCTGCTGCGCCGTGGCCAGTA
Tc1200   CGAAGGCCGGTCTGATCTTCCCCGTGGGCCGTGTGGGCTCGCTGCTGCGCCGCGGCCAGTA
Tc760    CGAAGGCCGGTCTGATCTTCCCCGTGGGCCGTGTGGGCTCGCTGCTGCGCCGCGGCCAGTA
Tb       TGAAGGCGGGGTTGATCTTCCCTGTGGGTCGCGTTGGTACGCTGCTGCGCCGCGGACAGTA
          ****** **  ********** ***** ** ** **  ************* ** *****

H14      CGCCCGTCGTGTCGGTGCGTCTGGCGCCGTGTACATGGCGGCGGTGCTGGAGTACCTGACA
C23      CGCCCGTCGTGTCGGTGCGTCGGGCGCCGTGTACATGGCGGCGGTGCTGGAGTACCTGACA
Tc1200   TGCCCGCCGCATCGGCGCGTCGGGCGCTGTGTACATGGCGGCGGTGCTGGAGTACCTGACA
Tc760    TGCCCGCCGCATCGGCGCGTCGGGCGCTGTGTACATGGCGGCGGTGCTGGAGTACTTGACA
Tb       TGCCCGCCGCATCGGTGCTTCTGGCGCTGTGTACATGGCGGCTGTGCTGGAGTACTTGACT
          ***** **  **** ** ** ***** ************** ************ ****

H14      GCTGAGCTGCTGGAGCTCTCCGTGAAGGCGGCGAGCCAGCAGGCCAAGAGGCCCAAGCGCC
C23      GCTGAGCTGCTGGAGCTCTCCGTGAAGGCGGCGAGCCAGCAGGCCAAGAGGCCCAAGCGCC
Tc1200   GCCGAGCTGCTGGAGCTGTCCGTGAAGGCGGCGAGCCAGCAGGCAAAGAAGCCGAAGCGCC
Tc760    GCCGAGCTGCTGGAGCTGTCCGTGAAGGCGGCGAGCCAGCAGGCAAAGAAGCCGAAGCGCC
Tb       GCCGAACTGCTGGAGCTATCCGTGAAGGCTGCTGCCCAACAGACGAAGAAGACGAAGCGCT
         ** ** ********** ********** **   *** *** * **** * * ******

H14      TGACACCCCGCACGGTGACGCTCGCTGTGCGCCACGACGACGACCTCGGCACCCTGCTGCG
C23      TGACACCCCGCACGGTGACGCTCGCTGTGCGCCACGACGACGACCTCGGCACCCTGCTGCG
Tc1200   TGACACCCCGCACGGTGACGCTTGCCGTGCGCCACGACGACGACCTCGGCATGCTCCTGAA
Tc760    TGACACCCCGCACGGTGACACTTGCCGTGCGCCACGACGACGACCTCGGCATGCTCCTGAA
Tb       TGACGCCACGCACAGTAACCCTTGCTGTACGCCACGACGACGACCTTGGTGCGTTGCTGCG
         **** ** ***** ** ** ** ** ** ***************** **     * ***

H14      GAACGTGACCCTGTCGCGCGGTGGCGTGATGCCGAGTCTGAACAAGGCGCTGGCGAAGAAG
C23      GAACGTGACCCTGTCGCGCGGTGGCGTGATGCCGAGTCTGAACAAGGCGCTGGCGAAGAAG
Tc1200   GGATGTGACGCTGTCGCGTGGTGGTGTGATGCCGAGCCTGAACAAGGCGCTGGCGAAGAAG
Tc760    GGATGTGACGCTGTCGCGTGGTGGTGTGATGCCGAGCCTGAACAAGGCGCTGGCGAAGAAG
Tb       CAACGTGACCATGTCCCGCGGAGGTGTGATGCCGAGCCTCAACAAAGCTCTGGCGAAGAAG
           * *****  **** ** ** ** ********** ** ***** ** ************

