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Oral-examination:

**Proteins and RNA sequences regulating gene expression in
*Trypanosoma brucei***

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Summary

Trypanosoma brucei, the agent causing African trypanosomiasis or sleeping sickness, constitutes one of the best-studied biological models so far. In order to adapt to different environments, this parasite mainly regulates gene expression by post-transcriptional mechanisms such as mRNA stability and translation. These processes are mediated by the interaction of sequences located in the 3'-UTR with *trans* acting factors.

RRM-proteins are involved in many aspects of the RNA metabolism. However, the function of most of them is unknown. The first part of this work deals with the characterisation of RBP3 and its role in developmental control of gene expression. Orthologues of this protein are found in *T. cruzi*, *T. congolense* and *L. major*. Over-expression and depletion of *TbRBP3* by RNAi suggest a stage-specific role for this protein in bloodstream cells. Microarray studies comparing wild-type cells with cells where *TbRBP3* levels have been perturbed (either by RNAi or over-expression) were unsuccessful in revealing putative mRNA targets, suggesting that this protein is not involved in control of mRNA levels. Pull down of *TbRBP3*-RNP complexes allowed the identification of several transcripts that selectively bind this protein. Specifically, the interaction of cyclin F-box and ZFP-mRNAs was confirmed by RT-PCR and the role of the over-expression of *TbRBP3* at mRNA levels was determined by Northern blot analysis. Unfortunately, attempts to identify *TbRBP3* interaction partners have failed. Studies to determine the role of this protein are in progress.

Specific sequences in the 3'-UTR of stage-specific transcripts are involved in mRNA turnover and translation. In this work, several regulatory elements involved in the developmental regulation of the amino acid transporter 11 are reported. A region between nt 290-618 of this 3'-UTR is required to down-regulate mRNA levels of the CAT reporter gene in bloodstream forms. Moreover, the presence of 2 elements involved in translational repression was also established. Several approaches used to identify interacting proteins of the AATP 11 3'-UTR under physiological conditions were unsuccessful. Approaches using *in vitro* conditions are suggested.

Zusammenfassung

Trypanosoma brucei, der afrikanische Trypanosomiasis oder Schlafkrankheit verursacht, ist einer der bisher am besten untersuchten biologischen Modellorganismen. Um sich an unterschiedliche Umgebungen anzupassen, reguliert dieser Parasit die Genexpression post-transkriptionell, hauptsächlich über mRNS Stabilität und Translation. Diese Prozesse werden durch die Interaktion zwischen Sequenzen, die sich in der 3'-UTR befinden und Proteinen reguliert.

Trotz ihrer Rolle in vielen Aspekten des RNS-Metabolismus ist die Funktion der meisten RRM-Proteine unbekannt. Der erste Teil dieser Arbeit beschäftigt sich mit der Charakterisierung von RBP3 und seiner Rolle in der post-transkriptionellen Kontrolle. Orthologue dieses Proteins findet man in *T. cruzi*, *T. congolense* und *L. major*. Überexpression und Hemmung von *TbRBP3* mittels RNAi zeigen eine stadium-spezifische Rolle für dieses Protein in den Blutstromzellen an. Microarraystudien, die Wildtypzellen mit *TbRBP3*-RNAi oder –überexprimierenden Zellen vergleichen, führten nicht zur Identifikation einer Ziel-mRNS. Diese Ergebnisse weisen darauf hin, dass dieses Protein keine Funktion bei der Kontrolle von mRNS Mengen hat. Isolation der *TbRBP3*-RNP Komplexe erlaubte die Identifizierung von verschiedenen mRNS, die selektiv dieses Protein binden. Insbesondere wurden die Interaktion der cyclin F-Box und ZFP-mRNS durch RT-PCR bestätigt und die Rolle der Überexpression von *TbRBP3* auf mRNA Levels wurde durch Northernblotanalyse untersucht. Versuche, *TbRBP3*-Interaktionpartner in *T. brucei* zu finden, blieben erfolglos. Versuche, um die Rolle dieses Proteins festzustellen werden durchgeführt.

Spezifische Sequenzen, die sich in der 3-UTR von stadiumspezifischen Genen befinden, sind an mRNS Stabilität und Translation beteiligt. In dieser Arbeit wurden regulierende Elemente identifiziert, die an der Entwicklungskontrolle des Aminosäuretransporters 11 beteiligt sind. Die Region zwischen nt 290-618 dieser 3'-UTR ist für die geringere Abundanz der mRNS eines CAT Reportergens in der Blutstromform verantwortlich. Außerdem wurden noch zwei Elemente gefunden, die in translationelle Repression verwickelt sind. Versuche, um AATP 11-3'-UTR-Interaktionsproteine unter physiologischen Konditionen zu finden, waren erfolglos. Experimente unter *in vitro* Bedingungen werden vorgeschlagen.

1 Introduction

The kinetoplastids are a widespread group of flagellated protozoa. They are mainly known by their ability to parasitize virtually all animal groups as well as plants and insects. However, there are also free-living kinetoplastids which feed on bacteria in aquatic, marine and terrestrial environments. These parasites are named after one of their most unusual features, a mitochondrial DNA known as kinetoplast DNA (kDNA). The kinetoplast is unique in its structure, function, and mode of replication (Renger and Wolstenholme 1971; Simpson 1973; Shapiro and Englund 1995; Liu, Liu et al. 2005).

The kinetoplastids are one of the best-studied examples of ancient eukaryotes. Phylogenetic studies based on data from rRNA (18S rRNA gene) and protein-coding genes established the monophyletic origin of this group (Stevens and Gibson 1999; Stevens, Noyes et al. 2001; Ginger 2005). In particular, three distinct species of this parasite have been studied intensively because they are the main agents for diseases of underprivileged people: *Leishmania* spp. (leishmaniasis), *Trypanosoma cruzi* (Chagas' disease) and *Trypanosoma brucei* complex (African sleeping sickness).

Leishmaniasis is a group of visceral, muco-cutaneous and cutaneous diseases, caused by protozoa of the genus *Leishmania*. This parasite invades and multiplies inside the macrophages of many vertebrates such as humans and dogs. The primary vectors for the transmission of *Leishmania* are female sandflies (*Phlebotomus* and *Lutzomyia*). There are 20 species and subspecies of this parasite that infect humans. The disease is prevalent in 88 countries and the 350 million people live at risk in endemic areas. The World Health Organization (WHO) estimates that 12–14 million people are affected and 1.5–2 million new cases occur per year. Visceral leishmaniasis (VL or Kala azar) caused by *L. donovani* spp. is the most severe form of the disease and it is fatal if left untreated. There are 400.000–500.000 of new cases every year mainly in South Asia (Bangladesh, India and Nepal). The most common form of leishmaniasis is the cutaneous form (CL) and it is due to infection with *L. major*. There are between 1'000.000 to 1'500.000 of people infected every year, most of them in Asia (Afghanistan, Iran and Saudi Arabia) and South America (Brazil and Peru). Other form of the disease is the mucocutaneous leishmaniasis (MCL) which is due to *L. braziliensis* and *panamensis* infection (www.who.int) (Alvar, Croft et al. 2006).

Trypanosomiases are caused by parasites of the genus *Trypanosoma* affect people in Africa, Central and South America. Chagas disease is found only in Latin America and is caused by *T. cruzi*. 13 million people are currently estimated to be infected with this parasite. The

primary vector is the reduviid bug *Triatoma infestans*. About 25% of infected people show disease symptoms. Chronically infected patients eventually suffer from irreversible damage to the heart and digestive tract. This disease causes considerable morbidity resulting in approximately 14,000 annual deaths. The control of Chagas disease in the last two decades has been a substantial public health achievement. The incidence of this disease was reduced by over 65% between 1990 and 2000, from an estimated 700,000 cases per year to fewer than 200,000. This is mainly due to elimination of the insect vector in many countries in South America (www.who.int) (Yamagata and Nakagawa 2006).

Human African trypanosomiasis (HAT) or sleeping sickness is caused by *Trypanosoma brucei* and transmitted by tsetse flies (*Glossina spp.*). The disease is fatal if not treated and affects individuals in over 30 sub-Saharan countries. Recent estimates indicate that over 60 million people living in some 250 foci are at risk of contracting the disease, and there are about 300,000 new cases each year. However, less than 4 million people are under surveillance and only about 40,000 are diagnosed and treated, mainly due to the lack of resources to control the disease properly and to improve methods of diagnosis and chemotherapy (www.who.int) (Barrett 1999; Barrett 2006; Brun and Balmer 2006).

Sleeping sickness occurs in two forms: a chronic one caused by *T. b. gambiense*, which occurs in west and central Africa; and an acute form, caused by *T. b. rhodesiense*, which occurs in eastern and southern Africa. *T. b. brucei*, a third subspecies of *T. brucei* causes Nagana in cattle but does not infect humans and is commonly used as a laboratory model organism. Uganda is the only country harbouring both forms of HAT. There are indications that this may change in the near future because areas with *T. b. gambiense* and *T. b. rhodesiense* are expanding (Brun and Balmer 2006).

Early symptoms, which include fever, headache and enlarged lymph glands and spleen, are more severe and acute in *T.b. rhodesiense* infections. Advanced symptoms include neurological and endocrine disorders. As the parasites invade the central nervous system, mental deterioration begins, leading to coma and death (www.who.int).

1.1 *T. brucei* life cycle

As most of the protozoa parasites, *Trypanosoma* has to adapt to several environmental conditions (insect vector and mammal host), which requires morphological, biochemical and basic cell biological changes. Figure 1.1 shows the life cycle of *T. brucei*. The parasite is cyclically transmitted from mammal to mammal by the tsetse fly, *Glossina spp* (1). In the bloodstream of mammalian hosts the parasite proliferates as morphologically slender form

(2) and survives free in the bloodstream by evading antibody responses through antigenic variation. This involves the sequential expression of antigenically distinct variable surface glycoproteins (VSGs). These proteins are linked to the outer membrane by a glycosylphosphatidylinositol (GPI) anchor (Young, Donelson et al. 1982; Hajduk 1984; McCulloch 2004; Pays, Vanhamme et al. 2004) (3). As parasite number increase they differentiate into non-proliferative stumpy forms. These cells are arrested in G1 of the cell cycle and are preadapted for transmission to tsetse flies. Upon ingestion by the vector, bloodstream trypanosomes transform into the insect midgut form (procyclics) and switch the VSG coat to a coat composed of EP and GPEET procyclins (4, 5, 6). This form contains a well developed mitochondrion that is required to generate energy. After proliferation in the tsetse midgut, the parasite (epimastigotes) migrates to the salivary gland where after further divisions (7) generates non-proliferative metacyclic forms which reacquire the VSG coat and are released into the salivary gland lumen, in preparation for inoculation into a new mammalian host (8) (Matthews 2005).

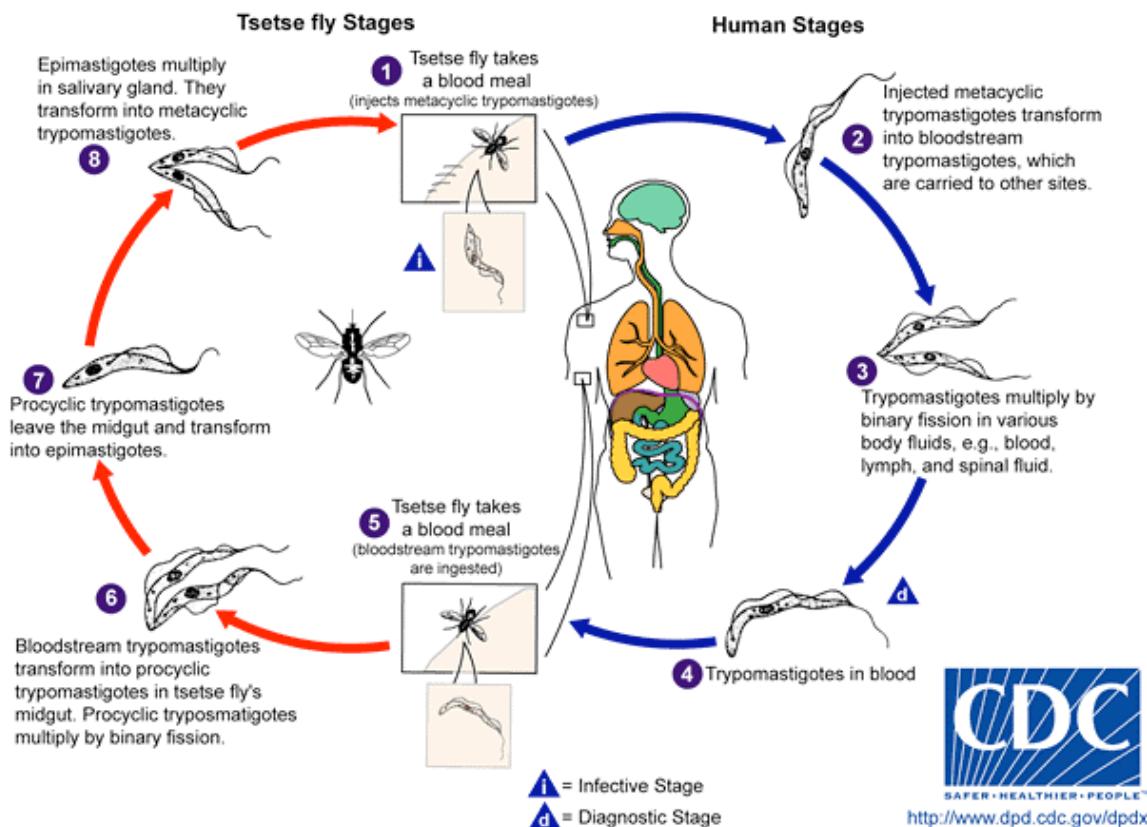


Fig. 1.1 Life cycle of *Trypanosoma brucei* (taken from Center for Diseases Control and Prevention, www.cdc.gov/)

1.2 Why is *Trypanosoma* special?

The presence of a number of unusual characteristics such as antigenic variation, kinetoplast DNA, glycosomes, and *trans* splicing has focussed the attention of many people in this parasite.

1.2.1 Genome organisation

Trypanosoma genome contains 2 different DNAs: nuclear DNA and the mitochondrial DNA or kinetoplast. The haploid nuclear genome of the three main species of kinetoplastids (*T. brucei*, *T. cruzi* and *L. major*) was completely sequenced in 2005 (Berriman, Ghedin et al. 2005; El-Sayed, Myler et al. 2005; Ivens, Peacock et al. 2005). The nuclear DNA of *Trypanosoma brucei* is about 35 Mb and is distributed among three size classes of chromosomes: most of the coding information is present in 11 pairs of megachromosomes (MC), which are numbered I to XI from smallest (1 Mb) to largest (> 6 Mb) and they contain 9068 predicted genes. Other genome elements are the Intermediate chromosomes (200±900 kb) and minichromosomes (50±150 kb), which usually contains repetitive elements such as the 177-bp repeat and silent VSG sequences (Ersfeld, Melville et al. 1999; El-Sayed, Hegde et al. 2000; Berriman, Ghedin et al. 2005)

The mitochondrial DNA, known as the kinetoplast (kDNA) is composed of interlinked DNA rings (maxicircles and minicircles) and constitutes as much as 20% of the total DNA in the organism. Maxicircle gene products include rRNAs and subunits of respiratory complexes. However, these transcripts are cryptic and require editing to form a functional mRNA. RNA editing is the process by which mitochondrial transcripts are transformed into translatable mRNAs by the insertion and deletion of uridines. The specificity of this mechanism is controlled by minicircle-encoded guide RNAs that serve as templates for uridylylate-insertion and deletion. (Benne, Van den Burg et al. 1986; Shapiro and Englund 1995; Stuart, Allen et al. 1997; El-Sayed, Hegde et al. 2000; Ginger 2005; Liu, Liu et al. 2005; Stuart, Schnaufer et al. 2005)

1.2.2 Metabolism

In the mammalian host, *T. brucei* relies upon glucose metabolism for energy generation. Uniquely, the first seven enzymes of the glycolytic pathway are compartmentalised within a specialized peroxisome, the glycosome (Opperdoes 1987). The mitochondrion of the bloodstream trypanosome plays no direct role in ATP production (apart from reoxidising glycerol-3-phosphate), and the end product of glycolysis is excreted from the cell. Therefore

this organelle is underdeveloped. On the other hand, procyclic trypanosomes rely on mitochondrial energy generation for their viability and their energy metabolism is characterised by the aerobic fermentation of carbohydrates (glucose) and amino acids mainly exogenous proline (Berriman, Ghedin et al. 2005; Ginger 2005). According to this, the presence of a family of 38 amino acid transporters has been reported in *T. brucei* (Berriman, Ghedin et al. 2005).

1.2.3 Gene expression in Trypanosomatids

1.2.3.1 Transcriptional control:

One of the main features in *Trypanosoma* is that the genes are transcribed in large polycistronic precursor RNAs. Only few of the different ORFs contain introns and the coding sequences are separated by short intergenic regions. This precursor is processed into mature monocistronic mRNAs through the excision of a coding region, capping upstream of the ATG start codon by *trans*-splicing, and polyadenylation. *Trans*-splicing is a reaction in which a 39-nucleotide spliced leader sequence is added at an AG dinucleotide downstream of a polypyrimidine tract (Agabian 1990; Liang, Haritan et al. 2003). This mechanism is coupled to the addition of a poly (A) tail to the gene immediately upstream in the cluster. Polyadenylation usually occurs 200–500 nucleotides upstream of the spliced leader addition site (Gilinger and Bellofatto 2001; Martinez-Calvillo, Yan et al. 2003; Benz, Nilsson et al. 2005). Only four examples of genes containing introns are evident in the trypanosomatid genomes, the poly (A) polymerase (PAP) genes of *T. brucei* and *Trypanosoma cruzi* (Mair, Shi et al. 2000), possibly one ATP-dependent DEAD/H RNA helicase and two hypothetical RNA binding proteins and (Ivens, Peacock et al. 2005).

Little is known about the mechanism of transcription initiation in trypanosomatids, and only a few promoters have been functionally analyzed. *T. brucei* RNA polymerase I (RNAP I) transcribes the pre-rRNA (18S, 5.8S and 28S) gene cluster, the mRNAs from the bloodstream-form variant surface glycoproteins (VSGs) and the procyclic-form procyclins (EPs and GPEETs). These promoters for these transcripts have been already characterised (Biebinger, Rettenmaier et al. 1996; Clayton 2002; Pays 2005). RNA polymerase III transcribes most U RNAs in addition to tRNAs (Palenchar and Bellofatto 2006).

In *T. brucei*, the genes are bidirectionally distributed on the chromosome and oriented to both telomeres. RNA polymerase II (RNAP II) is responsible for the transcription of the majority of mRNAs, as well as the SL RNA gene (Gilinger and Bellofatto 2001). Although the SL

RNA promoter has been well-studied (Gilinger and Bellofatto 2001; Das and Bellofatto 2003), RNA polymerase II promoters for protein coding genes have not been identified. Experimental evidence suggests that polycistronic transcription by RNA polymerase II (RNAP II) initiates bidirectionally in the region located between the two gene clusters. Transcription usually terminates in the region containing tRNA, rRNA, and/or snRNA genes (Andersson, Aslund et al. 1998; Martinez-Calvillo, Yan et al. 2003; Martinez-Calvillo, Nguyen et al. 2004). The *T. brucei* RNA pol II has been characterized (Das, Li et al. 2006) and the presence of few basal transcription factors found in other eukaryotes has been reported (Das, Zhang et al. 2005; Ivens, Peacock et al. 2005; Palenchar and Bellofatto 2006). Due to absence of regulation of transcription initiation by RNA polymerase II at the level of transcript initiation, gene expression is predominantly regulated through post-transcriptional mechanisms (Clayton 2002).

1.2.3.2 Post-transcriptional regulation and role of the 3'-untranslated region (3'-UTR):

In order to adapt to the different environments provided by both the insect vector and the mammalian host, *Trypanosoma* relies on post-transcriptional mechanisms to control gene expression. This is achieved mainly through differential RNA processing, mRNA stability and translation. These activities involve interactions between *cis* elements –mostly located in the 3'-UTR of the transcript and *trans* acting factors –RNA binding proteins. These regulatory sequences determine the mRNA abundance by modulating RNA degradation and often also translation efficiency (Clayton 2002).

The role of the 3'UTR in control of mRNA stability and in particular, the presence of specific sequences which confers developmental regulation, has been elucidated by using chloramphenicol acetyl transferase (CAT) or luciferase reporter genes (Hehl and Roditi 1994; Berberof, Vanhamme et al. 1995; Furger, Schurch et al. 1997; Teixeira and daRocha 2003). These are some examples about the regulation mediated by 3'-UTRs in *Trypanosoma*: in *T. cruzi*, the abundance of *Gp72*, *Gp85* and amastin mRNA is upregulated in amastigotes (Nozaki and Cross 1995). In particular, steady state levels of amastin mRNA are 60 fold higher and this upregulation is mediated by a region in the 3'-UTR that stabilizes the transcript (Teixeira, Kirchhoff et al. 1995; Coughlin, Teixeira et al. 2000; Teixeira and daRocha 2003). In mammalian cells AU-rich elements (ARE) present in the 3'-UTR are involved in destabilization of short life mRNAs such as interleukins and oncogenes (Chen and Shyu 1994; Chen, Xu et al. 1995). Similar destabilizing elements have been found in the 3'-

UTR of *SMUG*-mucin transcripts. These sequences promote rapid degradation of mucin mRNA upon differentiation to metacyclic trypomastigotes. This is mediated by interactions with the RNA binding proteins *TcUBP1* and *TcUBP2* (D'Orso and Frasch 2001; D'Orso and Frasch 2001). The higher amount of tubulin mRNA in epimastigotes is also related with the presence of positive and negative elements in this region (Bartholomeu, Silva et al. 2002).

In *Leishmania donovani*, the 3'-UTR is involved in the expression levels of *A2* transcripts in amastigotes (Charest, Zhang et al. 1996). The accumulation of *Hsp70* and *Hsp83* transcripts upon heat shock is mediated by the 3'-UTR in *L. infantum* (Zilka, Garlapati et al. 2001; Quijada, Guerra-Giraldez et al. 2002; Folgueira, Quijada et al. 2005). A translational effect of these elements has also been observed (Quijada, Guerra-Giraldez et al. 2002; Folgueira, Quijada et al. 2005). The selective accumulation of the variant S of *Gp63* in the stationary promastigote (the infective stage) also relies on specific sequences located in the untranslated region (Ramamoorthy, Swihart et al. 1995; Myung, Beetham et al. 2002).

Regulatory 3'-UTR elements have been best characterized in *T. brucei*. Berberof was the first to identify sequences in the 3'-UTR of the VSG mRNAs of *T. brucei* involved in expression in bloodstream forms (Berberof, Vanhamme et al. 1995). Another transcript regulated by this mechanism is the fructose bisphosphate aldolase mRNA, which is 6-fold more abundant in bloodstream trypomastigotes than in procyclic forms (Clayton 1985; Hug, Carruthers et al. 1993; Hotz, Lorenz et al. 1995). Similarly, two hexose transporters (*THTs*) mRNAs are developmentally regulated by their 3'-UTRs. *THT1* mRNA is expressed exclusively in the bloodstream and *THT2* mRNA in the procyclic stage (Hotz, Lorenz et al. 1995).

The presence of U-rich elements (UREs) in the 3'-UTR of stage-specific mRNAs such as *EP1* procyclin, *PGKB* and *PPDK* has been reported (Quijada, Guerra-Giraldez et al. 2002). They are similar to AU-rich elements found in the 3'-UTRs of transiently expressed proto-oncogenes, lymphokines and cytokines in mammalian cells (Chen and Shyu 1994; Chen, Xu et al. 1995). The role of these sequences in mRNA destabilization in bloodstream forms has been demonstrated using reporter genes under the control of these 3'-UTRs or the corresponding URE-deleted version (Hotz, Hartmann et al. 1997; Schurch, Furger et al. 1997; Quijada, Guerra-Giraldez et al. 2002).

The genes for the cytosolic and glycosomal phosphoglycerate kinases (*PGKs*) of *T. brucei* are found in a compact tandem array together with a third *PGK*-related gene, *PGKA*, which is expressed at low level. Expression of the two PGK genes (*PGKB* and *PGKC*) is differentially regulated in the life cycle of *T. brucei*: the glycosomal PGK (*PGKC*) protein and its mRNA are abundant in the mammalian stage of the cycle but not in the insect stage, whereas the

reverse is observed for the cytosolic PGK (*PGKB*) protein and its mRNA (Gibson, Swinkels et al. 1988). The sequences responsible for this regulation are located in the 3'-UTR of the different transcripts (Blattner, Helfert et al. 1998; Quijada, Guerra-Giraldez et al. 2002; Colasante, Robles et al. 2007).

The 3'-UTRs of the mRNA encoding the surface proteins *EP* and *GPEET* in *T. brucei* procyclic forms are the best characterized regulatory motifs in *Trypanosoma*. These proteins are undetectable in bloodstream forms, and the regulation occurs at several levels, mainly at mRNA abundance and translational levels. The transcript is 11-fold more abundant in procyclic forms compared to bloodstream and in this stage the translation is suppressed by 10-fold relative to the insect forms (Furger, Schurch et al. 1997; Hotz, Hartmann et al. 1997). The presence of 2 specific sequences in the 3'-UTR are responsible for this control: a 16-mer stem-loop which functions as a stabilizing element in procyclic forms (Furger, Schurch et al. 1997; Schurch, Furger et al. 1997) and a 26 U-rich element which is involved in decreasing the stability of the transcript and translation repression in bloodstream forms (Furger, Schurch et al. 1997; Hotz, Hartmann et al. 1997; Schurch, Furger et al. 1997). Secondary structure analysis showed that this 3'-UTR can be divided into three domains. Domains I and III that form stable structures, and a central domain (domain II) containing the 26-mer. Studies using enzymatic digestion and lead hydrolysis showed that domain II is normally in single-stranded conformation (Drozdz and Clayton 1999).

Other developmentally regulated genes that have been studied include *GPI-PLC* (Webb, Burns et al. 2005; Webb, Burns et al. 2005) (*GPI-PLC* mRNA steady state levels are higher in procyclic cells) and *COX* complex which is present in procyclics. *COX V* mRNA contains a U-rich element in the 3'-UTR which is involved in translational control, *COX VI* 3'-UTR also confers translational regulation and finally *COX IX* 3'-UTR is involved in controlling mRNA abundance (Mayho, Fenn et al. 2006).

The mechanism of mRNA turnover in *T. brucei* has been established by mapping of degradation intermediates of reporter mRNAs bearing different 3'-UTR (Irmer and Clayton 2001). *T. brucei* has at least two different mRNA degradation pathways. The first one is involved in degrading constitutive mRNAs (such as the mRNAs encoding actin or histone H4) and stable mRNAs (such as *PGKC* in bloodstream forms). It is not present in developmentally down-regulated. It involves deadenylation and decapping, followed by bidirectional degradation mediated by both 5' to 3' exonucleases (XRNA) and 3 to 5' exonucleases (exosome) (Irmer and Clayton 2001; Haile, Estevez et al. 2003; Li, Irmer et al. 2006).

The second pathway deals with developmentally downregulated mRNAs that need to be rapidly destroyed (such as the mRNAs encoding *EP* procyclin through the 26-mer element) and it involves bidirectional degradation mediated by both 5' to 3' exonucleases (XRNA) and 3 to 5' exonucleases (exosome) (Irmer and Clayton 2001; Haile, Estevez et al. 2003; Li, Irmer et al. 2006). This mechanism is delayed after down-regulation of either XRNA (Li, Irmer et al. 2006) or the exosome (Haile, Estevez et al. 2003). This pathway affects ~70% of the mRNA and does not require deadenylation (Irmer and Clayton 2001). The remaining 30% of the RNA is apparently subjected to the default pathway initiated by deadenylation (Li, Irmer et al. 2006).

1.3. RNA binding proteins

RNA binding proteins can be grouped into families based on a common sequence motif that interacts directly with RNA (Mattaj 1993; Chen and Varani 2005). In many proteins from bacteria and viruses, RNA recognition is mediated by small highly basic stretches rich in Arg and Lys residues. Two examples are the regulators of gene expression in HIV viruses Tat and Rev (Weeks, Ampe et al. 1990; Heaphy, Finch et al. 1991). In eukaryotes, many proteins involved in RNAi and micro-RNA processing pathway contain the Piwi Argonaut and Zwille (PAZ) domain (Lingel, Simon et al. 2003; Lingel, Simon et al. 2004). Although it is mainly found in DNA-binding proteins, the zinc-finger motif (CCHH-type) also binds to RNA and participates in protein–protein interactions. The tandem zinc finger domain (TZF) of the protein TIS11d binds to an AU-rich element in the 3'-UTR of the *TNF α* mRNA to promote its deadenylation and degradation (Hudson, Martinez-Yamout et al. 2004). HnRNP proteins and many nucleolar proteins such as nucleolin are characterized by the presence of RGG (Arg-Gly-Gly) boxes (Ghisolfi, Joseph et al. 1992; Kiledjian and Dreyfuss 1992). The generation of the small nuclear ribonucleoprotein particles (snRNPs) involved in pre-mRNA splicing requires the association of the seven Sm proteins in a heteromeric core that is able to bind snRNAs (Kambach, Walke et al. 1999; Wang, Palfi et al. 2006). RNA modifying enzymes also interact with RNA through their catalytic domain. RNase III proteins such as Drosha and Dicer, involved in processing RNAi and micro-RNA precursors belong to this category (Chen and Varani 2005). Proteins containing PUF repeats (a 40 amino acids motif) (Zamore, Williamson et al. 1997) bind the 3'-UTR of hunchback mRNA in *Drosophila* (Wickens, Bernstein et al. 2002) and the 3'UTR of *fem-3* mRNA in *C. elegans*, leading to an increase in the rate of deadenylation and/or repression of translation of these mRNAs (Zhang, Gallegos et al. 1997). In eukaryotes, the three most common $\alpha\beta$ RNA binding motifs are the

RNA recognition motif (RRM), the double stranded RNA-binding domain (dsRBD) and the K-homology (KH) domain. RRM and KH proteins interact primarily with single-stranded RNA, while the dsRBD binds to structured RNA and is involved in dsRNA metabolism (Staufen protein) (Chen and Varani 2005).

The RNA recognition motif (RRM), also known as RNA-binding domain (RBD) or ribonucleoprotein domain (RNP) is found in all life kingdoms. In eukaryotes, this motif is one of the most abundant protein domains. It consists of 90 amino acids containing a central sequence of eight conserved residues that are mainly aromatic and positively charged (Lys / Arg- Gly-Phe / Tyr-Gly / Ala-Phe / Tyr-Val / Ile / Leu-X-Phe / Tyr, where X can be any amino acid) called RNP-1. Sometimes, a second conserved motif of 6 amino acids is also present (RNP-2) (Kenan, Query et al. 1991). According to the Pfam protein families database, a total of 6768 RRM motifs have been identified in 3541 different proteins (<http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF00076>). They are often found as multiple copies within a protein (44% of eukaryotic RRM proteins contain two to six RRMs) and / or together with other domains (21% of eukaryotic RRM proteins) as the zinc fingers of the CCHC and CCCH type (21% of those with an additional domain), the polyadenylate binding protein C-terminal domain (PABP or PABC, 10%), and the WW domain (9 %) (Chen and Varani 2005; Maris, Dominguez et al. 2005).

In *Trypanosoma*, most of the RNA binding proteins characterised so far are involved in editing, rRNA processing and splicing. BLAST analysis predicts 75 RRM-type proteins in *T. brucei* genome, 80 in *Leishmania* and 135 in *T. brucei* (De Gaudenzi, Frasch et al. 2005). Many of them are associated with other motifs as zinc fingers and RGG domains.

NRBD1 and NRBD2 (previously p34 and p37), two RRM-type proteins are involved in the import and/or assembly pathway of 5S rRNA during ribosome biogenesis in *T. brucei* (Pitula, Ruyechan et al. 2002, Zhang, 1997 #130). They interact with NOPP44/46, the first RGG-type protein characterized that has affinity for poly (U) sequences in the nucleolus (Das, Peterson et al. 1996; Pitula, Ruyechan et al. 2002). RBP11 has domains suggestive of involvement in rRNA processing or transcription (De Gaudenzi, Frasch et al. 2005). Many RNA binding proteins are involved in editing. The guide RNA binding protein gBP21 that promotes the annealing of gRNAs to the pre-edited mRNA has been identified in *T. brucei*, *L.tarentolae* and *C. fasciculata* (Koller, Muller et al. 1997; Allen, Heidmann et al. 1998; Muller, Lambert et al. 2001; Muller and Goringer 2002; Brecht, Niemann et al. 2005; Vondruskova, van den Burg et al. 2005). *TbMP42*, a zinc finger protein that binds dsRNA, is involved in the editing reaction cycle through its exo- and endoribonuclease activity (Brecht, Niemann et al. 2005).

RBP16 interacts with gRNA through a cold shock domain (CSD) and a RRG domain. Depletion of this protein selectively affects the editing of apocytochrome B (CYb) and also has a role in maintaining mRNA stability of several non edited mRNAs (Hayman and Read 1999; Pelletier, Miller et al. 2000; Pelletier, Xu et al. 2001). Other RNA binding proteins involved in this process are: the oligo (U) binding protein *TbRRG1* (Vanhinne, Perez-Morga et al. 1998) and the RNA editing associated protein 1 (*TbEAP1*) (Madison-Antenucci and Hajduk 2001). Both serine/arginine (SR)-rich proteins, TSR1 and TSR1IP have been implicated in the recognition of the 5' and 3' splicing sites in *T. brucei* (Ismaili, Perez-Morga et al. 1999; Ismaili, Perez-Morga et al. 2000). Homologues have been identified in *T. cruzi* (TcSR1) (Portal, Lobo et al. 2003). The U2 snRNP auxiliary factor 35 (U2AF35) has already been characterized in *T. cruzi*, *T. brucei* and *L. major* (Xu, Wen et al. 2001; Vazquez, Atorrasagasti et al. 2003).

The Poly (A)-binding protein (PABP1), a RRM-type protein, was characterized in *T. cruzi*, *T. brucei* and *Leishmania spp.* Its role in translational control and mRNA turnover by interaction with the poly (A)-tail at the 3'-end of all mRNAs has been established (Batista, Teixeira et al. 1994; Hotchkiss, Nerantzakis et al. 1999; Bates, Knuepfer et al. 2000).

RNA binding proteins are also involved in the 3' to 5' mRNA degradation pathway: *TbRRP4*, *TbRRP40*, and *TbCSL4* all have S1 RNA-binding activity and are part of the exosome complex (Estevez, Kempf et al. 2001). *TbZFP1* and *TbZFP2* are involved in differentiation of *T. brucei* (Hendriks, Robinson et al. 2001; Hendriks and Matthews 2005). The presence of 10 PUF protein family members in *T. brucei* has been reported and their role in post-transcriptional control is being investigated (Luu, Brems et al. 2006).

Although several *cis* elements involved in RNA degradation have been found in *Trypanosoma*, just few interacting proteins have been identified. Only two proteins have been characterized so far: *TcUBP-1* and *TcUBP-2* (U-rich binding protein) control stabilization of small mucin mRNAs in *T. cruzi* through interactions with the 3'-UTR (D'Orso and Frasch 2001; D'Orso and Frasch 2002; De Gaudenzi, D'Orso et al. 2003). Their *T. brucei* homologues *TbUBP1* and *TbUBP2* are also implicated in post-transcriptional gene regulation in *T. brucei* (C. Hartmann, unpublished data).

1.4 Amino acid transporters

While the metabolism of *Trypanosoma* bloodstream forms relies exclusively on glucose. Although proline is the main energy source in insect forms, other amino acids are also needed: glutamine is required for several biosynthetic pathways, cysteine as an additional

sulfur source, and tyrosine for protein synthesis. So far, three specific amino acid transporters systems, one for proline, one for arginine and one for aspartate were described in *T. cruzi* (Canepa, Silber et al. 2004; Geraldo, Silber et al. 2005; Silber, Rojas et al. 2006). A family of wide-spectra amino acid transporters composed of 29 genes in *L. major*, 38 in *T. brucei*, and 42 in *T. cruzi* is also present (Opperdoes 1987; Berriman, Ghedin et al. 2005).

This family belongs to the eukaryotic-specific amino acid/auxin permease (AAAP) family (TC #2.18) and it is one of the most divergent families of transporters found in eukaryotes due to its extensive divergence at protein sequence (Young, Jack et al. 1999). One of the main features of these proteins is the presence of 11 transmembrane domains and the broad substrate specificity of some of its members. Many of them are able to transport all 20 natural amino acids found in proteins while other members apparently exhibit absolute specificity for a single amino acid (Young, Jack et al. 1999). 8 classes of these amino acid transporters are expressed in *Leishmania donovani* promastigotes (Akerman, Shaked-Mishan et al. 2004) and 12 of them are reported in *T. cruzi* (Akerman, Shaked-Mishan et al. 2004; Bouvier, Silber et al. 2004). One developmentally regulated amino acid transporter was characterized in *L. amazonensis*; it has homology to PAT6 in *T. cruzi*, and amino acid transporter 11 in *T. brucei* (Geraldo, Silber et al. 2005). Despite their presence in African trypanosomes, none of them has been studied so far.

