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T7 polymerase-driven transcription is downregulated in metacyclic promastigotes and amastigotes of *Leishmania mexicana*

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Abstract: In our previous work we established a T7 polymerase-driven Tetracycline-inducible protein expression system in *Leishmania mexicana* (Biagi, 1953). We used this system to analyse gene expression profiles during development of *L. mexicana* in procyclic and metacyclic promastigotes and amastigotes. The transcription of the gene of interest and the T7 polymerase genes was significantly reduced upon cell differentiation. This regulation is not locus-specific. It depends on untranslated regions flanking open reading frames of the genes analysed. In this paper, we report that the previously established conventional inducible protein expression system may not be suitable for studies on differentiation of species of *Leishmania* Ross, 1903 and protein expression systems might have certain limitations.

Keywords: gene expression, untranslated regions, Tet-inducible system, *Leishmania mexicana*

Leishmania mexicana (Biagi, 1953) is a flagellated protist of the family Trypanosomatidae causing human cutaneous leishmaniosis, a disease that represents a public health risk in many tropical and subtropical countries (Magill 1995). The genome sequence of this medically important parasite is available (Rogers et al. 2011), but our better understanding of its biology, as well as the evaluation of suitable drug targets still critically depends on functional analysis of proteins of *L. mexicana* (see Myler 2008). Recently, we have established a T7 polymerase-driven, tetracycline (Tet)-inducible expression system in *L. mexicana* that was considered suitable for such analyses (Kraeva et al. 2014). Importantly, the application of Tet in trypanosomatid flagellates is not associated with deleterious effects, as is the case in most other eukaryotes (Hashimi et al. 2016).

Leishmania Ross, 1903 and related trypanosomatid parasites utilise an unusual mechanism of gene expression, as a varying number of tandemly arranged genes is transcribed into a single polycistronic precursor (Myler 2008). Subsequently, individual mRNAs are cleaved from this precursor and further processed by *trans*-splicing at the 5' end and polyadenylation at the 3' end of each mRNA molecule (Campbell et al. 2003). Due to the polycistronic transcription and the lack of conventional RNA polymerase II promoters, these protists rely on regulating their gene expression post-transcriptionally (Fernandez-Moya and Estevez 2010, Requena 2011). For example, 3' untranslated regions (UTRs) of the protein-coding genes are implicated in regulation at the level of mRNA stability and/or translation (McNicoll et al. 2005, Haile et al. 2008).

Gene expression in dixenous (= two hosts) trypanosomatids must be tightly regulated to allow parasites' fast adaptation to the drastically different environmental conditions they encounter in the vertebrate and invertebrate hosts (Lukeš et al. 2014). The life cycle of *Leishmania* spp. consists of three main developmental stages: extracellular procyclic and metacyclic promastigotes colonising intestinal tract of the female sandflies of the genera *Phlebotomus* Loew or *Lutzomyia* França, and intracellular amastigotes multiplying in the phagolysosomes of mammalian mac-

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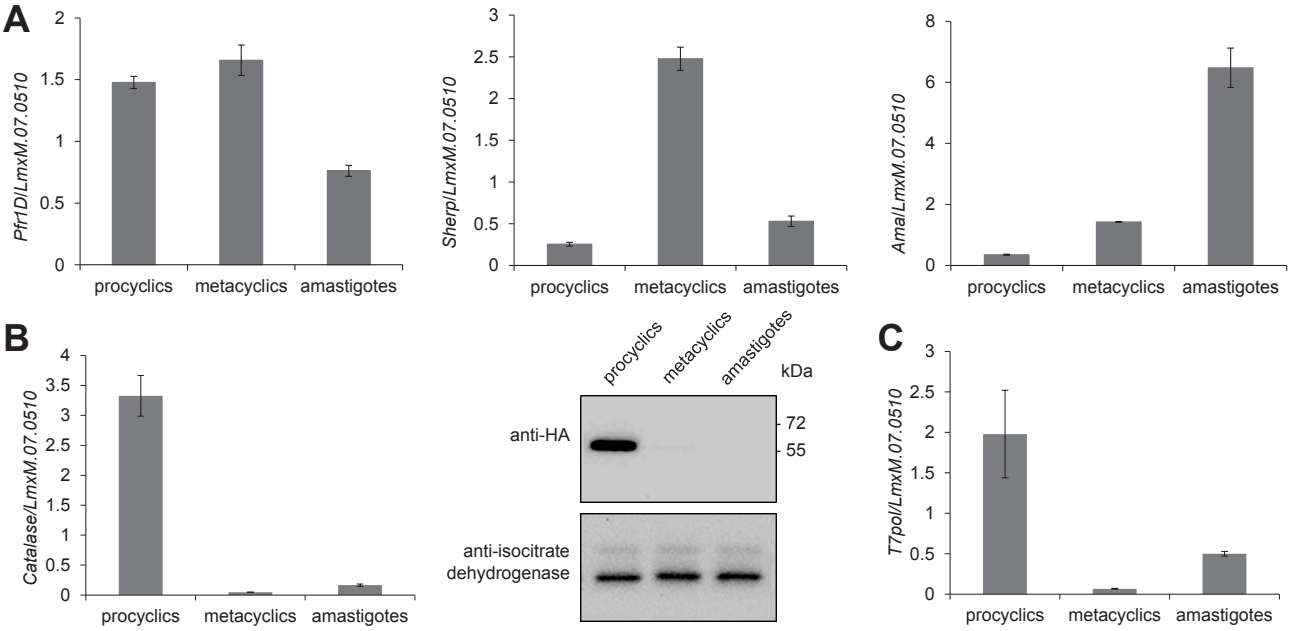

