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Analysis of the *Leishmania donovani* transcriptome reveals an ordered progression of transient and permanent changes in gene expression during differentiation

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Abstract

Leishmania donovani is an intracellular protozoan parasite that causes kala-azar in humans. During infection the extracellular insect forms (promastigotes) undergo rapid differentiation to intracellular amastigotes that proliferates in phagolysosomes of mammalian macrophages. We used microarray-based expression profiling to investigate the time-course of changes in RNA abundance during promastigote-to-amastigote differentiation in a host-free system that mimics this process. These studies revealed that several hundred genes underwent an ordered progression of transient or permanent up- and down-regulation during differentiation. Genes that were permanently up-regulated in amastigotes were enriched for transporters and surface proteins, but under-represented in genes involved in protein and other metabolism. Most of these changes occurred late in the differentiation process, when morphological differentiation was essentially complete. Down-regulated genes were over-represented in those involved in cell motility, growth and/or maintenance, and these changes generally occurred earlier in the process. Genes that were transiently up- or down-regulated during differentiation included those encoding heat shock proteins, ubiquitin hydrolases, RNA binding proteins, protein kinases, a protein phosphatase, and a histone deacetylase. These results suggest that changes in mRNA abundance may be important in signal transduction, as well as protein and mRNA turnover, during differentiation. In addition to these mRNA changes, other transcripts including one or more rRNAs and snoRNAs, and non-coding RNAs from several telomeres, also showed substantial changes in abundance during the differentiation process. This paper provides the first genome-scale quantitative analysis of gene expression during the transition from promastigotes to amastigotes and demonstrates the utility of the host-free differentiation system.

Keywords: *L. donovani*; Differentiation; Axenic amastigotes; Gene expression; Transcript abundance

1. Introduction

Intracellular parasitism is a process by which microorganisms cycle between vectors (that transmit the parasites) and hosts (to whom they are pathogenic). As a result, the parasites encounter extreme environmental changes during their lifecycle, to which they respond by differentiating into highly adapted forms that enable them to invade and proliferate inside their hosts. *Leishmania donovani*, the causative agent of visceral leishmaniasis (known as kala-azar in humans), is a parasitic protozoan that cycles between the alimentary tract of sand flies and mammalian macrophages. In the insect vector, the parasites grow as extracellular flagellated promastigotes, which differentiate into intracellular aflagellate amastigotes upon entering the phagolysosome of the host macrophages [1,2]. The amastigotes are adapted to grow and proliferate in the hydrolytic environment inside phagolysosomes [3,4].

Abbreviations: LdoS, *L. donovani* MHOM/SD/00/1SR; PGC, polycistronic gene cluster; GSS, genome survey sequence; THR, telomere hexamer repeat; TAS, telomere associated sequence; PSA2, promastigotes surface antigen; HSP83, heat shock protein 83

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Promastigote-to-amastigote differentiation is a complex process that is accompanied by a number of morphological and biochemical changes. Parasites change shape from elongated to spherical and lose most of their flagellum. They undergo a major shift in metabolism, especially in the rate and pH optima for several processes, including DNA synthesis [5] and nutrient uptake [6]. A number of amastigote-specific genes have been identified, including a 3′-nucleotidase [7], the *A2* gene family [8,9], *HSP100* [10], and a MAP kinase, *LMPMK* [11]. In addition, certain members of the *GP63* and *PSA-2* gene families are differentially expressed in amastigotes, and there are differences in the GPI anchor of the latter [12]. In contrast, some processes are down-regulated in amastigotes [13,14], most notably lipophosphglycan (LPG) biosynthesis, resulting in its replacement by glycoinositol phosholipid (GIPL) as the major component of the parasite surface coat [13].