1.5 Aims of the work described in this thesis

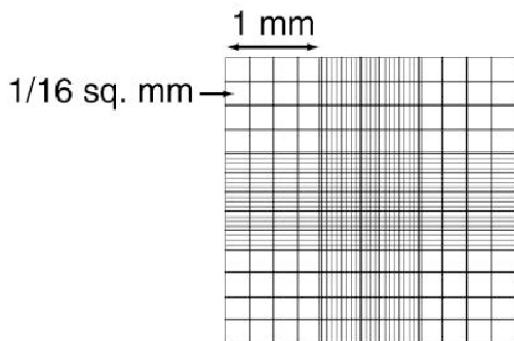
This thesis is the result of three different projects. Considering the role of some RRM-type proteins in post-transcriptional control, acting as regulators of developmentally expressed genes in *T. cruzi* and in *T. brucei*, the aim of the first work was the characterisation of *TbRBP3*, trying to establish its role in gene expression control. For this, microarray analysis was used. The other 2 parts of this manuscript rely on the *cis* elements present in the 3'-UTR of the mature mRNA that are involved in mRNA stability and translation. The identification of the sequences involved in the differential expression of the amino acid transporter 11 – a protein upregulated in the insect stages of this parasite-, was assessed by using a reporter gene and deletion mutants. Several strategies were employed to identify the proteins involved in this regulation. Finally, I tried to determine the role of T7 promoter on the toxicity of a reporter construct containing the chloramphenicol acetyl transferase gene under the control of *PGKC* 3'-UTR in bloodstream forms.

2 Materials and methods

2.1 Trypanosoma cell culture

2.1.1 Determination of cell density

The *T. brucei* culture was briefly homogenised by shaking the culture flask. 10 μ L was placed under a cover slip on a Neubauer improved counting chamber (Migge). The cells from two crossing 16-square-fields were counted, and their average was multiplied by 1×10^4 , which gave the amount of cells in 1mL of the culture. Dilutions were made to maintain the cultures below 7×10^6 and $1-2 \times 10^7$ /mL for bloodstream and procyclics respectively.



2.1.2 Bloodstream-form (BS) trypanosome culture

Bloodstream-form trypanosomes were kept in an incubator (Heraeus Instruments) at 37°C, 5% CO₂, in a humified atmosphere. The bottle caps were loosely tied to allowed gaseous exchange. Cells were harvested at densities up to $2,5 \times 10^6$ cells/mL * in supplemented HMI-9 medium. All culture work was done under sterile conditions in a laminar flow hood. For cryopreservation, aliquots of culture in log phase of growth ($1-2 \times 10^6$ cells/aliquot) were resuspended in 1 mL of HMI-9 with 10% glycerol in cryovials. The vials were wrapped in a thick layer of soft tissue paper and stored overnight at -80°C. This treatment allowed gradual freezing after which the tubes were transferred to cryoboxes in a liquid nitrogen tank. To restart cultures the cells were thawed at room temperature (RT) and transferred to 3 mL HMI-9 medium.

HMI-9 medium

^a IMDM (Gibco)	17.66g/L
NaHCO ₃ (Roth)	36mM
Hypoxanthine (Serva)	1mM
Na-pyruvate (Serva)	1mM
Thymidine (Sigma)	160mM
Bathocupron sulphonate (Serva)	50 mM

Supplemented HMI-9 medium, 500mL

HMI-9 supplemented with:

^bHeat-inactivated FCS 10% (v/v)

Penicillin/Streptomycin 50U/L (5mL of Penicillin-Streptomycin mix, Sigma)

L-Cysteine-HCl.H₂O 1.5mM (5mL of stock solution)

β-mercaptoethanol (Sigma) 0.14% (7.2μL in 5mL, filtered, added fresh)

^a*Iscove's Modified Dulbecco's Medium, no supplements*

^b*FCS was heat-inactivated by incubating it at 56°C for 30min.*

* Bloodstream trypanosomes grow at low densities compared to procyclic trypanosomes, but easily die if the culture is overgrown and usually take long to restart growing.

2.1.3 Stable transfection of bloodstream form trypanosomes

All centrifugation steps were done at 2,000 rpm for 10min at RT (Heraeus). 1 x 10⁷ cells from a log phase culture (1-2 x 10⁶ cells/mL) were pelleted, resuspended in 5 mL of Cytomix and centrifuged again. The pellet was resuspended in 0.5mL of Cytomix and transferred to a BTX cuvette at RT. About 10μg of sterile linearised plasmid DNA were added and mixed by pipeting up and down and subjected to a single electric pulse on a BTX electroporator set for peak discharge at 1.6kV, and resistance timing mode R2 (24 Ohm). The cells were then transferred to 25mL of warm (supplemented) HMI-9, distributed in 24-wells microtiter plates (0.5mL/well) and incubated overnight at 37°C. The next day, 0.5mL of medium with the appropriate antibiotics (depending on the background of the cells and twice the concentration of the selection antibiotic) was added to each well. Stable clones were established between 5-7 days with adequate cell densities. The new clones were scaled up to 5mL of supplemented HMI-9 for further culture and downstream analysis.

Cytomix

EGTA	2mM
KCl	120mM
CaCl ₂	0.15mM
K ₂ HPO ₄ /KH ₂ PO ₄	10 mM (pH 7.6)
HEPES	25 mM
MgCl ₂	5 mM
Glucose	0.5% (w/v)
BSA	100µg/mL
Hypoxanthine (Serva)	1mM
pH 7.6 (with NaOH)	

The medium was filter sterilized and stored at 4°C

2.1.4 Procyclic form trypanosome culture

Procyclic form trypanosomes were cultured in tightly closed flasks in a 30°C-room at densities between 0.1-10 x 10⁶ cells/mL* in supplemented MEM-Pros. All work was done under sterile conditions in a laminar flow hood. Cryopreservation and thawing was done as for bloodstream form but in supplemented MEM-Pros medium with 10% glycerol and at about 2 x 10⁶ cells/aliquot.

MEM-Pros

CaCl ₂	265mg/L	NaH ₂ PO ₄	0.14 g/L
KCl	0.4g/L	HEPES	7.14g/L
MgSO ₄ .7H ₂ O	0.2g/L	L-Arg-HCl	126mg/mL
NaCl	6.8g/L	LCys-Cys	24mg/L
L-His-HCl.H ₂ O	42mg/L	L-Gln	292mg/L
L-Ile	52mg/mL	L-Tyr	100mg/L
L-Leu	52mg/L	L-Val	46mg/L
L-Lys	73mg/L	L-Pro	600mg/L
L-Met	15mg/L	Adenosine	12mg/L
L-Phe	100mg/L	Ornithine-HCl	10mg/L
L-Thr	48mg/L		pH: 7.4
L-Try	10mg/L		

10mL of MEM non-essential amino acids (Gibco), 10mL of MEM Vitamins (Sigma) and 10mg of Phenol Red were added to 1L of medium and the pH adjusted. The medium was filter sterilised, aliquoted at 450mL and kept at 4°C.

Supplemented MEM-Pros medium, 500mL

MEM-Pros with:

Heat-inactivated FCS (Gibco)	10% (v/v)
Hemin	7.5 mg/L (1.5mL of stock solution)
Penicillin/Streptomycin	50U/L (5mL of Penicillin-Streptomycin (5000U/ml), Sigma)

*Hemin stock: 0.25% in 0.1M NaOH, autoclaved and stored at 4°C. *Cultures of procyclic trypanosomes under 1 x 10⁵ cells/mL do not grow unless in conditioned medium, which is obtained after centrifugation and sterile filtration of a well-established culture of procyclic cells. It contains undetermined factors that enable growth of low-density cultures.*

2.1.5 Stable transfection of procyclic trypanosomes

All centrifugation steps were done at 2,000 rpm for 10 min at RT (Heraeus).

Cells were grown to log phase of culture (2-4 x 10⁶cells/mL). 2 x 10⁷ cells were washed once in 10mL of ice cold ZPFM (Zimmerman post-fusion medium). The pellet was resuspended in 0.5mL of ice-cold ZPFM and transferred to a cold 1mL cuvette (BTX) on ice. 10µg of sterile linearized plasmid DNA was added and mixed by pipeting up and down. The cells were electroporated as described for bloodstream forms (Section 2.1.4.). The cells were then transferred to 10mL MEM-Pros with appropriate antibiotics according to cell background and incubated overnight at 30°C. The following day, selection antibiotic was added to this culture and 1.5mL distributed to each well on the top row of a 24-well microtiter plate. 500µL of conditioned medium (i.e., medium obtained from a previous culture, centrifuged and sterile-filtered) with appropriate antibiotics, was aliquoted into each of the remaining wells. The cells were distributed vertically following the principle of limiting dilution: 0.5mL of the culture in the first well on the first row was transferred to the second well. The dilution was done consecutively for the third and fourth well, always with cells from the immediate-upper well. Stable clones were established between 7-14 days with adequate cell densities. The cultures were often scaled up in volume (e.g. 5mL) for further culture and downstream analysis.

5X ZPFM

NaCl	123mM
KCl	8mM
Na ₂ HPO ₄	8mM
KH ₂ PO ₄	1.5mM
MgAc	1.5mM
Ca(OAc) ₂	90μM
pH:	7.0 (with NaOH or acetic acid)

The medium was filter-sterilized and stored at 4°C.

2.1.6 Antibiotics used for selection of recombinant trypanosomes

- **Phleomycin (Cayla):** Stock solution: 5mg/mL, stored at 4°C.
Concentrations used in culture: 0.2μg/mL (bloodstream); 0.5μg/mL (procyclics).
- **Hygromycin B (Life technologies):** Stock solution: 50mg/mL, stored at 4°C.
Concentrations used in culture: 15μg/mL (bloodstream); 50μg/mL (procyclics).
- **Neomycin (G418, Gibco).** Stock solution: 50mg/mL, stored at 4°C.
Concentrations used in culture: 0.5μg/mL (bloodstream); 12μg/mL (procyclics);
- **Blastidicin (Invitrogen).** Stock solution: 5mg/mL, stored at -20°C.
Concentration used in culture: 10μg/mL (procyclics); 5μg/mL (blood stream).
- **Puromycin (Sigma).** Stock solution 1mg/mL, stored at 4°C.
Concentration used in culture: 0.2ug/mL (bloodstream); 1 μg/mL (procyclics)

2.1.7 Tetracycline-inducible cells

The 427-449 and 427-1313-cell lines (both bloodstreams and procyclics) have integrated the *Tn10 tet* repressor gene and express it constitutively. These cells can be transfected with inducible trypanosome expression vectors i.e., constructs containing a promoter with two operator sites where repressor molecules bind hence preventing promoter driven transcription. When Tetracycline (Tet) is added to the culture medium, it binds the repressor molecules, which then fall off the operator. This allows transcription of a given gene under the control of the (inducible) promoter. Tet-inducible vectors used in this work were pHd676 (with the PARP RNA pol 1 promoter), pHd918, p2T7-177-Hyg and p2T7^{TA}Blue (with the bacteriophage T7 promoters), pHd1484, pHd1485.

2.2 Basic methods for nucleic acids and proteins analysis

2.2.1 Phenol extraction

One volume (vol) of a 1:1 mix of Roti®-Phenol (Roth, equilibrated in TE buffer) and chloroform was added to DNA or RNA solution in TE or water respectively. The samples were gently mixed by inversion and centrifuged for 5 min at 13,000 rpm at RT. The aqueous phase was then ethanol precipitated.

TE buffer

Tris-HCl	10mM (pH 7.5)
EDTA	1mM

2.2.2 Ethanol precipitation and washes

To a DNA or RNA sample, 10% of its volume of 3M NaAc (pH 5.2) and 2.5 volumes of 100% ethanol were added. After 30 min at -20°C, the samples were centrifuged at 13,000 rpm for 15 min at 4°C. The pellet was washed with 70% ethanol and air-dried. The pellet was then resuspended (TE/water) and incubated at 55°C for 10 min to fully dissolve the sample. Samples were then stored in NaAc/ethanol mix at -20°C (DNA) or at -80°C (RNA). The samples were centrifuged and washed just before use.

2.2.3 TCA precipitation

If needed, proteins in aqueous samples were concentrated adding TCA at a final concentration of 10% and DOC (deoxyholic acid) at a final concentration of 0.15%. Then, the sample was mixed and incubated overnight on ice. The sample was vortexed briefly and centrifuged at 13,000 rpm for 15 min at RT. The pellet was washed with 3 volumes of original sample volume of ice-cold acetone, incubated at RT for 10 min, then centrifuged at 13,000 rpm for 15 min. The pellet was air-dried at room temperature for 10 min and stored at -20°C or dissolved in 1 X Laemmli buffer (Section 2.8.4), denatured for SDS-PAGE (Section 2.8.3).

100% TCA

TCA 100g in 41mL H₂O

100% TCA + DOC

100% TCA	10mL
DOC	40mg

2.3 Recombinant DNA technology

2.3.1 PCR

100ng of plasmid or genomic DNA were used as template in a 50 µL-PCR reaction. The reaction mix included 1X PCR-buffer, 0.2 mM dNTPs, 1pmol each reverse and forward primers and 0.5 U of *Taq*-DNA pol (Amersham). A reaction volume of 25µL was used for colony PCR (Section 2.4.3.3.). The tubes were placed in a thermocycler (RoboCycler®Gradient 96, Stratagene); the standard PCR steps were: DNA denaturation at 94°C for 1-5 min, 20-30 cycles of denaturation, primer annealing and elongation, and 10 min of elongation at 72°C. The annealing temperature depended on the primer pairs used.

The PCR reaction was analyzed by running 5 µL in an ethidium-bromide-stained agarose gel. If there were a single PCR product, the PCR product was then purified using a QIAquick PCR purification Kit (Qiagen Inc.). If there were more than one product, the whole sample was electrophoresed and the specific product (determined by size) purified from the agarose gel (NucleoSpin Extract, Macherey Nagel). For cloning purposes, the primers were often designed with restriction endonuclease sites. These enabled the ligation of the product into a vector DNA cut with the same restriction enzymes that generate compatible ends (sticky end-ligation).

10X *Taq*-DNA pol buffer

Tris-HCl	100 mM (pH 9.0)
KCl	500mM
MgCl ₂	15mM
Triton X-100	1% (v/v)

dNTPs

2µL of each of 100mM dATP, dGTP, dTTP and dCTP were added to an eppendorf tube and the volume made up to 100µL with sterile water. This resulted in a final concentration of 200µM for each dNTP. 5µL of this solution was used in a 50µL PCR reaction.

Forward and reverse primers

1µL of each primer (100pmol/µL) was used in a 50µL PCR reaction

2.3.2 Restriction endonuclease digests

Each enzyme was used in conditions recommended by the manufacturer - Amersham or New England BioLabs. Usually, 2-3U of enzyme were used to digest 1µg of DNA, taking care that the enzyme (usually stored in glycerol-containing buffers) constituted less than 10% of the

total reaction volume. Restriction enzyme digests were incubated for 2 h, at the optimum temperature. In case of digestion at DNA-ends (like PCR products), the reactions were incubated overnight. Cleavage of DNA substrate with two restriction endonucleases simultaneously (double digestion) was done in the buffer that resulted in the maximal activity for both enzymes. If no one buffer met the buffer requirements of both enzymes, the reactions were performed sequentially. First, cleavage was done with the restriction endonuclease that requires the lower salt reaction conditions, then salt concentration was adjusted (using a small volume of a high concentration salt solution) to approximate the reaction conditions of the second restriction endonuclease. The second enzyme was added and incubated to complete the second reaction.

2.3.3 Creation of blunt ends in DNA fragments

Following restriction enzyme digestion that generated 5'- or 3'-protruding ends, the desired fragment was gel purified (section 2.3.6.) and the overhangs filled-in as described below:

2.3.3.1 Removal of 3'-overhangs

About 3-5 μ g of digested DNA was incubated (30 sec, 37°C) with 1 μ L of T4-DNA polymerase (Amersham, 4U/ μ L), 1X DNA polymerase buffer and 1 μ L of dNTPs in a 20 μ L-reaction was added for further 5min of incubation. The reaction was stopped by heat inactivation (75°C for 10min).

2.3.3.2 Fill-in of 5'-overhangs

Approximately 3-5 μ g of DNA was incubated (15min at RT) with 1 μ L of the Klenow fragment of DNA Polymerase (USB, 5U/ μ L), 2 μ L of 10X buffer L (Amersham) and 1 μ L of dNTPs in a 20 μ l reaction volume. The enzyme was heat-inactivated (75°C for 10min).

dNTPs

dATP, dGTP, dTTP and dCTP 2mM (final concentration of each)

10X Buffer L (Amersham)

Tris-HCl	100mM (pH 7.5)
MgCl ₂	100mM
DTT	10mM

2.3.4 Dephosphorylation of 5'-ends

The removal of a phosphate group from a linearised plasmid with Calf Intestinal Phosphates (CIP, Boehringer Mannheim, 1 U/ μ L) prevents it from self-ligation and lowers background. About 2 μ L of CIP were added to the restriction digest and incubated at 37°C for 15 min. The tube was transferred to 55°C for 15 min. The reaction was stopped by addition of 2 μ L of 500mM EDTA and heat inactivation (75°C for 10 min). The DNA was purified by using the Qiagen Miniprep kit, or by gel extraction.

2.3.5 Agarose gel electrophoresis

Gels were prepared by melting 0.8-1.8 g agarose (Gibco) in 100mL of TAE buffer in a microwave oven. Ethidium bromide (approximately 30 μ g, from a stock solution at 10mg/mL) was added for UV visualization. DNA molecules fractionated on 1 X TAE buffer for 30-90 min at 100-120V, and were then visualized and photographed under UV light.

TAE buffer

Tris-HCl	40mM
EDTA	1mM (pH 8.0)
Acetic acid	0.11%

2.3.6 Purification of DNA fragments from agarose gels

All steps were at RT. The purifications were done using a NucleoSpin® Extract kit (Macherey Nagel) following the manufacturer's instructions.

2.3.7 Ligation of DNA fragments

DNA inserts excised from plasmid or PCR-generated were cloned into a vector prepared as described in sections 2.3.2 to 2.3.6. DNA fragments were ligated using 1U of T4-DNA ligase, with molar vector:insert DNA ratios of about 1:3. 1 μ L each of 10X ligation buffer and T4-DNA-ligase were added and the volume made upto 10 μ L with Nuclease-Free water. The ligation reactions were incubated at 16°C overnight.

10X Ligation buffer

Tris-HCl	0.5M (pH 7.6)
MgCl ₂	0.1M
ATP	10mM
DTT	10mM

2.4 Amplification of recombinant DNA in bacteria

2.4.1 Preparation of competent cells

All centrifugation steps were carried out at 4°C.

Escherichia coli DH5 α cells were taken from a frozen stock at -80°C and incubated overnight at 37°C on an antibiotic-free LB-agar plate. A single colony was picked and used to inoculate 5mL of ψ B medium. The culture was incubated at 37°C with vigorous shaking for about 2 h ($OD_{550} \sim 0.3$). The cells were then transferred to 100mL of medium pre-warmed to 37°C and incubated for another 2 h ($OD_{550} \sim 0.48$). The culture was transferred into a Corex® tube, incubated on ice for 5 min, and then centrifuged at 6,000 rpm for 5 min (Sorvall, SS34 rotor). The bacterial pellet was re-suspended in 40mL of cold TfbI buffer, incubated on ice for 5 min and centrifuged again at 6,000rpm for 5min. The pellet was re-suspended in 4mL of cold buffer TfbII, incubated on ice for 20min then aliquoted 200 μ L/tube for storage at -80°C.

DH5 α Cells (Promega)

Genotype: *supE44, ΔlacU169(Δ80lacZΔM15), hsdR17, gyrA96, thi-1, relA1*

ψ B medium

Trytone peptone (Difco)	20 g/L
Yeast extract (Difco)	5 g/L
MgSO ₄ (20mM)	5 g/L
pH 7.6 (with KOH)	
The medium was autoclaved and stored at RT	
For agar plates: Bacto™ AGAR (Difco)	15 g/L

TfbI buffer

KAc	30mM
RbCl ₂	100mM
CaCl ₂	10mM
MnCl ₂	50mM
Glycerol	15%
pH 5.8 (0.2M acetic acid)	

TfbII buffer

MOPS	10mM
CaCl ₂	75mM
RbCl ₂	10mM
Glycerol	15%
pH 6.5	(0.1 M KOH)

Both buffers were sterile-filtered and stored at 4°C.

2.4.2 Transformation of competent cells with recombinant DNA

Approximately 5 μ L of a ligation reaction were mixed with a 25 μ L aliquot of competent *E.coli* DH5- α (thawed on ice). The mix was incubated on ice for 30 min and heat-shocked in a water bath at 42°C for 45 sec. 200 μ L of ψ B medium was added and the cells incubated at 37°C for 45 minutes. An aliquot of the transformation was spread out on LB-agar plates containing appropriate antibiotic (section 2.4.3.1.).

<u>Luria Bertani (LB) Medium</u>	<u>LB Medium + Agar (LB-Agar)</u>
Tryptone	10g/L
Yeast extract	5g/L
NaCl (170mM)	10g/L
	LB Medium
	Bacto-Agar 15g/L

2.4.3 Selection of transformants

2.4.3.1 Antibiotic selection

To select for transformants, an aliquot of the transformation reaction was spread on LB-Agar plates containing appropriate antibiotic. Ampicillin was used at a final concentration of 100 μ g/mL; kanamycin at 30 μ g/mL and chloramphenical at 20 μ g/mL. The antibiotics were added after the medium had cooled to about 50°C.

2.4.3.2 Blue–White selection of recombinant bacteria

Whenever a cloning plasmid contained a polylinker (MCS: multiple cloning site) within the β -galactosidase gene, the cloning of an insert into this site disrupted the β -galactosidase gene. Bacteria that are transformed with this recombinant DNA do not metabolize galactose, whose metabolic product is blue in the presence of IPTG. These colonies appear white after an overnight growth at 37°C. Colonies transformed with wild type plasmid (plasmid without insert) give blue colonies. Plasmids with this color-selection system used in the work presented in this thesis are pBlueScript (Stratagene), pGEM-T (Promega) and p2T7^{TA}Blue.

The transformed bacteria were streaked on LB-Agar plates supplemented with 1.5mg of X-Gal (a metabolisable analog of galactose) (80µL of a 2% stock solution was spread on a plate ready to use) and 1mg of IPTG (5µL of a 830mM stock solution was spread on the plate). Development of blue color of colonies was sometimes enhanced by a 1-2h incubation at 4°C. A number of white colonies were screened for DNA inserts by colony PCR (Section 2.4.3.3).

DNA mini-preps (see 2.5.1) or restriction digests and agarose gel analysis. Putative clones were then confirmed by DNA sequencing.

2% X-Gal

2 mg/mL in Dimethylformamide
Stored in the dark at -20°C

830 mM IPTG

2 g/10mL
Stored at -20°C

2.4.3.3 Screening bacterial transformants by colony-PCR

A PCR cocktail composed of PCR buffer, dNTPs, primers, and *Taq*-DNA polymerase and sufficient for the number of colonies to be screened was prepared. Twenty-four bacterial colonies were picked with the aid of a 10µL-pipette tip and resuspended individually in 20µL of the PCR cocktail. Each colony was also patched onto a separate plate for reference. The reaction was incubated at 94°C for 10 min to lyse the cells and to inactivate nucleases. The amplification was done for 20-30 cycles in the RoboCycler® as follows: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The final extension was performed at 72°C for 10 min after which the reaction was held at 4°C. The products (10µL) were electrophoresed on a 1-1.2% ethidium bromide stained-agarose gel and visualized under UV. Clones were identified, larger quantities of DNA made (mini- or maxi DNA preps) and 8% glycerol stocks made and kept at -80°C.

2.5 Analysis of transformants

2.5.1 Plasmid DNA mini-preps

All centrifugation steps were performed at RT.

Up to 24 bacterial colonies were picked and resuspended individually into 3mL of LB supplemented with appropriate antibiotic. The cultures were incubated at 37°C with vigorous shaking for 12-16 hours. 1.5mL of the overnight cultures were centrifuged in an eppendorf tube at 13,000 rpm for 1min. The pellet was resuspended in alkaline lysis buffer of the Eppendorf FastPlasmid™ Mini kit (Eppendorf AG, Hamburg, Germany) and processed according to the manufacturers instructions. The DNA minipreps were screened for the desired clones by restriction endonuclease digestion, agarose gel analysis and confirmed by DNA sequencing at Medigenomix GmbH (Martinsried, Germany). A 100µL aliquot of bacterial culture was mixed in a ratio of 1:1 with 16% glycerol in LB and kept at -80°C.

2.5.2 Midi-preparation of plasmid DNA

All centrifugation steps were performed at 4°C.

After an overnight growth at 37°C, up to 100 mL of bacterial culture were centrifuged at 6,000 rpm for 10min (Sorvall, GSA rotor). The resulting pellet was carefully re-suspended in 6mL of cold buffer P1 and transferred to an SS34-rotor centrifuge tube. 6mL of freshly prepared 0.2N NaOH/1% SDS were added, mixed and allowed to lyse the cells for 5 min. The lysate was mixed with 6mL buffer P3 and incubated on ice for 20 min. Large cellular fragments were separated by centrifugation at 13,000 rpm for 30min (Sorvall, SS34 rotor). The clear supernatant was passed through a column (Tip100, Qiagen) previously equilibrated with 4 0mL of buffer QBT. The column was washed twice with 20mL of buffer QC and the DNA eluted with 5 mL of buffer QF into a clean SS34-tube. The DNA was precipitated with 3.5 mL of isopropanol and centrifuged at 11,000 rpm for 30min. The pellet was air-dried and re-suspended in 300 μ L of TE buffer and transferred into an eppendorf tube. The midi-prep DNA was used for a variety of downstream processes, e.g. transfections.

Buffer P1

Tris-HCl	50mM (pH7.8)
EDTA	10mM (pH8.0)
RNase A	100mg/L (Sigma)

Buffer P3

KAc	3M
Glacial Hac	(50% v/v)
pH 4.8 (acetic acid)	

Buffer QBT

NaCl	0.75M
MOPS	50mM
Ethanol	15%
TritonX-100	0.15%
pH 7.0	

Buffer QC

NaCl	1M
MOPS	50mM
Ethanol	15%
pH 8.2	

Buffer QF

NaCl	1.25M
MOPS	50mM
Ethanol	15%
pH 7.0	

2.5.3 Extraction of *T. brucei* genomic DNA

All centrifugation steps were performed at RT.

Between 3-10 x 10⁷ cells were centrifuged at 2000 rpm for 10min. The cell pellet was resuspended in 150 μ L of Tris-EDTA-LiCl-Triton X-100 (TEL-T) buffer, mixed gently by inversion and incubated for 5 min. 150 μ L of equilibrated phenol-chloroform was added, mixed by vigorous shaking and centrifuged at 13,000 rpm for 5 min. The upper aqueous phase was aspirated into a new tube containing 500 μ L of 100% ethanol. The tube was swirled gently for 15 seconds, incubated for 5min and centrifuged at 13,000 rpm. The DNA pellet was

washed with 70% ethanol and air-dried. The pellet was then resuspended in 30-100 μ L of TE and incubated with RNase A for 30min. Ethanol precipitation was repeated to remove DNase A.

TELT Buffer

Tris.Cl	50mM (pH 8.0)
EDTA	62.5mM (pH 9.0)
LiCl	2.5mM
Triton X-100	4% (v/v)

2.5.4 Southern blotting and hybridization conditions

Between 5-10 μ g of genomic DNA was subjected to a single or double restriction enzyme digest in a total volume of 100 μ L in conditions recommended by the suppliers. After an overnight digestion, a fresh aliquot of enzyme(s) was added and the reaction continued for 2 h to ensure complete digestion. The reaction was concentrated to 20 μ L by ethanol precipitation and loaded onto an 0.8% agarose gel. Electrophoresis was run overnight at about 20V in 1 X TAE. The gel was photographed under UV light, rinsed briefly in water and depurinated in a tray of 0.25 M HCl with gentle agitation for 10 min. The gel was transferred into denaturing solution and gently agitated for 30 min. It was rinsed briefly in water and placed in neutralisation solution for 30 min with gentle agitation. The DNA was then transferred overnight to a nylon membrane (Hybond-N⁺, Amersham) by downward capillary blotting using a Turbo blotterTM apparatus (Schleicher & Schuell) and 10X SSC as transfer buffer. The blot was rinsed in 5 X SSC for 5min, air-dried and UV-crosslinked (UV Stratalinker 1800, Stratagene). The blot was prehybridized for 2 h in pre-hybridization solution. Hybridization was done overnight at 60°C with 100ng of denatured α -[³²P]-labeled probe (Section 2.6.4) in hybridization solution. After hybridisation the membrane was washed 3 times each for 15 min in 10mL of wash buffer at 50°C. The blot was exposed on an X-Omat X-ray film with intensifying screens and kept at -80°C for 2-5 days.

Denaturing Solution

NaCl	1.5 M
NaOH	0.5M

Prehybridisation Solution

5X Denhardt's reagent
6X SSC
0.5% SDS
Herring Sperm DNA (Serva) 10 μ g/mL

Neutralizing Solution

NaCl	1.5 M
Tris-HCl	0.5 M (pH7.2)
EDTA	1mM

Hybridization solution

5X Denhard's reagent
6X SSC
0.5% SDS (w/v)
Herring Sperm DNA 10 μ g/mL

20X SSC

NaCl	3M
Na-Citrate	0.3M
pH 7.2	

50X Denhardt's Solution

BSA	1% (w/v)
Ficoll	1% (w/v)
Polyvinilpyrrolidone	1% (w/v)
Filtered and stored at -20°C	

Southern blot wash buffer

1X SSC
0.5% SDS (w/v)

DNA for probes or Herring sperm DNA was denatured by incubation at 95-100°C for 5-10min and immediately put on ice. The probe was saved and stored at -20°C and again denatured before use.

2.5.5 Cloning of *TbRBP3* and AATP 3'-UTR

The open reading frames of *TbRBP3* gene was amplified by PCR using 427 pc genomic DNA and primers listed here.

***TbRBP3* (Tb11.03.0550)**

cz 1685

Sense primer used to amplify of *TbRBP3* ORF for over-expression.

cz 1686

Antisense primer used to amplify of *TbRBP3* for over-expression.

cz 2335:

GCTAAGCTTATGGAACCGGAGTGGAAAGTTCCAGTAG

Sense primer with a Hind III site used to amplify *TbRBP3* for myc- and TAP tagging. It has a point mutation from G to T at 21 nt. to eliminate an internal Hind III site.

cz 2336:

GCTGTTAACATTCTGCTGCTGCTGC

Antisense primer with a Hpa I site and without stop codon used to amplify *TbRBP3* for TAP tagging.

cz 2342.

GCAGGATCCATTCTGCTGCTGCTCT

Antisense primer with a Bam HI site and without stop codon used to amplify *TbRBP3* for myc tagging,

cz 2143

CATCTCGAGTGGAACCGGAGTGTGAAAG

Sense primer with an Xho I site for amplifying a 526 bp fragment of *TbRBP3* for RNAi.

cz 2144

AGTGGATCCGCATTGTAAACATGGCGG

Antisense primer with a Bam HI site for amplifying a 526 bp fragment of *TbRBP3* for RNAi.

cz 2425

CAGGAGCTCCGTCACACATGCCTGAG

Sense primer with a Sac II site to amplify 5'-UTR of *TbRBP3* for V5 *in situ* tagging.

cz 2430

CAGCCGCCGGCGTCACACATGCCTGAG

Antisense primer with a Xba I site to amplify 5'-UTR of *TbRBP3* for V5 *in situ* tagging.

cz 2427

GTACTCGAGATGGAACCCGGAGTGTG

Sense primer with an Apa I site to amplify 5' CDS of *TbRBP3* for V5 *in situ* tagging.

cz 2431

GTAGGGCCCACGCTCTCAATGGAC

Antisense primer with a Xho I site to amplify 5' CDS of *TbRBP3* for V5 *in situ* tagging.

AATP 11 3'-UTR

cz 1948

ATATGTTAACCTCGAG(T)₁₈(AGCT)₃

Oligo dT primer with Hpa I and Xho I sites used to define the AATP 3'-UTR.

cz1951

TTGGCTTATATTCCCCGCACTCTTCATC

Sense primer used to define the AATP 3'-UTR.

cz 1952

GTAAGGATCCGGAGGAGGCAGCGTAGCAGGAG

Sense primer with a Bam HI site used to define and to amplify the AATP 3'-UTR.

cz 2085

TGAAGCATCCCCCTTATTCAACACCTC

Antisense primer to amplify the region between 40-630 nt of the AATP 3'-UTR.

cz2086

TATTGTTAACACAACGAAAATAAGACAAGTGG

Sense primer with a Bam HI site to amplify the region between 40-630 nt the AATP 3'-UTR.

cz 2087

AAATCAATGCGTACCAATTATTGATGCAA

Antisense primer to amplify the region between 1-141 nt the AATP 3'-UTR.

cz 2088

AATTGGTAGGCATTGATTATAGAGAC

Sense primer to amplify the region between 260-833 nt the AATP3'-UTR.

cz 2089

ATATGTTAACCCGAAAGTATCGCCTG

Antisense primer with a Hpa I site to amplify deletions of the AATP3'-UTR.

cz 2090

TAAGAGCTCAGTGTCTCTATAATC

Antisense primer to amplify the region between 1-290 nt the AATP 3'-UTR.

cz 2091

GACACTGAGCTCTATTCGTTCTGTTGC

Sense primer to amplify the region between 618-833 nt the aatter 3'-UTR.

cz 2184

TCCTCACGCAGCTCAGTGTCTTATAA

Antisense primer to amplify the region between 1-290 nt the AATP 3'-UTR.

cz 2185

GAGCTGCGTGAGACTCAACCAATAC

Sense primer to amplify the region between 350-833 nt the AATP 3'-UTR.

cz 2186

GAAAAGTTAGAAAGTATTGCTTGAGTCC

Antisense primer to amplify the region between 350-833 nt the AATP 3'-UTR.

cz 2187

CTTTCTTAACCTTTCTCTTTAATCTTG

Sense primer to amplify the region between 420-833 nt the AATP 3'-UTR.

cz 2190

CGCGCTAGCAACTCACCAAGATTA

Antisense primer to amplify the region between 610-833 nt the AATP 3'-UTR.

cz 2191

GAGTTGCTAGCGCGGCTCAATGGA

Sense primer to amplify the region between 1-535 nt the AATP 3'-UTR.

cz 2240

ATCGTTAACAAATGTTCCCCAATCA

Antisense primer used to amplify the intergenic region of the AATP 11.

cz 2389

GCTGGATCCCGTCTTCCGTCCACTGAG

Sense primer with a Bam HI site to amplify the region between 290-618 nt of the aatter 3'-UTR. To use for cloning in pBS SKII.

cz 2390

GCTGTTAACACAAGTGGAGCAAATAAGAA

Antisense primer with a Eco RI site to amplify the region between 290-618 nt of the aatter 3'-UTR. To use for cloning in pBS SKII.

cz 2436

AGCTGGATCCGGCTTAGTATAGCGAGGTTAGCTACACTCG

Sense primer used to amplify J6f1f aptamer.

cz 2437

GCATGGATCCGAATTGGCTCAGCACGAGTGTAGCTAAACC

Antiense primer used to amplify J6f1f aptamer.

cz 2489

GCAAAGCTTATGTCCCCTATACTAGG

Sense primer with a Hind III site to amplify ORF from GST.

cz2490

GCAGATCTCTCACGAGCGGCCGCT

Antisense primer with a Hpa I site to amplify ORF from GST.

cz 2497

GCAAAAGCTTATGGCTTCTAACTTACTCAG

Sense primer with a Hind III site used to amplify MS2 coat protein.

cz 2498

GCAGGATCCATTAGTAGATGCCGGAGTTG

Antisense primer with a Bam HI site used to amplify MS2 coat protein.

cz 2514

GCTAGTTAACCTTGTCCCGCTCGTCCAT

Antisense primer with a Hpa I site for amplifying EGFP.

cz 2585

GCTGGGCCATGGTGAGCAAGGGCGAGGAGTCT

Sense primer with an Apa I site for amplifying EGFP

cz 2583

GCTAAGCTTATGGCCGCACACACGACG

Sense primer with a Hind III site used to amplify λ N peptide.

cz 2584

GCTGGGCCGGTGGCTAGCCGCCGTTGCAGC

Antisense primer with an Apa I site used to amplify λ N peptide.

ORF-PCR products were cloned into pHD615 (for expression in pc cells, (Biebinger, Wirtz et al. 1997)), pHD617 (bs, (Biebinger, Wirtz et al. 1997)), and into pHD918 (for TAP-tagging,

(Estevez, Kempf et al. 2001)). RNAi-fragments were cloned either into p2T7-177 (LaCount, Bruse et al. 2000).

