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SCIENCE DIRECT
Experimental Parasitology 112 (2006) 126–129
Experimental Parasitology
www.elsevier.com/locate/yexpr

Research brief

Leishmania (*Viannia*) *panamensis*: Cloning of the histone H1 genes by representational difference analysis

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Received 3 March 2005; received in revised form 22 September 2005; accepted 24 September 2005
Available online 9 November 2005

Abstract

We report the use of representational difference analysis to identify genes that have up-regulated expression in the amastigote life-cycle stage of *Leishmania* (*Viannia*) *panamensis.* This simultaneous process of selection and amplification allowed the cloning of several specific DNA fragments. One of them shows a high percentage of similarity with histone H1 genes from other Trypanosomatids and, as expected, is up-regulated in the amastigote life-cycle stage. The gene is present in two copies that are expressed at different levels in promastigotes and also in amastigotes, which seems to be a consequence of their different 3′ untranslated regions.

Index Descriptors and Abbreviations: Trypanosomatidae; *Leishmania* (*Viannia*) *panamensis*; Subtractive PCR; Differential expression; Histone H1; UTR, untranslated region; kDa, kilodalton; cDNA, complementary DNA; RDA, representational difference analysis; SSH, suppression subtractive hybridization

Parasites of the genus *Leishmania* are the causative agents of a wide spectrum of diseases that affect more than 80 countries throughout the world (Ashford et al., 1992). The *Leishmania* (*Viannia*) subgenus is restricted to Central and South America and produces the American Cutaneous Leishmaniasis (ACL). Some species such as *L. panamensis* and *L. braziliensis*, can cause the disfiguring mucocutaneous form of the disease. Whereas Old World *Leishmania* species have been extensively characterized and described at the molecular level, very few molecules from New World *Leishmania* species have been reported in the data bases. *Leishmania* parasites cycle between two morphological forms: the promastigotes in the invertebrate host and the amastigotes in mammalian tissues. Amastigotes are taken up by sand flies, where they transform into promastigotes. Promastigotes replicate and differentiate to infective metacyclic forms in the invertebrate host and are subsequently injected to the mammalian host. Metacyclic forms are then phagocytosed by macrophages where they rapidly transform into amastigotes. This process has been mainly studied in Old World *Leishmania* species, for which proteomic and genomic approaches permit rapid characterization of the changes in protein expression. In contrast, the lack of genomic sequencing data for the New World *Leishmania* subgenus makes the application of the new technologies in these species difficult. This stresses the importance of the use of other methodologies capable of isolating differentially expressed genes without the need of genomic sequence information.

Representational difference analysis (RDA) has been designed to specifically amplify cDNAs that are preferentially expressed in a cell population (Hubank and Schatz, 1994). cDNA fragments from the tester population (amastigotes derived from infected murine J774

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0014-4894/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2005.09.009

macrophages) that are also found in the driver population (exponentially growing promastigotes) are excluded from the amplification process as a consequence of crosshybridization. Briefly, specific adaptors were ligated to the cDNAs from the amastigote population in the flanking *Bgl*II sites: 1 μg of purified tester cDNA *Bgl*II-digested was incubated with 0.5 nmol of primers 24–1 and 12–1 (See below) and 0.5 U of ligase at 16 °C for 2 h. Then 0.5 μg of the amastigote cDNA was mixed with 40 μg of the cDNAs derived from promastigotes, which had previously been ethanol precipitated, dried and re-suspended in 4 μL of 3× EE buffer (30 mM EPPS (Sigma), pH 8.0; 3 mM EDTA). The sample was covered with 30 μL of mineral oil and denatured for 15 min at 95 °C then 1 μL of 5 M NaCl was added and the mixture was allowed to re-anneal for 20 h at 67 °C. One microliter of the hybridization product was diluted in 200 μL of PCR mix without primer (0.2 mM dNTPs, 2 mM $MgCl_2$) with 15 U of *Taq* polymerase and incubated at 72 °C for 5 min (Filling step). Then 0.5 μL of the 100 μM specific adapter/oligo stock were added and the first 10 cycles of the PCR were run (1 min 95 °C, 90 s 72 °C). Forty microliter of this PCR product were digested with 20 U of mung-bean nuclease at 37 °C for 30 min. Subsequently, mung-bean nuclease was heat inactivated at 95 °C and the subtracted material was subjected to 20 further PCR cycles at the same conditions described above. The subtracted cDNA was digested again with *Bgl*II and new adapters were ligated. The procedure was repeated three times.

