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The PUF proteins in *Trypanosoma brucei*

Supervisors: **Prof. Dr. Christine Clayton**

Prof. Dr. Bernd Bukau

Zentrum für Molekulare Biologie (ZMBH)

Universität Heidelberg

Im Neuenheimer Feld 282

69120 Heidelberg

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Summary

The protozoan parasite *Trypanosoma brucei*, which causes human sleeping sickness, has to adapt to rather different environments as it cycles between the mammalian host and the tsetse fly vector. This adaptation is mediated by changes in trypanosome gene expression, which are mainly regulated at the post-transcriptional level. Proteins with an RNA-binding "Puf" domain are important for post-transcriptional control by modulation of mRNA stability and regulation of translation in other species. This suggested that Puf domain proteins might also have a similar role in trypanosomes. In addition to the previously characterized *Tb*PUF1 (Hoek, Zanders et al. 2002) I have identified eight more *PUF* genes in the *T. brucei* genome. A comparison of the characteristic RNA binding domain of *T. brucei* PUF proteins suggested that they bind related but distinct targets. Interestingly, each of the PUF protein has an orthologue in *Trypanosoma cruzi* and *Leishmania major*. Phylogenetic analysis suggested that there were several kinetoplastid PUF proteins before separation from the eukaryotic lineage. Depletion of each of the nine *T. brucei* PUF proteins by RNA interference did not result in an obvious phenotypic change. Furthermore, PUF1 knock out procyclic cell lines were viable, indicating that this PUF protein is not essential for in vitro growth. Additionally, double RNAi analyses suggested that the proteins tested did not share redundant functions in *T. brucei*. Microarray studies comparing wild-type cells with cells where PUF levels have been perturbed (either by RNAi or overexpression) revealed one putative mRNA target for PUF5: *CAP17* (corset associated protein 17) mRNA is downregulated upon overexpression of PUF5 in the insect form of the parasite. Interestingly, PUF5 overexpression was also lethal for procyclic cells. Multiple mRNAs which associated with PUF proteins in mRNP complexes were identified. PUF5 for example, selectively binds to mRNAs encoding for amino acid transporters in bloodstream form cells. Attempts to identify PUF interaction partners have so far failed. The effect of PUF proteins on global protein expression level was also investigated using two-dimensional gel electrophoresis approach. A few proteins which were differentially regulated upon RNAi or knockout of PUF proteins were identified. These results indicated that PUF proteins in *T. brucei*, as is true for PUF proteins in general, are involved in regulating gene expression.

Zusammenfassung

Die Schlafkrankheit wird durch den Parasiten *Trypanosoma brucei* hervorgerufen. Dieser einzellige Erreger wird durch die Tsetse-Fliege von einem Säugerwirt zum nächsten übetragen. Die hohe Adaptionsfähigkeit des Erregers beruht auf der Regulation der Genexpression, die bei Trypanosomen hauptsächlich post-transkriptionell abläuft. Proteine mit einer RNS-bindenden „Puf“ Domäne spielen in anderen Eukaryonten eine wichtige Rolle in der post-transkriptionellen Kontrolle; sie modulieren die Stabilität der RNS und regulieren die Translation. Es ist zu erwarten, dass PUF Proteine diese Rolle auch in Trypanosomen haben. Zusätzlich zum charakterisierten *Tb*PUF1 (Hoek, Zanders et al. 2002) wurden während dieser Arbeit acht weitere PUF Proteine in *T. brucei* identifiziert. Ein Vergleich der RNS-bindenden Domäne lässt vermuten, dass PUF Proteine in *T. brucei* ähnliche aber unterschiedliche mRNS binden. Interessant ist, dass orthologe PUF Proteine auch in *Trypanosoma cruzi* und *Leishmania major* vorkommen. Phylogenetische Analysen deuten darauf hin, dass kinetoplastide PUF Proteine schon vor der Abspaltung von der eukaryotischen Abstammungslinie existierten. Die Expression jedes einzelnen PUF Proteins wurde mitttels RNAi gehemmt. Dies führte jedoch nicht zu offensichtlichen phänotypischen Veränderungen in *T. brucei*. Die gleichzeitige Hemmung der Expression von zwei PUF Proteinen hatte ebenfalls keinen ersichtlichen Phänotyp zur Folge und lässt vermuten, dass PUF Proteine keine redundanten Funktionen in *T. brucei* ausüben. Auch die gezielte Inaktivierung des PUF1 Gens mittels „Knockout“ hatte keine Folgen. Desweiteren konnten Microarraystudien, die Wildtypzellen mit PUF-RNAi oder -überexprimierenden Zellen verglichen, eine Ziel-mRNA für PUF5 identifizieren. Die Menge an *CAP17* (corset-associated protein 17) mRNA wird bei Überexpression von PUF5 in prozyklischen Zellen reduziert. Auch ist die Überexpression von PUF5 für *T. brucei* tödlich. Zudem konnte gezeigt werden, dass verschiedene mRNS assozieren mit PUF Proteinen in mRNP Komplexen. PUF5 zum Beispiel bindet spezifisch RNS, die Aminosäuretransporter kodieren. Versuche, PUF-Interaktionspartner in *T. brucei* zu finden, blieben erfolglos. Der Einfluss von PUF Proteinen auf die globale Proteinexpression wurde mittels zweidimensionaler Gelelektrophorese untersucht. Die Expression einiger Proteine verändert sich in PUF-RNAi und PUF1 knockout Zell-Linien. Diese Resultate lassen darauf zurückschliessen, dass PUF Proteine auch in Trypanosomen in die Kontrolle der Genexpression involviert sind.

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1. Introduction

1.1. Trypanosomes

Trypanosomes are flagellated protozoa belonging to the order of Kinetoplastida. The name of this group is derived from the kinetoplast, a microscopically visible structure of thousands of interlocked circles formed by their mitochondrial DNA, known as kinetoplast DNA (kDNA) (Shlomai 2004; Liu, Liu et al. 2005). Phylogenetic analyses based on 18S rRNA genes indicate that kinetoplastids branched very early from the eukaryotic lineage and therefore display some peculiar and unique features. Members of the family compartmentalize the first seven glycolytic steps in a peroxisome-like organelle, called the glycosome (Opperdoes 1987). Polycistronic transcription and the subsequent *trans*-splicing of spliced leader (SL) RNA to the pre-mRNA is another characteristic feature of trypanosomatids (Parsons, Nelson et al. 1984). Furthermore, there is no reproducible evidence for defined RNA polymerase II promoters (with the exception of the SL RNA promoter) or for developmental regulation of polymerase II transcription (Clayton 2002). Mitochondrial RNA in trypanosomes undergoes dramatic modifications by editing (Stuart, Schnaufer et al. 2005). The ability to escape the humoral immune response by expressing a changing repertoire of variant surface glycoprotein (VSG), a process called antigenic variation, is another characteristic trait (Borst 2002). Interestingly, the glycosyl phosphatidyl inositol membrane anchor (GPI anchor), relevant to all eukaryotes, was first discovered in trypanosomatids (Ferguson, Homans et al. 1988). The study of these organisms has revealed novel systems which were subsequently found to be widespread and important to general eukaryotic biology.

1.1.1. Genus *Leishmania*

Members of the genus *Leishmania* are the causative agents of leishmaniasis, which affects many vertebrates, including humans, dogs and several rodent species. The primary vectors transmitting *Leishmanias* are sandflies. Leishmaniasis currently threatens 350 million men, women and children in 88 countries around the world, with 1-2 million people infected every year. The various types of leishmaniasis (cutaneous, mucocutaneous and visceral) are confined primarily, but not exclusively, to Central and South America, central Africa, and parts of southern and central Asia (from WHO internet site: www.who.int).

1.1.2. *Trypanosoma cruzi*

Trypanosoma cruzi causes Chagas disease (also called American Trypanosomiasis) and is widely distributed throughout South and Central America. 1-2 million people are infected every year. The insect vector (Triatoma) for Chagas disease ingests amastigotes or trypomastigotes when it feeds. In the vector, the parasite reproduces asexually and metacyclic trypomastigotes reside in the vector's hindgut. The vector defecates on the host's skin while it feeds, and the metacyclic trypomastigotes enter the host's body, most often being "rubbed in" to the vectors bite or the mucous membranes of the eye, nose, or mouth (from www.who.int).

1.1.3. *Trypanosoma brucei*

Three subspecies of *Trypanosoma brucei* have so far been recognized; *T. b. brucei, T. b. gambiense* and *T. b. rhodesiense*. They are all widely distributed in tropical Africa, where their vectors, Tsetse flies (*Glossina spp.*) can also be found. *T. b. brucei* is not pathogenic to humans but causes a disease called nagana in African wild and domestic ruminants. *T. b. brucei* is routinely used as a laboratory model organism for other kinetoplastids. *T. b. gambiense* and *T. b. rhodesiense* are the causative agents of sleeping sickness (Human African Trypanosomiasis). *T. b. gambiense* is found in central and West Africa. It causes chronic infection, which does not mean benign. A person can be infected for months or even years without obvious symptoms of the disease emerging. But when the symptoms finally emerge, the disease is already at an advanced stage. *T.b. rhodesiense* is found in southern and east Africa. It causes more acute infection that emerges after a few weeks. These parasites are more virulent than the *T. b. gambiense* and the disease develops more rapidly, which means that it can be more quickly detected clinically. The incidence of sleeping sickness may approach 300,000 to 500,000 cases per year, and it is fatal unless treated (from www.who.int).