RT-PCRs and IPs

cz 2478

GAATGCCCTTATGTAAGTAA

Sense primer to amplify Cyclin F box domain CDS.

cz 2481

CAGCAAATGGAGCACCACTG

Antisense primer to amplify Cyclin F box domain CDS.

cz 2578

ATGTCGGACGAGGAACAAAC

Sense primer to amplify actin CDS.

cz 2579

CGACTCGTCGTATTCACTCT

Antisense primer to amplify actin CDS.

cz 2719

ATGTTATGCAGGTGGGAAC

Sense primer to amplify tubulin CDS.

cz 2581

CCTTGCGACAACGTCACCACGG

Anntisense primer to amplify tubulin CDS.

cz 2232

ACGCTGGTGCTACGCCCTGAA

Sense primer that anneals at the end of CAT ORF.

czZFP-Fw

TATAAAGCTTATGTCCTCTCAATGAACACAG

Sense primer to amplify CDS from Tb07.11L3.90.

czZFP-Rev

TAATGGATCCCTGCCCTAAAGAAGTTGCGC

Antisense primer to amplify CDS from Tb07.11L3.90.

cz2795

GACAAGCTTATGCACATTCAACACCC

Sense primer to amplify CDS from ZFP1.

cz2796

GACGGATCCTCAATCCTCCAACCTTAC

Antisense primer to amplfy CDS from ZFP1.

2.6 Isolation and analysis of RNA

2.6.1 Extraction of *T. brucei* total RNA

Total RNA was isolated from $2\text{-}4 \times 10^7$ cells from cultures with densities between $1\text{-}2 \times 10^6$ cells/mL (bloodstream form) or $3\text{-}4 \times 10^6$ cells/mL (procyclics). The cells were centrifuged at 2,000 rpm for 10 min at RT and the pellet resuspended in 1mL of pegGOLD TriFastTM (Peqlap, GmbH). 200µL of chloroform was added and shaken vigorously and the tube stood at room temperature for 5 min. The mix was centrifuged at 13,000 rpm for 15min at 4°C. The aqueous phase was pipeted into 500µL of isopropanol and RNA precipitated for about 30 min at RT. For use, the RNA pellet was redissolved into appropriate volume of RNase-free water. Alternatively total RNA was isolated using RNeasy Mini/Midi Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

2.6.2 Northern blotting

Approximately 25µg of total RNA or 4µg of poly(A)⁺ RNA were precipitated with 1/10 volumes of 3M NaAc (pH 5.4) and 2.5 volumes of ethanol for 15 min on ice. The tube was centrifuged at 13,000 rpm for 20 min at 4°C and the pellet washed with 750µL of 70% ethanol. The RNA pellet was dried on a heat block at 37°C for 5 min. 15µL of loading mix was added to the pellet and incubated at 37°C for 10 min. The tube was vortexed briefly to dissolve the RNA and incubated at 65°C for 10min, chilled on ice for 5 min and loaded on a gel. 5µg of molecular size marker (Invitrogen's 0.24-9.4kb marker) were treated as the samples and loaded along side the samples. The RNA was resolved on a 1% agarose/formaldehyde gels at 18-20V in 1X MOPS. After electrophoresis, the gel was photographed under UV light and the RNA transferred onto a neutral nylon membrane (Nytran® Scheicher and Schuell) using the Turbo-blotter apparatus. The RNA was cross-linked in UV light in a stratalinker (Stratagene). Prehybridisation was done for 2 hours at 65°C in Northern blot Prehybridisation Buffer. Hybridisation was done overnight at 42°C with 100ng of $\alpha\text{-}[^{32}\text{P}]$ -random-prime-labelled DNA probe. The blot was washed once in Wash Buffer I at room temperature for 30 min, then once in Wash Buffer II at 42°C for 45 minutes, and finally once in Wash Buffer III at 42°C for 30 minutes. The blot was exposed on an X-Omat X-ray film with intensifying screens and kept at -80°C for 1-5 days.

<u>10X MOPS buffer</u>	<u>RNA gel</u>	<u>RNA loading Mix</u>
MOPS 200mM	Agarose 1% (w/v)	Formamide 50% (v/v)
Na-acetate 80mM	Formaldehyde 0.5M	Formaldehyde 6.5% (v/v)
EDTA 10mM	1X MOPS	1X MOPS
pH: 7.0		EtBr 10µg/mL

<u>Prehybridisation Buffer</u>	<u>Hybridisation Buffer</u>
5X Denhard's reagent	Formamide 50%
6X SSC	6X SSC
1% SDS	1% SDS
Herring Sperm DNA 100µg/mL	Herring Sperm DNA 100µg/mL

<u>Wash Buffer I</u>	<u>Wash Buffer II</u>	<u>Wash Buffer III</u>
10X SSC	1X SSC	0.1X SSC
1% SDS	0.5% SDS	0.2% SDS

2.6.3 Random prime labeling of DNA probes

DNA probe labeling was done using the Prime-It® II Random Primer labeling Kit (Stratagene) following the manufacturers instructions. Briefly, the DNA fragment to be labelled was diluted to 25ng/mL in TE. 4µL (100ng) was made up to 20µL with sterile water and the probe denatured at 95°C, 5 minutes and chilled on ice. To 1.5mL eppendorf tube in an ice bath, the following reagents were added in the following order:

- 10µL Nucleotide mix
- 5µL Primers
- 20µL Denatured DNA (100ng)
- 1µL Enzyme solution (Klenow 5U)
- 3µL α-[³²P]-dCTP (30 µCi)
- 11µL Sterile distilled water

The contents were mixed gently by pipetting up and down and spun briefly to collect the contents at the bottom of the tube. The reaction was incubated at 37°C for 1 hour. The probe

was then purified using the Nucleotide Removal Kit (Qiagen), following the manufacturer's instructions.

2.7 Isolation and analysis of *T. brucei* proteins

2.7.1 Extraction of total protein

All centrifugation steps were done at 4°C. 2×10^6 cells were centrifuged at 2,500 rpm for 10 min and washed with PBS (with complete protease inhibitor, Roche Applied Science) and re-centrifuged. The supernatant was carefully aspirated leaving about 10-15 μ L of buffer. This was either stored at -20°C till used or an equal volume of 2 X Laemmli buffer was added and the sample denatured (by boiling for 95°C for 5 minutes) for SDS-PAGE.

2.7.2 Determination of protein concentration by the Bradford protein assay

The Bradford Assay is a rapid and accurate method commonly used to determine the total protein concentration of a sample. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. Within the linear range of the assay (~5-25 μ g/mL), the more protein present, the more Coomassie binds. Protein standards containing a range of 0 to 25 μ g BSA were prepared from a stock of 1 μ g/ μ L to a standard volume of 1 mL (see Table 2.1).

Table 2.1 Preparation of a standard curve for protein determination

Tube	1	2	3	4	5	6
BSA (μ L)	0	5	10	15	20	25
1M NaOH (μ L)	50	50	50	50	50	50
Dist. Water (μ L)	950	945	940	935	930	925
BSA (μ g/mL)	0	5	10	15	20	25

The Bradford reaction was initiated by adding 800 μ L, from the dilution series above, to 200 μ L of Bradford dye reagent in corresponding tubes labeled 1B to 6B. The reaction was incubated at room temperature for 5 min and the absorbance measured at 595 nm against the blank (tube 1B). A standard curve of absorbance versus BSA concentration was prepared and used to determine protein concentration in the test samples taking into consideration any dilution factor.

2.7.3 SDS-PAGE

About 8mL 12% running gel were poured into a mounted mini protean electrophoresis apparatus (BioRad) overlayed with water and allowed to polymerise. 2.5 mL of stacking gel were then added and a comb slid between the glass plates. Once polymerised the gel was placed in electrophoresis chamber and filled up with 1 X running buffer. Trypanosome samples (2×10^6 cells/lane) were loaded, the chamber connected to a power supply and proteins fractionated at 150V for about 45 min.

12% Separating gel (15mL)

Gel 30	6mL	Gel 30	0.65mL
1.5M Tris-HCl (pH 8.8)	3.75mL	0.5M Tris-HCl (pH 6.8)	1.25mL
Water	5.25mL	Water	3.05mL
10% APS	50 µL	10% APS	25µL
TEMED	10µL	TEMED	10µL

10X Running buffer, 1L

Tris (Roth)	30.3g
Glycine (AppliChem)	188g
10% SDS	100mL
pH 8.3	

2.7.4 Western blotting

All incubations and washes were at RT and with gentle agitation. Cell pellets of about 2×10^6 cells each were resuspended in 1 X Laemmli buffer and denatured on a heating block at 95°C for 5 minutes. The samples were fractionated by SDS-PAGE and transferred to a polyvinyl diflouropyrolidol (PVDF) membrane (Hybond-P, Amersham). Blotting was done at 100V for 1h at 4°C in blotting buffer using a BioRad apparatus. The membrane was then incubated in blocking buffer (blotto) for 1 hour (RT), washed once for 15 min and then twice for 5min with fresh changes of wash buffer. It was then incubated for 1h in blocking solution supplemented with antibodies at appropriate dilution. The blots were again washed as before to remove unbound primary antibody. The filter was then incubated for 30 min with Horseradish peroxidase (HRP)-conjugated secondary antibody diluted at 1:1000 in blocking solution. Unbound antibody was washed off with PBS-T (15 min x1, then 5 min x4). Specific protein bands were visualized by incubating the membrane in ECL™ detection reagents (Amersham) for 1 min and exposing for 10 sec to 60 min on ECL films. The membranes were

stripped for re-probing by incubating in stripping buffer for 30 min at 50°C and washing for 10 min in excess PBS-T.

<u>Blotting buffer</u>	<u>PBS-T buffer pH 7.6</u>
Tris	25mM
Glycine	192mM
Methanol	20%
<u>Blocking solution</u>	Tween-20 (Serva) 0.3%
Skimmed milk 10% (in PBS-T)	

Stripping buffer

Tris	62.5mM (pH 6.7)
SDS	2%
β-mercaptoethanol	100mM

2 X Laemmli buffer

Tris.Cl	0.25M (pH 6.8)
SDS	4%(w/v)
Glycerol	20%(v/v)
β-Mercaptoethanol	3%(v/v)
Bromophenol blue	Trace

2.7.5 Coomassie blue staining of SDS-PAGE gels

All steps were performed at RT. The gel was soaked for 30 min in Coomassie blue staining solution (with gentle agitation). Gels were destained at least three times, for 1 h each, in high Methanol solution and further in Low Methanol solution for up to 12 h. For record purposes, the gel was transferred to a solution with 20% ethanol/10% glycerol for 30-45 min and then vacuum-dried.

Coomassie blue staining solution

Coomassie Brilliant Blue R250	0.25%(w/v)
Methanol	45.4%(v/v)
Glacial acetic acid	9.2%(v/v)

High Methanol solution

Methanol	45.4%(v/v)
Glacial acetic acid	7.5%(v/v)

Low Methanol solution

Methanol	5%(v/v)
Glacial acetic acid	7.5%(v/v)

2.7.6 Preparation of dialysis tubes

Dialysis tubes (MWCO 12000-14000) were cooked 10 min in 2% NaHCO₃/1mM EDTA pH 8,0 and rinsed with dH₂O, and cooked 10 min in 1mM EDTA. Storage was in the same solution at 4°C and rinsed with H₂O before use.

2.7.7 *In vivo* labeling with [³⁵S]-Methionine

All centrifugation steps were at RT. 2x10⁷ cells were centrifuged for 10 min at 2,000 rpm and washed with 500µl labeling medium by centrifugation at 6'000 g for 2 min. The cell pellet was resuspended in 400µl and 150 mCi of [³⁵S]-Methionine (Amersham, *in vivo* labeling grade, 10 mCi/ml) was added. Cells were incubated for 1hr at 37°C (bloodstream) or 30°C (procyclics) and washed 2x with normal medium (6'000g for 2 min). Pellets were stored at –80°C until immunoprecipitation.

Labeling medium

ISCOVE's medium (Gibco) lacking Methionine, supplemented with 10mM glucose, 1.5mM L-Cysteine (only for bloodstream), 0.14% b-mercaptoethanol (only for bloodstream), and 10% heat-inactivated FCS (previously dialysed against 30mM HEPES pH 7.3 /150mM NaCl).

2.7.8 Immunoprecipitation

All centrifugation steps were at 4°C, 5 min at 13'000 rpm; incubations were also at 4°C. Each [³⁵S]-Methionine-labeled sample, kept at –80°C as pellets, was solubilised with 750µl solubilising buffer, placed for 30 min on ice (vortexing every 5-7 min) and centrifuged. The supernatant was put to rotate for 2 hrs or overnight with 40µl of Sepharose-protein A. After centrifugation, the supernatant was transferred to a new tube and incubated with the chosen antibody for 1 hr, also rotating, 40µl of Sepharose-protein A were added and centrifuged after 2 hrs. The Sepharose pellet was washed six times: three times with 500µl of buffer A, two times with 500ml of buffer B, and once with 500ml of buffer C. The last pellet was resuspended in 45ml of Laemmli buffer and denatured for SDS-PAGE; 15 ml (2x10⁶ cells) were loaded in each lane. Following fixation with Low Methanol Solution, the gel was soaked in 5x gel volume EN3HANCE (NEN, PerkinElmer) for 1 hr under gentle agitation. The used enhancer solution was discarded into an appropriate radioactive waste container. The gel was soaked in cold water and agitated for 45 min at 4°C, vacuum-dried (2 hrs at 80°C) and exposed to an X-ray film at –80°C.

solubilising buffer (10ml)

50mM Tris-HCl pH 7.4
 500mM NaCl
 2% IGEPAL CA-630 (Sigma)
 1 tablet complete protease inhibitor (Roche)

<u>buffer A</u>	<u>buffer B</u>	<u>buffer C</u>
10mM Tris-HCl pH 7.5	10mM Tris-HCl pH 7.5	10mM Tris-HCl pH 7.5
150mM NaCl	500mM NaCl	
0.2% IGEPAL CA-630	0.2% IGEPAL CA-630	
2mM EDTA	2mM EDTA	

Sepharose-protein A (Amersham Biosciences)

0.5 g were hydrated for 1 hr in 200 ml of 100 mM Tris-HCl at 4°C and then washed with the same buffer. For storing, 0.02% Na-azide was added. Before use, the slurry was equilibrated in solution A.

2.7.9 Immunoprecipitation to isolate mRNP complexes

The protocol of Tenenbaum et al. was adapted to isolate mRNP complexes (Tenenbaum, Carson et al. 2000; Tenenbaum, Lager et al. 2002). To this end, 3×10^7 cells expressing the tagged protein of interest and the CAT-ATTP 3'-UTR reporter construct were lysed in 400 μ L of polysome lysis buffer (100 mM KCl, 5 mM MgCl₂, 10 mM Hepes, pH 7.0, 0.5% Nonidet P-40), including one tablet of complete inhibitor (without EDTA, Roche) and 200 units RNaseIn (Promega) and 5 μ L Vanadyl Ribonucleoside complexes (Sigma). Pre-swollen IgA beads were preincubated with anti-I antisera for 2 hrs at 4°C in NT2 buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM MgCl₂, 0.05% Nonidet-P 40) and washed 6 times with 1 ml of NT2 buffer. 50 μ L of the pre-coated beads were incubated with the lysate for 2 hr at 4°C with rotation. This step was repeated, and then the beads were collected by centrifugation and the lysate was incubated with 50 μ L of either anti-Myc sepharose or anti-GFP precoated IgA beads for 2 hr. The supernatant was collected, the beads were washed 6 times with 1 mL of NT2 buffer and the mRNPs were eluted either by adding myc-peptide in NT2 buffer supplemented with 200 units RNaseIn and 5 μ L Vanadyl Ribonucleoside complexes (Sigma) and incubation for 20 min at 4°C, or just by adding 100 μ L of NT2-buffer. The eluate was supplemented with 0.1% SDS and 30 μ g proteinase K, and incubated for 30 min in a 55°C water bath. The immunoprecipitated RNA was isolated by phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation.

2.8 Tobramycin affinity chromatography to isolate mRNPs.

The protocol of Hartmuth et al. was followed (Hartmuth, Urlaub et al. 2002; Hartmuth, Vornlocher et al. 2004).

2.8.1 Derivatization of the matrix

1mL of a 5-mM tobramycin solution (0.2 M NaHCO₃, 0.5 M NaCl, pH 8.3 (NaOH)) were the incubated by head-over-tail rotation overnight with 2 mL (packed volume) of *N*-hydroxysuccinimide-activated Sepharose 4 Fast Flow (Amersham) previously washed four times with 9 mL 1mM HCl by gentle resuspension and brief centrifugation at 250g at 4⁰C. After centrifugation at 250g (5 min) the supernatant was removed and 8 ml of blocking buffer A (0.2 M NaHCO₃, 0.1 M NaCl, 1 M ethanolamine, pH 8.0 (HCl)) were added and incubated for 2 hours at room temperature, washed 3 times with PBS and twice with PBS containing NaN₃ at 4⁰C, and stored.

2.8.2 Testing of the Tobramycin matrix

For affinity selection, 4X binding buffer 4X BP (80 mM Tris-HCl, pH 8.1, 4 mM CaCl₂, 4 mM MgCl₂, 2 mM DTT) was freshly prepared. The specificity of the interaction between the tobramycin aptamer present on the *CAT-AATR* 3'-UTR reporter mRNA and the matrix-bound tobramycin was monitored as follows: 50 µL of the tobramycin matrix was blocked with 400 µL of blocking buffer B (1X BP, 300 mM KCl, 0.1 mg/ml tRNA, 0.5 mg/ml BSA, 0.1% NP-40) by head over tail (HOT) rotation overnight. The matrix was collected and 400 µL of a mixture containing 1 µg of total RNA containing the tobramycin-tagged RNA and 0.1 mg/mL tRNA in binding buffer (1X BP, 145 mM KCl), were added to 15 µL of matrix and incubated for 1.5 h at 4⁰C. The matrix was collected, the supernatant saved. The matrix was then washed three times with 750 µL ml of W145 buffer (1X BP, 145 mM KCl, 0.1% NP-40) and the bound RNA was eluted by incubating with 100 µL of E145T elution buffer (1X BP, 5mM tobramycin, 145 mM KCl, 2 mM MgCl₂) with HOT rotation for 10 min. The RNA present in the supernatant and the eluted RNA were reversed transcribed and the cDNAs obtained were used as a template in a PCR reaction to amplify a fragment encompassing part of the CAT reporter gene, the tobramycin aptamer and part of the *AATR* 3'-UTR.

2.9 Tandem Affinity Purification (TAP)

TAP purification was done as described in (Puig, Caspary et al. 2001) with minor modifications.

2.9.1 Preparation of cell lysate for TAP purification

Reserve and pre-cool centrifuge and rotor SS34 and ultracentrifuge 75Ti, all other tools pre-cooled. For each TAP purification spin down 5×10^9 cells at 2000rpm at 4°C. Wash cells twice with 50ml ice-cold PBS. Snap freeze pellets in liquid Nitrogen and store at – 80°C or directly proceed with extract preparation. Break cells in a final volume of 6ml breakage buffer (10mM Tris-Cl, 10mM NaCl, 0.1% IGEPAL, adjusted to pH 7.8 with HCl) including one tablet of complete inhibitor (without EDTA, Roche) by passing 15-20 through a 21-25 gauge needle. Check on a glass slide to ensure complete breakage. Spin cell lysate at 10'000g for 15 min to remove cell debris. Transfer supernatant to pre-cooled Polycarbonate Thick Wall Beckman centrifuge tubes (13.5ml capacity, 355630 rec. No.). Spin at 35'000 rpm, 4°C, for 45 min. After centrifugation transfer supernatant to 15 ml Falcon. Measure volume and add NaCl to a final concentration of 0.14M (note that breakage buffer is already 0.01M NaCl). Mix and take 25µl aliquot (start material, #1).

2.9.2 TAP-Purification

200µl IgG sepharose bead suspension (Amersham Biosciences) is transferred into a 0.8x4-cm Poly-Prep column (Bio-Rad) and washed with 10ml IPP150 (10mM Tris-Cl, pH 7.8, 150mM NaCl, 0.1% IGEPAL). The cleared cell lysate is adjusted to 10mM Tris-Cl, pH 7.8, 150mM NaCl, and 0.1% IGEPAL) and transferred into the column containing the washed beads and rotated for 2hrs at 4°C. Elution is done by gravity flow. 25µl of flow through (IgG flow through, #2) is taken for subsequent analysis. The beads are washed three times in 10ml of IPP150 and once with 10ml of TEV cleavage buffer (IPP150 adjusted to 0.5mM EDTA and 1mM DTT). Cleavage is done in the same column by adding 1ml of TEV cleavage buffer and 100 units of TEV protease (Gibco). The beads are rotated for 2hrs at 16°C and the eluate is recovered by gravity flow. Take 15µl aliquot (IgG eluate, #3).

200µl of calmodulin affinity bead suspension is transferred to a column and washed three times with 10ml of IPP150 calmodulin binding buffer (10mM Tris-Cl, pH 7.8, 10mM β-mercaptoethanol, 150mM NaCl, 1mM magnesium acetate, 1mM imidazole, 2mM CaCl₂, 0.1% IGEPAL). Three mililiters of IPP150 calmodulin binding buffer and 3 µl of 1M CaCl₂ are added to the 1 ml of eluate recovered after TEV cleavage. This solution is transferred to

the column containing washed calmodulin beads and rotated for 1 h at 4°C. Beads are washed three times with IPP150 calmodulin binding buffer. The bound proteins are eluted with 1 ml of IPP150 calmodulin elution buffer (10mM Tris-Cl, pH 7.8, 10mM β-mercaptoethanol, 150mM NaCl, 1mM magnesium acetate, 1mM imidazole, 2mM CaCl₂, 0.1% IGEPAL, 2mM EGTA). The eluate is concentrated by TCA precipitation.

2.9.3 TAP purification to isolate mRNP complexes

The TAP purification method was also used to isolate mRNP complexes. The protocol of (Gerber, Herschlag et al. 2004) was adapted. To this end, 1x10⁹ cells expressing the TAP-tagged protein of interest were lysed in 6 ml breakage buffer (see 2.10.1.), supplemented with 200 units RNaseIn (Promega) and 5μl Vanadyl Ribonucleoside complexes (Sigma). Binding, washing, and TEV cleavage were done as described in 2.10.1., but TEV cleavage buffer was supplemented with 200 units of RNaseIn and 5μl Vanadyl Ribonucleoside complexes (Sigma). The eluate after the TEV cleavage was supplemented with 0.1% SDS and 30 μg proteinase K, and incubated for 30 min in a 55°C water bath. The immunoprecipitated RNA was isolated by phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation. Note, that mRNP complexes can be isolated from all cells which express epitope-tagged mRNA binding proteins using immunoprecipitation (see, (Tenenbaum, Carson et al. 2000; Tenenbaum, Lager et al. 2002)). Negative controls were applied either by using cell lines expressing only the TAP-tag or using cell lines expressing an untagged version of the mRNA binding protein of interest.

2.10 Electrophoresis mobility shift assay (EMSA)

EMSA was done as described in (D'Orso and Frasch 2001) with minor modifications.

2.10.1 *In Vitro* Transcription

The region encompassing the nt 290-618 of the *AATR* 3'-UTR, was amplified by PCR and cloned into the *Bam*HI and *Eco*RI sites of the vector pBS(KS) (Stratagene). Transcription of sense and antisense sequences was performed with the MAXIscript *In vitro* transcription kit (Ambion) following the instructions of the manufacturer. 1 μg of *Xho*I and *Xba*I-digested plasmid was transcribed using T3 or T7 RNA-polymerase in the presence of [α -32P]UTP (800 Ci/mmol, Amersham) and 500 μM ATP, CTP, and GTP and incubated for 30 min at 4°C. The transcripts were purified on an 8 M urea-6% polyacrylamide gel and eluted overnight in RNA elution buffer (0.3 M NaOAc, 10 mM MgCl₂, and 1 mM EDTA).

2.10.2 Protein Extract Preparation

For total protein extract preparation, 2.5×10^8 parasites were resuspended in 1 ml of lysis buffer (25mM Hepes pH 7.6, 5 mM MgCl₂, 1.5 mM KCl, 2 mM DTT, 10 mM, 0.1% Nonidet-P40) supplemented with one tablet of complete inhibitor (without EDTA, Roche). After 30 min on ice, the extract was centrifuged at 13,000 rpm and the supernatant was stored at -70 °C.

2.10.3 Heparin-Agarose Chromatography

A column containing 2 ml of Affi-Gel heparin gel (Bio-Rad) was equilibrated with 10 bed volumes of Buffer D (20 mM Hepes pH 7.9, 20% Glycerol, 0.2 mM EDTA, 0.5 mM DTT and 100 mM KCl). 1 mL of total protein extract was applied and the column was washed with Buffer D and the protein was eluted with a KCl linear gradient (0.1 to 2M KCl in application buffer). 1 mL fractions were collected and O.D.₂₈₀ nm measured. Peaked fractions were pooled, desalting using Millipore columns and used mobility assays.

2.10.4 Analysis of RNA-Protein Interactions

Binding reactions were performed with 30 µg of trypanosome total extract (prepared as above), 10,000 cpm of RNA probe, 10 mM Tris-HCl (pH 7.6), 5% glycerol, 100 mM KCl, 5 mM MgCl₂, 1 µg/ml bovine serum albumin, 500 ng/µL tRNA (Sigma) in a 20 µL final volume. The incubation time was 20 min at 25 °C. Each reaction was loaded directly onto a 4% acrylamide-bisacrylamide (38:2), 0.5% TBE non-denaturing gel to perform an electrophoresis mobility shift assay (EMSA). The gels were dried and exposed to film at -70 °C.

2.11 Microarray

Microarray analysis was performed as described in (Diehl, Diehl et al. 2002) with some modification.

2.11.1 Genomic *T. brucei* microarray

Genomic *T. brucei* microarrays were generated by Stefanie Brems at the DKFZ (Deutsches Krebsforschungszentrum, Heidelberg, Germany). In brief, 24,567 random shotgun clones representing the genome of *T. brucei brucei* strain TREU927/4 were provided by Najib El-Sayed of the Institute for Genomic Research (TIGR, Rockville, USA). These clones were PCR amplified in 384- or 96-well microtitre plates using primers d(TTGTAACGACGCCAGTG) and d(GCGGATAACAATTTCACACAGGA), which

are flanking the multiple cloning site of vector pUC18. The PCR products were checked by agarose gel electrophoresis.

DNA fragments were spotted onto poly-L-lysine coated glass slides as described in (Diehl, Diehl et al. 2002). Spotting was done with an SDDC-2 DNA Micro-Arrayer from Engineering Services Inc. (Toronto, Canada) and SMP-3 pins (TeleChem International Inc., Sunnyvale, USA). Subsequently, the slides were cross-linked using UVC-500 (Hoefer Scientific Instruments, San Francisco, USA).

2.11.2 Sample preparation, labeling and hybridisation

Total RNA from 4×10^8 exponentially growing cells (below 1×10^6 cells/ml for bloodstream forms and 2×10^6 cells/ml for procyclics) was isolated using Qiagen RNeasy Midi Kit. 15 μ g of total RNA was reverse transcribed using SuperScriptTM III (Invitrogen). The following protocol for a 40- μ l reaction volume was used:

1. Add the following components to a nuclease-free microcentrifuge tube:

5 μ l of oligo(dT)₁₂₋₁₈ (100ng/ μ l) (500ng)

15 μ g of total RNA

3 μ l dAGT-mix (10mM)

2 μ l dCTP (1mM)

2 μ l Cy3/Cy5-dCTP (1mM)

RNase free water to 27 μ l

2. Heat mixture to 65°C for 5 min. and incubate on ice for at least 1 min.

3. Collect the contents of tube by brief centrifugation and add:

8 μ l 5x First-Strand Buffer

2 μ l 0.1 M DTT

1 μ l RNaseInTM (Promega)

2 μ l SuperScriptTM III RT (200 units/ μ l)

4. Mix by pipetting gently up and down. If using random primers, inc. tube at 25°C for 5 min.

5. Incubate in the dark at 50°C for at least 3hrs (or overnight).

6. Inactivate the reaction by heating at 70°C for 15 min.

7. Add 1 μ l RNaseH (Roche).

8. Incubate 20 min. at 37°C.

9. Use QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany).

10. Elute cDNA with 2 x 50µl RNase free water.
11. Measure cDNA concentration.
12. Ethanol-precipitate cDNA.
13. Resuspend cDNA-pellet in 5µl TE, vortex.

Prehybridisation/Hybridisation:

14. Wash DNA chip 10 sec. in 0.2% SDS, 10 sec. in dH₂O, and 3 min in 95°C dH₂O.
15. Place slide into Atlas Glass Hybridization chamber (DNA-Array side up!) and fill with 2ml Prehybridisation Buffer (5x SSC, 0.1% SDS, 1% BSA)
16. Incubate for 45 min at 55°C.
17. Wash the slide by dipping 3x in dH₂O at RT.
18. Dip the slide in Isopropanol (abs.) and dry. Slides should be used immediately after prehybridisation.
19. Mix Cy3- and the corresponding Cy5-labelled cDNA and denature at 95°C for 5 min. Immediately put sample on ice. Quickspin.
20. Add 60µl of hybridisation buffer (50% formamide, 3x SSC, 1% SDS, 5x Denhardt's reagent and 5% dextran sulfate).
21. Spread hybridisation mix over glass slide. Cover with cover slip, and hybridize o/n in a dark humidified chamber at 62°C.
22. Wash the slides at RT: 10 min in Washing Buffer 1 (2x SSC, 0.2% SDS), 10 min Washing Buffer 2 (2x SSC), and 10 min in Washing Buffer 3 (0,2x SSC). Dry slides.

2.11.3 Image acquisition and data analysis

DNA chips were scanned with ScanArray 5000 (Packard BioScience, Dreieich, Germany) subsequently with Cy3- and Cy5-channel at a resolution of 10µm. Analysis of resulting image were performed using GenePix software (Axon Instruments, Union City, USA) to generate numerical values of spot signal intensities. The software package MCHIPS (Fellenberg, Hauser et al. 2002) was used for data quality assessment and normalisation. Differentially regulated transcripts were confirmed by Northern blot analyses.

2.12 Indirect immunofluorescence assay (IFA)

All steps were done at RT; washes are for 5 min, with gentle agitation. About 1×10^6 cells were centrifuged for 10 min at 2,000 rpm and resuspended in 4 mL of 1X PBS containing 4% Paraformaldehyde. This parasite suspension was distributed in a four-chambered slide (Falcon) for fixation. After 25 min, the fixation solution was aspirated and the chambers washed with PBS. If the experiment had to be suspended at this point, the slides were kept at 4°C with the cells immersed in PBS containing 0.2% sodium azide. On resumption, this buffer was discarded and the slides rinsed with PBS. Cells were then permeabilised by incubating in 0.2% Triton X-100 in PBS for 20 min. After washing, the cells were incubated in blocking solution (0.5% gelatin in PBS) for 20 min. Fixed, permeabilised cells were then incubated 1h in an appropriate dilution (1:500 to 1:1000) of the appropriate primary antibody in gelatin/PBS solution. The cells were washed twice with Triton X-100/PBS and incubated with the corresponding secondary antibody (anti-rabbit or anti-mouse IgG, Alexa-flour conjugated) in gelatin/PBS solution 30min. After two washes with 0.2% Triton X-100/PBS solution, the nucleic acids of the cells were stained for 10 min with DAPI (4', 6'-diamidino-2-phenylindole, 200ng/mL in PBS). The slides were washed once with PBS and air-dried. Controls were stained with antibodies or dyes individually to confirm that no fluorescence light was bleeding into the other channels. The chamber walls were peeled off and the slides mounted with a drop of 84% glycerol in PBS on each field. Cells were examined with a Leica DM RXA microscope equipped with Deconvolution Software Openlab (Improvision). Images were recorded using a Hamamatsu digital CCD camera (Hamamatsu Photonics K.K., Japan). Processing and pseudo-colouring and merging of images were performed using Adobe Photoshop software (version 7, Adobe Systems Incorporated).

3 Results

3.1 Characterisation of *TbRBP3*

3.1.1 Identification of *TbRBP3*

One of the main topics in the lab is to study the mechanisms involved in mRNA degradation in *Trypanosoma brucei*. This aspect is relevant for the control of developmentally regulated mRNAs. The role of RRM-type RNA binding proteins in these mechanisms is well established and although BLAST search on the *T. brucei* genomic database predicts the presence of 75 of these proteins, the role of most of them has not been established. This work started with the characterisation of the *T. brucei* homologues of *T. cruzi* U- rich binding proteins 1 and 2 that bind the 3'-UTR of *T. cruzi* small mucin genes (Tcsmug) mRNAs and target them for degradation. By searching the *T. brucei* genome database (<http://www.genedb.org/>) it was possible to find another homologue of this family of proteins, that we named RNA Binding Protein 3 or *TbRBP3* ([Tb11.03.0550](#)).

The *TbRBP3* gene is located on chromosome 11 approximately 10 kb upstream of the *TbUBP2* gene. *TbRBP3* is a predicted protein composed of 200 amino acids and with a molecular weight of 22.3 kDa. The RRM recognition motif is located between the protein residues 80-151. The protein shares 59.8% identity with *TcRBP3* ([Tc00.1047053507093.250](#)). Using the amino acid sequence of the *TbRBP3* as a query in BLASTP analysis, 2 other homologues of the protein were also found in other Trypanosomatids ([congo1375b07.q1k_4](#) in *T. congolense* and [LmjF25.0520](#) in *Leishmania major*). These sequences and *TbUBP1* ([Tb11.03.0620](#)), *TbUBP2* ([Tb11.03.0580](#)), *TcUBP1* ([Tc00.1047053507093.220](#)) and *TcUBP2* ([Tc00.1047053507093.229](#)) were chosen and compared using ClustalW. The corresponding alignment is shown in figure 3.1.1. The presence of 3 different domains stands out: the highly conserved RRM recognition motif in the central part of the proteins and 2 poorly conserved domains at the N- and C- terminal regions that might be involved in protein-protein interactions.

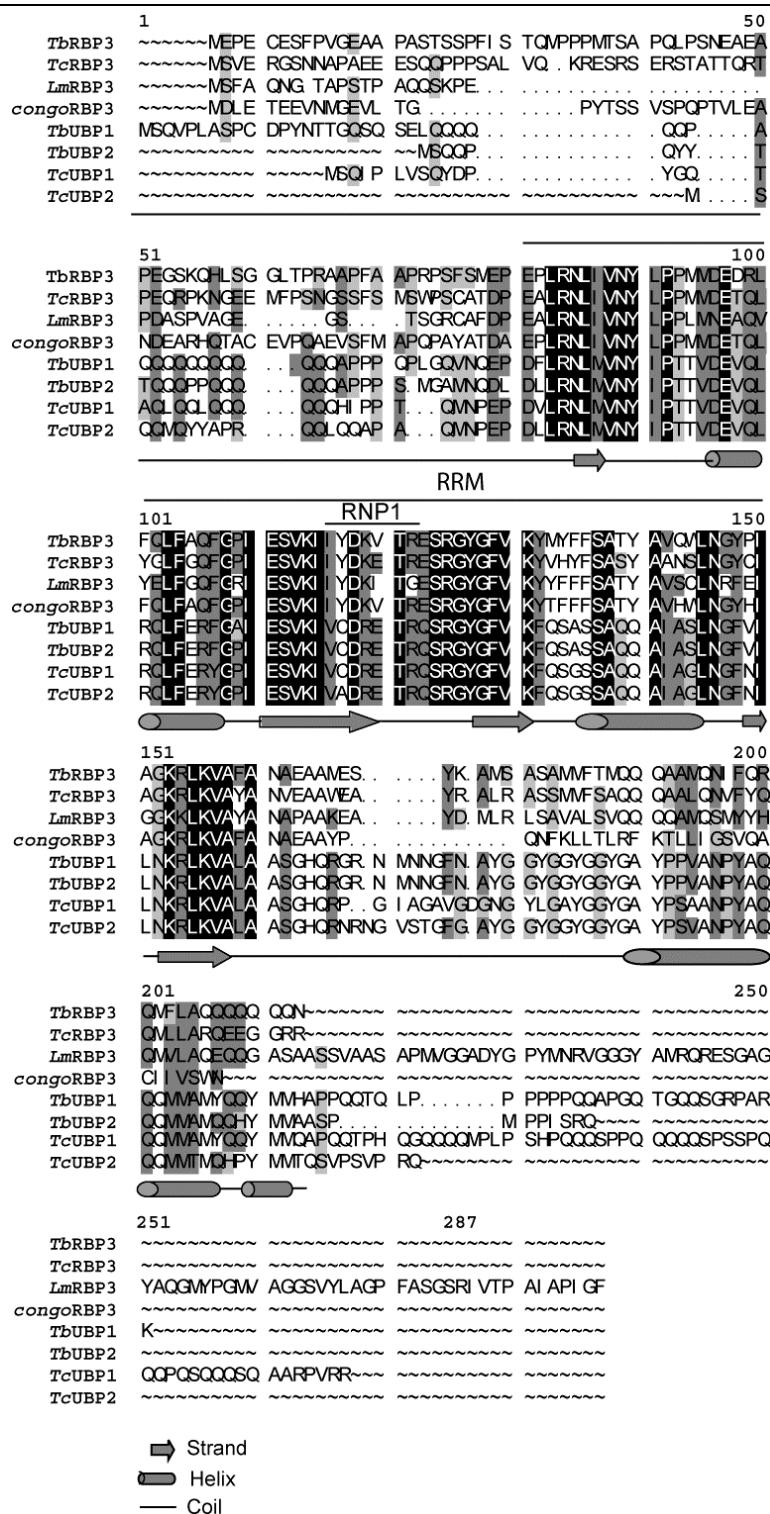


Fig. 3.1.1 RBP3 is present in different Trypanosomatids. ClustalW alignment of RBP3 homologues present in different species of trypanosomatids (*T. brucei* RBP3 accession number Tb11.03.0550, *T. cruzi* (Tc00.1047053507093.250), *L. major* (Lm.jF25.0520) and *T. congolense* (congo1375b07.qlk_4)). *TbUBP1* and 2 and *TcUBP1* and 2, -which are involved in developmentally regulated gene expression control- were also compared. Conserved residues (the RRM-motif and in particular the RNP1 binding motif is underlined) are colored black. Less conserved amino acids are highlighted grey (weak homology is observed at the N- and C-terminal domains of the protein).