Under these conditions, several DNA fragments that were expected to be preferentially expressed in amastigotes were amplified. Northern blot analyses revealed that one of the fragments, named GDE2, hybridizes to a single 1100 nucleotide mRNA that shows a 2-fold increase in its steady state levels during transition from promastigotes

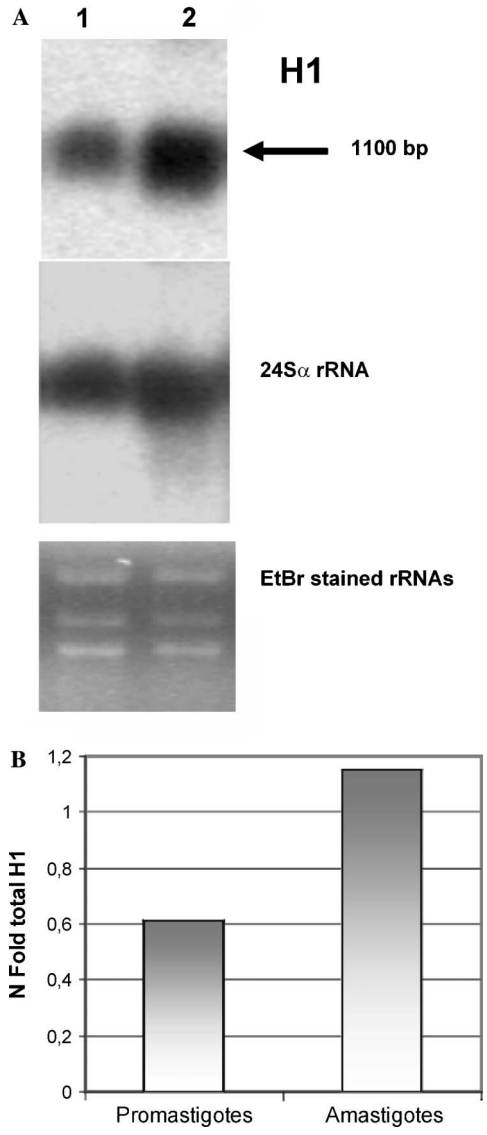

Fig. 1. Northern blot analysis of clone GDE2. (A) Total RNA from *Leishmania* (*Viannia*) *panamensis* promastigotes (1) and axenic amastigotes (2) hybridized with the insert of clone GDE2 (top panel) or a 24Sα rRNA probe (middle panel). The size of the H1 mRNA is indicated. rRNA bands stained with ethidium bromide are shown in the lower panel. (B) Ratio between the intensities of the bands obtained for H1 and 24Sα rRNA probes when hybridized with total RNA from promastigotes and amastigotes. Results are representative, in all cases, of four independent experiments.

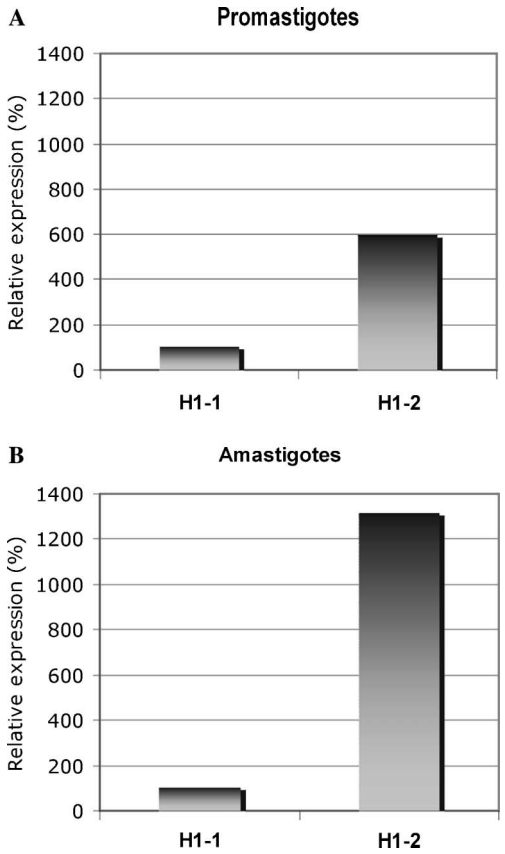

Fig. 2. Quantitative real-time PCR analysis of the relative expression of both gene copies. (A) Relative expression of the large (H1-1) and the short (H1-2) gene copies in promastigotes. H1-1 gene expression was given an arbitrary value of 100% expression. (B) Relative expression of the large (H1-1) and the short (H1-2) gene copies in amastigotes derived from infected murine J774 macrophages. H1-1 gene expression was given an arbitrary value of 100% expression.