1.1.4. *Trypanosoma brucei* life cycle

The life cycle of *T. brucei* involves transmission between a mammalian host (bloodstream form) and an insect vector (procyclic form). Trypanosomes undergo a series of differentiation events in order to adapt to these distinct environments (Fig.1.1.). Apart from morphological restructuring, changes in surface coat and biochemical adaptation, the parasites also alternate between replicating and cell cycle arrested forms.

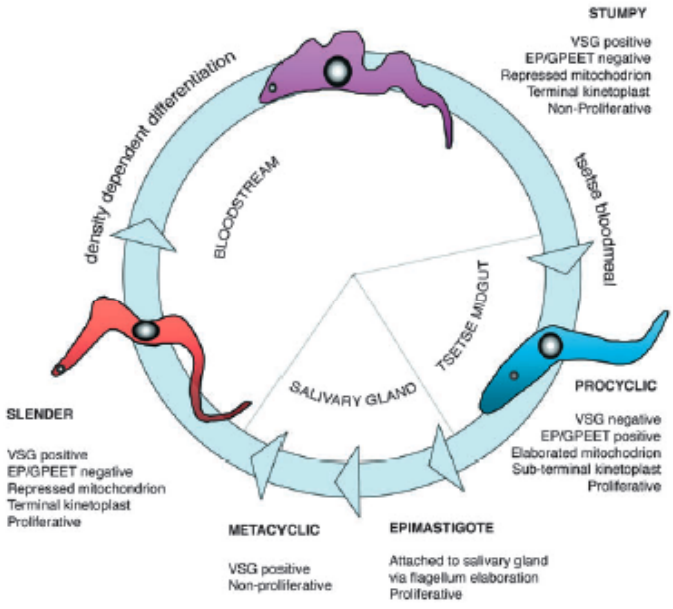


Fig.1.1. Schematic of the *T. brucei* life cycle, taken from (Matthews 2005).

1.2. Gene expression

Gene expression is a multi-step process which begins with gene transcription and RNA processing. Translation of the messenger RNA (mRNA) is then followed by folding, post-translational modification and targeting of the protein. Mechanisms of transcription have been analyzed in great detail in some model organisms such as the budding yeast *Saccharomyces cerevisiae*, mouse and human. Whereas prokaryotes have a single DNA-dependent RNA polymerase, eukaryotic organisms harbor three such enzymes in the nucleus (RNA pol I-III). Transcription regulators and co-factors have been described which modulate transcription efficiency at the level of transcription initiation. Chromatin organization can also modulate the efficiency of transcription by allowing or restricting access to distinct genomic loci. After transcription initiation, the nascent mRNA undergoes three types of processing events: a special nucleotide is added to its 5' end (capping), intron sequences are removed from within the mRNA molecule (splicing), and the 3' end of the mRNA is generated (cleavage and polyadenylation). Some of the RNA processing events that modify the initial mRNA transcript (for example, those involved in RNA splicing) are carried out primarily by special small RNA molecules. Once an mRNA has been correctly processed, it is escorted to the cytosol by proteins. To initiate translation in eukaryotes, initiation factors recognize and bind to the cap structure, and a small ribosomal subunit binds to the mRNA molecule at a start codon. Subsequently, a large ribosomal subunit will bind to form a functional ribosome, and thereby initiates the elongation phase

of protein synthesis. The mRNA molecule progresses codon by codon through the ribosome in the 5'-to-3' direction until one of three stop codons is reached. A release factor then binds to the ribosome, terminating translation and releasing the newly synthesized polypeptide.

Many steps in the pathway from gene to protein are regulated to control gene expression. Control of transcription initiation usually predominates (transcriptional control). However, post-transcriptional and –translational regulatory processes are also important. These processes include (1) attenuation of the RNA transcripts by its premature termination, (2) splicing, (3) control of 3'-end formation by cleavage and poly-A addition, (4) RNA editing, (5) nuclear export, (6) localization of certain mRNAs to particular compartments of the cell, (7) mRNA translation, (8) regulated mRNA degradation, (9) protein folding, (10) protein modification and localization, and (11) protein degradation.

1.2.1. Gene expression in Trypanosomatids

1.2.1.1. Introduction

Regulation of gene expression is a means to adapt to different environments, with different temperatures, nutrients and defence systems (e.g. of the host). Control of gene expression can be exerted on several different layers; in trypanosomatids however, most regulatory pathways act at post-transcriptional level. To understand this phenomenon, one has to take a closer look at the trypanosome gene organization.

1.2.1.2. Gene organization, transcription and RNA processing

In trypanosomatids, protein coding genes are tandemly linked, almost never interrupted by introns, and separated by short intergenic regions; exceptions which have introns are the genes encoding poly(A) polymerase (Mair, Shi et al. 2000), ATP-dependent DEAD/H RNA helicase, and two hypothetical proteins that are predicted to be capable of RNA binding (Ivens, Peacock et al. 2005). The majority of genes are transcribed polycistronically by an α-amanitin sensible RNA polymerase II. Surprisingly, despite enormous effort to identify RNA pol II promoters, no sequences displaying typical characteristics of such promoters have been found in trypanosomatids, the only exception being the SL RNA promoter (Luo, Gilinger et al. 1999; Gilinger and Bellofatto 2001). Analyses of the *Leishmania major* 269-kb chromosome I reveal a striking organization of the 79 genes found on this chromosome. Fifty genes are lined up one after another on one

strand and the other remaining 29 are packed adjacent to each other on the opposite strand (Myler, Audleman et al. 1999). Similar types of genomic organization are found in other chromosomes of *T. cruzi* and *T. brucei* (Andersson, Aslund et al. 1998; El-Sayed, Hegde et al. 2000; Berriman, Ghedin et al. 2005). Following transcription the polycistronic RNA precursor has to be cleaved to create the individual mature mRNA transcripts. This process involves two coupled cleavages: one at the 5'-end, with associated *trans*-splicing, and a further downstream cleavage with polyadenylation of the 3'-end (Ullu, Matthews et al. 1993; Matthews, Tschudi et al. 1994; Liang, Haritan et al. 2003). The process of *trans* splicing was discovered more than two decades ago when analyzing different variant surface glycoprotein (*VSG*) mRNAs in *Trypanosoma brucei*. It was found that all these *VSG* mRNA carry a common 39-nucleotide sequence, namely the spliced leader (SL) sequence (Boothroyd and Cross 1982). This SL sequence derives from a small capped RNA, the SL RNA, and has later been found on all trypanosome mRNAs (Milhausen, Nelson et al. 1984; Agabian 1990).

1.2.1.3. mRNA stability and degradation

Regulatory sequences in 3'-untranslated regions (UTRs) are involved in mediating either mRNA stability or degradation of the respective mRNA. Many genes with regulatory 3'-UTRs have been investigated in trypanosomatids. Examples from *T. cruzi* include the genes coding for amastin, tuzin, GP72, GP85, GP82, and the genes encoding small and large mucins (Nozaki and Cross 1995; Teixeira, Kirchhoff et al. 1995; Di Noia, D'Orso et al. 2000; Brittingham, Miller et al. 2001); in *Leishmania* protein A2 and the major surface proteins MSPL, MSPS, and MSPC contain 3'-UTR regulatory elements (Ramamoorthy, Swihart et al. 1995; Charest, Zhang et al. 1996; McCoy, Beetham et al. 1998; Myung, Beetham et al. 2002). Regulatory 3'-UTR elements have been best characterized in *T. brucei*. Most differentially regulated transcripts analyzed to date encode for proteins involved in energy metabolism or for surface proteins of the parasite, these two categories being fundamentally different in the two life cycle stages. Thus, the fructose bisphophate aldolase mRNA is at least 6-fold more abundant in bloodstream trypomastigotes than in procyclic forms and the 3'-UTR is responsible for the regulation (Clayton 1985; Hug, Carruthers et al. 1993; Hotz, Lorenz et al. 1995). The genes for the cytosolic and glycosomal phosphoglycerate kinases (PGK) 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) and its mRNA are abundant in the mammalian stage of the cycle but not in the insect stage, whereas the reverse is found for the cytosolic PGK (PGKB) and its mRNA (Gibson, Swinkels et al. 1988). Similarly, two hexose transporters (THTs) are developmentally regulated by their 3'-UTRs (Hotz, Lorenz et al. 1995), with THT1 being expressed exclusively in the bloodstream and THT2 in the procyclic stage. A large group of genes with regulatory 3'-UTRs include the major surface proteins VSG, EP and GPEET. A VSG 3'-UTR has been shown to cause a 20-fold higher expression of reporter gene in bloodstream forms than in procyclic forms (Berberof, Vanhamme et al. 1995) and the 3'-UTRs of *T. brucei* procyclic form surface protein mRNAs, EP and GPEET, are examples with well-characterized regulatory motifs (Furger, Schürch et al. 1997; Hotz, Hartmann et al. 1997; Schürch, Furger et al. 1997; Drozdz and Clayton 1999; Quijada, Guerra-Giraldez et al. 2002).