3.1.2 *TbRBP3* has a role in bloodstream stage *T. brucei*

3.1.2.1 Over-expression of *TbRBP3*

In order to determine if *TbRBP3* is differentially expressed in both life stages of *Trypanosoma*, 10 µg RNA of each were run on a formaldehyde gel, transferred to a nylon membrane and hybridized with a ³²P-labeled probe corresponding to *TbRBP3* ORF. As a loading control, the membrane was also hybridized with SRP (signal recognition particle). The results of this Northern blot are shown in figure 3.1.2. A band of 4 kb corresponding to *TbRBP3* RNA was obtained. After normalization, it can be observed that the mRNA steady-state levels are equal in both life stages of the parasite (figure 3.1.2). This figure is part of the northern blot shown in figure 3.1.3. It was not possible to determine if the protein was also differentially expressed due to the lack of a specific antibody, but later studies using *in situ* V5 tagged-RBP3 demonstrated that this protein is expressed equally in both life stages of the parasite (see figure 3.1.6).

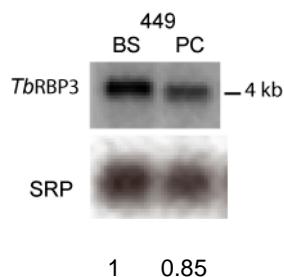


Fig. 3.1.2 *TbRBP3* mRNA is approximately equally expressed in both life stages of *T. brucei*. Northern blot analysis with 10µg of total RNA from *T. brucei* 449 BS and PC and hybridised with a ³²P labeled probe recognising *TbRBP3*. SRP was used as a loading control.

To get insight into the function of *TbRBP3*, the protein was first over-expressed in both procyclic and bloodstream forms. For this, Claudia Hartmann, a member of the lab, amplified by PCR the entire *TbRBP3* ORF using the primers cz1685 and cz1686, gel purified it and cloned it HindIII/BamHI into pHD617 (containing actin 3'UTR, for expression in PC) or pHD615 (containing VSG 3'UTR, for expression in BS) generating the plasmids pHD1323 and pHD1324. Then I continued the experiments. 10 µg of each DNA were cut with *Not I*, and the linearised plasmids were used to transfect *T. brucei* 449 PC and BS cells which contain the *Tet* repressor. The selection of the transformed parasites was done by adding hygromycin to the growth media.

After selection the recombinant expression of *TbRBP3* was induced by addition of tetracycline (100 ng/mL) and total RNA was prepared after 48 hours. The presence of the new transcript was assessed by northern blot using a *TbRBP3* specific probe. The results are shown in figure 3.1.3. Two additional transcripts were detected apart from the endogenous one: one of the expected size (0.9 kb in BS and 1 kb in PC) and a shorter one (150 bp lower), which suggests that the transcripts were alternatively processed. The size of the recombinantly expressed transcript was different between PC and BS. This difference was due to the size of the 3'-UTR which was used for optimal expression in the two cellular stages.

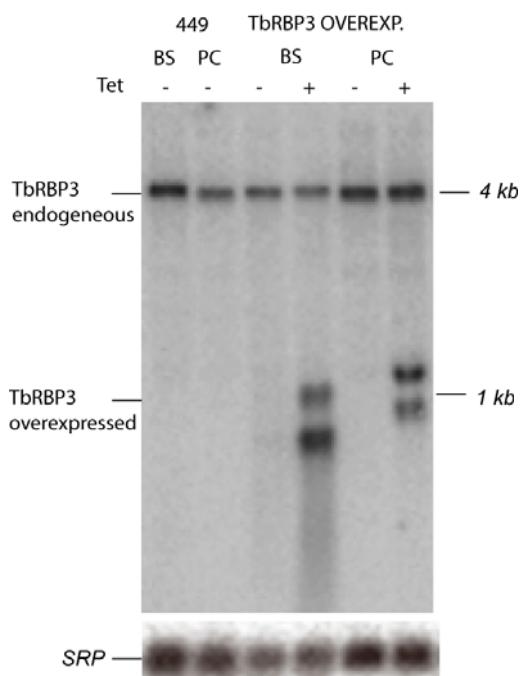


Fig. 3.1.3 Over-expressed *TbRBP3* mRNA is present in bloodstream forms as well as in procyclic forms. Northern blot analysis with 10 μ g of total RNA from *T. brucei* 449 BS and PC and hybridized with a 32 P labeled probe recognizing *TbRBP3*. SRP was used as a loading control.

To assess if the increased levels of *TbRBP3* had a visible phenotypic effect, over-expression of the protein was induced and the cell growth monitored every 24 hours during one week (Figure 3.1.4). If *TbRBP3* is over-expressed in procyclics, it does not affect growth and also has no apparent effect on morphology. In contrast, the cell growth of bloodstream forms was impaired. The doubling time of these cells increased by 50% when the exogenous *TbRBP3* was present (doubling time was 11.3 hrs vs. 8 hrs in the wild type). This indicated that *TbRBP3* might play an important role in this life stage.

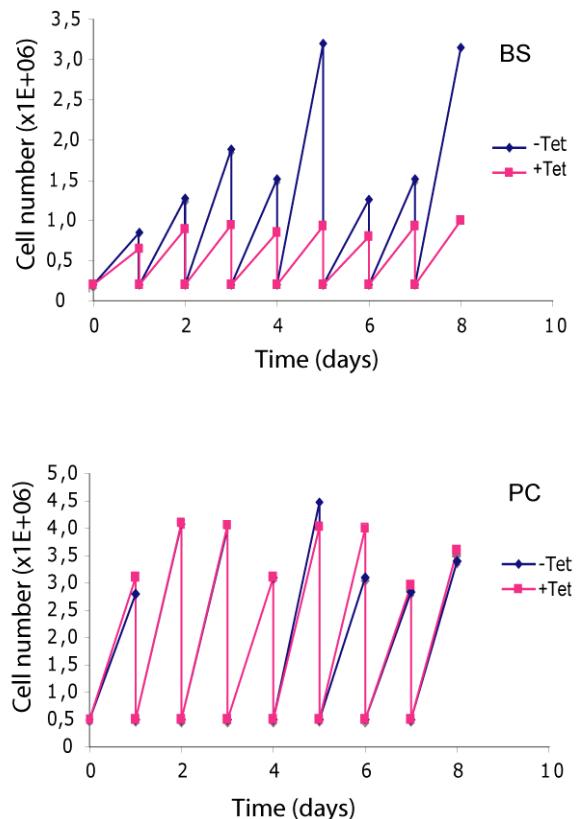


Fig. 3.1.4 Effect of over-expression of *TbRBP3* on cell growth. PC and BS cell lines over expressing *TbRBP3* were grown in the absence (blue line) or presence (pink line) of tetracycline and growth monitored for one week. Cells were diluted every day to a density of 2×10^5 cells/ml for BS and to 5×10^5 cells/ml for PC.

3.1.2.2 RNAi

RNA interference is a potent tool for studying gene function. This mechanism is used by *T. brucei* to regulate the transcript abundance of retroelements and the process currently represents the method of choice in gene function studies of the parasite (Djikeng, Shi et al. 2001; Ullu, Djikeng et al. 2002; Motyka and Englund 2004; Ullu, Tschudi et al. 2004).

As a second approach to investigate the role of *TbRBP3*, the protein level was decreased by RNAi in bloodstream and procyclic cells according to the procedure of the *T. brucei* functional genomics project (TrypanoFAN <http://trypanofan.path.cam.ac.uk/cgi-bin/WebObjects/trypanofan>). To select the region of the transcript to be targeted, RNAi software was used (Redmond, Vadivelu et al. 2003) and the primers were selected using the MIT's primer3 program (<http://www.genome.wi.mit.edu/cgi-bin/primer/primer3-www.cgi>) and NCBI blast (<http://www.ncbi.nlm.nih.gov/BLAST/>). A 525-bp fragment was amplified by PCR using the primers cz2143 and cz2144 and the PCR product was cloned between two opposing and inducible T7 promoters in p2T7-177 XhoI/BamHI (LaCount, Bruse et al. 2000). The resulting plasmid (pHD1638) was transfected into *T. brucei* 427 1313/514 bloodstream and procyclic cell lines.

To check the depletion of *TbRBP3* Northern blot analysis was performed. After 24 hours of RNAi induction the *TbRBP3* mRNA levels decreased in both procyclic and bloodstream forms, although approximately 30% of the transcript still remained (Figure 3.1.5 A). RNAi induced cells were grown in the presence of tetracycline for one week and growth was monitored every 24 hours (figure 3.1.5 C). Silencing of *TbRBP3* had similar effects on cell growth as it was observed for the over-expression: the growth of bloodstream cells was impaired, while the growth rate in procyclics did not change. This suggests that this protein might play a functional role in this specific stage. The depletion of the protein in BS was confirmed by Western blot of cell extracts from BS containing an *in situ* V5-tagged RBP3 and cotransfected with the dsRNAi RBP3 plasmid. After 24 hours, the levels of the protein were decreased (Figure 3.1.5 B).

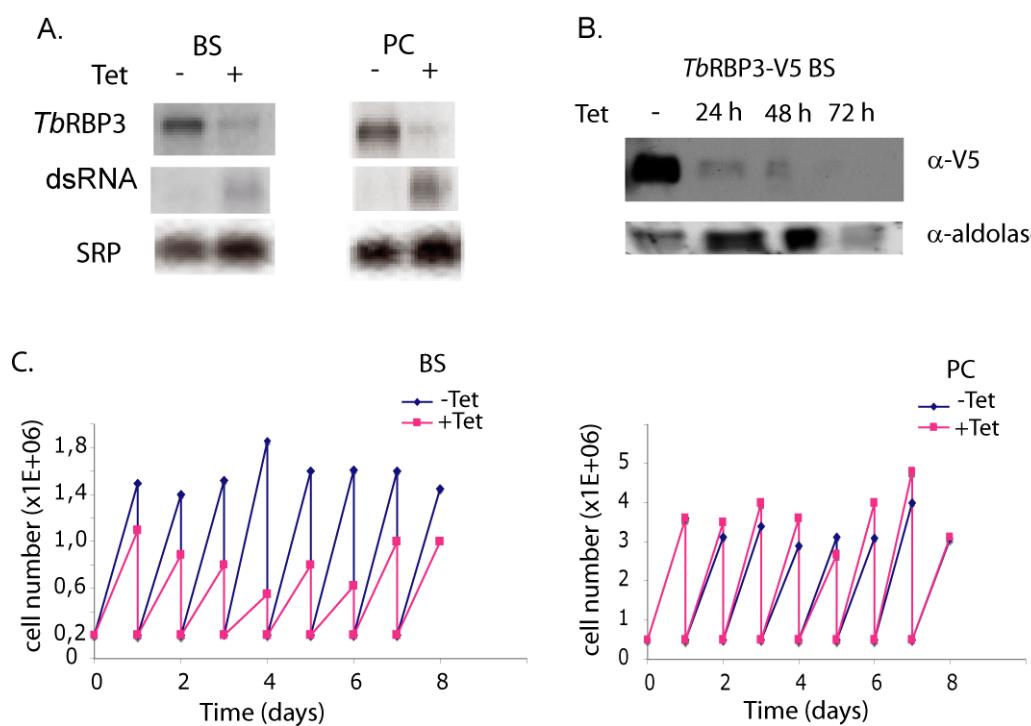


Fig. 3.1.5 Knock down of *TbRBP3* affects BS growth. A. Northern blot of BS and PC after induction of the dsRNA. B. Western blot of an *in situ* tagged V5-*TbRBP3* in BS after induction of dsRNA RBP3. C. Cell growth curve.

3.1.3 Cellular localisation of *TbRBP3*

Due to lack of an antibody specific for *TbRBP3*, I over expressed both a myc- and a TAP-tagged version of the protein and then assessed the localisation by cellular fractionation and immunofluorescence. For this purpose, the plasmids pHD1484 (C-terminal myc-tag) and pHD918 (C-terminal TAP-tag) were used. In order to eliminate an internal Hind III cutting site located at 20 nt in the coding sequence of *TbRBP3*, a silent point mutation was introduced by PCR. Then the ORF without the stop codon was cloned between HindIII/BamHI sites for the myc-tagged, and between HindIII/HpaI sites for the TAP-tagged version. BS and PC cells were transfected and selected by adding hygromycin. Western blot analysis after cellular fractionation showed that the myc-tagged *TbRBP3* is present in the cytoplasm of PC. This was confirmed by immunofluorescence microscopy using an anti-myc antibody. Attempts to express the fusion protein in BS of the parasite were unsuccessful (data not shown), although resistant-clones were obtained, the protein was not expressed. To look at the localisation of the protein in BS, we used a cell line containing a C-terminal TAP-tagged *TbRBP3*. The TAP-tag consists of two IgG binding units of Protein A (ZZ domains), a tobacco etch virus (TEV) protease cleavage site, and a calmodulin binding domain (Rigaut, Shevchenko et al. 1999; Puig, Caspary et al. 2001). Western blot analysis using PAP antibody (peroxidise anti-peroxidase) showed that the over expressed protein is mainly located in the cytosol of the parasite. Immunofluorescence microscopy showed that the protein is concentrated at the perinuclear space. This could however also be a false impression because the cells are thickest around the nucleus.

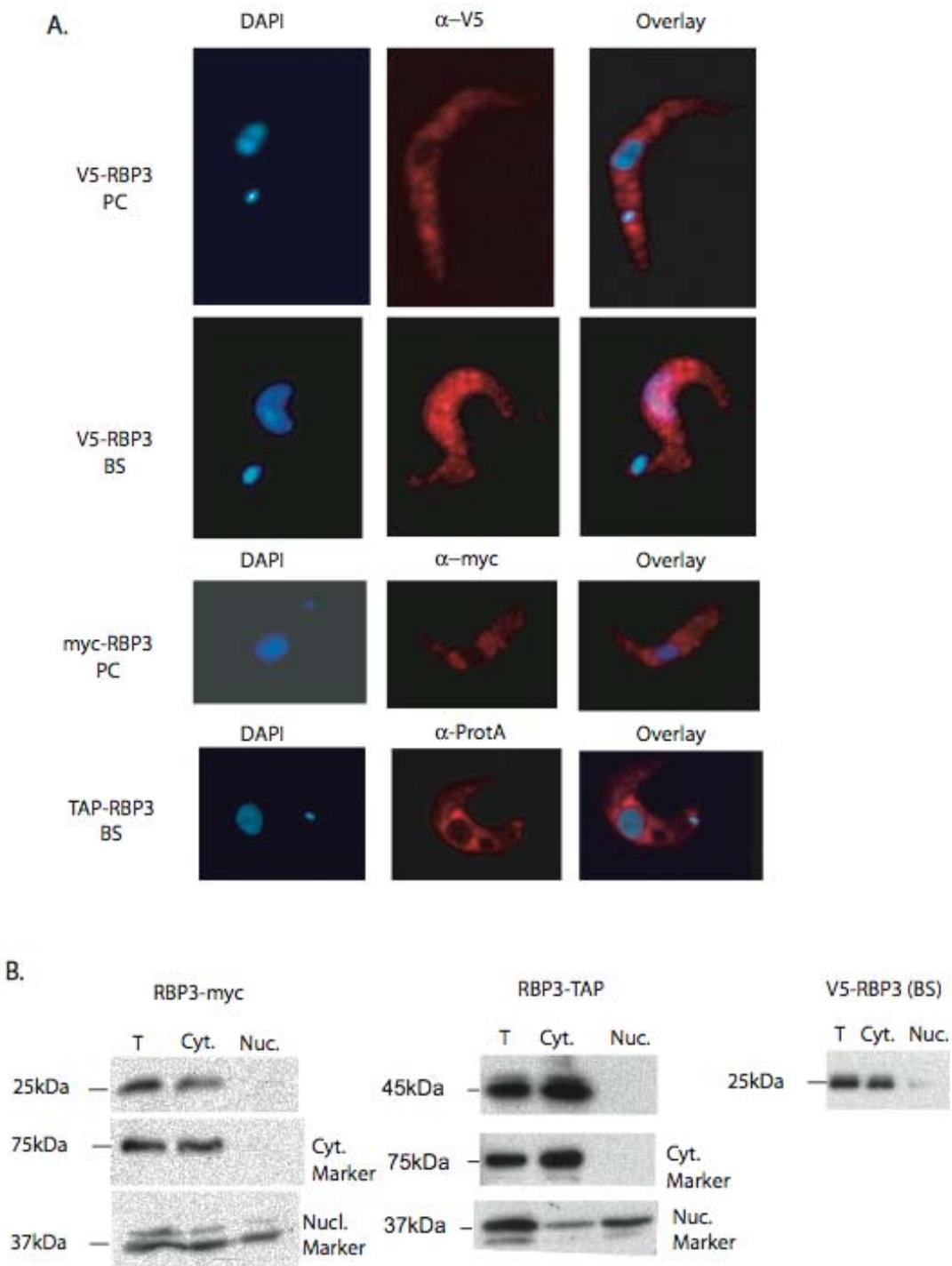


Fig. 3.1.6 *TbRBP3* is mostly localised at the cytosol of *T. brucei* in procyclic and bloodstream forms.
 A. Immunofluorescence of BS and PC cell lines containing either *V5 in situ*-RBP3 or over-expressed RBP3.
 B. Cell fractionation.

To avoid any artefact due to the over-expression of *TbRBP3*, a V5- *in situ* tagging of the protein was performed in BS and PC by homologous recombination. For this purpose, a cassette containing the blasticidin resistance marker (BSD) and a V5 tag (Shen, Arhin et al. 2001) was inserted between the 5'-UTR and the *TbRBP3* coding region. Briefly, 2 DNA fragments- one corresponding to 200 bp upstream of the ATG start codon and another spanning the first 300 bp of the *TbRBP3* ORF were amplified by PCR using *T. brucei* genomic DNA as a template. These fragments were sequentially ligated into the plasmid pBS:BSD-V5 to flank the blasticidin (BSD) marker and the V5 tag cassette to generate pHD1682. The flanking homology regions allowed a locus-specific recombination between wild-type chromosomal DNA containing the *TbRBP3* gene and the linear DNA fragment containing the resistance marker. Bloodstream form 1313/514 cells were transfected with SacII/ApaI linearised pHD1682 (*TbRBP3* 5'-UTR-Bsd-V5-*TbRBP3* CDR) and selected for resistance to blasticidin.

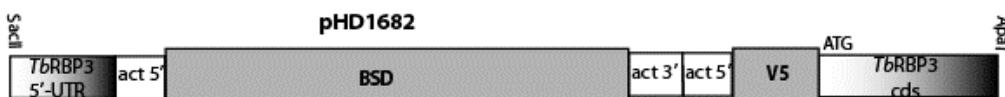


Fig. 3.1.7 Construct for V5-*TbRBP3* *in situ* tagging.

This allowed us to confirm the data obtained with the over-expressed constructs. Therefore we conclude that *TbRBP3* is mainly located in the cytosolic compartment.

3.1.4 Looking for possible roles of *TbRBP3*

3.1.4.1 Microarray analysis of *TbRBP3* over expressing or knock-down cells:

Considering the fact that both over-expression and knock-down of *TbRBP3* impairs the growth of bloodstream forms, it is possible that this RNA binding protein is involved in the regulation of specific mRNAs in this life stage. To test this, we used *T. brucei* specific genomic DNA microarrays (Diehl, Diehl et al. 2002; Brems, Guilbride et al. 2005) to compare the transcript levels of *T. brucei* wild-type bloodstream cells with BS cells where *TbRBP3*-levels were either elevated or depleted. Total RNA was isolated from uninduced and induced cells (for either *TbRBP3*- over-expression or the RNAi) and reverse transcribed with Cy3 and Cy5-labeled dCTP. After purification these cDNAs were used as a probe to hybridise the microarrays. Six independent hybridisations were performed for each *TbRBP3* RNAi or

over-expression cell line. The data was analyzed using M-CHIPS software (**M**ulti-**C**onditional **H**ybridization **I**ntensity **P**rocessing **S**ystem) (Fellenberg, Hauser et al. 2002). The spots that were more than 2.5 fold up or down-regulated were considered and some of them were sequenced (table 3.1). Surprisingly, the transcript corresponding to *TbRBP3* was not found in the analysis: it was neither upregulated in the microarrays in which the RNA from the over-expressed cell line was used as a probe nor down-regulated in the RNAi cell line. Since the microarrays used randomly cover 1.3 times the genome of *T. brucei*, it is possible that the *TbRBP3* gene is not represented on the slides.

Over-expression of *TbRBP3* in bloodstream cells resulted in differential regulation of several transcripts, 2 of them were derived from VSG expression sites, and an amino acid transporter which has previously been found to be upregulated in bloodstream forms. Northern blots were done to confirm these data, but unfortunately they failed to show the differential expression of these transcripts. Similar results were obtained when cDNA derived from *TbRBP3* RNAi cells was used. Of the several clones we selected only one, corresponding to Tb927.1.3250 (clone 1M10). It was affected in both cases: Its mRNA levels increased 2.5 fold when *TbRBP3* was over-expressed and decreased 2.7 fold after the knock-down. However, the transcript levels did not show any alteration when assessed by Northern blot. Figure 3.1.8 shows the results for this target with the microarray picture and the Northern blot. In contrast to previous analysis done in the lab suggesting that the integration of the over-expression plasmid into the rRNA spacer regions affects the transcripts nearby, just one of my transcripts (1M10) was located in chromosome 1.

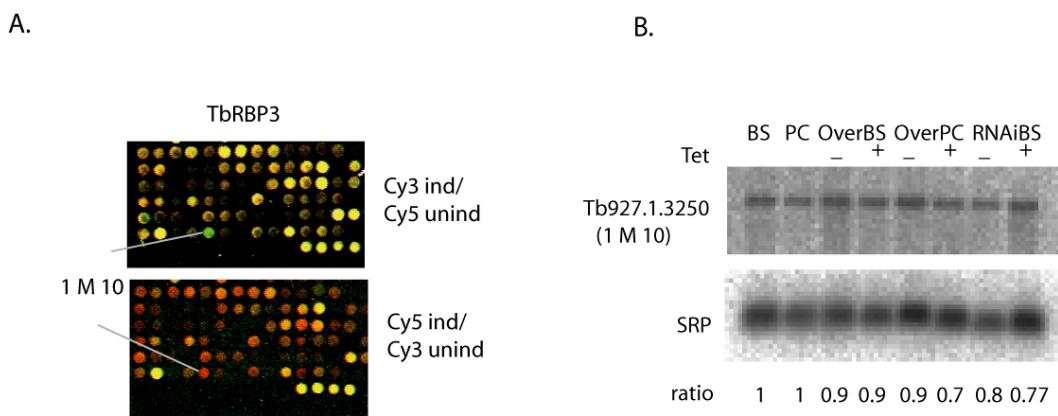


Fig. 3.1.8 Is 1M10 a false positive? A. Microarray showing upregulation of the spot 1 M 10 (putative sugar transporter after over-expression of *TbRBP3*). B. Northern blot showing that the mRNA levels of 1 M 10 did not change after over-expression of *TbRBP3*.

Clone	Accession number	Characteristics	Location	Array data	Northern data	Other
Over-expressed <i>TbRBP3</i>						
1_M_10	Tb927.1.3250	Sugar transporter protein	chr. 1	+2,4	1	RNAi growth defect in BS (Subramaniam, Veazey et al. 2006) 5307 bp
1_E_4	Tb11.01.2260	Intermediate filament protein	chr. 11	+3,05	N.D	1818 bp
2_P_20	Tb927.4.2750	Hypothetical protein	chr. 4	+4,49	1	900 bp
11_C_1	Tb927.6.1860	Rybosome recycling factor	chr. 6	+2,8		1029 bp
15_C_9	Tb11.0360	Gp63-3 surface protease homolog, putative	chr. 1	+3,18	1	1893 bp +1,64 (in microarray analysis of <i>TbRBP3</i> -RNAi BS but not confirmed by northern blot)
20_C_10	Tb927.7.3260	expression site-associated gene 7 (ESAG7) protein, putative	chr. 7		1	1020 bp +1,73 (in microarray analysis of <i>TbRBP3</i> RNAi BS but not confirmed by northern blot)
47_D_10	Tb927.3.1470	variant surface glycoprotein (VSG)-related, putative	chr. 3	+2,8	+1,26	1098 bp +2,64 (in microarray analysis of <i>TbRBP3</i> RNAi BS but not confirmed by northern blot)
48_K_13	Tb927.4.4850	amino acid transporter, putative	chr. 4	+2,5	1	1353 bp -4.2 (downregulation in BS)

Table 3.1 Positive clones for the microarray analysis of over-expressed and knocked down *TbRBP3*.

Clone	Accession number	Characteristics	Location	Array data	Northern data	Other
knocked-down <i>TbRBP3</i>						
1_M_10	Tb927.1.3250	Sugar transporter protein Intermediate filament protein	chr. 1	-2,74	1	RNAi growth defect in BS(Subramaniam, Veazey et al. 2006)5307 bp
13_E_9	Tb10.6k15.2000	chaperone protein DNAJ, putative	chr. 10	-3,5		1011 bp Upregulated in PC
13_P_7	Tb10.70.4060	60S acidic ribosomal protein P2, putative	chr. 10	-3,12	0,7	345 bp
29_P_4	Tb927.7.2630	GTP-binding protein	chr. 7	-2,9		2703 bp
38_L_23	Tb927.7.6050	receptor-type adenylate cyclase GRESAG 4, putative	chr. 7	-3,7	1	3810 bp
41_B_24	Tb11.57.0043	variant surface glycoprotein (VSG, pseudogene), putative	chr. 11	-2,5		1485 bp
47_F_3	Tb927.6.2300	adenosine kinase, putative	chr. 6	-3,8	1	
53_J_12	Tb927.8.5250	coatomer delta subunit, putative delta-COP, putative	chr. 8	-2,67		1596 bp
57_K_13	Tb09.211.0960	protein kinase, putative	chr. 9	-2,4		1095 bp

Table 3.1 continued.

3.1.4.2 Microarray analysis of RNAs bound to TAP-TbRBP3:

Immunoprecipitation methods have been successfully used for isolating different types of RNP s (ribonucleoprotein complexes) (Tenenbaum, Carson et al. 2000; Tenenbaum, Lager et al. 2002; Gerber, Herschlag et al. 2004; Lal, Mazan-Mamczarz et al. 2004) and in particular in our group the TAP-tag (Rigaut, Shevchenko et al. 1999) (Puig, Caspary et al. 2001) has allowed the identification of mRNAs bound to *T. brucei* Puf domain-proteins. (Luu, Brems et al. 2006). As an alternative to identify mRNAs bound to TbRBP3, I used the BS cell line containing the C-terminal TAP-tagged RBP3. Total cell extracts of BS cells over-expressing TAP-RBP3 were used to purify the ribonucleoprotein complexes by binding to IgG sepharose. The RBP3-RNPs were recovered after digestion with TEV (tobacco etch virus) protease. The samples were then treated with proteinase K and total RNA was phenol-chloroform extracted and processed as described above.

The cDNA obtained was applied to the genomic microarrays (Diehl, Diehl et al. 2002; Brems, Guilbride et al. 2005). As a control we used samples from cells expressing the TAP-tag alone. Six microarray hybridisations for three independent RBP3 affinity isolations were performed. To prevent bias caused by preferential label incorporation, the two CyDyes were swapped between the two types of RNA, TbRBP3-TAP and TAP alone (pHD918). 47 clones were found to be enriched by more than 2.5 fold in the TAP-RBP3 cells. 3 of them (2 corresponding to cyclin F box and another to ZFP1) were already annotated and I chose another 20, considering also that they were present in all the slides analysed and sent them for sequencing. These results are summarized in table 3.2.

spot	identity	location	characteristics	Array levels	RT-PCR
7_M_23	<i>Tb927.5.810</i>	Chr 5	Hypothetical protein, Zinc finger C-x8-C-x5-C-x3-H type (and similar)	+6.32	
47_E_14				+4.67	
28_E_3	3'UTR of <i>Tb927.6.3490</i>	Chr 6	TbZFP1 (Zinc finger protein)	+4.49	✓
54_J_21				+2.96	
21_K_21	3'UTR of <i>Tb927.4.1000</i>	Chr 4	Hypothetical protein with an endoplasmic reticulum targeting sequence	+10.90	
22_C_22				+4.13	
30_A_19				+6.73	
21_O_5	Tb927.1.4600	Chr 1	Hypothetical protein with a Cyclin F box domain	+6.07	
46_E_19	Tb927.1.4620	Chr 1	Hypothetical transmembrane protein	+4.54	
36_A_12				+3.81	
19_F_12	Tb927.1.4630	Chr 1	Hypothetical protein with a Cyclin F box domain	+5.84	
32_O_4	Tb927.1.4650	Chr 1	Hypothetical protein with a Cyclin F box domain	+3.68	
16_L_6				+4.93	
20_P_20	Tb927.1.4580	Chr 1	Hypothetical protein with a Cyclin F box domain	+6.15	✓
8_N_6	Tb09.244.2400	Chr 9	BARP (bloodstream stage alanine-rich protein)	+3.80	
14_B_21	3'UTR and ORF <i>Tb927.4.1010</i>	Chr 4	Hypothetical protein (signal anchor). Orphan sequence.	+4.03	
16_N_18	Tb10.70.2120	Chr 10	Hypothetical protein (signal anchor).	+3.25	
17_A_4	3'UTR of <i>Tb11.01.6380</i>	Chr 11	hypothetical protein with PSP1 C-terminal conserved region	+5.67	
17_B_16	Tb927.8.7820	Chr 8	hypothetical protein, conserved weakly similar to Cold shock protein.	+6.32	
38_C_5				+7.20	
24_F_12	Tb927.6.1780	Chr 6	mitogen-activated protein kinase,	+3.54	
32_L_10	Tb11.03.0570	Chr 11	5' end of CDS, including 5'UTR, and RBP3 3'UTR?	+3.99	

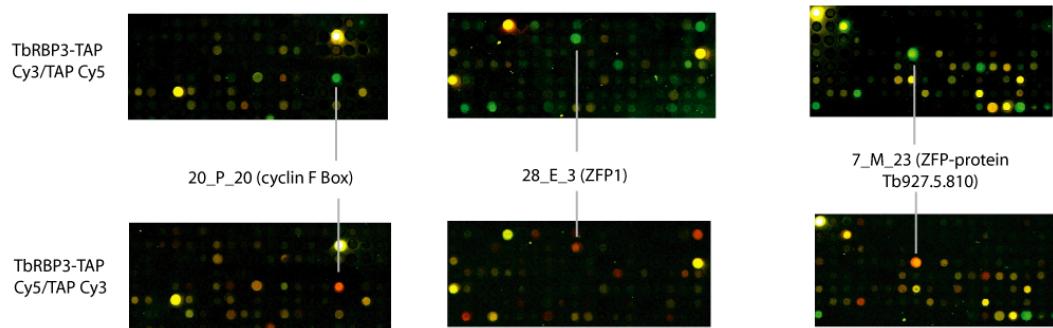
Table 3.2. Positive clones for TAP-*TbRBP3* overexpression.

Comparison of the previously described microarray data (from cells over-expressing or knocking-down RBP3) with the data obtained for the transcripts that specifically co-purify with the over expressed *TbRBP3* showed several differences. First, in most of the cases (65%) at least 2 clones represented the same transcript showing the high integrity of the DNA-chips. Transcripts corresponding to proteins containing either a zinc finger- or a cyclin F-box domain were overrepresented (9 clones). An enrichment for a transcript derived from the same cluster as the cyclin F box domain proteins was also observed.

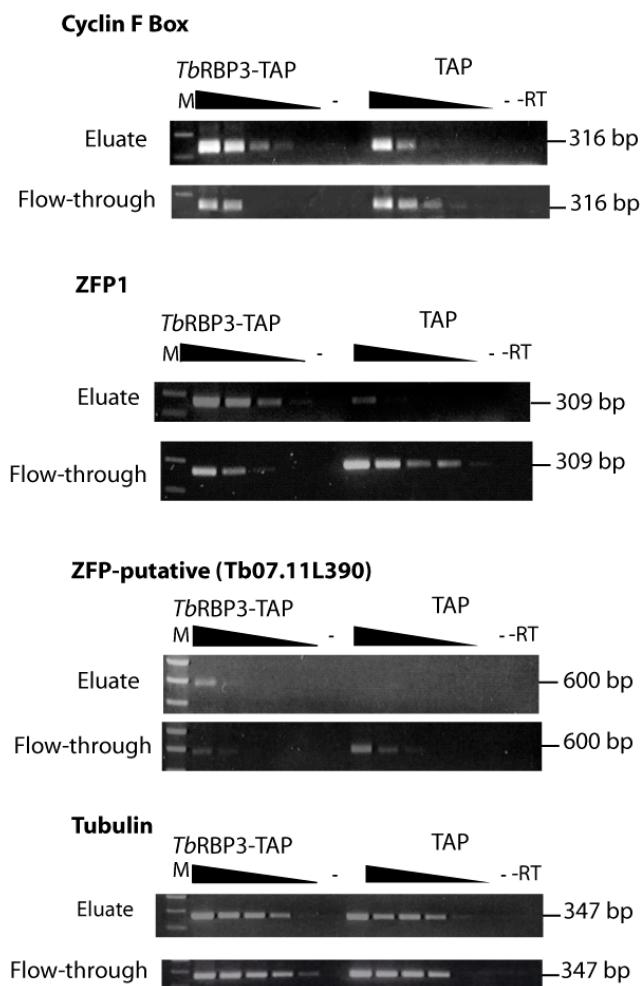
In order to confirm some of these data, RT-PCR was performed. Briefly, total RNA from *TbRBP3*- and 918- flow-throughs and from the eluates after TEV cleavage were extracted and reverse transcribed using oligo-dT. 1:10 serial dilutions (until 1:10000) of the cDNA generated were used as a template for the PCR, using specific primers for each transcript. After 30 amplification cycles, 10 µl of each sample were run on an agarose gel. Tubulin was used as a control. Figure 3.1.9 (upper part) shows an example of the microarray results for the 2 transcripts tested and the PCR products obtained (lower part left). As expected, a product of 315 bp for cyclin F-box (*Tb927.1.4580*) and a band of 309 bp for ZFP1 (*Tb927.6.3490*) were obtained. Both products were detected at higher dilutions of template cDNA in the eluate from *TbRBP3*-TAP compared to the control. This indicates that these transcripts really bind this protein preferentially and therefore co-purify with the TAP-tagged *TbRBP3*. The results of the PCRs for the flow through confirmed this – the products were seen at higher dilutions of template in the fraction corresponding to the control. Tubulin (380 bp) was equally present in the different samples. It was decided to look at the spots corresponding to these transcripts on the slides for the over-expressed *TbRBP3* in the previous arrays. The transcripts containing a cyclin F-box domain were between 1.5- and 2-fold up-regulated, but this increment was not consistent in all the slides analysed, probably this is why they were eliminated as targets by the M-CHiPS analysis. Northern blot analysis of the over-expressing cell line confirmed that the increment of cyclin F-box mRNA was not more than 2-fold; the same was true for ZFP1 (figure 3.9.C).

Two other RNAs encoding for zinc finger-containing proteins were enriched in the RBP3 eluate: *Tb927.05.810* and *Tb07.11L3.90* (Figure 3.9.1.C). The latter one is a protein interaction partner of 14-3-3 proteins in *T. brucei* (C. Benz, manuscript in preparation) and it was found in this microarray analysis. The binding of this transcript to TAP-*TbRBP3* was established by RT-PCR (Figure 3.1.9.B). Interestingly, the mRNA levels of both transcripts were 3-fold up-regulated in bloodstream forms upon over-expression of *TbRBP3* (Figure 3.1.9.C).

A.



B.



C.

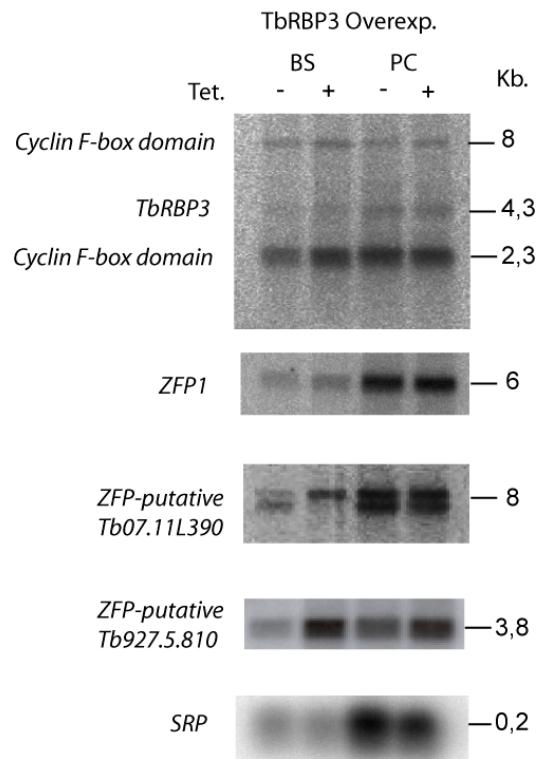


Fig. 3.1.9 Cyclin F box and ZFP mRNAs are enriched in the eluate of *TbRBP3-TAP*. A. Microarray slides showing positive spots. B. RT-PCR from eluates and flow-throughs after the first step of TAP purification. C. Northern blot showing mRNA levels of cyclin F box and ZFP after *TbRBP3* over-expression.

