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Short communication

Role of the stage-regulated nucleoside transporter *Tb*NT10 in differentiation and adenosine uptake in *Trypanosoma brucei*

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Trypanosoma brucei undergoes complex metabolic and morphological changes during its life cycle in order to adapt and survive inside the mammalian host and the tsetse fly vector. One of these adaptations to different environments is reflected by the expression of various nucleoside transporters. As protozoan parasites are unable to synthesise the purine ring *de novo*, they have to salvage preformed nucleosides and nucleobases from their hosts [1]. To date, several nucleoside transporters of *T. brucei* have been identified and characterised, all of which belong to the equilibrative nucleoside transporter family. Depending on their substrate specificities they can be assigned to two types of transport activities, P1 and P2 [2]. P1 activity is specific for the uptake of adenosine, guanosine and inosine and has been found in both bloodstream and procyclic forms. P2 activity is specific for adenosine and adenine uptake. Only one P2 transporter, *Tb*AT1, has been described so far. *Tb*AT1 is expressed in bloodstream forms and has been shown to transport the trypanocidal drugs melarsoprol and pentamidine [2,3]. Additionally, purines can be salvaged by the hypoxanthine transporting activities H1–H4 [4,5].

Although many nucleoside transporters and their substrate specificities have been characterised, the reason for the parasite to employ many related transporters with the seemingly redundant function of purine uptake, is still unclear. Furthermore, very little is known about the contribution of single transporters to purine uptake in different life cycle stages of the parasite.

The nucleoside transporter *Tb*NT10 [6] (Tb09.160.5480, also known as *Tb*AT-B [7]), displays a P1-type transport activity with high affinities for adenosine, guanosine and inosine and marginal affinities for hypoxanthine and adenine [6,7]. *TbNT10* mRNA was shown to be stage-regulated in *T. brucei rhodesiense EATRO* 2340, with highest expression in short stumpy bloodstream forms and lowest expression in long slender bloodstream and procyclic forms [6]. This regulated expression during the life cycle might point to a function of the transporter in the process of differentiation.

In this study a possible role of *Tb*NT10 in differentiation from long slender to short stumpy bloodstream forms, and in the following differentiation step to procyclic forms, was investigated. Northern blot analysis with mRNA from different life cycle stages of *T. brucei brucei* strain AnTat 1.1 [8,9] confirmed that the *TbNT10* mRNA is expressed at a low level in long slender bloodstream forms and is up-regulated in short stumpy bloodstream forms (Fig. 1A). In this strain, however, the highest level of *TbNT10* mRNA was found in procyclic forms. Strain-specific differences have been observed previously for the expression of another P1-type transporter *Tb*NT2 [10]. In addition, culture conditions might influence expression levels of certain transporters. The *TbNT10* mRNA is 4.2 kb long, with an open reading frame (ORF) of 1.4 kb. In order to determine the length of the untranslated regions (UTRs) a cDNA library [11] was screened and cDNAs containing spliced leader sequences were analysed. The 5′ UTR was found to be 42 bases. We could not determine the precise length of the 3′ UTR, as this region contains several

Abbreviations: UTR, untranslated region; SIF, stumpy inducing factor; GFP, green fluorescent protein; *Tb*NT10, nucleoside transporter 10 of *Trypanosoma brucei*; ORF, open reading frame; IC, inhibitory concentration

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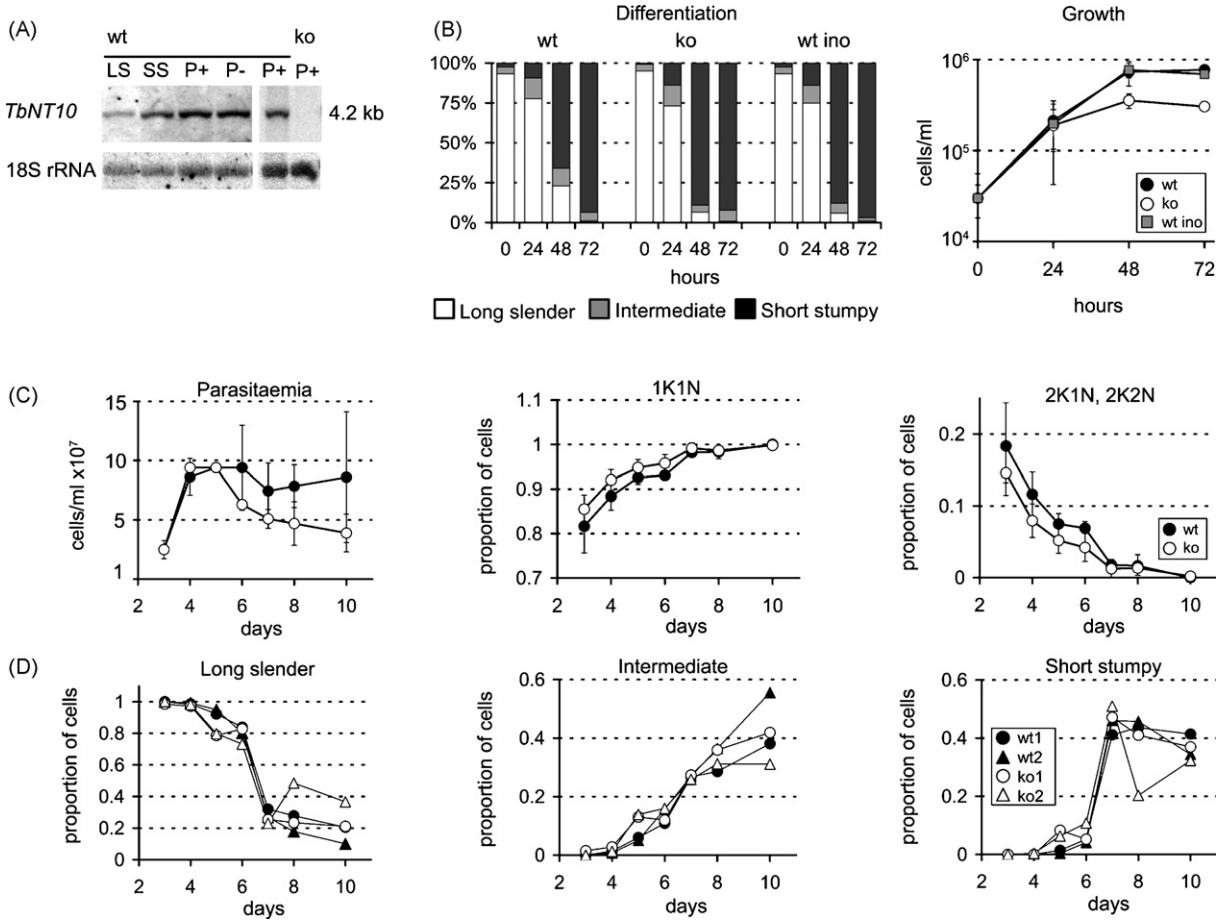