1.2.1.4. Post-translational control of gene expression in trypanosomes

First studies on the protein degradation machinery (i.e. proteasome) in trypanosomes already started a decade ago (Hua, To et al. 1996). It was demonstrated that the proteasome is implicated in stage-specific transformation in *T. cruzi* (Gonzalez, Ramalho-Pinto et al. 1996) and *T. brucei* (Mutomba and Ching 1998). Furthermore, regulation of the cellular concentration of cyclins, important regulators of the cell cycle, is also mediated by proteasome activity (Van Hellemond and Mottram 2000; Van Hellemond, Neuville et al. 2000).

1.2. RNA binding proteins

Throughout its lifetime, from biogenesis to translation and finally degradation, a changing repertoire of RNA binding proteins (RBPs) can bind to a particular mRNA. In this sense, mRNAs generally exist as messenger ribonucleoprotein (mRNP) complexes, rather than as single entities. A nascent mRNA is already bound by RBPs that co-transcriptionally mediate 5' end capping, splicing, and editing; 3' cleavage and polyadenylation; and quality control of the mRNA (Gott and Emeson 2000; Neugebauer 2002; Reed 2003). Translocation of mRNAs trough nuclear pores is also mediated by RBPs (Vinciguerra and Stutz 2004), and once in the cytoplasm, some mRNAs are localized to specific cellular regions by an interplay of various motor and mRNP adaptor proteins (Singer 2003; Van de

Bor and Davis 2004). In general transcripts are translationally repressed during localization (Huang and Richter 2004). Many RBPs that are involved in translation and regulation of translation also control mRNA transport and stability (Kuersten and Goodwin 2003; Kuersten and Goodwin 2005). Finally, an exonuclease-mediated degradation machinery destroys mRNAs either by normal, nonsense-mediated (NMD), and nonstop decay (NSD) pathways (Parker and Song 2004).

While many classes of RBPs have been described in higher eukaryotes, only a few *trans*-acting factors have been identified in trypanosomes. Poly(A)-binding protein (PABP1), a major cytoplasmic mRNA binding protein, was characterized in *T. cruzi*, *T. brucei* and *Leishmania spp*. (Batista, Teixeira et al. 1994; Hotchkiss, Nerantzakis et al. 1999; Bates, Knuepfer et al. 2000). PABP1 has been implicated in translation control and mRNA turnover by interaction with the poly(A)-tail at the 3'-end of all mRNAs. A role in ribosomal RNA (rRNA) biogenesis was suggested for the two proteins p34 and p37, that associate with 5S rRNA in *T. brucei* (Zhang and Williams 1997; Pitula, Ruyechan et al. 2002). Interestingly, both proteins interact with NOPP44/46, the major tyrosine-phophorylated nucleolar RBPs family in *T. brucei* (Das, Peterson et al. 1996; Pitula, Park et al. 2002). Furthermore, a nuclear serine/arginine (SR)-rich trypanosomal protein (TSR1), that contains two RNA recognition motifs (RRMs), has been implicated in *trans*-splicing in *T. brucei* (Ismaili, Perez-Morga et al. 1999; Ismaili, Pérez-Morga et al. 2000). SL RNA binding activity was also shown for XB1 and *Tc*SR, which were suggested to be involved in *trans*- (XB1) and *cis*-splicing (*Tc*SR) in *T. cruzi* (Xu, Wen et al. 2001; Portal, Espinosa et al. 2003). Many mRNA binding proteins were shown to be involved in RNA editing in *T. brucei*: these are the guide RNA binding proteins gBP16 (Hayman and Read 1999; Pelletier, Miller et al. 2000; Pelletier and Read 2003) and gBP21 (Koller, Muller et al. 1997; Allen, Heidmann et al. 1998; Muller, Lambert et al. 2001; Muller and Goringer 2002), the oligo(U) binding protein *Tb*RRG1 (Vanhamme, Perez-Morga et al. 1998), the RNA editing associated protein 1 (*Tb*EAP1) (Madison-Antenucci and Hajduk 2001), the endo-exoribonuclease *Tb*MP42 (Brecht, Niemann et al. 2005), and the mitochondrial RBPs MRP1 and MRP2 (Vondruskova, van den Burg et al. 2005). Two RBPs with an RRM, *Tc*UBP-1 and *Tc*UBP-2 (U-rich binding protein), were shown to have a destabilizing effect on specific mRNAs in *T. cruzi* (D'Orso and Frasch 2001; D'Orso and Frasch 2002). The *T. brucei* homologues of *Tc*UBP-1 and *Tc*UBP-2, *Tb*UBP1 and *Tb*UBP2, are also implicated in posttranscriptional gene regulation (C. Hartmann,

unpublished data). The recently completed genomes of *T. brucei*, *T. cruzi* and *L. major* (referred to here as the tri-tryp) contain many predicted proteins with RRM domains. *Trypanosoma cruzi* has nearly twice as many RRM-type proteins (139) as *T. brucei* (75) and *L. major* (80) (De Gaudenzi, Frasch et al. 2005). RNA binding proteins are also involved in the 3'-5' mRNA degradation pathway: *Tb*RRP4, *Tb*RRP40, and *Tb*CSL4 all have S1 RNA-binding activity and are part of the exosome complex (Estévez, Kemp et al. 2001). A role in differentiation from bloodstream to insect form stage of *T. brucei* was demonstrated for the two zinc finger proteins *Tb*ZFP1 and *Tb*ZFP2 (Hendriks, Robinson et al. 2001; Hendriks and Matthews 2005). And finally, *Tb*PUF1, a member of the PUF family of RNA binding proteins was identified in *T. brucei* (Hoek, Zanders et al. 2002). What are PUF proteins?

1.3. PUF proteins

Over the years, investigation of gene expression regulation in eukaryotes has mainly focused on the control of transcription initiation, which is the essential step for nearly all organisms. However, it has become evident that post-transcriptional control of gene expression is also of importance. Alternative splicing generates different mRNAs which then give rise to proteins with divergent functions (reviewed in (Black 2003)). Aberrant mRNAs need to be eliminated before they are translated into erroneous proteins (Wagner and Lykke-Andersen 2002; Weischenfeldt, Lykke-Andersen et al. 2005). And some mRNAs are only needed transiently, and therefore their degradation has to be tightly controlled (Wilusz and Wilusz 2004). Localization of mRNAs is another level of regulation: some mRNAs are only functional in certain parts of the cell (St Johnston 2005). In eukaryotic cells, every part of an mRNA can contain sequence elements responsible for its regulation. Some 5'UTR regulatory elements are involved in translational control (for review, see (Wilkie, Dickson et al. 2003), (Meijer and Thomas 2002)). However, the region between the termination codon and poly(A) tail- the 3' untranslated region, or 3'UTR- has emerged as preeminent (for review, see (Wickens, Bernstein et al. 2002)). Sequences in the 3'UTR are bound by *trans*-regulatory proteins, which control mRNA stability, translation and localization. PUF proteins are one family of these 3'UTR regulatory proteins. The two founding members of this family are *Drosophila* Pumilio and *C. elegans* FBF (Barker, Wang et al. 1992), (Zamore, Williamson et al. 1997), but they

have now been found in virtually all eukaryotes examined, including vertebrates, plants, yeast, protozoan, and slime molds (Wickens, Bernstein et al. 2002). In *Drosophila*, Pumilio protein is known to bind the 3'UTR of *hunchback* mRNA, leading to an increase in the rate of deadenylation and repression of translation of this mRNA (Murata and Wharton 1995; Wreden, Verrotti et al. 1997). Similarly, in *C. elegans*, FBF protein binds the 3'UTR of *fem-3* mRNA and leads to repression of translation (Zhang, Gallegos et al. 1997). Furthermore, PUF proteins in yeast and slime molds also repress expression of target mRNAs by binding to sequences in the 3'UTR (Souza, da Silva et al. 1999; Olivas and Parker 2000; Tadauchi, Matsumoto et al. 2001). Thus, the PUF family proteins are commonly 3'UTR repressors.