3.1.5 Interacting partners of *TbRBP3*

It is well established that the role of many RRM-containing proteins is not only mediated by the direct interaction with RNA molecules but also with other proteins. In particular, the interaction of *TcRBP3* with other polypeptides is known (De Gaudenzi, D'Orso et al. 2003). To determine if this holds true for *TbRBP3*, the tandem affinity purification (TAP) procedure was used (Puig, Caspary et al. 2001). As explained before, a tetracycline inducible C-terminal TAP-RBP3 was generated by cloning the *TbRBP3* ORF into pHD918. For the TAP- purification extracts from cell lines over expressing the tagged protein were used and the presence of putative targets was detected by SDS-PAGE and silver staining. Unfortunately, it was not possible to identify interacting partners of *TbRBP3* using this strategy. *TbRBP3* itself was also not detected. It is possible that the protein is expressed at very low levels and therefore higher amounts of cells are required for the analysis. Other strategies such as immunoprecipitation using the *in situ* V5-RBP3 or the myc-tagged cell line should be considered.

3.2 Functional characterisation of the amino acid transporter 11 3'-UTR

As mentioned in the introduction, trypanosomatids mainly regulate gene expression through post-transcriptional mechanisms. In particular, sequences located in the 3'-untranslated regions (3'-UTRs) of the mRNAs are necessary to control mRNA levels of developmentally regulated genes. By microarray analysis of differentially transcribed genes in the bloodstream and procyclic stages of the parasite, a clone corresponding to the amino acid transporter 11 (Tb927.4.4730) was found to be 4.8 fold up-regulated in procyclic TREU/927 cells. The main aim of this work was to investigate whether the 3'-UTR of the mRNA is involved in this developmental regulation and to find the sequences involved in this control.

3.2.1 Identification of the Amino Acid Transporter (AATP) 11 3'-UTR

To map the 3'-UTR of this amino acid transporter (AATP 3'-UTR), a reverse transcriptase reaction was performed with RNA from both bloodstream and procyclic *T. brucei* (Hug, Hotz et al. 1994). Oligonucleotide cz 1948 (5'-(dT)₁₈(AGCT)₃-3') was used for cDNA synthesis followed by a PCR using CZ 1948 and the coding sequence (CDS)-specific primer cz 1951, which anneals 160 nt downstream of the AATP stop codon. 1/10 volume of the PCR was used as a template to perform a second PCR with oligonucleotides cz1948 and cz 1952, an intergenic region (IGR)-specific primer that anneals to the first 24 nucleotides after the AATP stop codon. Several products between 0.2 and 0.8 kb were amplified, cloned into pGEM-T, and sequenced. Figure 3.2.1 shows the location of the oligonucleotides used (A) and the PCR products generated in the second PCR (B). Although it is a developmentally regulated gene (C), amplification products of a similar intensity were obtained from cDNAs from BS and PC. Sequencing analysis revealed the presence of different polyadenylation sites: at nucleotides 64, 220 and 833, the last one was one of the most prominent bands shown in the PCR. To determine which one is used by the parasite, they were compared to the length of the AATP mature RNA. As can be seen in figure 3.1.1 C, the endogenous transcript has a size of 2.3 kb as estimated by Northern blot analysis: 1.4 kb corresponds to the CDS and 60 bp to the 5'-UTR. That means that the 3'-UTR has a length of approximately 0.8 kb. Therefore the higher product (833 nt) obtained in the PCR must signal the polyadenylation site that is used by the parasite. The shorter products obtained could be due to internal priming of the oligo dT on poly (A) sequences within the 3'UTR of the mature RNA.

A.

tcttcttgagatgaagaagcggtccgtactcgatgaccgcgtgcggcggtgagctgctccattgctgt
 gtgtctacctgctcaccggattttcggtacgcagaattcgcccaacgggtggagggtccgttctgaagctt
 acgaccctacgcaaatccgttcttgcattggcatcatcgtaactctgcgtgggttc
 ttaacatgcgtcgatgccgtacagccctttcagggtgcgtcggtggacatggataccatgtcataatgttaggc
 acagtatcgatcgactgtttcatttgcgtcggtcggttc
cz1951-fw-CDR
 tcggacttgggtgcctctcggtgggttcattggcttatattccccactttcatcatgtacgcgggtg
 gttggacacgccaagcggtgggtgcagttatcttgacatatgtattgcattctgggttgttgc
cz1952-fw-IGR
 ttgtcttggtagcggcgtctgttactacacaattaagaggattctAAggaggggaggcggttagcagg
agagggagtgcaaacagccatttcaacacctcacgaatgtaatgaagaaaaataataatatttacttc
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 TAATTTActgttgcatttgcatttgcattttgcattttgcattttgcattttgcattttgcattttgcatttt
cgtttatqtaacqqaaccgtqacqcagtgaggactcaaccaataactttctatttttttttgcatttt
tactactaccaggtctttcacttttcttttgcattttgcattttgcattttgcattttgcatttt
ggcaagcgtcgttacatgcatatagcgtgcgcgtcaatggattactgagaaaagactaaagaaatatc
tactttcaatacATTTATTACTTTTCCTTgtctgcactaactatggtaacttatttcctccactgtctta
 ttttcgttctgtttgttgccccttttttttgcattttgcattttgcattttgcattttgcatttt
ccaagccgttccgtgttattccactttgcgttatgcattcaacgcgttatcttttttgcatttt
actttcccaccctaccaccaccaccctgcagccgtgcgtgaggcaggcgataacttgcgg
 aaaaactatgcgc
 aagcgcgcaataaaataaaagatataatataatatgcgttatctccgtgcgtccctcgcttaa
 cgccttattccaacgaatttcttttttttacttcgtgcgtccaccgctacgccttgcggatgggtgcgtt
 taaacactacatttgcatttgcattttgcgttatgcattttgcattttgcattttgcatttt
 ATG **cz1948-rev-olgo(dT)18**

- | | |
|----------------------|----------------|
| ■ Δ1 (0-40, 630-833) | — Δ4 (290-350) |
| ■ Δ2 (141-260) | — Δ5 (380-420) |
| ■ Δ3 (290-618) | — Δ6 (535-610) |
| | — Δ7 (448-500) |

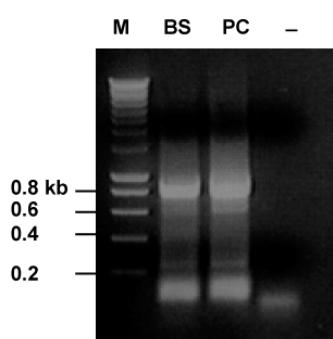
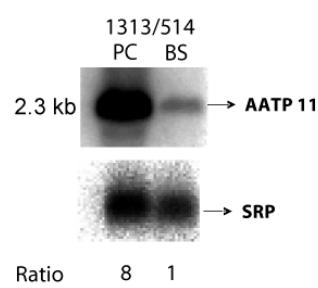
B.**C.**

Fig. 3.2.1 Determination of the AATP 11 3'-UTR. A. Part of coding sequence (CDS in italics) and intergenic region (IGR) sequence of AATTER 11 (Tb927.4.4730). The primers used for the PCRs (CZ1951 and CZ1952) are shadowed. Poly pyrimidine (poly(Y)) tract is underlined and the putative polyadenylation sites obtained by PCR are labelled with black arrows. The polyadenylation site for the AATTER 11 mRNA is labelled with a red arrow. B. Products obtained during the second round of PCR (oligos CZ1952 and CZ1948). The bands were cut and sequenced. The higher product (0.8 kb) corresponds to the 3'-UTR. C. Northern blot showing that this amino acid transporter is 8 fold upregulated in procyclic cells.

The results obtained agree with the mechanism that has been proposed to define a 3'-UTR in *Trypanosoma*, i.e. the polyadenylation site is roughly 100 nt upstream of a polypyrimidine (poly(Y)) tract (8-25 nt.) which signals *trans* splicing of the downstream mRNA (Hug, Hotz et al. 1994; Benz, Nilsson et al. 2005). The AG *trans* splicing acceptor, the poly(Y) tract and the polyadenylation sites are depicted in figure 3.2.1.

3.2.2 Role of the AATP 3'-UTR in developmental regulation of a reporter gene

To determine whether this untranslated region is involved in developmental control of gene expression, the 3'-UTR was amplified using the primers cz1952 and cz1948 and cloned downstream of the chloramphenicol acetyl transferase (CAT) reporter gene into the plasmid pHD1437 to generate the plasmid pHD1470. The plasmid pHD1437 is an optimised vector for analysis of 3'-UTRs: it contains a promoter for bacteriophage T7 polymerase to allow high levels of transcription (50 fold higher than RNA pol I) mediated by T7 polymerase, the 5'-UTR of the EP1 gene and the CAT gene that allows to measure the effect of the 3-UTR in an easy way. The main features of the vector are indicated in figure 3.2.2.

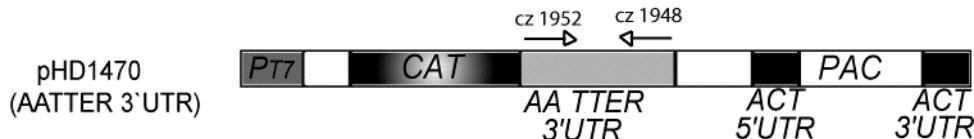


Fig. 3.2.2 Backbone plasmid used for analysis of AATP 3'-UTR

Both bloodstream and procyclic 427 1313/514 cell lines constitutively expressing the bacteriophage T7 polymerase (from pHD 514, G418 resistance) and the *Tn10* Tet-repressor (pHD1313 phleomycin resistance) were transfected with this plasmid. After selection with puromycin, the effect of the 3'-UTR on CAT mRNA levels was evaluated by Northern blot using CAT ORF as a probe. As a control the vector containing actin 3'-UTR instead of AATP 3'-UTR was used. Figure 3.2.3 shows the presence of a developmental regulated transcript of 1.8 kb corresponding to CAT- AATP 3'-UTR. It is approximately 7-fold more abundant in PC compared to BS (35% vs. 7%), while the levels of the reporter mRNA under the control of actin 3'-UTR were the same in both life stages. This difference is consistent with the values obtained for the endogenous AATP transcript. To measure the effect at the translational level, CAT assays of cellular extracts were performed. CAT activity in procyclic cells was 9.5-fold higher than in bloodstream, reflecting the regulation observed at the mRNA level and

indicating that this regulatory element is also involved in translational control, although at low level. Therefore the *AATP* 3'-UTR is involved in developmental control of gene expression.

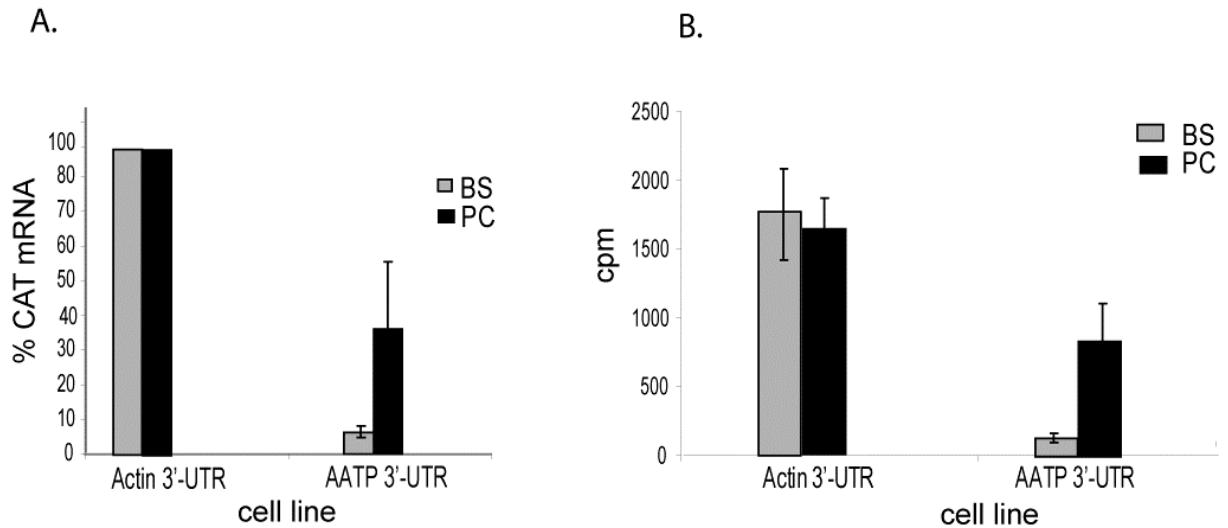


Fig. 3.2.3 AATP 3'-UTR is involved in developmental regulation of CAT. A. CAT mRNA levels from PC and BS under the control of AATTER-3'-UTR and actin 3'-UTR. B. CAT activity of the cell lines tested (cpm/ug protein). CAT-Actin 3'-UTR was used as positive control.

To study in more detail which sequences are involved in this regulation, several deletion mutants were created. In order to choose the regions to be deleted, the secondary structure of the 3'-UTR was predicted using M-FOLD, on the server Rensselaer Polytechnic Institute's School of Science (www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi) using the default values as parameters. The most probable structure considering the plot energy was chosen and 3 big deletions were done: $\Delta 1$ (the first 39 nt and the last 113 nt were deleted, 631 remained), $\Delta 2$ (between 142 and 257 nt, 718 remained) and $\Delta 3$ (between 290 and 618, 505 bp) generating the plasmids pHd1481, pHd1482 and pHd1483 respectively (Figure 3.2.4).

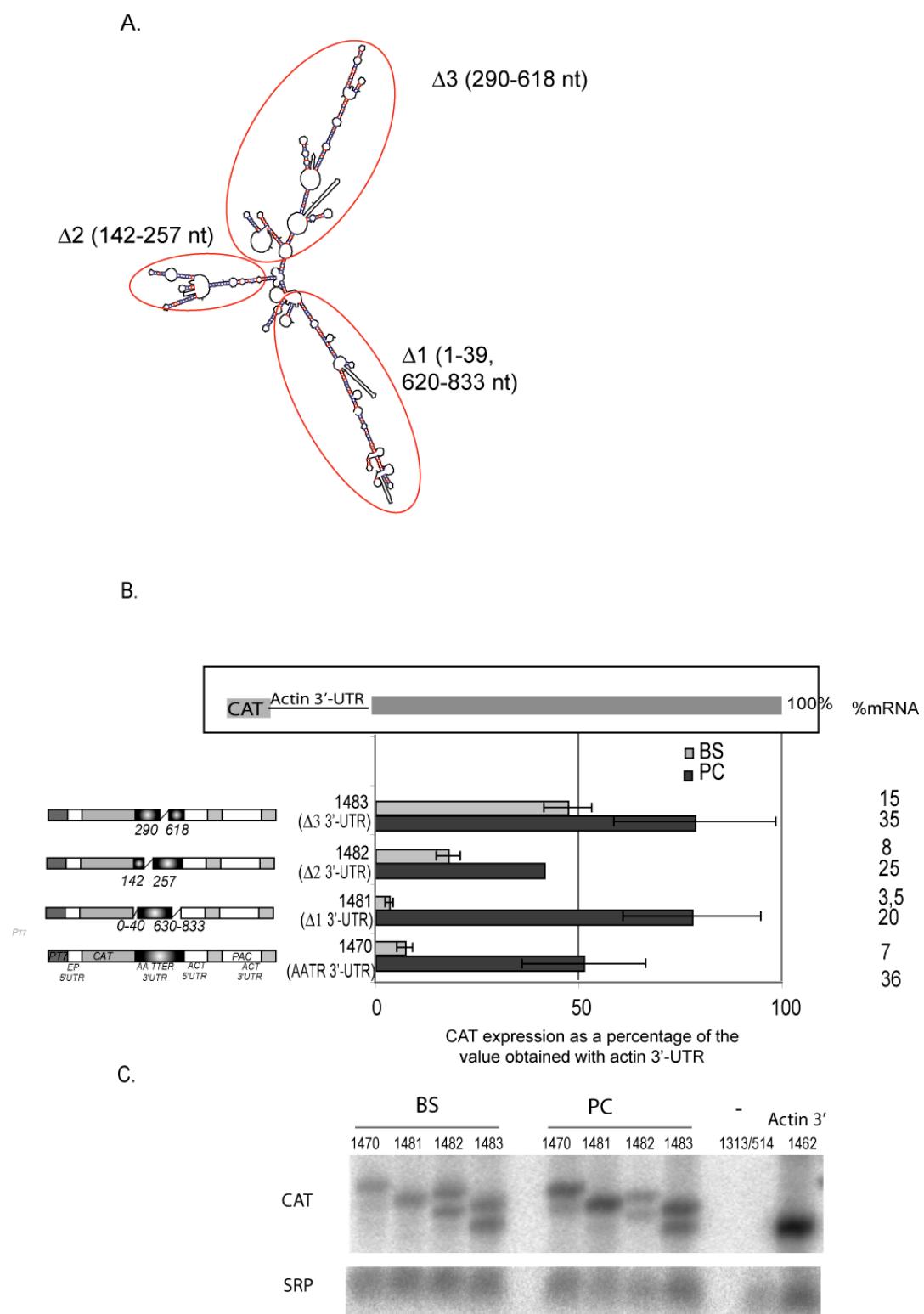


Fig. 3.2.4 A sequence contained in the Deletion 3 plasmid is involved in developmental control of CAT. A. Secondary structure of the AATP 3'-UTR. Regions selected for the deletions are shown. B. CAT activity (left chart) and mRNA levels (right column) expressed as percentage of actin (100%). C. Northern blot of the different mutants.

After transfection and selection with puromycin, total RNA was extracted and Northern blots were done. The CAT activity of the protein extracts was also measured. The values shown in figure 3.2.4 (Northern blot is shown in C and the values obtained are shown at the right side of the chart) are expressed as a percentage of CAT-actin 3'-UTR. For deletion 1, a transcript of about 1.5 kb in size was obtained, while for the other 2 deletions 2 different transcripts were detected: one of the expected size (1.7 and 1.2 kb for deletion 2 and 3 respectively) and another one 400 bp smaller, indicating the use of an alternative polyadenylation site. In procyclic cells, CAT mRNA levels were slightly down-regulated in the 3 constructs generated, but this reduction was less than 50% compared to the plasmid containing the entire 3'-UTR. In bloodstream forms, the mRNA levels decreased to 50% for the first deletion while for deletion 3, the amount of transcript was 2-fold higher compared to the whole 3'-UTR (pHD1470). However, these differences were not highly significant.

CAT assays were done to check the effect of the three deletions at the translational level (figure 3.2.4 part B). In contrast to what was observed with mRNA abundance, CAT activity increased for $\Delta 1$ and $\Delta 3$ in procyclic cells, resulting in an activity that was between 20-25% higher as that obtained for pHD1470. In bloodstream forms, the difference in CAT activity was only significant for deletion 3. For this construct, the CAT activity increased 7-fold compared to the entire 3'-UTR, which equals 90% of the value obtained for the whole AATP 3'-UTR in procyclics and indicates that there is a destabilising sequence between nt 290-618 that down-regulates gene expression at both transcript and translational levels in bloodstream forms.

To map in more detail this destabilising region, 4 smaller deletions (of 50 nt in average) encompassing structural elements in this region were done, generating plasmids pHD1575 ($\Delta 4$, 290-350 nt), pHD1576 ($\Delta 5$, 380-420 nt), pHD1577 ($\Delta 6$, 535-610 nt) and pHD1578 ($\Delta 7$, 448-500 nt). Drastic changes in CAT mRNA levels were however not observed. The effect at the translational level was more evident in procyclic cells (Figure 3.2.5). In particular, for the cells containing deletions 4 and 6 the levels of CAT activity increased by almost 3-fold compared to the cell line carrying the whole 3'-UTR and they were 20% higher than the actin 3'-UTR (positive control), suggesting the presence of elements that are normally inhibiting translation. In bloodstream cell lines, the protein activity was not considerably affected; only for deletion 4 a 4-fold increase in CAT activity was observed. However, it does not reach the levels previously observed for the large deletion in these cells. Therefore a smaller regulatory sequence could not be defined. Considering that the regions to be deleted were based on a

prediction, the possibility that the regulatory element was not included in the mutants cannot be entirely ruled out.

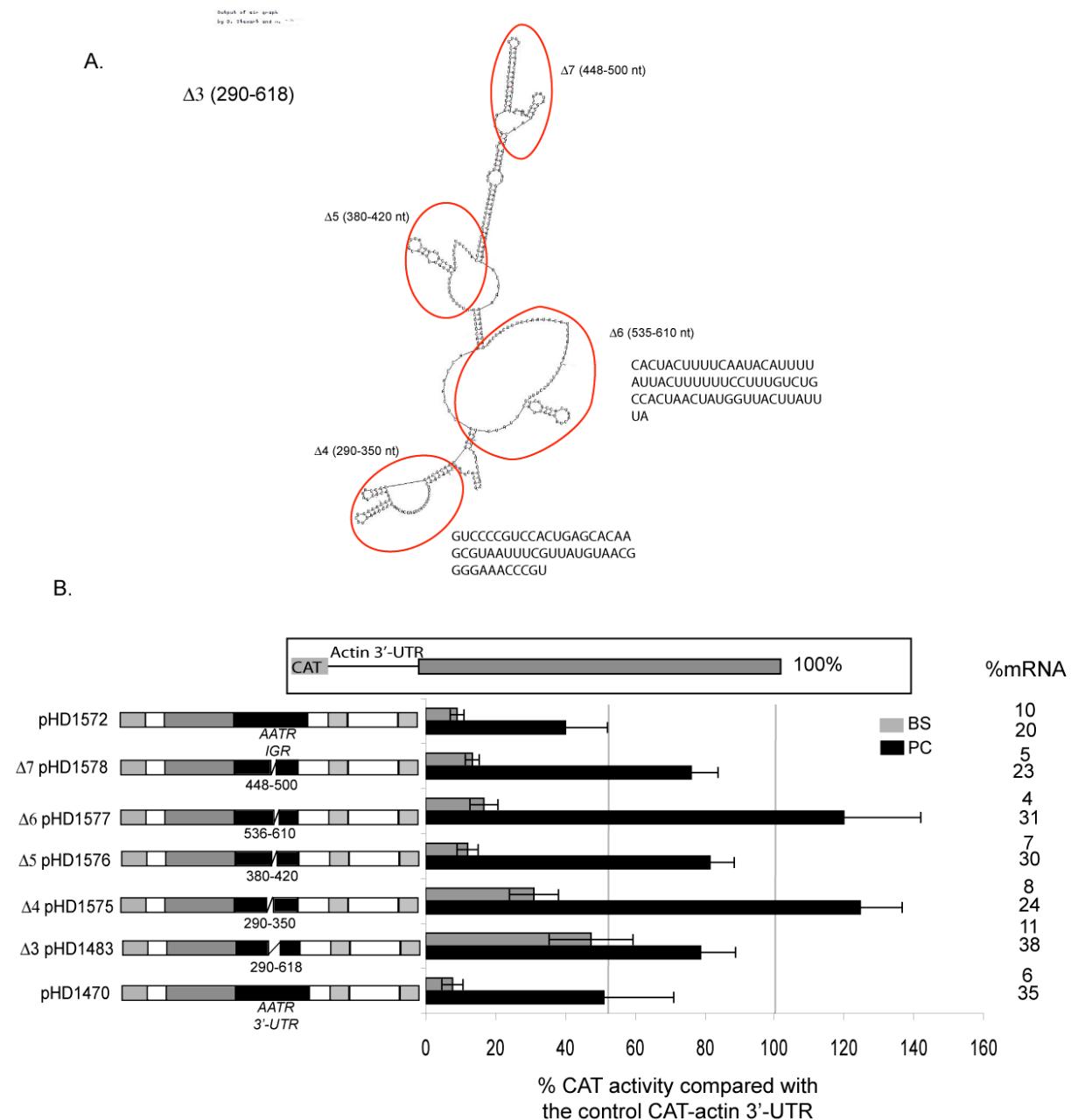


Fig. 3.2.5 Smaller deletions in region 3 affected translational activities. A. Secondary structure of deletion 3 and the regions chosen to be deleted. B. CAT activity and mRNA levels.

3.2.3 Looking for proteins involved in control of AATP 3'-UTR

3.2.3.1 Electrophoresis mobility shift assay (EMSA)

The effect obtained with deletion 3 indicated the presence of regulatory sequences in this region, so we decided to look for proteins that interact with this part of the 3'-UTR and therefore mediate this effect. The first approach was to check for direct protein:RNA interactions using electrophoresis mobility shift assay (EMSA). This technique is central to study gene regulation and is based on the observation that sequence-dependent protein:DNA or protein:RNA complexes migrate more slowly than free nucleic acid molecules when subjected to non-denaturing polyacrylamide or agarose gel electrophoresis. Because the rate of DNA migration is shifted or retarded upon protein binding, the assay is also referred to as a gel shift or gel retardation assay.

For the assay, first the region corresponding to the nucleotides 290-618 was amplified by PCR and cloned into pBlueScript SK as HindIII/BamHI. This allows *in vitro* transcription of the fragment using either T7 or T3 polymerase, generating either a sense or an antisense probe, the latter being used as a negative control. The *in vitro* transcription was done using Maxi Script II (Ambion) using ^{32}P -UTP, and the labelled probe purified. 10000 cpm of each transcript (sense and antisense) were incubated with 30 µg of cellular extracts from either 427 1313/514 procyclic or bloodstream forms in the presence of tRNA as a competitor for 15 min at 25°C. Then the samples were run on a non-denaturing 6% polyacrylamide gel, dried and exposed to a film at -70°C overnight. In the first attempts to this assay the probes were degraded during the incubation time, so we decided to select the nucleic acid binding proteins present in the extracts through heparin-sepharose chromatography. Using this approach a small band with slower migration was observed using the sense probe and procyclic extracts (Figure 3.2.6), however later attempts to reproduce and improve the results were unsuccessful.

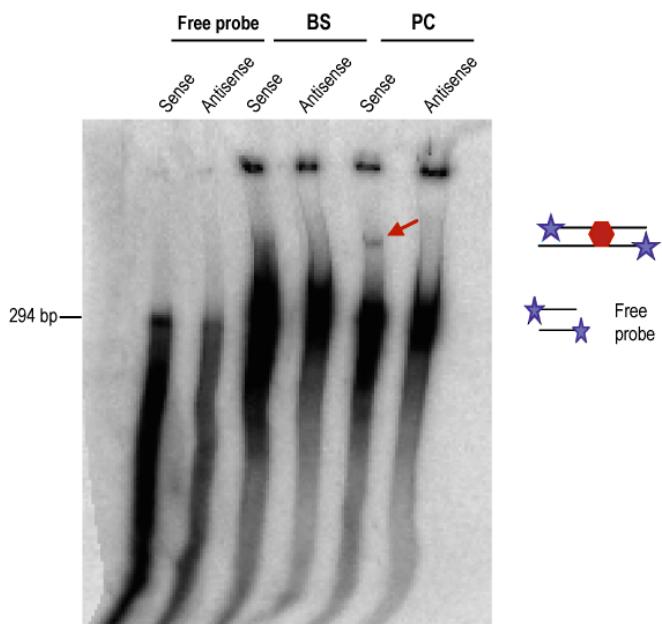


Fig. 3.2.6 EMSA to detect interacting partners of *TbRBP3*. Cell extracts from both BS and PC were incubated with an *in vitro* transcribed deletion 3 (sense) and run into a non denaturing PAGE 5%gel. Degradation of the free probe is observed. A small band with slower migration is seen in PC incubated with the sense probe.

3.2.3.2 Tobramycin affinity chromatography

As a second approach to identify *trans* elements interacting with the AATP 3'-UTR, I used an RNA affinity tag developed to isolate pre-mRNA-spliceosome complexes. It has previously been shown that a 40 mer aptamer stem-loop (named J6f1) stoichiometrically binds the aminoglycoside antibiotic tobramycin under physiological conditions. The dissociation constant is approximately 5 nM and it can strongly discriminate between similar aminoglycosides with respect to binding (for G-418 K_d is 5 μM). The sequence for the aptamer was generated using the partially overlapping primers cz2436 and cz2437, amplified by PCR and introduced between the CAT gene and the 3'-UTR BamHI of pHD1470 generating the plasmid pHD1644. This plasmid was used to transfect bloodstream and procyclic 427 1313/514 cells. A diagram of the purification procedure is depicted in figure 3.2.7. Briefly, cell extracts from cells containing and lacking the antibiotic binding site are incubated with a previously coupled tobramycin-derivatised Sepharose beads. After several washing steps, proteins bound to the 3'-UTR coupled to the RNA-aptamer molecule can be eluted by adding an excess of this aminoglycoside.

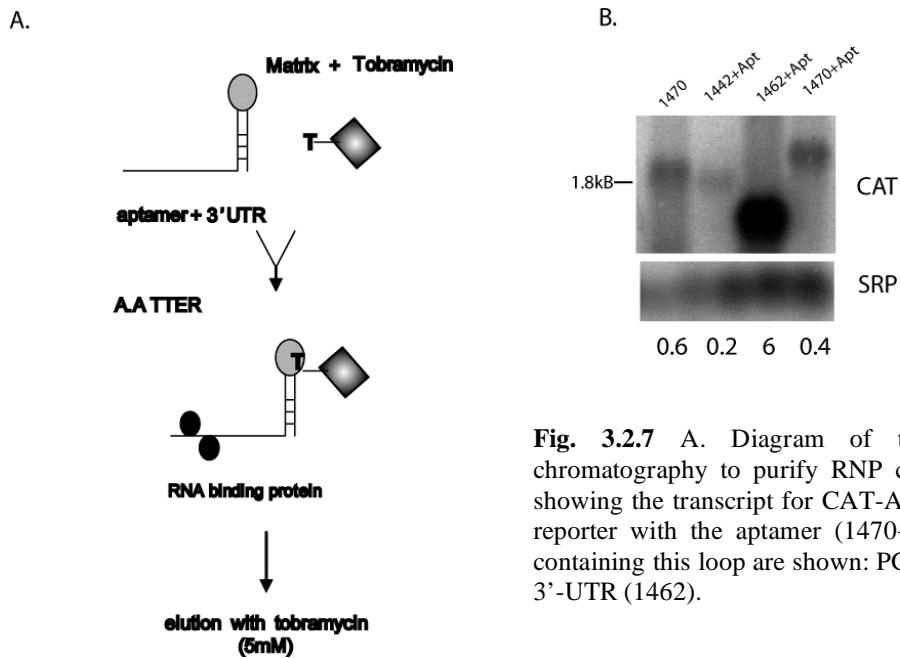


Fig. 3.2.7 A. Diagram of the aptamer based-affinity chromatography to purify RNP complexes. B. Northern blot showing the transcript for CAT-AATP 3'-UTR (1470) and the reporter with the aptamer (1470+Aptamer). Other constructs containing this loop are shown: PGKs 3'-UTR (1442) and actin 3'-UTR (1462).

After transfection, the presence of the aptamer-reporter mRNA was monitored by Northern blot and the ability of the transcript to bind to the tobramycin matrix assessed. For this, total RNA from cells containing and lacking the aptamer was extracted and incubated with the derivatized matrix. After elution, the RNA was reverse transcribed and the cDNA obtained used as a template for PCR. Unfortunately no product was observed (data not shown), suggesting that the RNA did not bind to the antibiotic. One possible explanation is that the stem loop structure is hidden and therefore not accessible to the beads. For this reason another approach was followed.

3.2.3.3 Pull down of RNP complexes using tethered-based systems

The tethered function analysis has been employed to test the function of many RNA binding proteins (Coller and Wickens 2002). It consists of an RNA:protein interaction that is mediated by a chimeric protein containing a peptide which binds a specific RNA stem loop with high affinity and a reporter mRNA carrying the recognition sites. I tried to adapt the system to pull down the proteins bound to the AATP 3'-UTR and the overall concept is summarized in figure 3.2.8. For this purpose several cell lines were generated that constitutively express the reporter containing the tether RNA between the CAT reporter gene and the 3'-UTR. Then these parasites were cotransfected with a plasmid containing a tetracycline inducible tethered protein (figure 3.2.8 upper part). After induction by adding tetracycline, the tethered protein will interact with the tether RNA bound to specific interacting proteins (lower part). These mRNPs can be isolated by immunoprecipitation. The success of the procedure can be assessed

by Western blot to detect the tethered protein and the specificity of the bound mRNA can be evaluated using RT-PCR. Most important, the interacting partner of the 3'-UTR can be identified by mass spectrometry. The constructs used for this approach are listed in table 3.3.

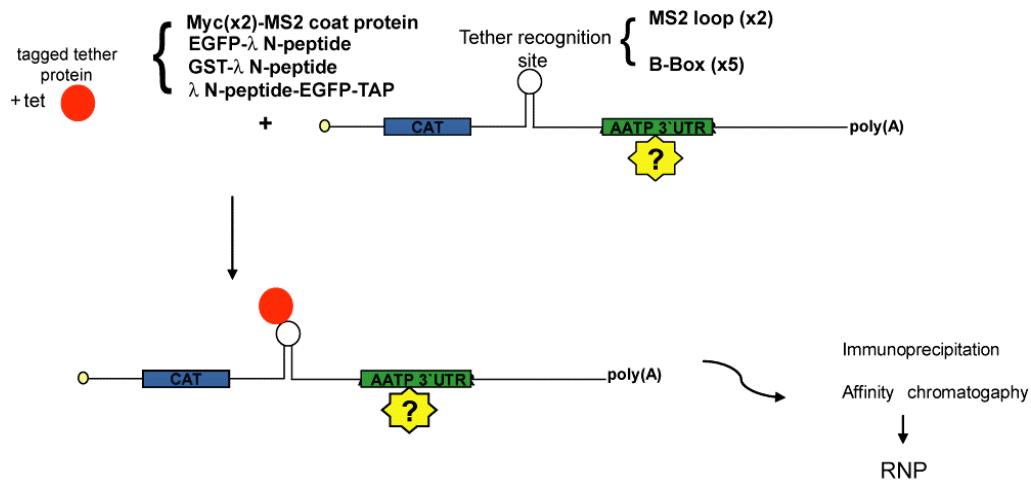


Fig. 3.2.8 Schematic representation of the tethering strategy to pull down AATP-3'-UTR RNP complexes. The RNA recognition site and the tethered proteins used are shown.

System	Plasmids	Features	Cloning sites
Tobramycin affinity	pHD 1644	pHD 1470 (AATP 3'-UTR) + aptamer Jf61 (Cho, Hamasaki et al. 1998; Hartmuth, Urlaub et al. 2002)	BamHI
	pHD 1668	pHD 1462 (Actin 3'-UTR) + aptamer Jf61 (Cho, Hamasaki et al. 1998; Hartmuth, Urlaub et al. 2002)	
MS2 tethered based	pHD 1706	pHD1470 (AATP 3'-UTR) + 2 MS2 binding sites (from pIII-MS2-4) (SenGupta, Zhang et al. 1996).	BamHI
	pHD 1709	pHD 918 + MS2 CP (DNA template from <i>S. cerevisiae</i> 140ura3pHybLex/Zeo-MS2) (invitrogen) (SenGupta, Zhang et al. 1996))	HindIII/BamHI
	pHD 1710	pHD1709 + 1 myc tag (from pHD1484).	NsiI/HindIII
	pHD 1711	pHD1709 + 2 myc tags (from pHD 1700).	
	pHD 1708 pHD 1693 pHD 1704 pHD 1743	pHD 1470 (aatter 3'-UTR) + 5 Box B sites pHD1502 (pHD924+ λN)+ EGFP pHD1502 (pHD 924 +λN) + GST pHD918+ λ-N +EGFP+TAP	BamHI HindIII/HpaI HindIII/HpaI λ-N: HindIII/ApaI EGFP: ApaI/HpaI TAP:HpaI/Bam HI

Table 3.3 Systems and plasmids used to purify RNP AATP 3'-UTR RNP complexes

3.2.3.3.1 MS2 coat protein system:

The first system employed was derived from the coat protein of the coliphage MS2 (MS2 CP), an RNA-binding protein of low molecular weight (13 kDa) involved in translational repression by binding to a 33 nt RNA secondary structure (MS2 loop) within the translational initiation region of the coliphage replicase gene (Peabody and Ely 1992; Peabody 1993). The interaction between these elements is cooperative, so adding more MS2 hairpin loops increases the affinity. Two MS2 binding sites were inserted between the CAT gene and the AATP 3'-UTR from pHD1470. The new construct was transfected into 427 1313/514 procyclic cells and selected by adding puromycin. A tetracycline inducible 2 myc-MS2 CP fusion protein was generated and used to co transfect procyclic cells carrying pHD1706 and selected with hygromycin. The expression of the protein was induced by addition of tetracycline (100 ng/ml). As a control I used a cell line containing the plasmid pHD1470 (without the RNA binding site) and the MS2 CP. The CAT-MS2-AATP 3'-UTR RNPs were pulled down by immunoprecipitation with anti-myc sepharose and eluted from the beads by adding myc-peptide. In order to standardize the conditions I performed an IP from 35 S-methionine metabolic labelled cell extracts. The myc-MS2 CP was detected as 17 kDa band in the eluates of both cell lines (Figure 3.2.9), suggesting that the IP parameters were accurate. This was also confirmed by Western blot. However, later attempts to precipitate the RNP complexes failed because the cell line lost the expression of the fusion protein during culture, and even initially it was produced at low levels.