Fig. 1. *TbNT10* mRNA expression and role in differentiation. (A) Northern blot analysis with 10 μg total RNA from long slender (LS) and short stumpy (SS) bloodstream forms, and early (P+) and late (P−) procyclic forms of AnTat 1.1 wild type (wt) and the *Tb*NT10 null mutant (ko). The complete ORF of *Tb*NT10 was amplified from genomic DNA by PCR with the primers *NT10*-orf1 5′-ATGCTCGGGTTTGAGTCGTT-3′ and *NT10*-orf2 5′-TTATGATTCTGGAAGAGCCG-3′ and used as template to synthesise a ^{32}P-labelled probe for hybridisation. The size of the *TbNT10* mRNA reported in the original publication [6] was incorrect, this being due to a misunderstanding between the two laboratories involved. The correct size of the transcript has been independently confirmed as being ∼4.2 kb (K. Matthews, unpublished data). The signals were normalised using a γ^{32}P-dATP end-labelled probe specific for 18S rRNA [17]. The *Tb*NT10 null mutant was constructed in early procyclic forms of *Trypanosoma brucei* AnTat 1.1 by sequentially replacing the ORFs by a neomycin- and a hygromycin-resistance cassette, respectively. The 5′- and 3′-flanking regions of the ORF were amplified by PCR using the primers: *NT10-Kpn*I 5′-CAGGTACCAAACTGACGAAAGTGC-3′, *NT10-Hin*dIII 5′-GGAAGCTTCTTGCTTAAATGACTCAG-3′, *NT10-Bam*HI 5′-TTGGATCCCTAAGAGGAGGTAA-3′ and *NT10-Xba*I 5′-TATCTAGACACATTTGTGGGCGCG-3′. The products were cloned into pBluescript upstream and downstream of a neomycin- or a hygromycin-resistance gene, respectively, using the restriction sites (underlined) introduced by the primers. Stable transformation was performed as described [18]. Inserts were excised by digestion with *Kpn*I and *Xba*I prior to electroporation. (B) *In vitro* differentiation from long slender to short stumpy bloodstream forms. Long slender bloodstream forms of the *Tb*NT10 null mutant and the wild type were thawed from frozen mouse blood stabilates and cultured with a starting density of 3×10^4 cells/ml in HMI-9 medium containing 0.65% low melting temperature agarose and 10% horse serum. The 1 mM inosine was added to wild-type cells where indicated (wt ino). After 0, 24, 48, and 72 h of incubation at 37 °C and 5% CO_2 the percentage of long slender, intermediate and short stumpy forms was determined by the diaphorase assay [14]. A representative experiment (from four independent experiments with similar results) is shown. Growth of the cells was monitored during differentiation and the means of three experiments ± standard deviation (S.D.) are shown. (C) *In vivo* differentiation from long slender to short stumpy bloodstream forms. Four MF1 mice were infected with bloodstream forms of wild type or *Tb*NT10 null mutant parasites, respectively. Parasitaemia was measured every 24 h from day 3 until day 10 post infection and the means ± S.D. are shown (two out of four mice infected with wild-type parasites died on day 9 of the experiment). At the same time points, cell division was monitored by DAPI staining and microscopic analysis of the numbers of nuclei (N) and kinetoplasts (K) per cell. The proportion of cells with 1K1N (comprising cells arrested in G1/G0 as well as proliferative cells early in their cell cycle) and dividing (2K1N, 2K2N) cells are shown. (D) The *in vivo* differentiation of the parasites described above was monitored by microscopic analysis of the morphology; 250 cells were analysed per sample and categorised as long slender, intermediate or short stumpy bloodstream forms. Data points represent the parasite populations in individual mice infected with the wild type (wt1, wt2) and the null mutant (ko1, ko2).

AU-rich stretches that supported internal priming. Considering the Northern blot data, however, we estimate the 3′ UTR to be ∼2.7 kb long.

As mentioned above, the regulated expression of *TbNT10* mRNA could point to several functions in the differentiation from long slender to short stumpy bloodstream forms. Short stumpy forms are pre-adapted to be taken up by the tsetse fly. They are unable to divide in the mammalian bloodstream and die within a few days. The differentiation from long slender into short stumpy bloodstream forms is triggered by a stumpy inducing factor (SIF) that accumulates in a cell-density dependent manner in culture medium (and presumably blood) containing long slender parasites [12]. SIF has been characterised as a small compound with a molecular weight of ≤500 Da. Its chemical