1.3.1. Sequence and Structural Similarity

Puf proteins are characterized by the presence of eight consecutive repeats (Puf repeats) of approximately 40 amino acids (Zamore, Williamson et al. 1997; Wharton, Sonoda et al. 1998; Tadauchi, Matsumoto et al. 2001; Wang, Zamore et al. 2001). This Puf repeat region is necessary and sufficient to bind to specific RNA sequences and to provide many of the protein's biological functions. The recently determined structures of human and *Drosophila* Pumilio reveal a striking, extended crescent (Fig.1.2.) (Edwards, Pyle et al. 2001; Wang, Zamore et al. 2001).

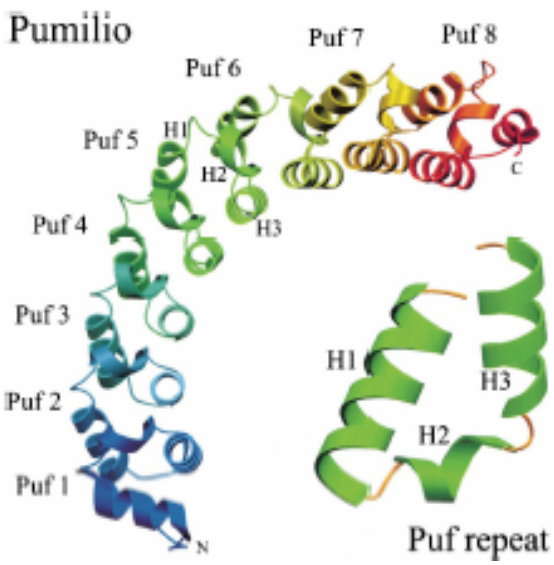


Fig.1.2. Structure of *Drosophila* Pumilio Puf domain taken from (Edwards, Pyle et al. 2001). The Puf domain contains eight tandem Puf repeats (shown in different colors), which are composed of three helices (H1, H2, and H3).

All of the individual Puf repeats form nearly identical three-helix triangles; these lie next to each other to form the elongated structure. The inner surface of the crescent carries the

conserved aromatic and charged amino acid residues that are likely to bind RNA, and the outer surface can contact other proteins. PUF proteins bind selectively to their target mRNAs. Individual PUF proteins bind to different RNA sequences and are affected differentially by base changes in a single sequence (Bernstein, Hook et al. 2005). Human and fly PUF proteins bind to mutant RNA derivatives of hunchback with different affinity, despite the two proteins being 80% identical (Zamore, Williamson et al. 1997). Nevertheless, all known PUF-binding sites posses a common tetranucleotide motif, UGUR (Murata and Wharton 1995; Zamore, Williamson et al. 1997; Zhang, Gallegos et al. 1997; Wharton, Sonoda et al. 1998; Tadauchi, Matsumoto et al. 2001). Using a yeast three-hybrid system (Bernstein, Buter et al. 2002) it was demonstrated that a single spacer nucleotide downstream of UGUR confers binding specificity of *C. elegans* FBF and PUF-8 (Opperman, Hook et al. 2005).

1.3.2. PUF interacting partners

PUF proteins do not act on their own to control mRNAs, but instead interact with other protein partners. To date, only three PUF binding partners have been found, one belonging to the Nanos family (Kraemer, Crittenden et al. 1999; Sonoda and Wharton 1999) and another being a member of the CPEB (cytoplasmic polyadenylation element binding protein) family (Luitjens, Gallegos et al. 2000). The third interaction partner *Drosophila* Brat, is a member of the NHL protein family and is recruited to *hunchback* mRNA through a ternary complex of Pumilio, Nanos and the mRNA (Sonoda and Wharton 2001). Co-immunoprecipitation approaches suggest a fourth group of PUF interacting partners: DAZ (Deleted in AZoospermia) and DAZ-like proteins were shown to interact with Human Pumilio-2 (Moore, Jaruzelska et al. 2003). These common PUF protein partners have so far been only detected in metazoans, but not in unicellular eukaryotes such as yeast, even though these contain multiple PUF proteins.

1.3.3. PUF targets

Although members of the PUF family are all found throughout the eukaryotic lineage, only few of their mRNA targets have been identified so far (Wickens, Bernstein et al. 2002). For example, in *Drosophila*, the PUMILIO protein binds to the Nanos response element (NRE) of maternal *hunchback* mRNA and represses its translation at the posterior part of the early embryo (Murata and Wharton 1995; Wreden, Verrotti et al. 1997). *Fem-3*-binding

factors (FBFs), the *C. elegans* Puf homologs, repress *fem-3* translation and thereby regulate the switch from spermatogenesis to oogenesis. FBFs are also involved in maintaining germline stem cells through binding and inhibition of *gld-1* mRNA expression (Camargo, Almeida et al. 1997; Zhang, Gallegos et al. 1997; Crittenden, Bernstein et al. 2002). Two specific mRNA targets have been described for yeast Puf proteins: Puf3p regulates turnover of *COX17* mRNA in vitro by binding to its 3'-UTR (Olivas and Parker 2000), and Puf5p interferes with HO endocluonease expression (Tadauchi, Matsumoto et al. 2001). Recently, work by (Gerber, Herschlag et al. 2004) has shown that the five Puf proteins in yeast each bind specifically to distinct and functionally related mRNAs. Puf1p and Puf2p bind preferentially to mRNAs that encode membrane-associated proteins; Puf3p selectively interacts with mRNAs encoding mitochondrial proteins; and Puf4p and Puf5p specifically bind to mRNAs encoding nuclear components (Gerber, Herschlag et al. 2004).

1.3.4. Protist Puf proteins

Members of the Puf protein family have so far been described only in a few protozoan organisms, namely *T. brucei*, *T. cruzi*, and *Plasmodium falciparum*. The first PUF protein described in *T. brucei* was *Tb*PUF1 (Hoek, Zanders et al. 2002), which is also the first member of the PUF family identified in an early branching eukaryote. *Tb*PUF1 was found in a two-hybrid screening to interact with *Tb*ESAG8. *PUF1* is expressed at equal levels in both insect and bloodstream-form parasites. Attempts to disrupt the two *PUF1* alleles in both forms of the life-stages by classical homologous recombination failed, suggesting that *PUF1* was an essential gene. However, growth analyses from conditional *PUF1* null mutant cell lines were not able to conclusively show that *PUF1* was essential. Cells that overexpressed a tagged version of PUF1 had a significant growth defect and a reduced infectivity in mice. Messenger mRNA stability assays showed that *Tb*PUF1 might regulate stability of specific expression site (ES) derived mRNAs (e.g. *ESAG8* and *VSG221*) in trypanosomes (Hoek, Zanders et al. 2002). These expression sites are found in subtelomeric regions and specifically harbor *VSG* and *ESAG* (expression site associated genes) genes that are transcribed by RNA pol I . Interestingly, the *Tb*PUF1 homologue in *T. cruzi*, *Tc*PUF6, and the two Puf proteins of *P. falciparum, Pf*Puf1 *and Pf*Puf2*,* were shown to specifically bind to the *Drosophila hunchback* NRE sequence in vitro (Cui, Fan et al. 2002; Fan, Li et al. 2004; Dallagiovanna, Perez et al. 2005).

To date, PUF proteins were shown to physically interact with members of three protein families (Nanos, CPEB, and Brat). Currently, no homologues of these PUF interactors are found in the genome of protozoa. (Hoek, Zanders et al. 2002) suggested that *Tb*PUF1 interacts with *Tb*ESAG8. However, the yeast two-hybrid assays as well co-immunoprecipitation studies demonstrating this interaction were somewhat preliminary. First, *Tb*PUF1 resides solely in the cytoplasm (Hoek, Zanders et al. 2002), whereas *Tb*ESAG8 is localized to the nucleolus (Hoek, Engstler et al. 2000). Moreover, previous yeast-two hybrid studies by (Perez-Morga and Pays 1999) suggested an interaction between *Tb*ESAG8 and *Tb*PIE8 (putative interacting with ESAG8), despite a mitochondrial localization of the latter. It is possible that *Tb*ESAG8 appears frequently as a false positive in yeast-two hybrid studies. Overall, only a few PUF binding partners are characterized and so far none have been conclusively found in protozoans.

1.4. Aims of the work described in this thesis

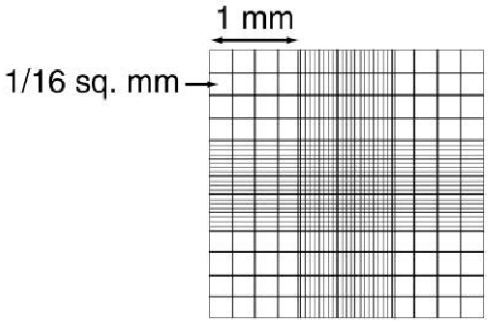
This work was started because post-transcriptional control of gene expression is crucial in Kinetoplastids, and very little is known about the regulatory proteins. The presence of a family of RNA binding proteins (Puf proteins), which are important for post-transcriptional control by modulation of mRNA stability and regulation of translation in other species suggested that they might also have this function in trypanosomes. Furthermore, *Tb*PUF1 had been suggested to be essential in *T. brucei* and to play a role in virulence. The aim of this thesis was to further investigate the function of *Tb*PUF1, and to identify other Puf proteins in *T. brucei* and to functionally characterize them. Another question to address was the identification of mRNA targets of the PUF proteins in *T. brucei*. The method of choice to identify these targets was to employ *T. brucei* microarrays. Another task was to look for PUF binding partners. Furthermore, I also took advantage of the advanced two-dimensional differential gel electrophoresis (DIGE) to study the role of PUF proteins in global protein expression, this being done towards the end of my thesis. A detailed understanding of PUF protein functions in *T. brucei* will certainly shed light on the evolutionary conservation (and hence importance) of these proteins throughout the eukaryotic lineage.