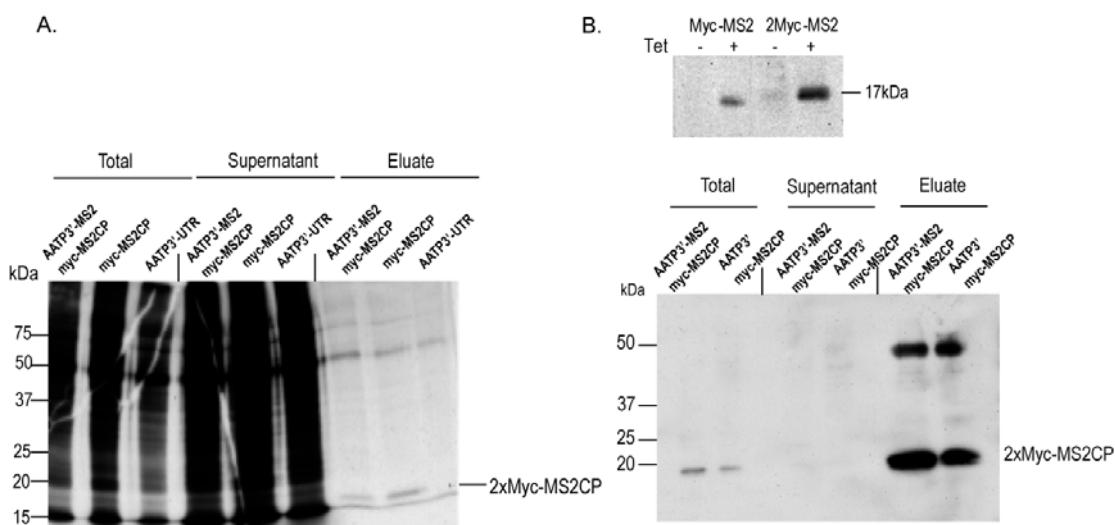


Fig. 3.2.9 Anti-myc antibody is able to immunoprecipitate MS2 CP. A. Immunoprecipitation with 35 S metabolically labelled procyclic cells containing either the reporter gene without binding sites or the protein alone or the over expressed protein and the reporter gene with MS2 binding sites. B. Western blot of procyclic cells expressing MS2 CP with 1 or 2 myc-tags and after immunoprecipitation Anti-myc antibody was used. Amount of cells: upper part: 1×10^6 cells. For the immunoprecipitation 1×10^6 cells were used and 7×10^6 cells.

3.2.3.3.2 λ-N peptide systems:

Due to the low expression of the MS2 CP, an alternative strategy consisting of a λ N-peptide was followed. This molecule is derived from the N protein of the lambda bacteriophage and it is involved in the antitermination mechanism of transcription of early genes through the interaction with the box B in the nascent RNA (Chattopadhyay, Garcia-Mena et al. 1995, Barik, 1987 #112). This is a 15 nt RNA stem loop frequently used for tether analysis (Coller and Wickens 2002). The approach is analogous to that described for MS2. Briefly, 5 copies of the box B were inserted in the reporter plasmid. Procytic cells containing the new construct were cotransfected with several tetracycline inducible proteins containing the λ-N peptide. Three different fusion proteins were tested: EGFP-λ (pHD1693), GST-λ (pH1704) and λ-EGFP-TAP (pHD1743). Cell lines carrying the protein and the reporter construct without the RNA binding sites were used as negative controls.

The first system employed consisted of the EGFP-λ protein. The presence of the GFP had the advantage of allowing easy detection of protein expression by immunofluorescence and available anti-GFP antibodies. The anti-λ-N peptide antibody was not used in any of the systems tested because of its low specificity and poor availability. The expression of the fusion protein was assessed by Western blot (Figure 3.2.10 B, upper panel). As expected, a band of 26 kDa is recognized by anti-GFP antibodies after induction of the protein in procytic forms. A cell line containing EGFP alone was used as a control

Immunoprecipitation of metabolically labelled cell extracts using anti-GFP antibody showed that the tagged protein was efficiently pulled down, and no signal was observed in the supernatant (Figure 3.2.10 A). After scaling up the system (to 3×10^8 cells) the protein could not be immunoprecipitated as efficiently as before and most of it was found in the supernatant but not in the eluate. This is shown in the lower part of figure 3.2.10 B (see third line in both supernatant and eluate fractions). The specificity of pull down of CAT-λ-AATP 3'-UTR mRNA was evaluated by RT-PCR after IP using a forward oligonucleotide that anneals to the CAT coding sequence and a reverse primer annealing to the 3'-UTR, and primers for actin and cyclin F box as controls. PCR analysis of the RNA present in the supernatant is shown in part C of the figure 3.2.10. Only signals for actin and cyclin F box were detected in the control (reporter without Box B). No PCR product was obtained when the PCR was done for detecting CAT-AATP 3'-UTR, although the construct is present in the cells. This result was not expected, as most of the RNA must be present in the supernatant fraction of the IP. It is

assumed that the RNA was degraded either during the IP or the purification step. It might also be that the interaction between the mRNA and the proteins is weak and easily disrupted during the procedure.

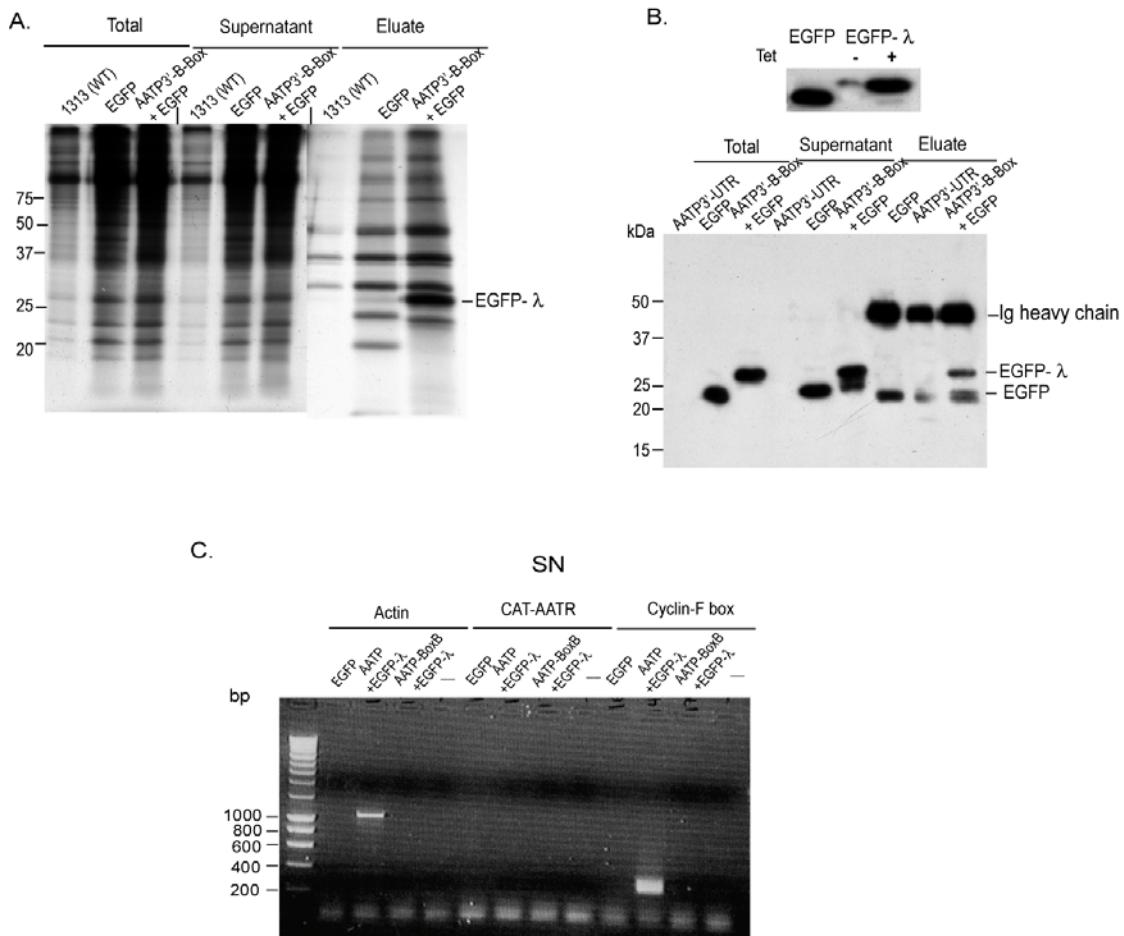


Fig. 3.2.10 Immunoprecipitation using EGFP-λ as tethered protein. A. Immunoprecipitation tithe ^{35}S metabolically labelled procyclic cells containing either the reporter gene without binding sites or the protein alone or the over expressed protein and the reporter gene with box B. B. upper panel: Western blot of procyclic cells expressing EGFP alone and with the tagged version. An increase of 2 kb is seen in the band recognized by anti GFP. Lower panel: Western blot after IP with Anti-GFP antibody. First line: PC with the reporter gene (without box B). Second line: PC containing EGFP. Third line: PC containing the reporter-B Box construct and over expressing EGFP-λ. C. To check the binding specificity RT-PCR was done. No amplification products were obtained.

In order to stabilise the interaction between the components of this RNP, a reversible chemical cross linking step using formaldehyde was performed. Briefly, procyclic forms over expressing EGFP-λ N peptide were incubated with 1% formaldehyde for 10 min to allow the formation of the cross-link and the IP was performed as usual. After the elution step, the crosslink was reversed by incubating the samples at 70 degrees for 45 min. Unfortunately no PCR products were obtained suggesting that the binding was not improved by this preliminary step (data not shown).

The last approach followed was to construct a fusion protein containing the λ N-peptide at the N terminus followed by EGFP and a TAP tag at the C terminus (pHD1743). The general idea was to pull down the RNA reporter and the interacting proteins using the λ N-peptide and then to purify the complexes performing the first step of the TAP purification, as explained before. The Western blot in figure 3.2.11 shows a procyclic cellular extract before and after induction of the protein by adding tetracycline. A band of 45 kDa is recognized by antibodies against the 3 different components of the construct.

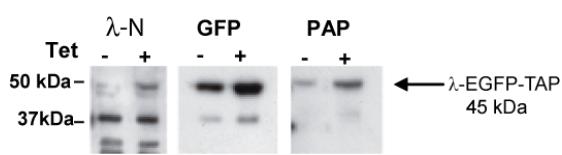


Fig. 3.2.11 over expression of λ N- EGFP – TAP in procyclic cells. The protein was over expressed after addition of tetracycline (+). Antibodies against the different “tags” were used: anti- λ N peptide (left), anti-GFP (middle) and PAP (right)O

Then a TAP purification using the accurate negative control, procyclic cells carrying the plasmid for the protein (pHD1743) and the reporter without the binding sites (1470) was done. Western blot analysis using anti-GFP antibody indicated that the purification was successful (Figure 3.2.12 lower part). RT-PCR was performed to check the binding specificity of the reporter to the protein. However a band of similar intensity was obtained in both eluates (test and negative control), suggesting that CAT-AATTER 3'-UTR was unspecifically sticking to the beads (Figure 3.2.12 upper part).

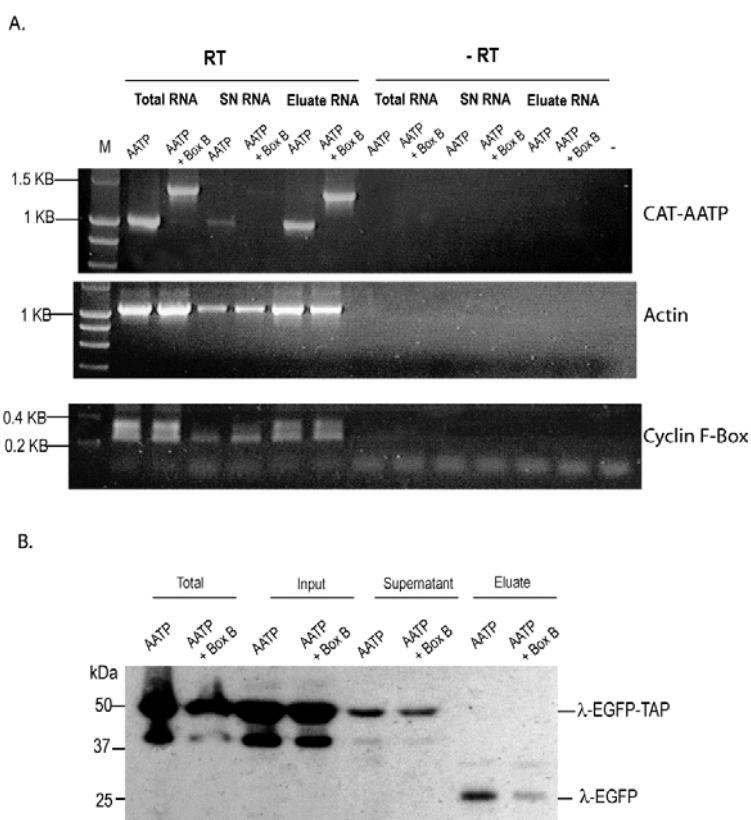


Fig. 3.2.12 Unspecific RNAs are pulled down during λ -EGFP-TAP purification. A. RT-PCR from total, supernatant and eluate RNA obtained after TAP purification of PC containing the CAT reporter construct with (AATP + Box B) or without box B (AATP) as a negative control. B. Western blot of (2×10^6 cells) the different fractions using anti-GFP antibody. After the cleavage by TEV protease the λ -EGFP segment is release in the eluate.

To reduce the unspecific interactions of other transcripts with the IgG-sepharose column, tRNA from yeast was used as RNA competitor. For this, IgG sepharose beads were preincubated for 2 hours with 100 µg/mL tRNA. However, this approach was not useful. The RNA from the control cell line (without the box B) was present in the elution fraction at similar levels to the reporter construct containing the specific binding sites (Figure 3.2.13 A). As a second alternative, heparin was included during the incubation of the extracts with the IgG beads. Two different heparin concentrations were tested: 0.5 and 1 mg/ml. In both situations PCR products for CAT-box B-AATP3'-UTR were obtained. However, PCR products were also obtained with the negative control (CAT-AATP 3'-UTR) indicating that the presence of heparin did not reduce unspecific binding of RNA to the beads. Figure 3.2.13 shows the PCR products obtained from eluates using 1 mg/ml heparin. In summary, several attempts to precipitate the RNPs interacting with the AATP-3'-UTR under “physiological” conditions were unsuccessful because the reporter mRNA was not bound with sufficient specificity to the IgG sepharose. An alternative method might be the coupling of *in vitro* transcribed reporter mRNA to an affinity column and subsequent incubation with cellular extracts. Although this is a totally artificial situation, this approach should be considered.

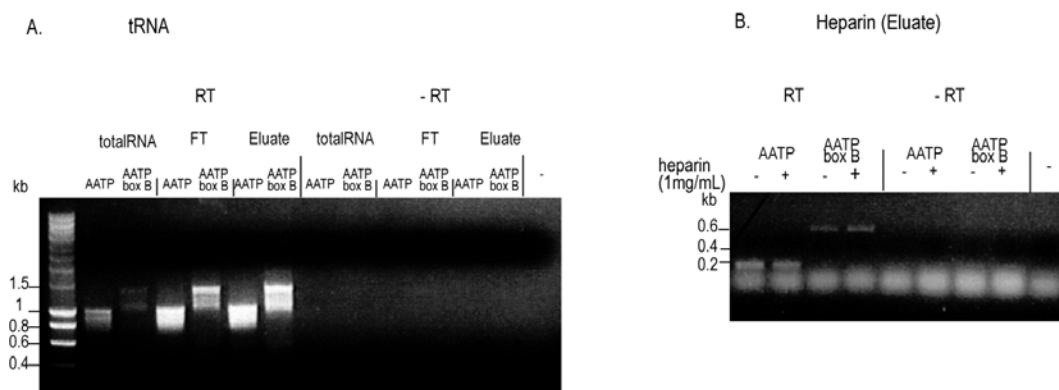


Fig. 3.2.13 Unspecific binding of AATP 3'-UTR. PCR from RNAs after TAP purification using tRNA as a RNA competitor (A) or heparin 1 mg/ml (B). Oligos CZ2232 and CZ2089 were used to amplify the 3'-UTR in A and in B CZ2232 and CZ2090 were used. In both cases the reporter RNA without the box-B element was pulled down.

3.3 Determination of CAT toxicity mediated by PGKC 3'-UTR in *Trypanosoma brucei* bloodstream forms

These results were published ((Colasante, Robles et al. 2007)). This is a summary of the work I did (see supplementary material.)

The PGK (phosphoglycerate kinase) cluster is the best studied model for polycistronic transcription in *Trypanosoma brucei*. It consists of 3 genes and 2 of them – *PGKB* and *PGKC* are developmentally regulated. *PGKC* is present in the glycosomes of bloodstream cells and its mRNA steady state levels are 20-fold higher than in procyclic cells. On the other hand, *PGKB* is located in the cytosol of insect cells and its mRNA levels are 10-fold upregulated. Previous studies done in the lab demonstrated that the 3'-UTR of these transcripts is involved in this regulation. In this work, Claudia Colasante went further into the mechanism of regulation of *PGKC* in BS using the same approach that I used for the characterisation of the AATP 3'-UTR: a CAT reporter gene under the control of the *PGKC* 3'-UTR.

While identifying the regulatory sequences of this 3'-UTR, an interesting fact was observed: it was not possible to get transfectants of BS with the CAT reporter construct containing the whole *PGKC* 3'-UTR when the transcription was driven by RNA T7 polymerase (a strong promoter) while they were easily obtained when a weak promoter (endogenous RNAPol II) was used. The difficulty in obtaining bloodstream trypanosomes expressing high levels of *CATPGKC* mRNA suggested that either the RNA or the protein might be toxic. I tried to determine which of these assumptions was true (see Figures 5 and 6 A of this manuscript).

For this, a reporter plasmid containing a tetracycline-inducible promoter was made. The plasmid (“j” (pHD 1667) in Figure 5A), was transfected into trypanosomes BS and *CAT* mRNA and enzyme activity were measured. The clones obtained did not show any phenotype in the absence of tetracycline (Figure 5B), but upon addition of the antibiotic, cell growth was impaired (Figure 5B). An increase in CAT activity and in *CAT-PGK* mRNA was observed after the induction (Figure 5C). Using a specific probe for *PGKC* 3'-UTR it was possible to establish that the *CAT-PGK* mRNA levels were ten-fold higher than that of the endogenous *PGK* mRNA (Figure 5C) and that the *PGKC* mRNA endogenous levels were unaffected by the CAT induction. *CAT-PGKC* mRNA levels were higher than the control (*CAT*-Actin 3'-UTR mRNA). Overall these results suggest that an excess of CAT is toxic for the cells. At the same time, I was able to get one cell line containing the constitutively expressed reporter

construct. Although this clone grew slowly, it was possible to perform CAT assays and Northern blots (Figure 5 C).

To determine whether the toxicity was due to CAT protein or *CAT-PGK* mRNA, translation initiation was inhibited by inserting a (G)30 sequence upstream of the *CAT* gene (*G-CATPGKC*) (plasmid “m” in figure 5). After transfection and selection by adding puromycin, the expression of G-CAT was induced by addition of tetracycline. In contrast to the data obtained before, no phenotypic effect was observed in these cells and CAT activity also decreased, suggesting that an excess of CAT protein was causing the toxicity. Unfortunately, *CAT* mRNA levels also decreased (even lower than the control). Therefore it was not possible to determine the cause of this toxicity.

4 Discussion

4.1 *TbRBP3*

RRM-type proteins belong to the most abundant class of RNA binding proteins in eukaryotes. They are required to control many aspects of RNA metabolism, from RNA synthesis of the nascent chain to its degradation. In Trypanosomatids, these proteins are involved not only in universal processes such as transcription and translation but also in very specialized events that are characteristic of this genus such as *trans* splicing and RNA editing of mitochondrial transcripts. Due to the unusual organisation of the genome, these parasites exclusively rely on post-transcriptional mechanisms to control gene expression (e.g., splicing, mRNA degradation and translation), and although the molecular bases of them have been elucidated, not much is known about the regulatory elements involved in developmentally regulated gene expression control. Therefore the search and characterisation of these proteins are required to gain a better and deeper understanding of the parasite.

As most of the RRM-type proteins, *TbRBP3* is a modular protein (Figure 3.1). The only region with high homology between the different RBP3s found in other species of Trypanosomatids and related proteins is the RRM-motif, in particular the RNP-1 motif, with a predicted secondary structure $\beta\alpha\beta\beta\alpha\beta$. *TcUBP1* also contains a glutamine-rich region at the C terminal domain present in an α helical conformation that might be involved in protein-protein interactions.

Two facts led us to believe that *TbRBP3* has a regulatory function in development in this parasite: both over-expression and RNAi knockdown of *TbRBP3* transcript in bloodstream forms considerably impaired the cell growth (Figures 3.1.4 and 3.1.5.) while the insect form was not affected. Although the depletion of the mature transcript was not complete (30% of the endogenous mRNA still remained after 24 hours in both life cycle stages), and therefore some amount of the protein is still present, this down-regulation was enough to observe a phenotypic effect. Considering that the most related proteins to RBP3 are involved in the control of mRNA steady state levels in *T. cruzi* (De Gaudenzi, D'Orso et al. 2003) and in *T. brucei* (C. Hartmann, manuscript in preparation), *TbRBP3* could be also implicated in a similar mechanism. To determine whether mRNAs were specifically up- or down-regulated in bloodstream forms with altered RBP3 levels, microarray analysis was performed (Diehl, Diehl et al. 2002; Brems, Guilbride et al. 2005). Although several transcripts were observed to be differentially regulated in these analyses (table 3.1), these results could not be confirmed by Northern blot analysis. This was unexpected as the Northern blot is more sensitive, and it

has been found that the observed changes in regulation are higher than the reported ones for microarrays (C. Benz, C. Hartmann, D. Luu, personal communication), so higher differences would have been expected. So far there is no good explanation for this inconsistency, apart from the fact that RBP3 might not be involved in the control of mRNA levels.

The interaction of members of the *TcRBP* family with different *T. cruzi* transcripts has been reported in *in vitro* RNA binding assays as well as *in vivo* by co-immunoprecipitation from RNP complexes. *TcUBP1* specifically interacts with *Tcsmug*, *tssa*, cruzipain and amastin transcripts, while *Tcsmug* mRNA is bound to *TcUBP2* *in vivo* (De Gaudenzi, D'Orso et al. 2003). In order to identify endogenous mRNAs associated with *TbRBP3* in bloodstream forms, a combination of affinity purification of TAP tagged *TbRBP3* and microarray analysis of RNAs associated with the mRNP complexes was used. Although the main purpose of the tandem affinity purification is to identify interacting proteins under native conditions, it has also been used to identify ligands (nucleic acids, lipids, peptides) that interact, directly or indirectly, with the target protein (Rigaut, Shevchenko et al. 1999; Puig, Caspary et al. 2001; Gerber, Herschlag et al. 2004). Preliminary results showed that five of the clones of the microarray analysis that copurify with TAP-RBP3 coded for proteins containing the cyclin F box domain, while another four were related to two zinc finger proteins. RT-PCR analysis confirmed the selective binding of one of the transcripts for each protein family (Figure 3.1.9).

Proteins containing cyclin F box domains play roles in very diverse cellular processes, such as polyubiquitination, transcription elongation, centromere binding and translational repression. Usually these proteins contain other associated motifs such as WD- and leucine-rich repeats that are also involved in the binding of the protein to its substrates (Kipreos and Pagano 2000). In *T. brucei*, the cyclin F box transcript is developmentally regulated, and about 2-fold more abundant in bloodstream forms. The role of these proteins in Trypanosomatids is under study (C. Benz, C. Hartmann, manuscript in preparation).

The recent completion of the *T. brucei* genome predicts the presence of 65 proteins containing zinc finger domains of the C-x8-C-x5-C-x3-H type (CCCH) many of them carrying further accessory domains. This type of proteins is characterised by its ability to interact with RNA and thereby affecting mRNA stability and localisation. In *T. brucei*, a group composed of three small CCCH zinc finger proteins has been involved in differentiation from bloodstream- to procyclic forms. *TbZFP1* is downregulated in bloodstreams and an enrichment in *TbZFP1* levels after initiation of differentiation to the procyclic form has been observed (Hendriks, Robinson et al. 2001; Hendriks and Matthews 2005). Two of the clones enriched in the pull

down experiments of TAP-*TbRBP3*-RNPs followed by microarray analysis contained the *TbZFP1* 3'-UTR. Although this does not mean that the interaction of the protein with this mRNA is mediated by the untranslated region, it would not be surprising considering that the interaction of most of the RNA binding proteins that are involved in gene expression control is mediated through this region of the mature RNA. In this sense, it could be that *TbRBP3* is involved in the post-transcriptional regulation of *TbZFP1*. One possibility is that the interaction of the protein with *TbZFP1* transcripts in bloodstream forms might affect the stability of the mature transcript promoting its degradation. However, the data obtained from the microarray analysis of both the over-expressed- and knocked down- cells lines contradict this hypothesis. This might be due to the fact that the filtering conditions selected were too high so that several putative targets were undetectable. And in fact, the levels of these transcripts (Cyclin F box domain-containing proteins and ZFP1) were not considerably affected in the former microarray analysis. In the case of cyclin F box, the transcript was not more than 2-fold upregulated when *TbRBP3* was over-expressed and *TbZFP1* was almost not affected. Northern blots from total RNAs showed that the difference was no more than 2-fold upregulation in both cases (3.1.9.C). This suggests that although *TbRBP3* interacts with the transcripts, it is not involved in the control of their steady state levels.

TbRBP3 might also be involved in translational repression of *TbZFP1* at this stage. In this sense, tethered function analysis might be useful to elucidate the role of this protein. It was not determined whether the role of *TbRBP3* in post-transcriptional control of *TbZFP1* is reflected in the differentiation process as monomorphic (that is differentiation incompetent) cell lines are commonly used for this kind of studies.

Interestingly, two other transcripts encoding proteins containing a single ZFP domain were pulled down with *TbRBP3*-TAP (table 3.2 and figure 3.1.9). One of them was found in this study. The other one, *Tb07.11L390*, was previously found as an interacting partner from 14.3.3 proteins by TAP purification (C. Benz). *Tb07.11L390* mRNA levels were also 3-fold upregulated by the over-expression of *TbRBP3* in bloodstream forms (figure 3.1.9). Since many transcripts containing ZFP domains seem to interact with RBP3, it is tempting to consider a general role of this protein in their regulation. Autoregulation mediated at the level of mRNA stability has been reported for transcripts encoding RRM-type proteins as AUF1/hnRNPD (Zhang, Wagner et al. 1993; Wilson, Sun et al. 1999; Banihashemi, Wilson et al. 2006) and for the zinc finger CCCH-type tristetraprolin (Brooks, Connolly et al. 2004), through AU-rich elements present in the 3'-UTR. However, a similar mechanism between different kinds of RNA binding proteins has not been observed, and it is difficult to assess the

functional significance of such an interaction. Forty seven clones of the genomic microarray showed enrichment in the binding to the protein, 23 of them were already sequenced and the binding of two of them was confirmed by RT-PCR. RT-PCRs still have to be done to confirm the interaction of the other transcripts. The identity of the other 24 clones remains to be determined.

TcRBP3, the homologue in *T. cruzi*, is a cytosolic protein only present in the epimastigote stages (De Gaudenzi, D'Orso et al. 2003). Using two different tagged versions of *TbRBP3* it was possible to establish that the protein is located in the cytoplasm of both procyclic and bloodstream forms and concentrated in the perinuclear space. In order to rule out any mislocalisation due to over-expression of the tagged protein, *in situ* tagging of the protein was also done. This allowed confirmation of the results obtained above, and it was also an indirect way to establish that unlike to what was found for the *T. cruzi* homologue, the protein is constitutively expressed in African trypanosomes.

RRM-type proteins can also establish protein-protein interactions either to recognise the RNA target or to mediate RNA-independent functions. For instance, the heterodimeric splicing factor U2snRNP auxiliary factor (U2AF), binds to SF1 through its RRM binding domain called UHF during the initial stages of pre-mRNA splicing in *S. cerevisiae* (Rain, Rafi et al. 1998; Kielkopf, Lucke et al. 2004). Although this kind of modification has not been observed in *Trypanosoma*, protein-protein interactions can be mediated through accessory domains. In *T. brucei*, TSRP1 binds to TSR and U2AF35 during *trans* splicing (Ismaili, Perez-Morga et al. 2000), and *TcUBP1* and *TcUBP2* bind to the poly(A) binding protein (PABP) to regulate the stability of *Tcsmug* and amastin transcripts in a stage specific manner (De Gaudenzi, D'Orso et al. 2003). Considering the results obtained from the TAP-*TbRBP3* microarray analysis, it would be interesting to determine whether this protein does not only interact with cyclin F-box- and ZFP-containing transcripts but also with the polypeptides. I looked for *TbRBP3* binding partners by using the tandem affinity purification (TAP) approach in bloodstream forms. The main advantage of this method is that it allows the purification of the protein under native conditions and the identification of protein complexes in stoichiometric amounts without previous knowledge of the complex composition, activity, or function (Rigaut, Shevchenko et al. 1999; Puig, Caspary et al. 2001). However, this approach has failed to identify *TbRBP3* interacting partners. It is possible that the protein levels of the targets are low, that they were not observed in the silver stained-SDS PAGE gel or that the presence of the TAP tag disrupts the putative interactions, as it has been reported that sometimes the addition of the C-terminal tag to a protein can impair its function (Puig,

Caspary et al. 2001). Another possibility would be that the protein-protein interactions are only transient or too weak and therefore not detectable with this method.

These results indicate that *TbRBP3* might have more than one function in regulation in *T. brucei*. At the posttranscriptional level, this protein is involved in the regulation of specific mRNA levels in this parasite. The fact that alterations in its abundance affect cell growth underlines its importance. *TbRBP3* might also be required to control other mechanisms involved in RNA homeostasis and the effect of RBP3 on translation should be investigated further.

4.2 Functional analysis of AATP 11 3'-UTR

One of the most interesting aspects of Trypanosomatids when compared to other eukaryotes is their control of gene expression: due to the lack of polymerase II promoters in these organisms and polycistronic transcription they cannot regulate this process at the classical level of transcription initiation. Therefore gene expression control is mainly mediated at post-transcriptional levels through pre-mRNA processing i.e., *trans*-splicing and polyadenylation, mRNA stability and translation. All of them require the presence of sequences located in the 3'-UTRs that recruit protein complexes in a precise moment when it is necessary for the cell. In this work I identified regulatory elements present in the amino acid transporter 11 (AATP) 3'-UTR. These elements are involved in the differential expression of this transcript during the *Trypanosoma* life cycle, and might provide information to get a deeper insight into the developmental control of gene expression in *Trypanosoma brucei*.

In bloodstream trypanosomes, glycolysis is the major source of ATP (Michels, Hannaert et al. 2000). In contrast, in the procyclic form, which grows in the Tsetse fly, the mitochondrial metabolism is more important than glycolysis (van Weelden, van Hellemond et al. 2005). In the insect, the parasite uses amino acids, mainly proline to generate energy. Therefore the amino acid transporters (AATP) are expressed in this life stage, as it has been demonstrated for *T. cruzi* and *Leishmania* (Akerman, Shaked-Mishan et al. 2004; Bouvier, Silber et al. 2004; Canepa, Silber et al. 2004; Geraldo, Silber et al. 2005). As an indicative of this, the steady state level of the endogenous *AATP 11* transcript is 8-fold higher in procyclic than in bloodstream forms (Figure 3.2.1); it is not known whether this difference is maintained at the protein level. The experimentally determined 3'-UTR fits to the *in silico* predicted one, which was generated by an algorithm that uses several distinct parameters to predict processing sites of mature mRNA transcripts (Benz, Nilsson et al. 2005).

The first impression if we consider just the sequence of this untranslated region is that this region might be involved in destabilization of the mature transcript. Three different kinds of destabilising elements are present in this 3'-UTR: five AU-rich elements type I (AUUUA) spread along the whole 3'-UTR, several U-rich sequences and one copy of the core motif of the 26-mer element of *EP* procyclin transcripts. In higher eukaryotes, AU-rich elements are involved in destabilising short-life transcripts such as oncogenes and interleukins and also in inhibiting or promoting translation (Barreau, Paillard et al. 2005). The presence of the 26-mer core element (UAUUUUUU at the RNA level) is not surprising. It is known that this element is significantly overrepresented among the 3'-UTRs of developmentally regulated genes, being present in the 3'-UTR of one-third of procyclic form enriched transcripts (like *PGKB* and *COX*) and 8% of 3'-UTRs for bloodstream-enriched transcripts (Hotz, Lorenz et al. 1995; Clayton and Hotz 1996; Hotz, Biebinger et al. 1998; Irmer and Clayton 2001; Mayho, Fenn et al. 2006). The fact that these sequences are found in the 3'-UTR suggested that AATP 11 expression could be regulated at the mRNA stability level, the translational level, or both (Zhang, Kruys et al. 2002). The predicted secondary structure of the *AATR* 3'-UTR (figure 3.2.4) showed that apart from 3 U-rich regions predicted to adopt a single stranded conformation, most of these elements were interacting with other nucleotides, and therefore might not be functional.

As has been observed for many developmentally regulated genes in *Trypanosoma*, the 3'-untranslated region is responsible for the differential expression of the AATP mRNA in insect forms. The mRNA levels of the reporter construct *CAT-AATP* 3'-UTR were 5-fold upregulated in procyclic forms compared to bloodstream forms. Although these values did not fully reflect the regulation level of the endogenous mRNA, they are very similar to those obtained for the endogenous transcript (8-fold). An artefact due to over-expression conditions cannot be discarded. Moreover, this region is also under a slight translational control as CAT activity was 10-fold higher in the insect forms.

There are several steps at which mRNA abundance can be regulated: at the stage of mRNA processing, export from the nucleus and degradation. For instance, there is a 5- to 10-fold increase in transcription initiation and in transcription elongation of the *EP* procyclin genes in procyclic compared to bloodstream forms (Furger, Schurch et al. 1997). As it was explained before, regulation of mRNA stability is the major way of controlling mRNA amounts in *Trypanosoma*. *EP* and *PGKB* mRNAs have half-lives of about 5 min in bloodstream forms and over an hour in procyclic forms (Hotz, Hartmann et al. 1997; Haile, Estevez et al. 2003). Preliminary results (data not shown) indicate that the same is true for the endogenous *AATP*

mRNA: the transcript is unstable in bloodstream cells (after addition of Actinomycin D the half life is less than 10 min compared to more than 1 hour in procyclic cells). Half life measurements of the reporter constructs have to be done to determine whether this model reflects the regulation of the endogenous mRNA. If the half-life of the *CAT-AATR* 3'-UTR mRNA is different from the value obtained with the *AATR 11* transcript, it could be reflected in the steady state levels of the transcript. It is known that transcript abundance is determined by RNA stability (Clayton 2002).

Deletion of the main domains of the secondary structure of the *AATP* 3'-UTR allowed the identification of a regulatory element located between nt 290-618. In bloodstream forms this deletion ($\Delta 3$) causes a 2.2-fold increase in the *CAT- $\Delta 3$ AATP* mRNA levels and a 7-fold upregulation in CAT activity. This represents 90% of the CAT activity observed in insect forms containing *CAT-AATP* 3'-UTR. This region contains two of the U-rich tracts that are predicted to be present in single stranded conformation. The results shown for the deletions were not consistent with those obtained with the reporter containing the whole 3'-UTR. Apparently, translational control plays a more important role in regulating the expression of the reporter gene. It could be that the regions deleted are too big, so that other regulatory elements are masking the effect at the mRNA level. It has been reported that the secondary structure of mRNA 3'-ends is critical in determining their effects on mRNA abundance (Hotz, Hartmann et al. 1997; Irmer and Clayton 2001). Although the predicted models of the secondary structure for the different deletions indicate that the overall structure was maintained, an alteration in the secondary structure of the 3'-UTR cannot be ruled out. If the folding of the 3'-UTR is affected, it might also be reflected in the mRNA levels obtained for *CAT- $\Delta 3$ AATP*. Half life measurements would be useful to establish if the stability of this reporter construct increases in bloodstream forms.