H14      CACAAGAGCAGCAAGAAGGCGCGGGCGACGCCCAGCGCTTAG
C23      CACAAGAGCAGCAAGAAGGCGCGGGCGACGCCCAGCGCTTAG
Tc1200   CACAAGAGCAGCAAGAAGGCGAGGGCGACGCCCAGCGCTTAG
Tc760    CACAAGAGCAGCAAGAAGGCGAGGGCGACACCCAGCGCTTAG
Tb       CAGAAGAGCGGAAAGCACGCGAAGGCGACGCCAAGCGTCTAG
         ** ****** * *** * ***  ****** ** ****  ***
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Fig. 2. Multiple alignment of the H2A histone nucleotide sequence from *T. rangeli* H14 strain, Accession no. AY147905 (H14); *T. rangeli* C23 strain, Accession no. AF169130 (C23); *T. cruzi* Y strain 1.2 kb unit Accession no. X67287 (Tc1200); *T. cruzi* Y strain 0.76 kb unit, Accession no. X83272 (Tc760), and *T. brucei* core histone H2A, temporary systematic id 185.t00098 (Tb). Stars indicate the positions that have identical nucleotides in all sequences.

exception of the *T. cruzi* Shubacbarina and Munanta strains where a single hybridization band of 0.6 Mb was observed.

3.3. LSSP-PCR

Based on the differences found between the histone H2A intergenic regions from *T. rangeli* KP1(+) H14 and KP1(−) C23 strains, we carried out a LSSP-PCR assay seeking for differences between *T. rangeli* KP1(+) and KP1(−) strains. Using genomic DNA from several *T. rangeli* strains and the H14INTF/R primers, one fragment of approximately 300 bp in all KP1(+) and KP1(−) analyzed strains was PCR amplified (figure not shown). However, as shown in Fig. 5, the pattern of bands generated after LSSP-PCR was clearly different in KP1(−) and in KP1(+) strains. Thus, two specific bands of 1300 and 400 pb were amplified with the Trs, Tre, and 5048 KP1(−) strains (bands a and b in Fig. 5), whereas in the H14, IRHO/CO/82/Ch, and P19 KP1(+) strains three specific fragments of 460, 1440, and 3400 pb were amplified (bands c–e in Fig. 5).

As expected, the *T. rangeli* C23 strains show a similar LSSP-PCR pattern than that observed for the KP1(−) strains (laboratory data).

4. Discussion

Histone H2A is an evolutionary conserved protein which presents different genomic organization among related species (Maxson et al., 1983). In *T. cruzi* some polymorphism between *T. cruzi* I and *T. cruzi* II strains has been described. These differences were observed in the histone H2A copy number, in its chromosomal location and in the level of