2. Materials and methods

2.1. *T. brucei* cell culture

2.1.1. Determination of cell density

The *T. brucei* culture was briefly homogenised by shaking the culture flask. 10µL 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 x 10^4, which gave the number of cells in 1mL of the culture. Dilutions were made to maintain the cultures below 7 x 10^6 and 1-2 x 10^7/mL for bloodstream and procyclics respectively.



2.1.2. Bloodstream-form trypanosome culture

Bloodstream-form (bf) trypanosomes were cultured in an incubator (Heraeus Instruments) at 37°C, 5% CO_2, in a humified atmosphere. The bottle caps were loosely tied to allowed gaseous exchange. Cells were harvested at densities up to 3 x 10^6 cells/mL* in supplemented HMI-9 medium. All culture work was done under sterile conditions in a laminar flow hood. For cryopreservation, 500µL aliquots of culture in log phase of growth (1-2 x 10^6 cells/aliquot) were added to an equal volume of 20% glycerol in HMI-9 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 5mL HMI-9 medium.

HMI-9

$^{\alpha}$IMDM (Gibco)	17.66g/L
$NaHCO_3$ (Roth)	36mM
Hypoxanthine (Serva)	1mM
Na-pyruvate (Serva)	1mM
Thymidine (Sigma)	160mM
Bathocupronsulphonate (Serva)	50 mM

Supplemented HMI-9 medium, 500mL

HMI-9 supplemented with:

$^{\delta}$Heat-inactivated FCS 10%(v/v)	
Penicillin/Streptomycin	50U/L (5mL of Penicillin-Streptomycin mix, Sigma)
L-Cysteine-HCL.H_2O	1.5mM (5mL of stock solution)
β-mercaptoethanol (Sigma)	0.14% (7.2µL in 5mL, filtered, added fresh)

$^{\alpha}$ *Iscove's Modified Dulbecco's Medium, no supplements*

$^{\delta}$ *FCS was heat-inactivated by incubating it at 56ºC 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 10^7 cells from a log phase culture (1-2 x 10^6 cells/mL) were centrifuged, suspended in 10mL 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 37ºC. 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_2HPO_4/KH_2PO_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 10^6 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 20% glycerol and at about 2 x 10^6 cells/aliquot.

MEM-Pros pH: 7.4

$CaCl_2$	265mg/L	NaH_2PO_4	0.14 g/L
KCl	0.4g/L	HEPES	7.14g/L
$MgSO_4.7H_2O$	0.2g/L	L-Arg-HCl	126mg/mL
NaC1	6.8g/L	LCys-Cys	24mg/L
L-His-HCl.H_2O	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	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 4ºC.

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 4ºC. *Cultures of procyclic trypanosomes under 1 x 10^5 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 (Beckckman). Cells were grown to log phase of culture (3-8 x 10^6cells/mL). 2 x 10^7 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 lmL cuvette (BTX) on ice. 10µg of sterile linearized plasmid DNA was added and mixed by pipetting 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 30ºC. 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_2HPO_4	8mM
KH_2PO_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 4ºC.

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 (bloodstream).
- **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. The cell line 427-1313-514 additionally expresess T7 polymerase. 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 pHD615, pHD617 (Biebinger, Wirtz et al. 1997), pHD918 (Estévez, Kemp et al. 2001), p2T7-177-Hyg (LaCount, Bruse et al. 2000), p2T7^{TA}-blue (Clayton, Esteacutevez et al. 2005), and pHD1621 (p2T7-177-Bsd, Corinna Benz).

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-HCI	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 -20ºC, the samples were centrifuged at 13,000 rpm for 15 min at 4ºC. The pellet was washed with 70% ethanol and air-dried. The pellet was then resuspended (TE/water) and incubated at 55ºC for 10min to fully dissolve the sample. Samples were then stored in NaAc/ethanol mix at -20ºC (DNA) or at -80ºC (RNA). The samples were centrifuged and washed just before use.

2.2.3. TCA precipitation

About 100µL of protein sample was diluted 1:6 in 1X PBS containing complete protease inhibitor, Roche Applied Science (total volume 600µL). One-quater volume (150µL) of 100% TCA containing deoxycholate (DOC) was added, the sample 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 H_2O

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 94ºC for 1-5 min, 20-30 cycles of denaturation, primer annealing and elongation, and 10 min of elongation at 72ºC. 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).

<u>10X *Taq*-DNA pol buffer</u>

Tris-HCl	100 mM (pH 9.0)
KCl	500mM
$MgCl_2$	15mM
Triton X-100	1% (v/v)

<u>dNTPs</u>

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.

<u>Forward and reverse primers</u>

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

2.3.3.2. Fill-in of 5'-overhangs

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

<u>dNTPs</u>

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

10X Buffer L (Amersham)

Tris-HCI	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/µL) prevents it from self-ligation and lowers background. About 2 µL of CIP were added to the restriction digest and incubated at 37ºC for 15 min. The tube was transferred to 55ºC for 15 min. The reaction was stopped by addition of 2µL of 500mM EDTA and heat inactivation (75ºC 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 µg, 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 µL each of 10X ligation buffer and T4-DNA-ligase were added and the volume made upto 10µL with Nuclease–Free water. The ligation reactions were incubated at 16ºC overnight.

<u>10X Ligation buffer</u>

Tris-HCl	0.5M (pH 7.6)
$MgC1_2$	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 4ºC.

Escherichia coli DH5α cells were taken from a frozen stock at -80ºC and incubated overnight at 37ºC on an antibiotic-free LB-agar plate. A single colony was picked and used to inoculate 5mL of ψB medium. The culture was incubated at 37°C with vigorous shaking for about 2 h (OD_{550} ~ 0.3). The cells were then transferred to 100mL of medium pre-warmed to 37°C and incubated for another 2 h (OD_{550} ~ 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 200µL/tube for storage at –80°C.

<u>DH5α Cells (Promega)</u>

Genotype: *sup*E44, ΔlacU169(Δ80lacZΔM15), *hsdR17, gyrA96, thi*-1, r*elA*l

ψB 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 4ºC.

2.4.2 Transformation of competent cells with recombinant DNA

Approximately 5µL of a ligation reaction were mixed with a 25 µL 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 42ºC for 45 sec. 200µL of ψB medium was added and the cells incubated at 37ºC for 45 minutes. An aliquot of the transformation was spread out on LB-agar plates containing appropriate antibiotic (section 2.4.3.1.).

Luria Bertani (LB) Medium

Tryptone	10g/L
Yeast extract	5g/L
NaCl (170mM)	10g/L

LB Medium + Agar (LB-Agar)

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 100μg/mL; kanamycin at 30μg/mL and chloramphenicol at 25μg/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 37ºC. 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) and p2T7^{TA}-blue.

The transformed bacteria were streaked on LB-Agar plates supplemented with 1.5mg of X-Gal (a metabolizable 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 4ºC. 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	830 mM IPTG
2 mg/mL in Dimethylformamide	2 g/10mL
Stored in the dark at -20ºC	Stored at -20ºC

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 1 min. The pellet was resuspended in alkaline lysis buffer of the Eppendorf FastPlasmid^{TM} 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 20% glycerol in LB and kept at -80ºC.

2.5.2. Maxi-preparation of plasmid DNA

All centrifugation steps were performed at 4°C.

After an overnight growth at 37ºC, 200mL of bacterial culture were centrifuged at 6,000 rpm for 10min (Sorvall, GS3 rotor). The resulting pellet was carefully re-suspended in 10mL of cold buffer P1 and transferred to an SS34-rotor centrifuge tube. 10mL 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 10mL 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 (Tip500, Qiagen) previously equilibriated with buffer QBT. The column was washed twice with 30mL of buffer QC and the DNA eluted with 15mL of buffer QF into a clean SS34-tube. The DNA was precipitated with 10.5mL of isopropanol and centrifuged at 11,000 rpm for 30min. The pellet was air-dried and re-suspended in 500µL of TE buffer and transferred into an

eppendorf tube. The maxi-prep DNA was used for a variety of downstream processes, e.g. transfections.