The differentiation process can be mimicked in axenic culture by shifting promastigotes from an insect-like (26 °C, pH 7) to an intralysosomal-like (37 °C, pH 5.5 and 5% CO_2) environment [15–19]. These axenic amastigotes resemble animal-derived amastigotes and have been widely used for investigating parasites activities without the complication of host cell material [20–23]. Time-course analysis of *L. donovani* differentiation showed that promastigotes expressed the amastigote-specific A2 protein family within an hour of exposure to the intralysosomal environment and at 5 h they start to transform to amastigote-shaped cells [19]. This morphological transformation occurs synchronously, while the cells are arrested at the Gl stage of the cell cycle, and is complete within 24 h. Differentiation proceeds for an additional 2 or 3 days until the parasites complete their shedding of LPG and begin expressing amastigote-specific activities [15,24]. Little is known about the molecular processes that mediate promastigote-to-amastigote differentiation, but it is likely that exposure to the higher temperature and lower pH of the intralysosomal environment initiates a series of changes in gene expression that lead to the morphological changes associated with amastigotes.

Regulation of gene expression in *Leishmania* is unusual because their protein-coding genes are transcribed as polycistronic RNAs with tens-to-hundreds of adjacent genes on the same DNA strand [25–29]. Mature mRNAs are subsequently obtained from coordinated polyadenylation and *trans*-splicing, which adds a 39-nt spliced leader (SL) sequence to the 5′ end of all mRNAs [30,31]. As a consequence of this unusual gene organization, *Leishmania* gene expression appears to not be regulated at the level of transcription [32], but stage-specific expression of a number of genes has been shown to be regulated *via* mRNA stability [8,33–38].

The goal of the present study was to use DNA microarray technology to investigate the possible role of changes in RNA abundance during promastigote-to-amastigote differentiation of *L. donovani*. Microarray expression profiling has been previously used to compare procyclics, metacyclics and amastigotes of *L. major* [39–41], *L. donovani* [42], *L. infantum* [43], and *L. mexicana* [44], but none of these studies examined changes in gene expression during the process of differentiation. The results of the present study indicate that there is an ordered progression of specific changes in gene expression during *L. donovani* promastigote-to-amastigote differentiation, with some genes changing expression within 5 h after exposure to the differentiation signal, and others changing only after 24 h. We also find that a significant number of genes are transiently up- or down-regulated between 5 and 24 h; an unexpected behavior given the unusual gene organization of the *Leishmania* genome. Interestingly, we also observed large changes in snoRNA abundance and telomeric transcripts during differentiation. These results suggest that changes in RNA abundance are important during differentiation, and raise the possibility that mechanisms other than changes in mRNA stability play a role in this process.

2. Materials and methods

2.1. Leishmania strain and growth conditions

A cloned line of *L. donovani* MHOM/SD/00/1SR (LdoS) was used in all experiments [15]. This cell-line was maintained as a clone by inoculating single colonies of promastigotes from medium 199 agar plates. Promastigotes were grown in medium 199 and supplemented with 10% fetal calf serum at 26 °C. Promastigote-to-amastigote differentiation in a host-free culture and the maintenance of axenic amastigotes were performed by inoculating late-log phase promastigotes in medium 199 at pH 5.5 containing 25% fetal calf serum and incubating them at 37 °C in 5% CO_2 environment [19]. Axenic amastigotes were routinely sub-cultured for 10 weeks before differentiation back to promastigotes, by changing the growth medium. For microarray analysis, RNA was harvested at 0 (promastigotes), 5, 10, 24 h during differentiation and from fully-formed (2 weeks) axenic amastigotes using Trizol reagent (Gibco BRL).

2.2. Preparation of labeled cDNA and microarray hybridization

Fluorescently labeled cDNA was prepared and hybridized to microarrays as previously described [39]. Multiple replicates of all hybridizations were performed to account for sample heterogeneity and possible variation due to hybridization and reciprocal labeling experiments were also carried out to identify dye-bias. The amastigote *versus* promastigote comparison was repeated nine times with Cy5-labeled amastigote RNA and Cy3-labeled promastigote RNA, and six times with reciprocal labeling; the 5 and 24 h *versus* promastigote comparisons were each carried out six times in the Cy5 *versus* Cy3 direction and four in the reciprocal; and the 10 h *versus* promastigote comparison was repeated three times in the Cy5 *versus* Cy3 direction. At least two different RNA samples were used for all comparisons except for 10 h *versus* promastigote. Slides were scanned using a GENEPIX Pro 4000 scanner (Axon Instruments) and the data was extracted and initially analyzed using the software supplied with the scanner. Local background was subtracted from the intensity value of each spot on the array and the data was exported as EXCEL files for further statistical analysis.