Attempts to delimitate the region involved in the control were unsuccessful as the mRNA levels were slightly lower than the values obtained for the *CAT-AATP* 3'-UTR transcript in both procyclic and bloodstream forms (Figure 3.2.5). However, it was possible to identify two regions involved in translational repression which are not required for differential expression at the transcript level in both life stages: between 290-350 nt, (*CAT- $\Delta 4$ ATTP*) and between 535-610 nt, (*CAT- $\Delta 6$ ATTP*). Both constructs showed enzymatic activities higher than the CAT-actin control in procyclics, whereas in bloodstream forms only the *CAT- $\Delta 4$ ATTP* activity was elevated. However, it did not reach the values obtained with *CAT- $\Delta 3$ ATTP* (35% vs 49%). Looking for special motifs in these regions, $\Delta 6$ bears the U-rich element plus

one AU-rich element type I partially present as a single strand AUUUA. It is unlikely that this motif is functional, so the translational effect might be mediated by the U-rich element.

Translational repression is involved in the regulation of many AU-rich containing mRNAs in eukaryotes. Using *in vitro* translation studies it has been determined that these sequences inhibit the stable association of the small ribosomal subunit with the TNF α mRNA (Wax, Nakamura et al. 2005) through their interaction with TIA-1(Piecyk, Wax et al. 2000). In *T. brucei*, the 26-mer polypyrimidine tract is required to down-regulate the few EP procyclin mRNA transcripts that are still produced in bloodstream forms (Hotz, Hartmann et al. 1997). Although the mechanism of degradation of this transcript has been well established (Li, Irmer et al. 2006), it is not known how the translational control is achieved. It might be that the translational repression observed for CAT-AATR and EP procyclin is similar to the control of the cytokine mentioned before. Interestingly, in mammalian cells, the increased expression of the cationic amino acid transporter (Cat-1) during amino acid deprivation is due to increased mRNA levels and enhanced translation mediated by the interaction of the RNA binding protein HuR with AU-rich elements located in the 3'-UTR (Aulak, Liu et al. 1996; Hyatt, Aulak et al. 1997; Aulak, Mishra et al. 1999; Yaman, Fernandez et al. 2002).

Since it was not possible to delimitate the region in $\Delta 3$ involved in the developmental regulation of *CAT-AATR 3'-UTR* mRNA it was considered that perhaps the whole region and/or a structure only formed when the entire region is present, is required to exert this control.

4.2.1 Does over-expression of *CAT-AATP* and *CAT-PGKC* transcripts affect gene expression control?

The best studied model of developmentally regulated genes in trypanosomes whose transcription is driven by pol II is the phosphoglycerate kinase (PGK) gene cluster. The developmental regulation is mediated by a poor *trans* splicing signal in the case of PGKA (Kapotas and Bellofatto 1993) and by the 3'-UTR for *PGKB* and *PGKC* (Blattner and Clayton 1995). There are approximately 20 molecules of *PGKC* mRNA in one bloodstream form cell, 0.006% of the total mRNA molecules present. At the translational level, there is a 50-fold upregulation: the parasite contains 300,000 molecules of PGKC protein, 0.35% of the total protein molecules in the cell (Jurgen Haanstra data, unpublished). If there were the same amount of *AATP* mRNA molecules as for *PGKC* transcript in procyclic cells (20 molecules), these would correspond to 0.003% of the total mRNA molecules. We do not have data about

the regulation at the protein level, but if the CAT-AATP reporter gene reflects the behaviour of the endogenous gene, 6,000 molecules of AATP 11 protein should be present in one insect form cell. The transcription of the *CAT-AATP* and *CAT-PGKC* construct is driven by T7 polymerase, which increases the amount of primary transcript about 20-fold, so it would be 400 mRNA molecules of either *CAT-AATR* or *CAT-PGKC* per cell and 120,000 protein molecules of CAT-AATR and 6×10^5 protein molecules of CAT-PGKC. The excess of CAT under the control of *PGKC 3'-UTR* in comparison to the protein produced with the *AATR 3'-UTR* construct was reflected in the difficulty to get a bloodstream cell line containing the CAT-PGKC reporter construct when the transcription was driven by a constitutive T7 polymerase promoter. This was only possible after inserting a tetracycline operator region in order to make the transcription tetracycline inducible. Moreover, the growth of these cells was impaired after induction of the reporter (Figure 5). It was not possible to determine whether this toxicity was either due to an excess of *CAT* mRNA or of CAT protein. We did a reporter construct containing a G30 track between the 5'-UTR and CAT ORF to inhibit translation initiation. Unexpectedly, the *CAT-G30-PGKC* mRNA levels were decreased as well as the CAT activity. The secondary effects seen using the strong T7 promoter were overcome when the transcription was mediated by polymerase I promoter (Colasante, Robles et al. 2007). If the mRNA turnover of *AATP* and *PGKC* were mediated by a regulatory protein which is involved in stabilisation of the transcripts in either procyclic or bloodstream cells by interacting with the 3'-UTR and this factor is present in limiting amounts, then it might not be enough protein to bind to the 3'-UTR of the over-expressed reporter and the endogenous transcript, and this might be reflected in the destabilisation of the mRNA and also in the mRNA steady state levels. *CAT-PGKC* mRNA half life was shorter when the transcription was driven by T7 polymerase than when driven by polymerase II (Colasante, Robles et al. 2007). However, it is not clear if this effect was mediated by the promoter itself or by an excess of RNA molecules.

4.2.2 Looking for a protein involved in the developmental regulation of AATP 11

We wanted to identify factors responsible for targeting the *-AATP* mRNA either for degradation and/or for translational repression. RNA stability and translation modulation usually involves the interaction of sequences in the 3'-UTR with regulatory proteins (as was described above) which recruits the degradation machinery (exosome and 5' to 3' exonucleases) or affects translation.

To date, no more than 10 AU-rich binding proteins (AUBP) with an established function in mRNA turnover and translation have been described in mammals and they are involved in the control of 5-8% of the total mRNA transcripts in humans (Bakheet, Williams et al. 2006). Most of them have different properties and only share their affinity to AU-rich elements and their role in mRNA turnover and/or translation (Lai, Stumpo et al. 1990; Zhang, Wagner et al. 1993; Aulak, Liu et al. 1996; Min, Turck et al. 1997; Carballo, Lai et al. 1998; Wilson and Brewer 1999; Chen, Gherzi et al. 2001; Stoecklin, Colombi et al. 2002). These properties were later found to be important for ARE-binding by mobility shift assays (using either the purified epitope tagged version of the protein or cytosolic extracts from AUBP overexpressing cells) and mRNA half life measurements of reporter genes (Zhang, Wagner et al. 1993; Aulak, Liu et al. 1996; Carballo, Lai et al. 1998; Wilson and Brewer 1999). So far none of these regulators has been identified through direct interaction with its target mRNA. There are two of these proteins in *Trypanosoma*: the ARE-binding proteins *TcUBP1* and *TcUBP2* in *T. cruzi*. *TbUBP1* and *TbUBP2* in *T. brucei* are involved in stabilisation of a transcript encoding a protein containing a cyclin F box domain (C. Hartmann, manuscript in preparation). The *T. cruzi* proteins were found by searching ESTs and Genome Sequence Surveys for clones having homologies with eukaryotic ARE binding proteins such as members of the ELAV family (D'Orso and Frasch 2001; D'Orso and Frasch 2002) and then by homology, *TbUBP1* and *TbUBP2* could be identified (C. Hartmann manuscript in preparation).

The interaction of *TcUBP1* with AU rich sequences present in the 3'-UTR of *Tcsmug* transcripts in epimastigotes of *T. cruzi* (D'Orso and Frasch 2001) and the interaction of the mammalian HuR with U-rich elements in the 3'-UTR of *PGKB*, *EP1* and *PPDK* has been established by electrophoretic mobility shift assay (EMSA) (Quijada, Guerra-Giraldez et al. 2002). In these examples the binding between the RNA probe and the protein was already suspected and purified protein was used in the reactions. I tried to adapt the technique using the region encompassing the 340 nt of the *AATR Δ3* 3'-UTR and total cell extracts of either procyclic or bloodstream forms. After selection of nucleic acid binding proteins by heparin-Sepharose chromatography, a weak-retarded migrating band was observed in extracts from the insect form (figure 3.2.6). However, attempts to reproduce the interaction failed. It is recommended to use RNA probes of less than 100 nt in length to minimize unspecific binding and to have good resolution during the running in the non-denaturing gel. However, this approach was used by (Coughlin, Teixeira et al. 2000) to detect a 36 kDa protein in cellular extracts from *Leishmania* amastigotes, using the in vitro transcribed amastin 3'-UTR, that has

a size of 700 nt as a probe (Coughlin, Teixeira et al. 2000). It might be that the complex between $\Delta 3$ -AATR RNA and the putative protein was too big to be resolved on a 4% PAGE non denaturing gel.

4.2.2.1 Attempts to identify regulatory factors under native conditions

One of the main disadvantages of shift binding assays is that they do not reflect physiological conditions. An ideal way to overcome this problem is to isolate the ribonucleoprotein (RNP) particles under native conditions. Aptamers selected against nucleic-acid binding proteins and small molecular-weight ligands have been used to investigate protein and nucleic-acid sequence specificity. Isolation of pre-splicesosome associated proteins, like U1 and U2 snRNP were done using an *in vitro* transcribed pre-mRNA containing an RNA aptamer which has high affinity to tobramycin (Hartmuth, Urlaub et al. 2002; Hartmuth, Vornlocher et al. 2004). I used this protocol to isolate CAT-AATP 3'-UTR RNP complexes under native conditions. Preliminary assays to demonstrate the binding of the reporter transcript bearing the J6f1 aptamer to the tobramycin derivatized Sepharose failed. Although M-FOLD analysis of the J6f1-AATP 3'-UTR indicated that the secondary structure was maintained, it is possible that the folding of CAT-aptamer-AATP 3'-UTR impaired the access of the affinity site to the tobramycin matrix. It is important to indicate that the studies done so far include the binding site either at the 3'-end (Hartmuth, Urlaub et al. 2002; Hartmuth, Vornlocher et al. 2004) or the 5'-end (Vazquez-Pianzola, Urlaub et al. 2005), and that *in vitro* transcribed RNA coupled to the tobramycin matrix was used, which means that only one RNA species is present in the assay.

TcUBP1 is expressed in the three different cell stages of the parasite and selectively binds transcripts in each stage. Its role in mRNA turnover is dual, either increasing mRNA stabilisation or promoting mRNA degradation, similar to AUF1 in mammalian cells. This suggests that *TcUBP1* could act in the formation of different RNP complexes in different developmental stages. In epimastigote stages, it interacts with PAB1 and *TcUBP2* to stabilise *TCSMUG* transcript. *TcUBP1* also binds *TSSA* mRNA in epimastigotes and destabilises the transcript. *TcUBP1* and *TcPABP1* bind *amastin* mRNA in both epimastigotes and trypomastigotes. The presence of few proteins involved in regulation of several transcripts might also be the case for *T. brucei* and probably they are part of different RNP complexes, in some cases interacting with an mRNA in a particular stage while in other situations recognising mRNAs in more than one parasite stage. If this were the case, the amount of protein would be a limiting factor in regulating the expression of *CAT-AATR* and *CAT-PGKC*

mRNAs. It might also be possible that the protein is part of another RNP complex involved in the regulation of other genes, and therefore not present in sufficient amounts to regulate all the over-expressed *CAT-AATP* transcripts.

Tethered function analysis approaches using AUBPs have been performed to determine their function in mRNA turnover. The roles of CUG-BP1 (CELF1) (Barreau, Watrin et al. 2006), TTP, BRF1 (Lykke-Andersen and Wagner 2005) and KSRP (Chou, Mulky et al. 2006) as stimulators of degradation and /or promoters of translation were assessed using fusion proteins containing the MS2 coat protein. Modifications of this assay, in which the tethering protein is used as a tag for mRNAs have been used to determine the cellular localisation of several transcripts (Coller and Wickens 2002; Kedersha, Stoecklin et al. 2005; Liu, Valencia-Sanchez et al. 2005; Sheth and Parker 2006). Recently, this methodology was adapted to isolate RNPs. By using a λ -GST affinity chromatography it was possible to isolate and identify 40LoVe, a protein involved in the localisation of *VGL* mRNA in *Xenopus* oocytes (Czaplinski, Kocher et al. 2005). I used two different tethering systems as a tag to identify associated proteins: an N-myc tagged version of MS2 coat protein and GFP bearing the λ -N peptide at the C terminus. Both systems were successful in immunoprecipitating the tethered protein as shown by western blot using anti-myc or anti-GFP antibodies, respectively. However, the low expression of the myc-tagged MS2 coat protein was a clear disadvantage to pull down proteins associated with the *AATP-3'-UTR*. On the other hand, the immunoprecipitation using the GFP- λ tethered protein was inefficient, as most of the tethered protein was found in the supernatant (Figure 3.2.9). It was not possible to detect any transcript in the supernatant of the λ -GFP by RT-PCR either, indicating that the RNA was degraded either during the immunoprecipitation or the RNA extraction, even in the presence of RNase inhibitors during the procedure. We decided to change the reporter and included a TAP tag segment at the C-terminal domain, which allows efficient recovery (about 80%) of the protein and also has other advantages as mentioned above (Rigaut, Shevchenko et al. 1999; Puig, Caspary et al. 2001), generating a λ -N peptide-EGFP-TAP fusion protein. Instead of purifying the RNPs by classical immunoprecipitation, the complexes were pulled down during the first step of the TAP purification and eluted by TEV protease cleavage.

By RT-PCR we detected the control transcript in the pulled down fraction (*CAT-AATP 3'-UTR*) as well as the reporter bearing the B-boxes. Unfortunately, it was not possible to eliminate the non-specific binding of the transcript control to the protein using different competitors (tRNA and heparin sulphate) (Figure 3.2.13).

Using metabolic labeling followed by TAP purification it was not possible to detect any protein specifically bound to the reporter containing the B-boxes (data not shown). Considering that the putative regulatory element binds to the 3'-UTR in the reporter construct but not to the tethering protein, this interaction might be disrupted during the TAP purification. *In vivo* cross-linking (chemical or UV-mediated) might be useful to stabilise these interactions. Moreover, although both the reporter transcript and the tagged protein are over-expressed in the cells, the amount of regulatory protein is the same, and the protein background that unspecifically interacts with any domain of the protein is high, making the detection by SDS-PAGE difficult. It can also not be ruled out that this polypeptide was part of a different RNP by the time the protein extracts were prepared. Therefore a higher amount of cells might be required to identify proteins bound to the AATP 3'-UTR.

Other methodologies to find RNPs have been also established, they include the rescue of mutants that are unable to degrade an unstable reporter mRNA (BRF1) (Stoecklin, Colombi et al. 2002) and the introduction of a modified nucleotide (biotin-dT) at a defined site into a synthetic oligoribonucleotide, protein-RNA cross-linking, isolation via the RNA-bound biotin and purification in high yield for analysis by mass spectrometry. However, many steps are required for this approach, the RNA must be produced with three modifications, that is, a position-specific cross-linker, a neighboring biotin, and a radioactive label, and the specific site for the binding of the protein needs to be intact in order to do the cross-link. Moreover, the amount of cross-linked product must be obtainable in a quantity sufficiently large to be analyzed by MS (Rhode, Hartmuth et al. 2003)

As an alternative, it is possible to do the assay under *in vitro* conditions, using an *in vitro* transcribed reporter gene coupled to the tether protein as an affinity ligand and cytosolic extracts from which interacting proteins can be pulled down. Although it is not the ideal solution, it might be useful to find proteins which are involved in the targeting of developmentally regulated transcripts to the degradation machinery and the translational apparatus of the cell. Another possibility is to do a complete TAP-tag purification and to look by mass spectrometry for associated complexes.

The identification of the elements involved in developmental regulation of the AATP in *T. brucei* confirms the relevance of the sequences located in the 3'-UTR in post-transcriptional regulation. Although the identification of *trans* acting factors involved in this control remains unsolved, their identification would be helpful to clarify the preliminary steps of developmentally regulated mRNA turnover.

5 General abbreviations

aa	amino acid
Amp	ampicillin
APS	ammonium persulfate
ARE	AU-rich element
ATP	adenosine-5'-triosephosphate
ACTR	amino acid transporter
bs	bloodstream form
bp	base pairs
BSA	Bovine Serum Albumin
bsd	blasticidin
CAT	chloramphenicol acetyl transferase
CIP	calf intestinal phosphatase
COX	cyclooxygenase
CP	coat protein
cpm	counts per minute
DAPI	4', 6'-diamidino-2-phenylindole
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
DEPC	diethyl pyrocarbonate
dGTP	2'-deoxyguanosine 5'-triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphates
dsRNA	double-stranded RNA
DTT	1,4-Dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenedinitrilo tetraacetic acid
EMSA	electrophoresis mobility shift assay
et al.	and others
ESAG	expression site associated gene
FCS	fetal calf serum
fig.	figure
HAT	human African trypanosomiasis
HOT	head over tail
Hyg	hygromycin
IPTG	isopropyl-β-D-thiogalactopyranoside
KAc	potassium acetate
kb	kilobase
kD	kilo Dalton
kDNA	kinetoplast DNA
LB	Luria-Bertani
MOPS	3-(N-morpholino) propane sulfonic acid
mRNA	messenger RNA
mRNP	messenger ribonucleoprotein
neo	neomycin
nt	nucleotide
OD	optical density
ORF	open reading frame

PABP	poly(A) binding protein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
pBS	plasmid Bluescript
pc	procyclic
PCR	polymerase chain reaction
PGKB(C)	phosphoglycerate kinase B (C)
pH	-log [H ⁺]
phleo	phleomycin
pI	isoelectric point
RBP	RNA binding protein
RNA	ribonucleic acid
RNAi	RNA interference
RNA pol	RNA polymerase
RNase	ribonuclease
rpm	rounds per minute
RRM	RNA recognition motif
rRNA	ribosomal RNA
RT	room temperature
SDS	sodium dodecyl sulfate
SL	spliced leader
SSC	saline sodium citrate
spp	subspecies
TAE	Tris-acetate-EDTA
TAP	tandem affinity purification
TCA	tricarboxylic acid
TE	Tris-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamide
tet	tetracycline
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
Tcsmug	<i>Trypanosoma cruzi</i> small mucin gene
Tssa	<i>Trypanosoma cruzi</i> small surface molecule
U, u	unit
UBP	U-rich binding protein
URE	U-rich element
UTR	untranslated region
UV	ultraviolet
Vol	volume
VSG	various surface glycoprotein
wt	wild-type
w/v	weight/volume
X-Gal	5-bromo-4chloro-3-indolyl-β-D-galactosidase

6 Supplemental material



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Regulated expression of glycosomal phosphoglycerate kinase in *Trypanosoma brucei*

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Abstract

In *Trypanosoma brucei*, the *PGKB* and *PGKC* genes-encoding phosphoglycerate kinase are co-transcribed as part of a polycistronic RNA. *PGKB* mRNA and the cytosolic PGKB protein are much more abundant in the procyclic life-cycle stage than in bloodstream forms, whereas *PGKC* mRNA and glycosomal PGKC protein are specific to bloodstream forms. We here show that a sequence between nucleotides 558 and 779 in the 3'-untranslated region of the *PGKC* mRNA causes low expression of the chloramphenicol acetyltransferase (*CAT*) reporter gene in procyclic trypanosomes. In procyclics, depletion of the RRP45 component of the exosome (3'→5' exonuclease complex) or the 5'→3' exonuclease XRNA increased the abundance of *CAT-PGKC* mRNA as a consequence of effects on the degradation of precursor and/or mature mRNAs. In bloodstream forms, inhibition of both *trans* splicing and transcription resulted in immediate exponential decay of *PGKC* mRNA with a half-life of 46 min. Inhibition of transcription alone gave non-exponential kinetics and inhibition of splicing alone resulted in a longer apparent half-life. We also found that production of mRNAs using T7 polymerase can affect the apparent half-life, and that large amounts of *CAT* enzyme may be toxic in trypanosomes.

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Keywords: *Trypanosoma*; mRNA degradation; mRNA turnover; Phosphoglycerate kinase

1. Introduction

Salivarian trypanosomes are protists which infect mammals in sub-Saharan Africa. The parasites multiply in the blood and tissue fluids of their mammalian hosts, and in the midgut of the insect vector, the Tsetse fly. In trypanosomes and other members of the order Kinetoplastida, the first seven to nine enzymes of glycolysis, and various other enzymes, are compartmentalised in a microbody called the glycosome which

is related to the peroxisomes of mammals and yeasts [1]. In bloodstream trypanosomes, glycolysis is the major source of ATP, and most phosphoglycerate kinase activity is found within the glycosome, where it is responsible for regenerating the ATP which is consumed by the hexokinase and phosphofructokinase reactions [2]. In contrast, in the procyclic form, which grows in Tsetse, mitochondrial metabolism is more important than glycolysis [3]. In procyclics, most phosphoglycerate kinase is found in the cytosol. Cytosolic phosphoglycerate kinase (PGKB) is encoded by the *PGKB* gene, which is located directly upstream of the gene-encoding glycosomal phosphoglycerate kinase, *PGKC*, on *Trypanosoma brucei* chromosome I [4]. The developmentally regulated expression of the two PGKs is essential for trypanosome survival, as expression of cytosolic PGK activity in bloodstream trypanosomes inhibits their growth [5].

Most Kinetoplastid genes are transcribed as long polycistronic precursors, which are subsequently cleaved to form monocistronic mRNAs by a *trans* splicing reaction in which a 39 nt-capped leader sequence is added to the 5'-end of each

Abbreviations: UTR, untranslated region; IGR, intergenic region; PGKC, glycosomal phosphoglycerate kinase; CAT, chloramphenicol acetyltransferase; PAC, puromycin acetyltransferase; SRP, signal recognition particle.

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mRNA, and by polyadenylation. There is as yet no evidence for any regulation of transcription by RNA polymerase II during trypanosome growth. The polycistronic mode of transcription of the *PGK* genes was demonstrated nearly 20 years ago by Gibson et al. [6], who documented the existence of precursor RNAs spanning the gap between *PGKB* polyadenylation site and the *PGKC* trans splicing acceptor site [6]. *PGKC* mRNA is at least 20-fold more abundant in bloodstream forms than in procyclic forms, while *PGKB* mRNA is at least 10-fold regulated [7,8]. By transient transfection of plasmids which encoded chloramphenicol acetyltransferase (CAT), we demonstrated that the 3'-untranslated regions (UTRs) of the *PGKB* and *PGKC* mRNAs were sufficient to cause procyclic- and bloodstream-form-specific expression, respectively [7]. A CAT reporter mRNA with a *PGKB* 3'-UTR was very unstable (half-life, 5–10 min) in bloodstream trypanosomes, and the region causing instability was mapped to a poly(U) tract [8].

To analyse regulation of *PGKB* mRNA degradation further, we assessed the effects of depletion of enzymes involved in mRNA degradation in bloodstream forms. A complex of 3'→5' exonucleases, the exosome, was shown to be limiting in the initiation of degradation of the CAT reporter RNA with a *PGKB* 3'-UTR [9], but there were no major effects on the abundance of the reporter mRNA, or on the abundances of native *PGKB* and *PGKC* mRNAs. In contrast, depletion of the 5'→3' exonuclease homologue XRNA was found to cause deregulation of both *PGKB* and *PGKC* mRNA: *PGKC* mRNA became detectable in procyclic forms, and *PGKB* mRNA levels increased in bloodstream forms [10].

In this paper, we concentrate on the mechanism of regulation of the bloodstream-specific *PGKC* mRNA.

2. Materials and methods

2.1. Trypanosome culture and transfection

Bloodstream and procyclic form *T. brucei* were cultured and transfected as previously described [11,12]. The strains used, which constitutively express bacteriophage T7 polymerase and the *Tn10 Tet*-repressor, are strain Lister 427 containing pHD514 (T7 polymerase, G418 resistance) and pHD1313 (*tet* repressor, phleomycin resistance) [13].

To obtain cells expressing CAT mRNAs, expression plasmids were linearised at a *NofI*-site within a segment of the plasmid from either an rRNA spacer region or the tubulin locus. Trypanosomes were transfected with 15–25 µg of plasmid DNA and clones were selected by addition of 0.2 µg µl⁻¹ puromycin for bloodstream cells and 10 µg µl⁻¹ for procyclic cells. Trypanosomes containing plasmids in which transcription of the marker depended on a tetracycline-inducible T7 promoter were obtained in the absence of tetracycline, presumably because "leakage" of the promoter was sufficient to give drug resistance. Chloramphenicol acetyltransferase (CAT) activity was measured [14] in the stable lines using logarithmic-phase cells (bloodstream forms: (0.5–1) × 10⁶ cells ml⁻¹; procyclic (2–4) × 10⁶ cells ml⁻¹).

For the RNAi knock-down of the RRP45 exosome subunit, procyclic trypanosomes containing pHD1425, 1427 or 1538 were transfected with plasmid pHD1116, which contains the *HYG* selectable marker and two copies of part of the *RRP45* gene, cloned in opposite orientations surrounding a fragment from the spliced leader array and preceded by an inducible RNA polymerase I promoter [15]. Transformants were selected with hygromycin and the efficiency of RNA interference against RRP45 was assessed by Northern and Western blotting, and by monitoring cell growth [15]. Clones showing clear growth inhibition and reduction of RRP45 expression upon addition of tetracycline were selected for further analysis. Cells with RNA interference against XRNA were previously described [10]. All XRNA RNAi experiments were done in cells containing RNAi plasmid pHD1532, except those shown with inverted triangles in Fig. 1, which were done in cells containing RNAi plasmid pHD1223 [10]; both of these plasmids effect RNAi using opposing T7 promoters, producing different ds RNA fragments which specifically knock down XRNA expression [10].

2.2. Plasmid constructs

All plasmids for analysis of the *PGKC* 3'-UTR were based on either pHD1424 [9] or pHD1437, which was constructed by replacing the rRNA spacer fragment in pHD1424 [9] with a segment of the beta-tubulin 3'-UTR (Fig. 2). The tubulin-locus segment from pHD449 [16] was amplified by mismatch PCR to destroy the *Sall* site using the primers CZ1868 and CZ1869 (Table 1). The resulting product was cut with *SacI* and *EcoRV*. The rRNA spacer removed from pHD1424 using *SacI* and *SacII*, and the *SacII* overhang in the plasmid was filled using T4 polymerase before insertion of the tubulin-locus fragment.

Different sequence areas of the *PGKC* 3'-UTR were amplified by PCR. To make internal deletions, four different primers (Table 1) were designed for each deletion: one outside 5'-primer, one outside 3'-primer and two overlapping internal primers which delimited the sequence to be removed. In the first 2 steps of the PCR, the 5'-piece and the 3'-segments were amplified independently. The overlapping region from the internal primers allowed annealing during a third PCR using the 5'- and the 3'-outside primers. The complete PCR products were then digested with *BamHI-Sall* and cloned into pHD1424 and pHD1437 [9].

The 3'-pieces of the internal deletions Δ311–407 and Δ429–510 were amplified using the 5'-primers CZ2332 and CZ2331, respectively, with 3'-primer CZ2304, and cloned into pGEMT-easy. The plasmid was then cut with *SmaI* to obtain blunt ends. The 5'-ends of the deletions were amplified with *Pwo*-polymerase using CZ2322 and CZ2323, respectively, with CZ1827 (see Table 1) and cloned into the linearised pGEMT-easy containing the 3'-piece. The *PGKC* UTR fragments were then excised from pGEMT-easy by *BamHI-XhoI* digest and cloned into pHD1437. All plasmids were checked by restriction digestion and sequencing.

To make inducible versions of some of these plasmids a duplicated *Tet* operator sequence (Table 1) was PCR-amplified and cloned into pGEM-T easy (Promega). pHD1643 was created

Table 1
Primers for standard and recombinant PCR, and the *tet* operator sequence

CZ number	Sequence	nt position
Amplification of PGKC 3'-UTR		
1827	TTTGGATCCAGGATTGTTCTTTTGTTG	66→86
1829	TCAT <u>GGATCC</u> CTGTCAACTGAAAAATGC	207→227
2327	CCAGCATCAACATGTCTCC	360→341
2332	GCAC <u>CCGGG</u> CTGAGTGGGAGATCATTCTC	454→473
2322	CTGAGTGGGAGATCATTCTC	457→476
2326	CGGAGAATGATCTCCCACTC	476→459
2323	GAAGACGCCAGAAAGCCGGC	558→576
2331	GCACCCGGGAAGACCCAGAAAGCCGG	558→575
1841	GATT <u>TGAC</u> AAAAGCATTACACCCCC	779→761
2304	ATTACTCGAGCTGAGGAAGGAATAGGGC	1156→1137
1832	GAAAAAGAAA-CAGC-ATCAACATGTCTCC	784→772 & 355→341
1833	GTGAT-GCTG-TTTCTTTCTTGCCTG	350→355 & 774→793
Mutation of tubulin region S_a/I site		
1868	CATGTTCTTCTCGCGTTATC	5' sense
1869	TCTTCGTTGACATGCC	Middle antisense
1870	GCGCGATGTCAACGAAGGA	Middle sense
1871	TATCCGCGGCTCGAGCATCTTCATTACCTAC	3' antisense
Double tet operator sequence		
<u>GGATCCCTCCATCGTGTAGAGATCTCCATCA GTGATAGAGATCCCTGGATCC</u>		

Restriction sites are underlined and the position of the oligonucleotide on the PGKC downstream sequence (Fig. 1) noted.

by cloning the J6f1 aptamer sequence [17] into the *Bam*HI site immediately downstream of the *CAT* gene of pHd1442 (Fig. 6A). The *Tet* operator fragment was excised with *Bam*HI and cloned into pHd1643 at the *Bgl*II site between the T7 promoter and the *EPI-5'*-UTR, creating the plasmid pHd1667 (Fig. 6A). As controls used plasmids with the *ACT* 3'-UTR replacing that of *PGKC* (Fig. 6A). Finally, a (G)₃₀ tract was introduced into pHd1667 by exchanging in a *Sma*l–*Nco*I fragment of pHd874 [18].

The aptamer was added in order to allow purification of the mRNAs along with bound proteins [17], but unfortunately, we could find no binding of the RNAs to tobramycin affinity columns.

2.3. Northern blots and RNA analyses

Total RNA was isolated from 4×10^7 log-phase cells (density not exceeding 10^6 ml $^{-1}$ for bloodstream forms and 5×10^6 ml $^{-1}$ for procyclics) either using a Rneasy® Mini Kit (QIAGEN, GmbH) or peqGOLD TriFast™ (peqlab, GmbH). Ten micrograms of the total RNA per lane were loaded on a 1% agarose-formaldehyde gel, resolved by electrophoresis, blotted on a nylon membrane (Nytran-N, Schleicher & Schuell, GmbH) and hybridised with 32 P-labelled probes. Resulting bands were quantified by PhosphorImager.

mRNA decay was analysed either by blocking transcription using Actinomycin D ($10 \mu\text{g ml}^{-1}$) or by blocking splicing using Sinefungin ($1 \mu\text{g ml}^{-1}$ for all experiments except those in Fig. 6 which used $2 \mu\text{g ml}^{-1}$), or by adding both drugs ($1 \mu\text{g ml}^{-1}$ Sinefungin for 5 min then $10 \mu\text{g ml}^{-1}$ Actinomycin D). After drug addition, total RNA was isolated at different time points and decay analyzed by Northern blot. Decay curves were plotted, half-lives measured, and statistical analysis done using Kaleidograph 3.6.4 (Synergy Software). Half-lives were

estimated using only the portions of the curves which fitted exponential decay; using Sinefungin or Actinomycin D alone, the measurement made at time 0 usually had to be excluded because there was a short delay before the mRNA level started to decrease.

3. Results

3.1. PGKC mRNA degradation in bloodstream and procyclic trypanosomes

We had previously shown that *PGKC* and *PGKB* regulation was disrupted by depletion of the 5'→3' exonuclease XRNA [10]. To analyse the mechanism of this effect, we treated bloodstream and procyclic trypanosomes with Sinefungin [19,20], which inhibits 5'-capping of the spliced leader RNA and therefore indirectly inhibits *trans* splicing [21–23]. Results are shown in Fig. 1. Thirty minutes after Sinefungin addition, two bands migrating at about 7 and 11 kb became visible (Fig. 1A and B). These may be dicistronic and tricistronic RNAs produced by partial splicing of the polycistronic precursor. Using this protocol, *PGKC* mRNA appeared to be quite stable in bloodstream forms, with half-life measurements of around 3 h (Fig. 1C). *PGKB* mRNA was 10–15-fold less abundant than *PGKC* mRNA in bloodstream forms (this work [8]) and accurate half-life estimation was impossible. After XRNA depletion, the abundance of *PGKB* mRNA increased, as expected, to about one-third of the *PGKC* mRNA abundance, with a corresponding decrease in the apparent degradation rate (Fig. 1A, quantitation not shown). The degradation of *PGKC* mRNA was, in contrast, not affected by XRNA depletion.

In procyclic forms, *PGKB* mRNA had a half-life of over 4 h, which was unaffected by XRNA depletion (Fig. 1B, quantitation not shown). *PGKC* mRNA was undetectable in

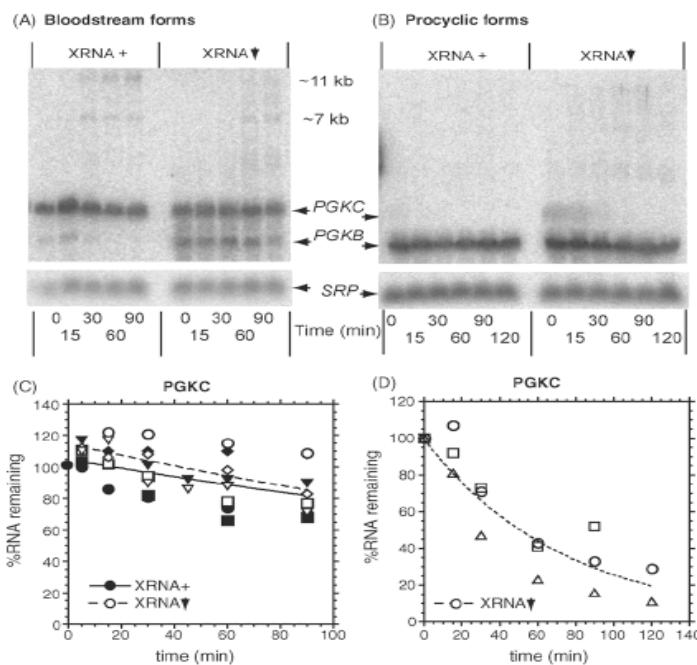


Fig. 1. Degradation of PGK mRNAs in bloodstream and procyclic trypanosomes. Sinefungin ($1 \mu\text{g ml}^{-1}$) was added to cultures and mRNA prepared at the times indicated. Levels of *PGKB* and *PGKC* mRNA were measured by Northern blotting, with *SRP* as a control. (A) Representative blot for bloodstream forms. The *PGKC* and *PGKB* transcripts are indicated, as are two higher-molecular-weight RNAs which appear after Sinefungin addition. "XRNA+" indicates wild-type levels of XRNA. The downward arrow indicates reduction of XRNA protein by RNA interference. To induce RNA interference the cells were grown for 24 h in the presence of tetracycline. (B) Northern blot showing degradation in procyclic forms. To induce interference, the cells were grown for 48 h in the presence of tetracycline. (C) Quantification of the signals from three independent bloodstream experiments. Filled symbols and continuous line: without tetracycline (normal level of XRNA); open symbols and dotted line: XRNA-depleted cells. Each type of symbol represents one experiment. The lines are fitted through the arithmetic mean values as exponential curves using the Kaleidograph programme. In all cases, curve-fitting was done including only values which were on the exponential part of the curve (usual threshold, Pearson's regression coefficient >0.95): early time points before degradation had started, and late time-points where the signal values were approaching background were excluded. (D) Degradation of *PGKC* mRNA after XRNA depletion in procyclic cells. Symbols as in (A). The half-life measurement is rather inaccurate due to the low signal.

procyclic forms (less than 5% of the *PGKB* signal). After XRNA depletion, *PGKC* mRNA was detectable (Fig. 1B) with a half-life of about 1 h (Fig. 1D).

3.2. A vector for analysis of 3'-UTRs

To find out which sequences in the *PGKC* 3'-UTR affected gene expression, we optimised a previous vector for analysis of multiple 3'-UTRs. The resulting vectors, pHD1424 and 1437, are illustrated in Fig. 3A (Table 2) and some relevant sequences are shown in Fig. 2. The vectors contain the following elements (Fig. 3A):

- (1) The pGEM plasmid backbone.
- (2) A segment to allow targeting into the trypanosome genome. For targeting to the tubulin locus (pHD1437-

based plasmids), the segment used is from the region between the beta- and alpha-tubulin open reading frames and contains an internal *NotI* site. If the plasmid is linearised at the *NotI* site before transfection into trypanosomes, the DNA integrates into the tubulin locus by homologous recombination. This allows transcription by RNA polymerase II reading through the tubulin repeats. Alternatively, for targeting into a silent part of the genome (pHD1424-based plasmids) a segment from the non-transcribed rRNA spacer region is used.

- (3) A promoter for bacteriophage T7 polymerase. If the construct is transfected into parasites expressing T7 polymerase, this promoter is active and the construct is transcribed by T7 polymerase. The amount of primary transcript is increased about 50-fold above the level obtained with RNA polymerase II.