Buffer P1		Buffer P3	
Tris-HCI	50mM (pH7.8)	KAc	3M
EDTA	10mM (pH8.0)	Glacial HAc	(50% v/v)
RNase A	100mg/L (Sigma)	pH 4.8 (acetic acid)	

Buffer QBT		Buffer QC		Buffer QF	
NaCl	0.75M	NaCl	1M	NaCl	1.25M
MOPS	50mM	MOPS	50mM	MOPS	50mM
Ethanol	15%	Ethanol	15%	ethanol	15%
TritonX-100	0.15%	pH 7.0		pH 8.2	
pH 7.0					

2.5.4. Extraction of *T. brucei* genomic DNA

All centrifugation steps were performed at RT.

Between 3-10 x 10^7 cells were centrifugued at 2000 rpm for 10min. The cell pellet was resuspended in 150µL of Tris-EDTA-LiCl-Triton X-100 (TELT) buffer, mixed gently by inversion and incubated for 5 min. 150µL 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 500µL 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 30-100µL 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)

2.5.5. Southern blotting and hybridization conditions

Between 5-10µg of genomic DNA was subjected to a single or double restriction enzyme digest in a total volume of 100µL 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 20µL 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 tranferred into denaturing solution and gently agitated for 30min. 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 blotter^{TM} 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 60ºC with 100ng of denatured α-[^{32}P]-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.

<u>Denaturing Solution</u>

NaCl	1.5 M
NaOH	0.5M

<u>Prehybridisation Solution</u>

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

<u>Neutralizing Solution</u>

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

<u>Hybridization solution</u>

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

20X SSC

NaCl 3M

Na-Citrate 0.3M

pH 7.2

50X Denhardt's Solution

BSA 1% (w/v)

Ficoll 1% (w/v)

Polyvynilpyrrolidone 1% (w/v)

Filtered and stored at -20ºC

Southern blot wash buffer

1X SSC

0.5% SDS (w/v)

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

2.5.6. Cloning of PUF constructs

The open reading frames of all nine *TbPUF* genes were amplified by PCR using 427 pc genomic DNA and primers listed in Table 2.1. Fragments for RNAi targeting were PCR amplified using RNAi primers listed in Table 2.1.

Table 2.1. Primers

ORF, size	primers	sequence	comments
TbPUF1 Tb10.70.2800 1701bp	CZ1769 5' CZ1770 3' CZ2136 3' CZ1811 3'	GATCAAGCTTATGTCGTCGGATGAGG CGAGGATCCCTATGTTCCTTTCTTG GATCGGATCCTGTTCCTTTCTTGCTT GATCCCCGGGTGTTCCTTTCTTG	HindIII BamHI BamHI, -stop SmaI, -stop
RNAi, 389 bp	CZ2210 5' CZ2211 3'	GATCctcgagGCGCCAGAACATCTTAAAGC GATCggatccCGACTTTGCCTCCACTCTTC	XhoI BamHI
TbPUF2 Tb10.389.0940 2544 bp	CZ2280 5' CZ2281 3' CZ2282 3'	GATCaagcttATGTCTGGTTGGGACG GATCggatccCTACAGCGTTGGCATG GATCgttaacCAGCGTTGGCATGCAG	HindIII BamHI HpaI, -stop
RNAi, 501bp	CZ2283 5' CZ2284 3'	CGAGCTAAAGGATTGCCTTG TCCTGCATCATAAGCACGAG	
TbPUF3 Tb10.100.0190 1758 bp	CZ2130 5' CZ2131 3' CZ2132 3' CZ2274 3'	GATCAAGCTTATGTGTTCCAGTTCCC GATCAGATCTTCAGCCGGAGAGCGGT GATCAGATCTGCCGGAGAGCGGTTGG GATCGTTAACGCCGGAGAGCGGTTGG	HindIII BglII BglII, -stop HpaI, -stop
RNAi, 555bp	CZ2206 5' CZ2207 3'	GATCGTCGACCGGACGATAATGAGCGAAATTG GATCAGATCTTGCAATCGTGTCAATGGTTTTG	SalI BglII

TbPUF4 Tb927.6.820 2967 bp RNAi, 459bp	CZ2133 5' CZ2134 3' CZ2135 3' CZ2258 3' CZ2208 5' CZ2209 3'	GATCAAGCTTATGGAGGCCAGTGCCGAGGTG GATCGGATCCTCATCCCTTCCTGCCGCGTTG GATCGGATCCTCCCTTCCTGCCGCGTTGCG GATCGTTAACTCCCTTCCTGCCGCGT GATCCTCGAGGCATCTGCAACGAACTCAAAAAGC GATCGGATCCAGCACATCAAGCATCGTCTGCAC	HindIII BamHI BamHI, -stop HpaI, -stop XhoI BamHI
TbPUF5 Tb927.7.4730 1293 bp RNAi, 436bp	CZ2259 5' CZ2260 3' CZ2261 3' CZ2529 3' CZ2262 5' CZ2263 3'	GATCAAGCTTATGCTTCGTAGGGGTG GATCGGATCCTCACTCACCGACTGCC GATCGTTAACCTCACCGACTGCCCCG GATCGGATCCCTCACCGACTGCCCCGG CTTGCTGTGAGTTCGCCATA TGACGGGATCACACACTGTT	HindIII BamHI HpaI, -stop BamHI, -stop
TbPUF6 Tb10.26.0140 2532 bp RNAi, 479bp	CZ2275 5' CZ2276 3' CZ2277 3' CZ2278 5' CZ2279 3'	GATCAAGCTTATGAGTTCAACCAAAG GATCGGATCCTCACTCGGCATCGAAG GATCGTTAACCTCGGCATCGAAGTGC TTATTCAGCGTGCAGTGGAG AAAAATGGCTTCCTCCTGGT	HindIII BamHI HpaI, -stop
TbPUF7 Tb11.01.6600 2115 bf RNAi, 583bp	CZ2285 5' CZ2286 3' CZ2287 3' CZ2288 5' CZ2289 3'	GATCAAGCTTATGCCAAAAATGCGTTTAG GATCTGATCATCATTCGGCCGTTTTG GATCGTTAACTTCGGCCGTTTTGAAAG GACCCTGTTTCGTCACCTGT TCATAAGATGCTTGCGTTGC	HindIII BclI HpaI, -stop
TbPUF8 Tb927.3.2470 1815 bp RNAi, 303bp	CZ2248 5' CZ2249 3' CZ2250 3' CZ2251 5' CZ2252 3'	GATCAAGCTTATGGGTAAAACTAACAC GATCGGATCCTTATTTCTTGGGGAGA GATCGTTAACTTTCTTGGGGAGAACC TCAACAGTCCCTTTGGACATC TTGCAATGAGACCCACGTAA	HindIII BamHI HpaI, -stop
TbPUF9 Tb927.1.2600 2004 bp RNAi, 371bp	CZ1857 5' CZ1858 3' CZ1889 5' CZ2137 3' CZ1886 3' CZ1920 3' CZ2037 5' CZ2038 3'	GATCAAGCTTATGGAAGTACGCGATG GATCGGATCCCTAACATTCTCCGTCA GATCGAGCTCATGGAAGTACGCGATG GATCAGATCTACATTCTCCGTCATCA GATCCTCGAGGTTAACACATTCTCCGTCATCA GAAGATCTGGCGTAGTCTGGGACGTCGTATGGG TAACATTCTCCGTCATC GATCGTCGACGGATGCCGCTTTAGTGG GATCAGATCTCAAGGCAACATGGGCGA	HindIII BamHI SacI BglII, -stop XhoI, HpaI, -stop BglII, -stop, +HA-tag SalI BglII

ORF-PCR products were cloned into pHD615 (for expression in pc cells, (Biebinger, Wirtz et al. 1997)), pHD617 (bf, (Biebinger, Wirtz et al. 1997)), and into pHD918 (for TAP-tagging, (Estévez, Kemp et al. 2001)). RNAi-fragments were cloned either into p2T7-177-Hyg (LaCount, Bruse et al. 2000), p2T7^{TA}-blue (Clayton, Esteacutevez et al. 2005), or pHD1621 (Corinna Benz). Following plasmids were generated (Table 2.2.):