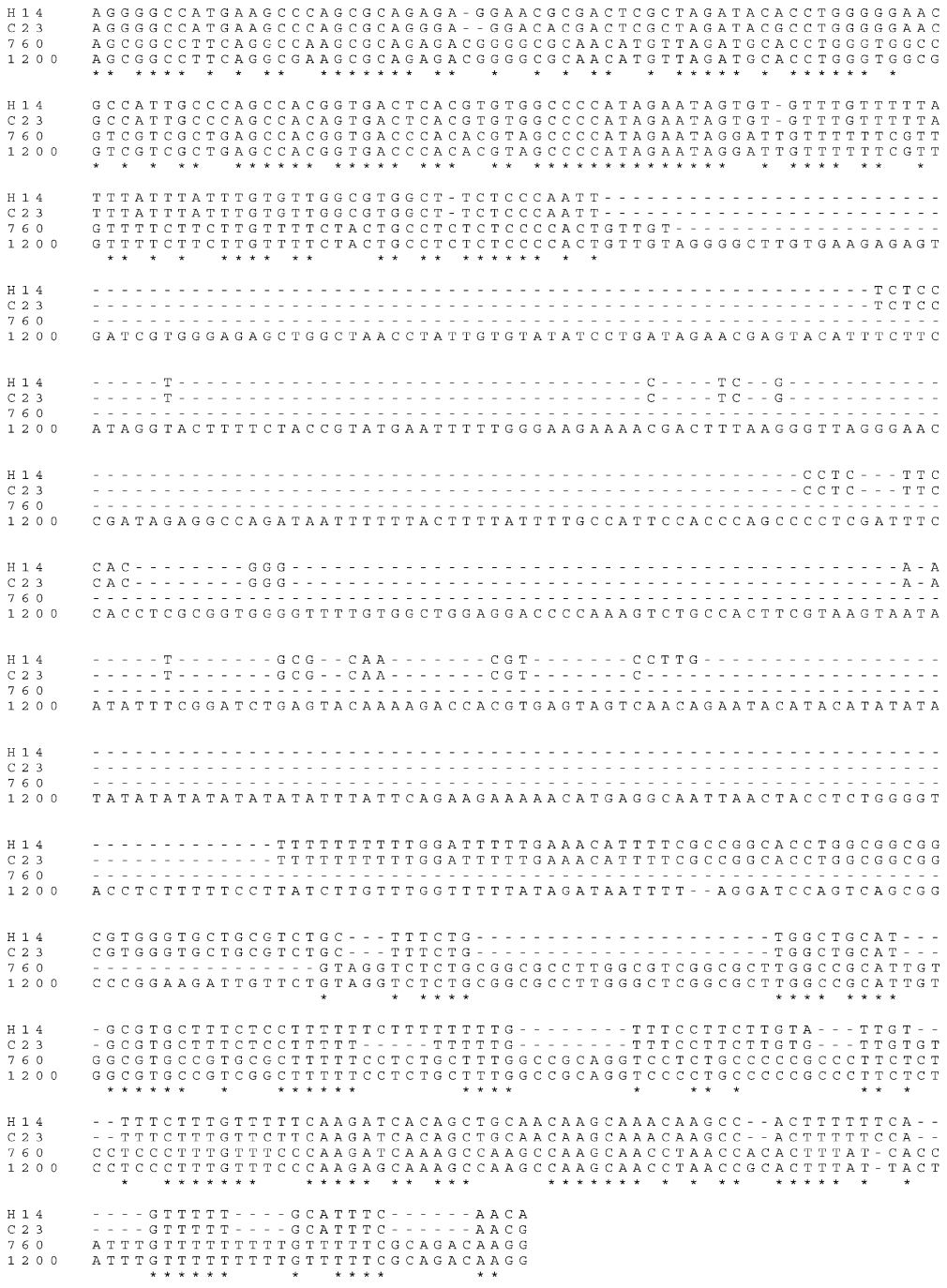

Fig. 3. Multiple alignment of the H2A histone intergenic regions from *T. rangeli* H14 strain, Accession no. AY147905 (H14), *T. rangeli* C23 strain, Accession no. AF169130 (C23); *T. cruzi* Y strain 1.2 kb unit Accession no. X67287 (Tc1200); *T. cruzi* Y strain 0.76 kb unit, Accession no. X83272 (Tc760). Stars indicate the positions that have identical nucleotides in all sequences.

expression of H2A mRNAs (Thomas et al., 2000). We have reported here the molecular characterization of the histone H2A gene from the KP1(+) *T. rangeli* H14 strain and analyzed specific divergences regarding *T. rangeli* KP1(−) type, in an effort to contribute to the molecular differentiation of the KP1(+) parasites versus KP1(−). We observed that although the *T. rangeli* H14 KP1(+) strain does not present significant differences neither in the genomic organization of the histone H2A genes nor in the H2A coding sequence relative to those previously reported for *T. rangeli* C23 KP1(−) strain (Puerta et al., 2000), there is a clear difference in their chromosomal location. Interestingly, these differences are extensive to other KP1(−) and KP1(+) strains. The loci containing the H2A genes are in two chromosomes of approximately 1.9 and 1.7 Mb in size for the Tre and 5048 KP1(−) strains. However, in the IRHO/CO/82/Ch and H14 KP1(+) strains the clusters for H2A genes are located in two chromosomes of 1.9 and 1.1 Mb. Thus, the chromosomal localization of histone H2A allows to distinguish between KP1(+) and KP1(−) *T. rangeli* strains.