Table 2
Plasmids used in this work

CAT 3'-UTR	Letter on figures	rRNA spacer plasmid	Tubulin-locus plasmid
Polylinker		1368	Has a tet operator adjacent to the T7 promoter
Polylinker		1424	pHD1368 without tet operator
Polylinker		1437	1424 with tubulin-locus fragment replacing rRNA spacer
PGKC 3'-UTR, IGR, dsORF 66–1156	a	1425	PGKC 3'-amplified with CZ1827 and CZ1828, cloned into pHD1368 (pHDI375), Δtet operator
PGKC 3'-UTR 66–779	b	1428	PGKC 3'-amplified with CZ1827 and CZ1841
PGKC 3'-UTR IGR, dsORF Δ1–207	c	1426	PGKC 3'-amplified with CZ1829 and CZ1828
PGKC 3'-UTR IGR, dsORF Δ355–773	d	1427	Hybrid PCR using bridging primer CZ1833
PGKC 3'-UTR IGR, dsORF Δ359–453	e	1539	Two fragments amplified with CZ2304 and CZ2332 and CZ2322 and 2327
PGKC 3'-UTR IGR, dsORF Δ476–557	f	1540	Two fragments amplified with CZ2304 and CZ2331 and CZ2326 and 1827
ACT 3'-UTR	g	1462	
PGKC 3'-UTR	h	1643	pHD1442 + Jf6 aptamer
PGKC 3'-UTR	j	1667	pHD1643 + Apt+tet operator
ACT 3'-UTR	k	1668	pHD1462 + Jf6 aptamer
PGKC 3'-UTR	m	1741	pHD1667 with (G) ₂₀ inserted at the <i>Hinc</i> III site

The tRNA spacer plasmids with PGK 3'-UTRs were either cloned into pHD1424, or were originally cloned into pHD1368, then subsequently digested with *Bgl*II and re-ligated to remove the tet operator.

Fig. 2. (A) Sequence of the *PGKC* locus from the termination codon to an *Xba*I site in the downstream open reading frame. The open reading frame is in upper case and possible *trans* splice sites (AG) are undefined. A polypyrimidine tract around n=900 which may signal *trans* splicing is in bold italics. Previously mapped polyadenylation sites [7] are indicated by A and an upward arrow, and the boundaries of deletions by vertical bars. This entire region was present in plasmid "a" (pHD1449 and 1425) shown in Fig. 3A. (B) The *ACT* intergenic region and 5'-UTR preceding the *PAC* open reading frame in all of the plasmid constructs shown in Fig. 2A. Symbols as in Fig. 1A.

- (4) A region situated 5' of the *EPI* procyclin gene containing a polypyrimidine tract, the acceptor AG for *trans* splicing and the 3'-untranslated region. This segment is sufficient to guarantee *trans* splicing of RNA.
 - (5) A *CAT* open reading frame with some flanking sequence, translated into the reporter enzyme chloramphenicol acetyl transferase.
 - (6) A polylinker.
 - (7) A region from the *ACT* (actin) locus. The actin locus contains tandemly repeated *ACT* genes. The region between one *ACT* open reading frame and the next includes the 3'-UTR downstream of the first *ACT* open reading frame, and a short region around a *Sall* site where polyadenylation occurs (Fig. 2B). After approximately 100 bp there is a polypyrimidine tract which signals *trans* splicing of the downstream *ACT* mRNA (bold italics in Fig. 2). In trypanosomes, polyadenylation of one mRNA is dependent on downstream *trans* splicing, which occurs approximately 100 nt upstream of the polypyrimidine tract in precursor mRNAs [24,25]. In the actin locus, this polypyrimidine tract is the signal for polyadenylation of the upstream *ACT* mRNA [26,27]. The polylinker in the vector is located at the *Sall* site, so the *ACT* polypyrimidine tract should ensure polyadenylation approximately at the polylinker sequence.
 - (8) A puromycin resistance cassette to enable selection of permanently transformed trypanosomes.
 - (9) An *ACT* 3'-UTR.
 - (10) Two T7 terminators.

3.3. The PGKC 3'-UTR can cause developmental regulation

We had previously found that the PGKC 3'-UTR (shown on Fig. 2A) is able to direct developmental regulation of a *CAT* reporter gene in transient transfection studies [7]. To find the

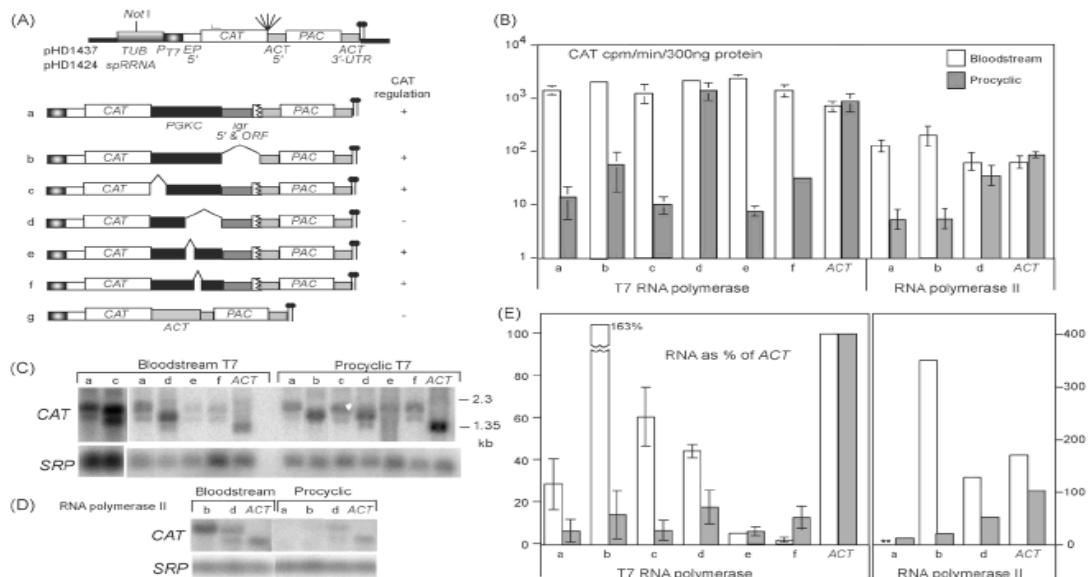


Fig. 3. (A) Plasmid constructs. The region used to target the plasmids into the genome (rRNA spacer, inverted relative to rRNA transcription direction, or tubulin intergenic region, “sense” orientation) has graded shading and the T7 promoter has bulls-eye (radial) shading. These segments are shown only on the top diagram of pHD1437 and 1424. The EP 3'-UTR is white, as is the CAT reporter gene. The two “lollipops” at the end of the ACT 3'-UTR are T7 terminators. In plasmids a–f, the PGKC 3'-UTR down to the native polyadenylation site is in black. The region which lies downstream of the polyadenylation site is filled dark grey, and a small fragment of the next downstream open reading frame is shown as a white fragment with a zigzag edge (labelled “igr 5’ and ORF”). For precise sequences present in each plasmid see Fig. 2A and for details see Table 2. The ability of the 3'-UTR to effect developmental regulation is also indicated next to each plasmid. A “+” sign indicates that the plasmid gives much lower CAT activity in procyclic forms than in bloodstream forms, while a “-” sign indicates that CAT activities are similar for both life-cycle stages. (B) Northern blot of mRNAs produced from the plasmids shown in (A). Results from the CAT probe were normalised using signal recognition particle (SRP) signals. (C) CAT activities of permanent cell lines containing the constructs shown in (A). Results are shown as cpm min⁻¹ µg protein⁻¹. Assays were done at least four times and are expressed as mean and standard deviation. The results are for single cell lines except for plasmids d and b, in which cases duplicate procyclic lines gave similar results. (D) Relative CAT RNA levels of permanent cell lines containing the constructs shown in (A). CAT mRNA levels were quantitated by phosphorimaging, and standardised relative to the SRP control. The amount from CAT-ACT in procyclic forms was assigned the value 100%. All mRNA levels were measured three times and are expressed as mean and standard deviation.

sequences responsible for PGKC regulation, we took as our starting point the 1156 bp region from the PGKC locus that had previously been tested [7]. This contains the stop codon and downstream DNA extending 75 nt into the next coding region (Fig. 2A). In Fig. 2A, the downstream ORF initiation codon is in upper case and possible AG acceptor dinucleotides for *trans* splicing of the downstream mRNA are underlined. A polypyrimidine tract, in bold italics and around 900 nt in the figure, lies approximately 100 bp downstream of the PGKC polyadenylation sites. This tract might signal both upstream polyadenylation and downstream *trans* splicing [24]. Basepairs 66–1156 (from now on referred to as PGKC-IGR) were inserted into the polylinker of pHD1437 (Fig. 3A, construct a), so that the PGKC-IGR was upstream of the ACT splice signal and 5'-UTR (Fig. 2B). We transfected this plasmid, “a” in Fig. 3A, into bloodstream and procyclic trypanosomes expressing T7 polymerase. Cells containing a similar construct with an ACT 3'-UTR (plasmid g) served as controls. The CAT activity obtained from

plasmid “a” in bloodstream forms was 100 times higher than that found in procyclic forms (note the logarithmic scale on Fig. 3B). All activity ratios were normalised for equal amounts of protein; since procyclic forms contain about twice as much protein as bloodstream forms, the ratio of CAT activity on a per cell basis is about 50:1. This confirmed that even at high expression levels, in permanent cell lines, developmental regulation by the PGKC 3'-UTR and intergenic region was present.

We next measured CAT mRNA levels in cell lines containing plasmid “a”. In contrast with endogenous PGKC mRNA, which shows at least 15-fold regulation, and also with the 100-fold regulation of CAT activity, the CAT mRNA level was only 5-fold regulated (Fig. 3D and E; note that the scale is linear). This result suggested two things: firstly, that translation of the mRNAs from plasmid “a” is more efficient in bloodstream forms than in procyclics; secondly, that the regulation of the abundance of the over-expressed CAT-PGKC-IGR mRNA did not fully reflect the regulation of endogenous PGKC mRNA.

In construct "a", there are two splice sites downstream of the *CAT* gene: one is from the *PGKC* locus, and the one further downstream is from the *ACT* locus. The presence of these two different splice sites results in polyadenylation at two different sites [28,29]. The shorter mRNA (1.8 kb) is expected to contain only the native *PGKC* 3'-UTR, and the longer one, which predominates, is extended by nt 300 (Fig. 3C, lane a).

We next attempted to define more closely the region responsible for low *CAT-PGKC-IGR* expression in procyclic trypanosomes. First, we deleted the region downstream of the polyadenylation site (Fig. 3A, plasmid b). This should give a *CAT* mRNA polyadenylated at the normal *PGKC* polyadenylation site (from now on referred to as *CAT-PGKC*), with processing determined by the downstream *ACT* trans splicing signal. Procyclic trypanosomes containing this construct had levels of CAT activity and mRNA that were similar to those seen with plasmid "a" (Fig. 3B and E), indicating that the sequences downstream of the polyadenylation site were not needed for depressed expression in procyclics. The major mRNA found was 1.7 kb long (Fig. 3C, lane b), as expected for use of the native polyadenylation sites. From the results of further deletions, we could see that 1–207 nt were dispensable for down-regulation of CAT protein expression in procyclic forms (plasmid c) but that deletion of 356–774 nt abolished it (plasmid d, *CAT-PGKCΔ356-774-IGR*). Two smaller deletions (plasmids e and f) did not restore expression in procyclics, suggesting that the regulatory region lies between nt 558 and 779. Attempts to obtain smaller deletions within this region by PCR, and to map polyadenylation sites on the *CAT-PGKC* mRNAs by RT-PCR, failed repeatedly, possibly because of strong secondary structures in the RNA. Overall, RNA levels did not correlate well with protein expression.

To control for non-stage-specific effects, we also tested the *CAT-PGKC* plasmids in bloodstream-form trypanosomes. All plasmids readily gave transformants except plasmid (b), for which we were able to obtain only one poorly growing cell line after multiple transfection attempts. Cells containing all plasmids had fairly similar levels of CAT activity, usually slightly higher than from the *CAT-ACT* control cells, but the amounts of RNA for plasmids "e" and "f" were lower than for the other plasmids.

All results so far had been obtained using plasmids integrated at the rRNA spacer region, and transcribed by T7 polymerase. Since, however, over-production of the *CAT-PGKC* transcripts by T7 polymerase might cause artefacts, we tested four of the plasmids again, targeting the DNA to the tubulin array in cells which did not express T7 polymerase. In the resulting cell lines, the transgenes should be transcribed by RNA polymerase II, which generally gives about 20-fold less expression than T7 polymerase in trypanosomes [18]. Results are shown in Fig. 3B and E, and a typical Northern blot is shown in Fig. 3D. The patterns of CAT and *CAT* mRNA expression were similar to those previously obtained except that values were around 10-fold lower. Notably, in the absence of T7 polymerase, bloodstream-form transformants with plasmid "b" (*CAT-PGK*) were obtained without difficulty; the RNA was twice as abundant as *CAT-ACT* mRNA. This suggested that very active tran-

scription of the *CAT-PGK* construct was toxic in bloodstream forms.

3.4. Sequences in the *PGKC* 3'-UTR affect mRNA decay

We had previously observed that in bloodstream forms, depletion of the exosome affected the onset of degradation of a *CAT-PGKB* mRNA, but did not affect steady state abundance of either this RNA or the endogenous *PGKB* and *PGKC* mRNAs [9]. The availability of cell lines expressing *CAT-PGKC* from the T7 promoter enabled us to now examine the effects of exosome depletion on degradation of mRNAs with the *PGKC* 3'-UTR. We therefore transfected selected cell lines with a plasmid designed for RNAi against the core exosome subunit RRP45 [15].

Results for procyclic forms expressing *CAT-PGKC-IGR* mRNA using T7 polymerase are shown in Fig. 4A. Cells with a normal amount of exosome exhibited a single, very weak band of *CAT-PGKC-IGR* mature mRNA (lane 8) which disappeared rapidly after Sinefungin addition (Fig. 4A, lanes 9–14 and filled symbols in the graph). Within 15 min of Sinefungin addition (lane 9), a larger band appeared. This band (*CAT-PAC*) hybridised with a *PAC* probe as well as the *CAT* probe (not shown) and was therefore likely to represent an unprocessed bicistronic precursor RNA terminating at the T7 terminator sequence. The half-life of the *CAT-PGKC-IGR* mRNA was impossible to measure accurately but was less than 10 min (Fig. 4A, filled symbols). After exosome depletion (lanes 1–7, and open symbols in the graph) there was a 3.6-fold increase in the steady-state level of *CAT-PGKC-IGR* mRNA (mean of three experiments). In addition, the abundance of the *CAT-PAC* precursor rose two- to three-fold. The rise in the precursor is likely to be due to a failure of RNA quality control in the nucleus as a consequence of exosome depletion. The Sinefungin dose used does not completely inhibit splicing, so the increase in *CAT-PGKC-IGR* mRNA could have been due either to increased precursor stability (allowing more time for splicing) or to a slight delay in degradation of the mature transcript. Exosome depletion in both bloodstream and procyclic trypanosomes did not detectably affect steady-state abundances of endogenous *PGKB* and *PGKC* mRNAs (not shown).

We also analysed whether the increased abundance of *CAT-PGKCΔ356-774-IGR* mRNA, relative to *CAT-PGKC-IGR* mRNA, was due to a longer mRNA half-life. The *CAT-PGKCΔ356-774-IGR* mRNA was indeed more stable than *CAT-PGKC-IGR* mRNA in procyclics (Fig. 4B, lanes 8–14 and filled symbols), exosome depletion did not have any reproducible effect on the apparent degradation rate (Fig. 4B, lanes 1–7 and open symbols), although precursor accumulation was increased two- to three-fold and the mature mRNA abundance had doubled.

We next measured the half-lives of *CAT-PGKC* mRNAs in bloodstream forms. To our surprise, the T7-polymerase-produced *CAT-PGKC-IGR* (Fig. 4C, lanes 1–6 and filled symbols) and *CAT-PGKCΔ356-774-IGR* mRNAs (Fig. 4C, lanes 8–12 and open symbols) were not much more stable than *CAT-PGKC* and *CAT-PGKCΔ356-774-IGR* mRNAs in procyclics. The instability of *CAT-PGKC-IGR* mRNA in bloodstream

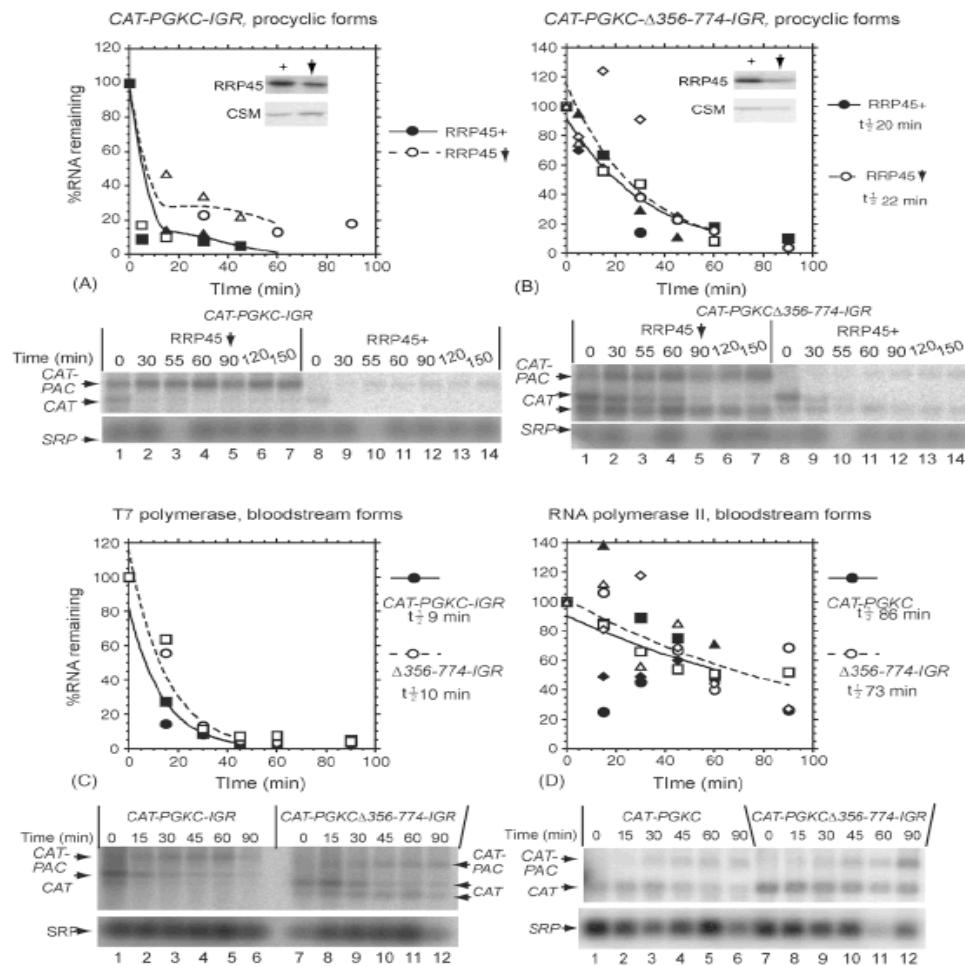


Fig. 4. (A) Degradation kinetics of *CAT-PGKC-IGR* (plasmid "a") Trypanosomes were treated with $1 \mu\text{g ml}^{-1}$ Sinefungin at time 0, and mRNA was detected by Northern blotting and quantitated by phosphorimaging using *SRP* mRNA as loading control. The amount of RNA at time 0 was set at 100%. The graph shows the results of individual experiments, each experiment being represented by symbols of one shape. The arithmetic mean values were calculated and individual points were joined to create the lines. A representative blot is also shown. To fit curves using the Kaleidograph programme, we included only values which were on the exponential part of the curve, excluding late time-points where the signal values were approaching background. RRP45+ cells were grown without tetracycline and have normal levels of RRP45 (filled symbols, solid line). RNA interference against RRP45 was induced by addition of tetracycline 2 days prior to the experiment (open symbols, dotted line). (Inset) Western blot of procyclic cells containing the CAT constructs before, and 24h after, induction of RRP45 knock-down. 2×10^6 cells per lane were loaded and protein depletion detected using an antibody against RRP45. Cytosolic marker (CSM) was used as a loading control. As expected [15], RRP45 depletion inhibited cell growth (not shown). (B) Degradation of *CAT-PGKCΔ356-774* (plasmid "d") mRNAs produced by T7 polymerase in procyclic trypanosomes. Details as in (A). Exponential curves were fitted through the arithmetic mean values and the resulting half-lives are shown. Curve fits: RRP45+ cells, $R = 0.88$; RRP45 depleted cells, $R = 0.99$. (C) Degradation kinetics of *CAT-PGKC-IGR* and *CAT-PGKCΔ356-774* mRNAs produced by T7 RNA polymerase in bloodstream trypanosomes. Exponential curve fits had $R > 0.95$. (D) Degradation kinetics of *CAT-PGKC-IGR*, *CAT-PGKC* (plasmid "b") and *CAT-PGKCΔ356-774* mRNAs produced by RNA polymerase II in bloodstream forms. Curve fits: *CAT-PGKC*, $R = 0.90$; *CAT-PGKCΔ356-774*, $R = 0.95$.

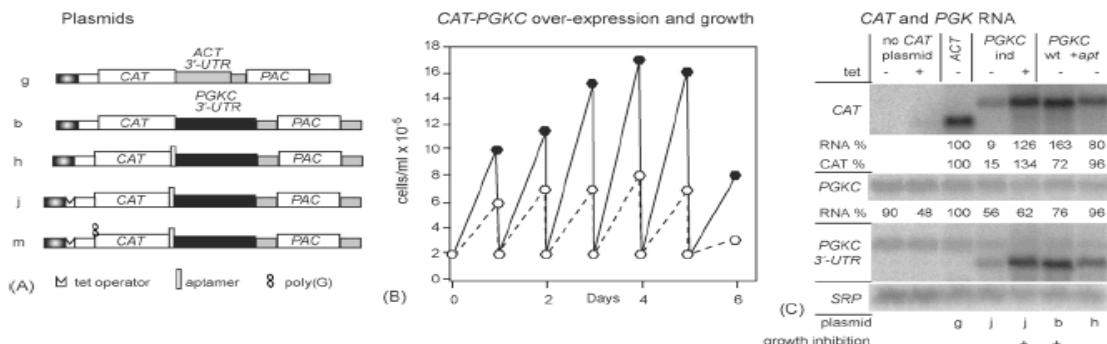


Fig. 5. Inducible over-expression of *CAT-PGKC* from plasmid b in bloodstream forms. (A) Plasmids used in the experiments. First, a short aptamer sequence was inserted between the *CAT* open reading frame and the *PGK* 3'-UTR in plasmid (b) (to give plasmid "h"), or the *ACT* 3'-UTR in the control plasmid (g) (to give plasmid "k"). Next, two tet operators were added downstream of the T7 promoter of plasmid "h" to give inducible plasmid "j". Finally, a poly(G) tract was inserted upstream of the open reading frame in plasmid "j", in order to inhibit translation (plasmid "m"). (B) Growth curve of cells containing plasmid "j" with and without tetracycline. (C) *CAT* and *PGKC* mRNAs in cells containing plasmids b, j, g and h. The 3'-UTR on the *CAT* genes are described briefly above the lanes ("ind" means "inducible") and indicated by letter underneath the lanes. Probes used are on the left of the blots. Underneath the blot probed with *CAT* are shown the *CAT* mRNA levels and *CAT* activities relative to the *CAT-ACT* control (mean of two measurements). The amounts of *PGKC* mRNA are shown beneath the blot probed with *PGKC*. Note that upon prolonged growth of the cells containing plasmid (b) *CAT* activity and RNA were lost altogether, suggesting that the population used for the measurements shown may have been a mixture of cells with and without *CAT-PGKC* expression.

forms explains why this RNA is only five times more abundant in bloodstream forms than in procyclic forms [19,20].

We next asked which aspect of the reporters was causing their misbehaviour in bloodstream forms. One obvious explanation was that the *CAT-PGKC-IGR* mRNA contained extra sequence downstream of the normal poly(A) site. Indeed, the bloodstream trypanosomes expressing *CAT-PGKC* mRNA (plasmid b) had five times more *CAT* mRNA than the line expressing *CAT-PGKC-IGR* mRNA (Fig. 3E), indicating that the additional 300 nt at the 3'-end reduced expression in bloodstream forms. In later experiments using an inducible construct (plasmid j, Fig. 5), we found a half-life for *CAT-PGKC* mRNA of 30 min in bloodstream forms (Sinefungin treatment, data not shown), confirming that the intergenic sequence was destabilising.

The other obvious possible culprit in causing instability was the T7 polymerase driving expression. To test this, we compared degradation of *CAT-PGKΔ356-774-IGR* mRNA (plasmid "d") transcribed by RNA polymerase II (Fig. 4D, lanes 7–9, open symbols) with degradation of the same RNA after transcription by T7 polymerase (Fig. 4C, lanes 7–9, open symbols). The mRNAs produced by RNA polymerase II were more stable than those produced by T7 polymerase.

3.5. Overexpression of *CAT* protein or *CAT-PGKC* mRNA is toxic

Our difficulty in obtaining bloodstream trypanosomes expressing high levels of *CAT-PGKC* mRNA suggested that either the RNA or the protein might be toxic. To investigate this, we made bloodstream trypanosome lines in which expression of *CAT-PGKC* mRNA was driven by a tetracycline-inducible T7 promoter. The plasmids (Fig. 5A) were transfected

into trypanosomes and *CAT* mRNA and enzyme activity were measured. Two cell lines containing inducible construct "j" (*CAT-PGKC* mRNA) grew normally in the absence of tetracycline (Fig. 5B) with relatively low *CAT* activity and RNA (Fig. 5C). Upon addition of tetracycline to these cells, growth was inhibited (Fig. 5B), and *CAT* protein and *CAT-PGKC* mRNA increased to a level which was approximately 10-fold higher than that of the endogenous *PGK* mRNA (Fig. 5C, compare signals in the panel hybridised with the *PGKC* 3'-UTR). The steady-state level of *PGKC* mRNA in the various cell lines was not affected by induced *CAT-PGKC* expression (Fig. 5C), and the *PGKC* mRNA half-life was also not significantly changed (not shown).

We have so far been unable to determine whether the toxicity was due to *CAT* protein or *CAT-PGK* mRNA. The levels of *CAT* protein in induced cells containing plasmid "m" were not sufficient to be visible in a Coomassie stain of total protein (data not shown). The bloodstream trypanosomes containing plasmid "b" gradually lost both the protein and the RNA, leading us to suspect that our measurements of RNA and protein levels had been made on mixed populations. Similarly, when the inducible cell line (j) was thawed after storage, we found that addition of tetracycline caused high *CAT* expression and some growth inhibition, but the *CAT-PGK* mRNA level was lower than before (not shown); although this would be consistent with *CAT* protein toxicity, interpretation is again difficult. As an alternative, we inserted a (G)₃₀ sequence just upstream of the *CAT* gene (*G-CAT-PGKC*) in order to inhibit translation [18] (Fig. 5A, plasmid m). The growth of two cell lines containing inducible *G-CAT-PGKC* (plasmid m) was unaffected by tetracycline addition (not shown) and they had very low *CAT* activity, but this was uninformative because the *CAT* mRNA levels were lower than in the *CAT-ACT* line.

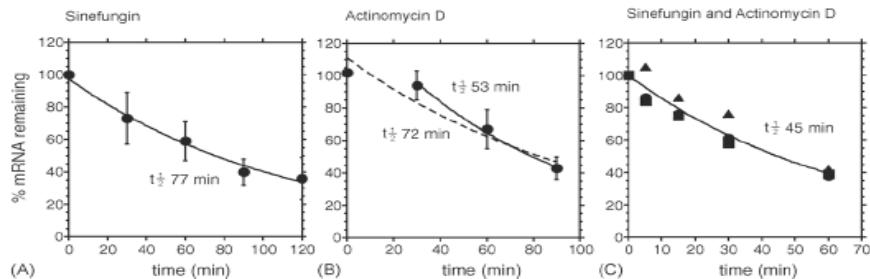


Fig. 6. Degradation of *PGKC* mRNA after inhibition of splicing or transcription alone, or of both together. (A) Sinefungin ($2 \mu\text{g ml}^{-1}$) was added at time = 0. RNA was analysed by Northern blotting using a *PGK* probe. Values are pooled from cells with an inducible CAT-PGKC mRNA grown without tetracycline (four experiments) and wild-type cells (one experiment) and are shown as arithmetic mean \pm standard deviation. (B) Actinomycin D ($10 \mu\text{g ml}^{-1}$) was added at time = 0. The cell lines used either contained no inducible RNAi construct (two experiments) or an RNAi construct targeting *CAF1* (A. Schwede, unpublished); they were all grown without tetracycline and had similar *CAF1* mRNA levels (not shown) and *PGKC* mRNA half-lives. The solid line is an exponential curve fitted without including values at time = 0, giving a linear correlation coefficient of 0.998. Including the zero time point when fitting the curve (dashed line) resulted in a correlation coefficient of 0.94. (C) Sinefungin ($1 \mu\text{g ml}^{-1}$) was added (time = −5 min), then Actinomycin D ($10 \mu\text{g ml}^{-1}$) was added (time = 0). The trypanosomes used were as in Fig. 1, and symbols are similar to those in Fig. 1. The *R* value was >0.998 . Down-regulation of XRNA had no significant effect on the half-life under these conditions (not shown).

3.6. Alternative measurements of the *PGKC* mRNA half-life

After addition of Sinefungin to inhibit mRNA maturation, we observed accumulation of minor high molecular weight bands which were presumably aberrantly spliced transcripts or precursors (Fig. 1), consistent with incomplete inhibition of spliced leader methylation [22]. We therefore repeated the measurement of *PGKC* mRNA degradation in bloodstream trypanosomes using $2 \mu\text{g/ml}$ Sinefungin, as described by Webb et al. [20]. Under these conditions, the average half-life of *PGKC* mRNA was 77 min (Fig. 6A) but some aberrant products still accumulated indicating incomplete inhibition of processing ([20] and data not shown). We compared this result with that obtained using Actinomycin D (Fig. 6B). Thirty minutes after Actinomycin D addition the level of *PGKC* mRNA was not much changed (Fig. 6B); only after this was exponential decay observed (half-life 55 min). Including the first time point resulted in a poor exponential curve fit and a longer apparent half-life (Fig. 6B, dashed line).

We next used a combined protocol, adding Sinefungin for 5 min to deplete the spliced leader mRNA, then Actinomycin D to inhibit transcription [10]. The half-life of *PGKC* mRNA was now measured at 45 min (Fig. 6C). We conclude that the addition of Sinefungin prior to Actinomycin D is the best protocol for complete and rapid inhibition of mRNA synthesis, and that the true half-life of *PGKC* mRNA is 45 min.

4. Discussion

The main aim of the work described in this paper was the identification of the 3'-UTR sequence responsible for the down-regulation of PGKC enzyme and *PGKC* mRNA in procyclic trypanosomes. To map the sequences, we used a reporter plasmid expressing *CAT* mRNAs with the *PGKC* 3'-UTR, transcribed by either T7 polymerase or RNA polymerase II. Procyclic try-

panosomes transfected with plasmids containing the complete *PGKC* 3'-UTR sequence expressed 100-fold less CAT activity than cells transfected with a plasmid containing a truncated version of the *PGKC* 3'-UTR lacking nt 356–774. Thus, nt 356–774 of the *PGKC* 3'-UTR suppressed CAT expression in procyclic trypanosomes.

Messenger RNA decay experiments revealed that the *CAT-PGKC* mRNA had a half-life of 10 min or less in procyclic forms, and that deletion of the regulatory region (nt 356–774) increased the half-life to about 20 min with a corresponding two-to three-fold increase in the mRNA. However, the increase of the mRNA level did not correlate with the 100-fold increase in CAT activity. This discrepancy between mRNA and enzyme levels suggests that the nt 356–774 sequence contains elements which control translation. Further deletions narrowed the likely regulatory region to nt 478–557. We screened the trypanosome genome with this region but found no matches apart from low-complexity AT-rich sequences which are common in non-coding regions irrespective of regulation [24]. In previous experiments, we have already seen that the secondary structure of mRNA 3'-ends is critical in determining their effects on mRNA abundance [18,19]. Future genomic comparisons will therefore have to include secondary structures in addition to primary sequence.

Bloodstream forms in which the *CAT-PGKC* reporter plasmid was transcribed by RNA polymerase II had at least 10-fold more CAT activity than equivalent procyclic forms and deletion of the nt 356–774 regulatory region did not increase in protein and RNA expression. Thus, the suppressive function of the nt 356–774 region is specific to procyclic forms.

Our further results indicated that production of *CAT-PGKC* mRNAs by T7 polymerase in bloodstream forms resulted in two artefacts. The first problem we observed was that the *CAT-PGKΔ356-774-IGR* mRNA (plasmid "d") was much less stable when produced by T7 polymerase than when produced by RNA polymerase II. A possible interpretation of this result is that the

stability of *PGKC* mRNAs in bloodstream forms depends on binding of a protein (or RNA) factor. It could be that the factor is present in limiting amounts; alternatively, it may not bind efficiently to mRNAs which are produced by T7 polymerase. The second problem was that high amounts of either *CAT-PGK* mRNA, or CAT protein, inhibited growth.

Depletion of XRNA caused increases in the amounts of *PGKB* mRNA in bloodstream forms and of *PGKC* mRNA in procyclics. Depletion of the exosome did not have major effects on the apparent half-life of *CAT-PGKC* mRNA in procyclic forms, but did cause an increase in the abundance of the *CAT-PAC* precursor RNA. The exosome and XRNA are located both in the cytoplasm and the nucleus [10,15]. The exosome is known to be involved in quality control of mRNA in *Saccharomyces cerevisiae*, by degrading aberrantly processed or unprocessed mRNAs [30–32]. We suggest that there is competition for the *CAT-PAC* precursor between the exonucleases and the *trans* splicing/polyadenylation machinery. If this were so, depletion of the exonucleases could tip the balance in favour of processing, and cause an increase in the amount of mature mRNA. The apparent delays in degradation of mature unstable mRNAs, and the increases in absolute amounts, which we observed after depletion of exonucleases could have been caused by effects on degradation of both cytosolic mature mRNA and nuclear precursors. To dissect this further, detailed studies on the precursor abundances and processing kinetics will be required.

Although the degradation and translation of *PGKB* and *PGKC* mRNAs are clearly developmentally regulated, *trans* splicing and polyadenylation have also been considered as possible contributors. Two previous studies have considered this. Siegel et al. [33] used luciferase expression as a surrogate for mRNA abundance after transient transfection of reporter constructs into procyclic trypanosomes. They concluded that the *PGKB* splicing signal was 50-fold more active than the *PGKC* splice signal, and that the *PGKA* signal had very low activity. Their constructs, however, did not include cognate 5'-UTRs, which may influence splicing efficiency [29]. Previously, Kapotas and Bellofatto had done similar experiments with CAT or luciferase reporters [34]. They found that *PGKA* splicing was very inefficient, but detected no difference between the *PGKC* and *PGKB* constructs. The design of the constructs was different in the two papers, and neither the accuracy of mRNA splicing nor the levels of mRNA were measured. A priori, regulation of splicing seems unlikely: since *trans* splicing and polyadenylation are mechanistically linked [25], one might expect inefficient *trans* splicing of *PGKC* mRNA to be detrimental to *PGKB* polyadenylation. Detailed measurements of splicing and polyadenylation kinetics are required to resolve this issue.

In yeast and mammalian cells, mRNA half-lives are now routinely measured using tetracycline-inducible reporter genes, but the kinetics of transcriptional shut-down in trypanosomes are too slow, relative to the RNA half-lives, to allow this approach (H. Irmer, then at ZMBH, unpublished). Most publications describing mRNA half-lives in Kinetoplastids have relied on the inhibition of transcription using Actinomycin D. When highly unstable mRNAs are examined the level of the mRNA starts to decrease almost immediately after Actinomycin D addition

[19]. Other mRNAs, however, show either a delay in degradation or, initially, a transient rise in abundance [19,35–38], which can lead to serious difficulties in estimating mRNA decay rates. Using Sinefungin to inhibit *trans* splicing [19,20], exponential degradation starts after a lag of 5–10 min, without an initial rise, but mRNA processing is not completely inhibited. After inhibition of transcription with Actinomycin D, the half-life of spliced leader RNA was 8 min [39], so splicing of pre-existing precursors may be able to continue after transcription has stopped. To prevent this we added Sinefungin 5 min before Actinomycin D. This protocol resulted reproducibly in immediate exponential decay and a shorter *PGKC* half-life than the other two protocols; similar results were obtained with actin mRNA [10]. Although it is possible that the combination of Actinomycin D and Sinefungin actually causes mRNA instability, we think that it is more likely that this is the only treatment which completely eliminates mRNA production.

In conclusion, the results presented here show that the *PGKC* 3'-UTR exerts strong regulatory effects in *T. brucei*, and that they are exerted at the levels of both mRNA stability and translation. Overall the results suggest that the *PGKC* 3'-UTR contains some elements which promote active degradation in procyclic forms, but that it also is responsible for an active stabilisation process in bloodstream forms.

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