Table 2.2. Plasmids

pHD1377	pHD615 + PUF1-cds @ HindIII/BamHI
pHD1378	pHD617 + PUF1-cds @ HindIII/BamHI
pHD1396	pHD918 + PUF1-cds (w/o stop) @ HindIII/(SmaI/HpaI)
pHD1538	p2T7-177 + PUF1-RNAi @ XhoI/BamHI
pHD1564	pHD615 + PUF2-cds @ HindIII/BamHI
pHD1565	pHD617 + PUF2-cds @ HindIII/BamHI
pHD1548	p2T7^{TA}-blue + PUF2-RNAi @TA-overhang
pHD1490	pHD615 + PUF3-cds @ HindIII/(BglII/BamHI)
pHD1491	pHD617 + PUF3-cds @ HindIII/(BglII/BamHI)
pHD1539	p2T7-177 + PUF3-RNAi @ (SalI/XhoI)/(BglII/BamHI)
pHD1650	pHD1621 + PUF3-RNAi @ (SalI/XhoI)/(BglII/BamHI)
pHD1492	pHD615 + PUF4-cds @ HindIII/BamHI
pHD1493	pHD617 + PUF4-cds @ HindIII/BamHI
pHD1540	p2T7-177 + PUF4-RNAi @ XhoI/BamHI
pHD1651	pHD1621 + PUF4-RNAi @ XhoI/BamHI
pHD1556	pHD615 + PUF5-cds @ HindIII/BamHI
pHD1557	pHD617 + PUF5-cds @ HindIII/BamHI
pHD1699	pHD1484 + PUF5-cds (w/o stop) @ HindIII/BamHI
pHD1652	pHD1621 + PUF5-RNAi @ XhoI/BamHI
pHD1702	pHD918 + PUF5-cds (w/o stop) @ HindIII/HpaI
pHD1544	p2T7^{TA}-blue + PUF5-RNAi @TA-overhang
pHD1562	pHD615 + PUF6-cds @ HindIII/BamHI
pHD1563	pHD617 + PUF6-cds @ HindIII/BamHI
pHD1547	p2T7^{TA}-blue + PUF6-RNAi @TA-overhang
pHD1653	pHD1621 + PUF6-RNAi @ XhoI/BamHI
pHD1554	pHD615 + PUF7-cds @ HindIII/(BclI/BamHI)
pHD1555	pHD617 + PUF7-cds @ HindIII/(BclI/BamHI)
pHD1543	p2T7^{TA}-blue + PUF7-RNAi @TA-overhang
pHD1654	pHD1621 + PUF7-RNAi @ XhoI/BamHI
pHD1552	pHD615 + PUF8-cds @ HindIII/BamHI
pHD1553	pHD617 + PUF8-cds @ HindIII/BamHI
pHD1542	p2T7^{TA}-blue + PUF8-RNAi @TA-overhang
pHD1411	pHD615 + PUF9-cds @ HindIII/BamHI

pHD1412	pHD617 + PUF9-cds @ HindIII/BamHI
pHD1456	pHD918 + PUF9-cds (w/o stop) @ HindIII/HpaI
pHD1489	p2T7-177 + PUF9-RNAi @ (SalI/XhoI)/(BglII/BamHI)

2.5.7. Knock-out constructs for *TbPUF1* and *TbPUF9*

A homozygous disruption of *TbPUF1* was performed in procyclic *T. brucei* strain 427. The knockout plasmids gim5::NEO and gim5::BSD were taken from (Maier, Lorenz et al. 2001) and modified as shown in Fig.2.1. *T. brucei* genomic DNA was used as a template to generate by PCR two DNA fragments: one spanning 200 bp (5'-UTR) upstream and 100 bp downstream of the ATG start codon (300 bp in total) of *Tb*PUF1. The primers used in this PCR amplification were CZ2369 and CZ2370 (see Table 2.3.). The second fragment was spanning the last 100 bp of ORF up 200 bp of the 3'-UTR (300 bp in total). The primers used for this PCR amplification were CZ2371 and CZ2372. These fragments were sequentially ligated into plasmids gim5::NEO and gim5::BSD (Maier, Lorenz et al. 2001) to flank the neomycin (neo) and blasticidin (bsd) marker cassettes, respectively (Fig.2.1.). These flanking homology regions allowed a locus specific recombination between wild-type chromosomal DNA containing the *TbPUF1* gene and linear DNA fragment containing the resistance markers. Procyclic form 427 cells were sequentially transfected with SacI/ApaI-linearised pHD1639 (PUF1-Bsd) and pHD1641 (PUF1-neo) plasmids and selected for double resistance to neomycin and blasticidin. The very same strategy was used to construct PUF2-knockout plasmids (pHD1640 and pHD1642).

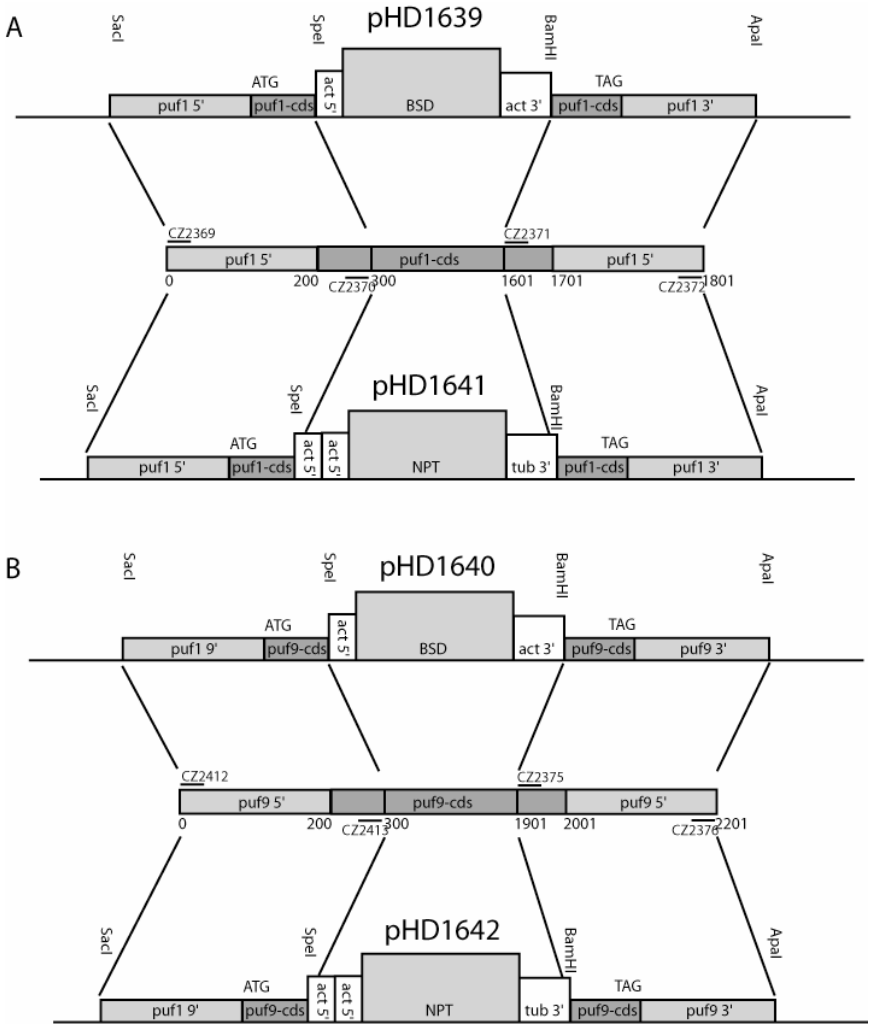


Fig.2.1. Constructs for deleting *TbPUF1* and *TbPUF9*, respectively.

Table 2.3. Primers for *Tb*PUF1 and *Tb*PUF9 knockout constructs:

CZ2369 5’	GATCGAGCTCAGAAGAGAAACGGCTC	SacI
CZ2370 3’	GATCACTAGTGTTCAGCAGTTCTTATC	SpeI
CZ2371 5’	GATCGGATCCACAGCAGTTTCCAATG	BamHI
CZ2372 3’	GATCGGGCCCAACAGTTTTTCTCTAAC	ApaI
CZ2412 5’	GTCAGAGCTCGGGTAGAAGTAAAGG	SacI
CZ2413 3’	CTAGGCTAGCAAGAGGGAATGGCCC	NheI
CZ2375 5’	GATCGGATCCAAATAGGCGGCAGAGC	BamHI
CZ2376 3’	GATCGGGCCCAAAAGTACATAAGTAC	ApaI

2.6. Isolation and analysis of RNA

2.6.1. Extraction of *T. brucei* total RNA

Total RNA was isolated from 2-4x10^7 cells from cultures with densities between 1-2 x 10^6 cells/mL (bloodstream form) or 3-4 x 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 TriFast™ (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 pipetted 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). Prehybridization was done for 2 hours at 65°C in Northern blot Prehybridisation Buffer. Hybridisation was done overnight at 42°C with 100ng of α-[^{32}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.

10X MOPS buffer		RNA gel		RNA loading Mix	
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

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

Wash Buffer I	Wash Buffer II	Wash Buffer III
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 intructions. Briefly, the DNA fragment to be labled 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- [^{32}P]-dCTP (30 µCi)
11µL Sterile distilled water

The contents were mixed gently by pipetting up and down and span 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 manufaturer's instructions.

2.7. Polyclonal antibodies

2.7.1. Peptide antibodies to *Tb*PUF1 and *Tb*PUF9

Generation of peptides and immunisation of rabbits were done by Eurogentec (Brussels, Belgium). To analyse *Tb*PUF1 and *Tb*PUF9 protein expression peptide-antibodies to a 16 amino acid region of *Tb*PUF1 and *Tb*PUF9 were generated. The two peptide sequences were selected with the help of the PROTEAN program, included in the 'Lasergene' program package (DNASTAR Inc). PROTEAN is a computer algorithm that can be used to predict the topological features of a protein directly from its primary amino acid sequence. The computer program generates values for surface accessibility parameters and combines these values with those obtained for regional backbone flexibility and predicted secondary structure. The output of this algorithm, the antigenic index, is used to create a linear surface contour profile of the protein (Jameson and Wolf 1988).