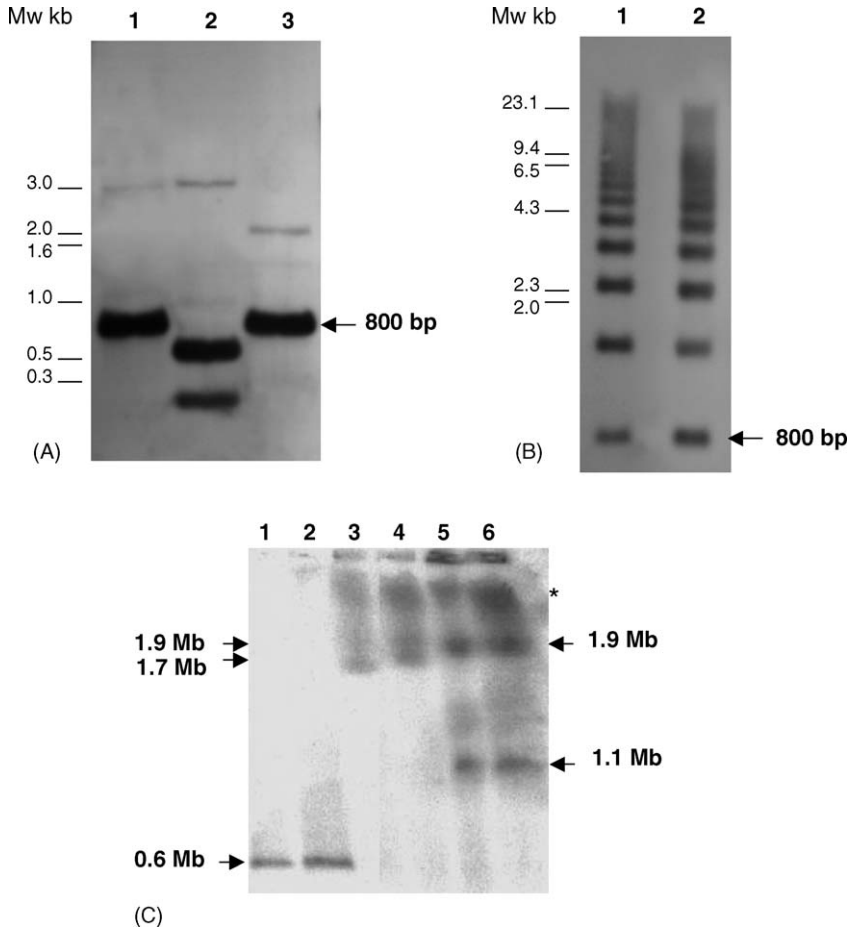

Fig. 4. Genomic organization and copy number of the histone H2A genes from *T. rangeli* H14 KP1(+) strain. (Panel A) Southern blot of *T. rangeli* genomic DNA digested with *Bgl*I, *Pvu*II, and *Sph*I restriction endonucleases and probed with the [α-^{32}P] dCTP labeled C1 clone. The numbers on the left side correspond to the *Hin*dIII-digested λ phage DNA used as molecular weight marker. (Panel B) Southern blot of *T. rangeli* genomic DNA partially digested with *Sph*I and *Bgl*I restriction enzymes and probed with the [α-^{32}P] dCTP labeled C1 clone. (Panel C) Pulsed-field gel electrophoresis of chromosomes from *T. cruzi* Shubacbarina (lane 1) and Munanta (lane 2) strains and from *T. rangeli* Tre (lane 3), 5048 (lane 4), IRHO/CO/82/Ch (lane 5), and H14 (lane 6) strains. After blotting, the PFGE-separated chromosomes were hybridized with the α-^{32}P-radiolabeled clone 1 sequence. The size of the hybridization bands, in Mb, is indicated at the left and right of the panel.