Fig. 1. Heterologous gene expression of the T7TR-HA_3-catalase in *Leishmania mexicana* (Biagi, 1953). **A** – *in vitro* differentiation of *L. mexicana*. qPCR quantification of *Pfr1D*, *Sherp* and *Amastin* gene expression used as markers for promastigotes (both pro- and metacyclics), metacyclics, and amastigotes, respectively; **B** – qPCR quantification and Western blot analysis of the HA_3-catalase expression in the differentiated cells after induction with 10 μg/ml of Tetracycline for 24 hours. Whole cell extracts were probed with α-HA and α-isocitrate dehydrogenase antibodies with the latter as a loading control; **C** – qPCR quantification of the T7 RNA polymerase expression analysed as above. Data for three independent biological replicates normalised to the *LmxM.07.0510* are shown in all cases. Detailed protocols were described previously (Kraeva et al. 2014, Záhonová et al. 2014). Primer sequences used for qPCR are available from authors upon request.

rophages (Bates 1994a). Metacyclics and amastigotes are pathogenic and thus can be used for identification of virulence factors of species of *Leishmania in vitro* and *in vivo*.

In this work we used the established transgenic *L. mexicana* T7TR system and followed the fate of the overexpressed protein during differentiation of parasites *in vitro* (Kraeva et al. 2014). The gene of interest was a catalase which is conspicuously absent from all species of *Leishmania* investigated thus far. This is one of the most potent enzymes on the Earth with Kcat reaching 10^7 molecules of H_20_2 per second. Its presence in all monoxenous relatives of *Leishmania* (species of *Leptomonas* Kent, 1880, *Crithidia* Léger, 1902 and *Lotmaria* Evans et Schwarz, 2014 of the subfamily Leishmaniinae – see Jirků et al. 2012) requires further investigation (Kraeva et al. 2015, Flegontov et al. 2016). The entire open reading frame of the catalase gene, PCR-amplified from DNA of the H10 isolate of *Leptomonas pyrrhocoris* Zotta, 1912 (Votýpka et al. 2012), was integrated into the β-tubulin locus and expressed in a T7 polymerase-dependent, Tet-inducible way.

Parasites were differentiated *in vitro* by changing the temperature and pH of the media (Bates 1994b). Proper separation of developmental stages of *L. mexicana* was confirmed by expression analysis of selected stage-regulated genes using qPCR as described earlier (Záhonová et al. 2014). The genes encoding PFR1D (LmxM.08_29.1750, LmxM.08_29.1760), SHERP (LmxM.23.1050, LmxM.23.1061) and Amastin (LmxM.08.0800, LmxM.08.0840, LmxM.08.0850) were used as promastigotes- (procyclics and metacyclics), metacyclics- and amastigotes-specific gene markers, respectively (Rochette et al. 2008, Sádlová et al. 2010) (Fig. 1A). A homolog of the 60S ribosomal protein L7a (LmxM.07.0510) was chosen as a reference based on our whole-transcriptome analysis of developmental stages of *L. mexicana* (Flegontov et al. 2016).

Other genes with similar expression patterns – ubiquitin hydrolase (UbH, LmxM.08_29.2300), short chain 3-hydroxyacyl-CoA dehydrogenase (LmxM.36.1140), serine acetyltransferase (LmxM.33.2850), and 60S ribosomal protein L17 (LmxM.24.0040) – were analysed in parallel. Normalisation to these genes did not significantly change the results (data not shown). The expression of catalase of *L. pyrrhocoris* in differentiated stages was analysed by qPCR and Western blotting. Both, the mRNA and protein levels were strikingly decreased in metacyclics and amastigotes (Fig. 1B). Comparison of the catalase and the T7 polymerase expression profiles at different developmental stages revealed a similar pattern (Fig. 1C). This is not surprising, provided that the transcription of catalase is T7 polymerase-driven.