This finding of karyotype variability between *T. rangeli* KP1(+) and KP1(−) strains was also observed for the KMP-11 genes (Urueña et al., 2004; Diez et al., 2005). Moreover, a polymorphism has been reported in the chromosomal localization of β-tubulin genes in *T. rangeli* strains of different geographical origin (Toaldo et al., 2001). The existence of two hybridization bands of different sizes in the PFGE after being hybridized with the C1 clone as a probe suggests the existence of homologous chromosomes of different sizes, a frequent feature in trypanosomatids (Thomas et al., 2000).

Comparison of the nucleotide sequence of H2A coding genes from H14 and C23 strains revealed an identity of 88%, which increases up to 99.5% when deduced amino acids sequences are considered. Thus, most of the changes are located on the codon third position, revealing for each mentioned *T. rangeli* strain a codon usage preference.

Since we found that the intergenic region of the H2A genes in H14 and C23 strains presented a 5% of sequence divergence, we developed a LSSP-PCR test looking for differential patterns which could allow to distinguish *T. rangeli* KP1(+) from KP1(−) strains. It has been reported that sequences that differ in a single base can be distinguished by the LSSP-PCR assay (Barreto et al., 1996).

Moreover, since in *T. cruzi* a similar percentage of identity exists between the intergenic regions of the histone H2A genes from parasites of *T. cruzi* I and *T. cruzi* II (laboratory data), we estimated that the percentage of deletions and/or insertions present in *T. rangeli* H2A intergenic region would be enough for this purpose.

The analysis of the results obtained in the LSSP-PCR assays allows to differentiate between KP1(+) and KP1(−) *T. rangeli* subpopulations. Thus, the intergenic region of histone H2A constitutes an additional genetic marker besides kDNA (Vallejo et al., 2002), non-transcribed spacer (Da Silva et al., 2004), and mini-exon gene (Grisard et al., 1999b), which can allow to differentiate KP1(+) and KP1(−) *T. rangeli* strains. In addition, these results extend the dimorphism between KP1(−) and KP1(+) strains to nuclear genes, and reinforce either clonal evolution or speciation postulated for *T. rangeli* strains (Vallejo et al., 2003).

Considering the discussion about the taxonomic position of *T. rangeli*, Salivaria or Stercoraria section, it is important

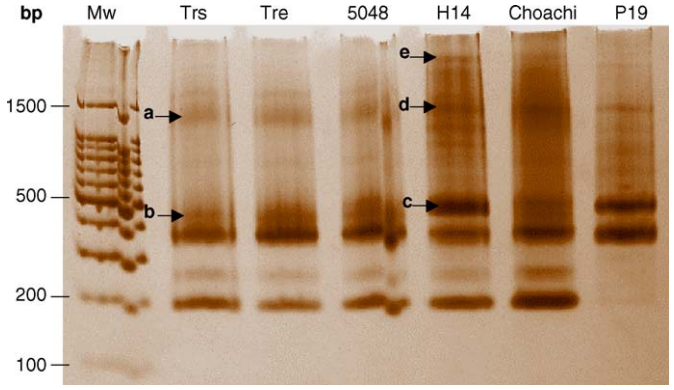

Fig. 5. LSSP-PCR amplified products from epimastigotes of several *T. rangeli* strains were separated in a 10% polyacrylamide gel and silver-stained. Each lane contains 10 μl of the PCR products. At the top of each lane the *T. rangeli* strains assayed are indicated; 100 bp ladder (Invitrogen) was used as molecular weight marker (MW) and the sizes of some fragments are indicated on the left hand side. Arrows (a and b) indicate the specific bands observed by the assays KP1(−) strains, bands of 1300 and 400 bp, respectively. Arrows c–e indicate the specific bands observed in the KP1(+) strains, size of 460, 1440, and 3400 bp, respectively. This figure shows one representative result out of three independent experiments.

to note that the percentage of identity of the histone H2A genes of *T. rangeli* is higher with *T. cruzi* than with *T. brucei*. Similar results were observed for genes encoding KMP-11, the kinetoplastid membrane protein 11 (Diez et al., 2005) and TrCaBP, a putative calcium binding protein (Porcel et al., 1996). These facts support a closer relationship between *T. rangeli* and *T. cruzi* than between *T. rangeli* and *T. brucei*.

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