To verify that the decrease of gene expression in metacyclics and amastigotes is not sequence-specific, we replaced the T7 polymerase open reading frame by that of mCherry as described previously (Kraeva et al. 2015). The construct was integrated into the same *18S* rRNA locus (Kushnir et al. 2005) and expression of the mCherry protein was confirmed by fluorescence microscopy (data not shown). The differentiation of the transgenic culture and proper separation of the life cycle stages were verified by

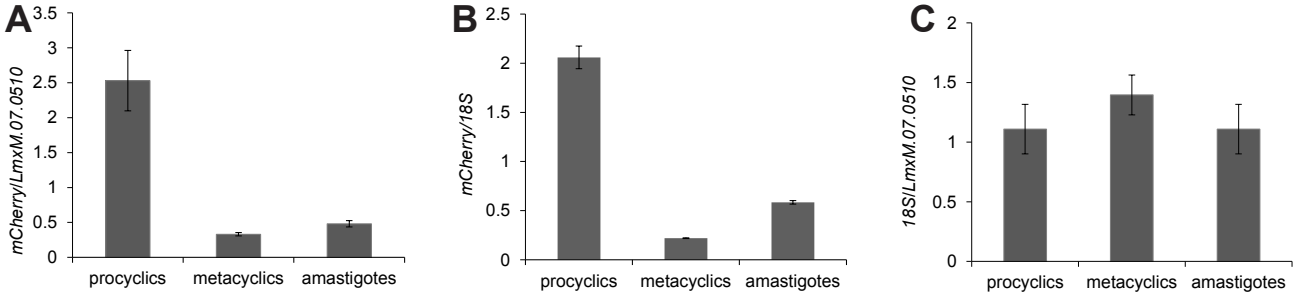

Fig. 2. A,**B** – mCherry gene expression in *Leishmania mexicana* (Biagi, 1953). mRNA expression level of mCherry in differentiated procyclics, metacyclics, and amastigotes. *LmxM.07.0510* and *18S* rRNA genes were used for normalisation in **A** and **B**, respectively; **C** – expression of the *18S* rRNA is stable throughout development of *L. mexicana*. Results of three independent biological replicates are presented. Detailed protocols were described previously (Kraeva et al. 2014, Záhonová et al. 2014). Primer sequences used for qPCR are available from authors upon request.

qPCR as described above. Next, we examined the expression profile of the mCherry transcript in differentiated procyclics, metacyclics and amastigotes and confirmed it to be similar to that of the catalase and T7 polymerase (Fig. 2A).

The mCherry, T7 polymerase and Tet-repressor genes were all integrated into the *18S* rRNA locus. Previous studies showed a 5 to 20-fold decrease in transcription of several RNAs derived from this locus during *Leishmania donovani* (Laveran et Mesnil, 1903) differentiation (Saxena et al. 2007). To exclude an influence of the rRNA locus-dependent regulation on the reporter genes expression, we compared mCherry expression profile normalised to the *18S* and *LmxM.07.0510* genes (Fig. 2A,B). Similar patterns of the mCherry mRNA expression suggest that gene expression regulation in our system is not locus-specific. As a control, we analysed expression of the 18S rRNA and confirmed it to be stable throughout development (Fig.2C).

Although the molecular mechanisms governing expression of developmental genes during the promastigote-to-amastigote differentiation are not well understood and characterised, a number of reports have demonstrated that protein abundance is chiefly controlled by mRNA stability. It depends on the sequences present in both 5' and 3' UTRs (Aly et al. 1994, Charest et al. 1996, Garcia-Estrada et al. 2008).

In our study, the mCherry, T7 polymerase, and Tet-repressor genes were flanked by the UTRs derived from the calmodulin (LtaP.09.0940) intergenic regions of *Leishmania tarentolae* Wenyon, 1921 (Breitling et al. 2002, Kushnir et al. 2005). Whole-transcriptome profiling revealed that calmodulin mRNA abundance in species of *Leishmania* is developmentally regulated. It is reduced by 1.5–2 fold upon differentiation from procyclics to metacyclics and amastigotes (Aslett et al. 2010, Dillon et al. 2015). Patterns of the expression of reporter genes upon differentiation of *L. mexicana* followed those of calmodulin. The discrepancy in the degree of downregulation can be explained by differences in calmodulin expression regulation in *L. tarentolae* and *L. mexicana.*

In summary, here we would like to draw attention of the parasitology community to the potential limitations that conventional gene expression systems might have in the studies of the development of species of *Leishmania*.

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