The C-terminal peptide sequences 'RELARKNGNQKNKKRW' (aa-residue 445-460 of *Tb*PUF1) and 'RQQNRRQSHSQPRRQP' (aa-residue 633-638 of *Tb*PUF9) showed the highest antigenic index. 20-30mg of each peptide were synthesized and 5mg were coupled to a carrier protein (Hemocyanin, BSA, OVA, THY). Two rabbits were immunized (500 ng each) with each peptide followed by three individual boosters (again 500 ng each) at day 14, 28, and 56. Peptide synthesis, KLH coupling and immunization of the rabbits, were done by Eurogentec (Belgium). Serum was taken at day 0 (preimmune, PPI), day 38 (small bleeding), day 66 (large bleeding), and day 87 (final bleeding). The specificity and titer of the antiserum were tested by Western blotting.

2.7.3. Affinity chromatographic purification of antibodies

The immuno-affinity matrix Affi-Gel 10 (BioRad 153-6064) was thawed in an ice water bath for 30 minutes. Meanwhile, 2mg of peptide was reconstituted in coupling buffer). 0.5mL of resin was placed in a 15mL falcon tube and the volume made to 10mL with water. The tube was inverted several times and centrifuged at 2000rpm for 1 min. The supernatant was aspirated and the wash repeated once more with water and then twice with coupling buffer. The supernatant was aspirated and the resin incubated with antigen

solution overnight at 4°C in a gentle tumbler. The resin was then centrifuged at 2000rpm for 1 min, the supernatant aspirated. The resin was washed again with 10mL of blocking buffer, centrifuged as before and resuspended in 2mL of blocking buffer. This was incubated for 1h at 4°C on a tumbler. The resin was centrifuged again and washed twice with PBS, twice with elution buffer and twice with 100mM Tris-HCl (pH 8). The resin was resuspended in 1mL of 100mM Tris-HCl (pH 8), transferred to a 10mL disposable column, washed with another 1mL of Tris.HCl and transferred to the column and washed with 10mL of Tris-HCl (pH 8). It was then washed with 10mL of PBS-500 and then with 10mL PBS. The serum was passed through the column; the collected flow-through was again passed through the same column and finally the resultant flow-through was kept frozen. The column was washed with 10mL of PBS and then with 10mL of PBS-250 and finally with 10mL of PBS-500. The antibody was eluted with 5X 0.5mL of elution buffer into a tube that already contained 90µL of 1M Tris-HCl (pH 9.5) and mixed well. The final pH was checked with a pH paper indicator and if necessary, adjusted to pH 7-8. An equal volume of cold, sterile glycerol was added, mixed well and the antibodies stored in aliqouts at –20°C.

Coupling Buffer

The coupling buffer should not contain primary amino groups e.g. Tris, glycine. The following buffers are recommended at 50mM: HEPES, MOPS, MES, acetate, bicarbonate, borate/borax. For the work reported in this thesis bicarbonate buffer was used.

AffiGels

AffiGel-10 couples proteins best at pH near or below the isoelectric point of the antigen.
AffiGel-15 couples proteins near or above their isoelectric point. It is best used for acidic proteins or peptides but can be used for basic antigens carrying out the coupling in very basic buffers, like borate-borax, at a pH 0.5units higher than the isoelectric point.

Blocking buffer

Ethanolamine-NaOH 100mM (pH 8.0)

Elution Buffer

Glycine 0.1M (pH 2.5)

PBS-250

PBS containing 250mM NaCl

PBS-500

PBS containing 500mM NaCl

2.8. Isolation and analysis of *T. brucei* proteins

2.8.1. Extraction of total protein

All centrifugation steps were done at 4ºC. 2 x 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 10-15µL 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.

2.8.2. 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 µg/mL), the more protein present, the more Coomassie binds. Protein standards containing a range of 0 to 25µg BSA were prepared from a stock of 1µg/µL to a standard volume of 1 mL (see Table 2.4).

Table 2.4. Preparation of a standard curve for protein determination

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

The Bradford reaction was initiated by adding 800µL, from the dilution series above, to 200µL 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.

2.8.3. 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 electrophresis chamber and filled up with 1 X running buffer. Trypanosome samples ($2x10^6$ cells/lane) were loaded, the chamber connected to a power supply and proteins fractionated at 150V for about 45 min.

12% Separating gel (15mL)		4% Stacking gel (5mL)	
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
H_2O	5.25mL	H_2O	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	

2.8.4. Western blotting

All incubations and washes were at RT and with gentle agitation. Cell pellets of about $2x10^6$ 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 4^0C 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 50^0C and washing for 10 min in excess PBS-T.

Blotting buffer		PBS-T buffer pH 7.6	
Tris	25mM	Na_2HPO_4	10mM
Glycine	192mM	KH_2PO_4	1.8mM
Methanol	20%	NaCl	140mM
		KCl	2.7mM
		Tween-20 (Serva)	0.3%

Blocking solution

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

2.8.5. 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)

2.8.9. Preparation of dialysis tubes

Dialysis tubes (MWCO 12000-14000) were cooked 10 min in 2% $NaHCO_3$/1mM EDTA pH 8,0 and rinsed with dH_20, and cooked 10 min in 1mM EDTA. Storage was in the same solution at 4°C and rinsed with H_2O before use.

2.8.9. In vivo labeling with [^{35}S]-Methionine

All centrifugation steps were at RT. $2x10^7$ 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 [^{35}S]-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% β-mercaptoethanol (only for bloodstream), and 10% heat-inactivated FCS (previously dialysed against 30mM HEPES pH 7.3 /150mM NaCl).

2.8.10. Immunoprecipitation

All centrifugation steps were at 4°C, 5 min at 13'000 rpm; incubations were also at 4°C. Each [^{35}S]-Methionine-labeled sample, kept at –80°C as pellets, was solubilized with 750µl solubilizing 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 Lämmli 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.

solubilizing buffer (10ml)
50mM Tris-HCl pH 7.4
500mM NaCl
2% IGEPAL CA-630 (Sigma)
1 tablet complete protease inhibitor (Roche)

buffer A	buffer B	buffer C
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.

2.9. Isolation of polysomes and polysomal RNA

Isolation of polysome were performed following a protocol adapted from (Djikeng, Shi et al. 2003).

2.9.1. Preparation of cytoplasmic extracts for polysomes

Materials required:
- Centrifuge tubes (SCI, 14x89mm Kat.-Nr.7030, Beckman)
- Gradient maker (Gradient Master, Biocomp)
- Leupeptin, 10mg/ml (Invitrogen)
- Cycloheximide, 100mg/ml (dissolved in 100% EtOH)
- Proteinase K, 20mg/ml

All steps at 4°C. Cycloheximide was added (100µg/ml) to exponentially growing cells to freeze translating ribosomes on the mRNA. The drug was present in all buffers throughout the entire procedure. $5x10^8$ – $2x10^9$ cells were collected by centrifugation at 2000 x g for 5 min and washed twice with ice-cold polysome buffer. Cells were resuspended in 0.5ml of polysome buffer and lysed by the addition of NP-40 (final 0.2%). The cell suspension was homogenized and the lysate was cleared by centrifugation at 10'000 x g for 4 min.

2.9.2. Sucrose density gradient

Gradients were prepared before cell extract preparation. Cleared lysate from $5x10^8$ cells were layered onto 15%-50% sucrose gradients prepared in polysome buffer and centrifuged at 4°C for 2 hrs at 36'000 rpm in a Beckman SW41 rotor. Then 1-ml fractions were collected using the ISCO gradient fractionation system. The OD_{254} profile was recorded using the ISCO UA-6 detector. For protein sample preparations, the eluates were precipitated with 1ml of 20% TCA and protein pellets washed three times with acetone. For RNA extraction, each fraction was precipitated with 1ml of isopropanol, and the material was collected by centrifugation. Pellets were resuspended in 0.3ml of a solution containing 10mM Tris-HCl (pH 7.5), 1mM EDTA, 100µg/ml proteinase-K, and 1% SDS and incubated at 65°C for 30 min. The sample was precipitated with 1 volume of isopropanol after addition of 20µg of glycogen and NaCl to 600mM. Samples were processed for Northern blot analysis as described in 2.6.2.

2.10. Indirect immunofluorescence assay (IFA)

All steps were done at RT; washes were for 5 min, with gentle agitation. About $1x10^6$ 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 4^0C 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 permeabilized 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, permeabilized cells were then incubated 1 hr 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 Deconvulsion 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 Fotoshop software (version 7, Adobe Systems Incorporated).

2.11. Tandem Affinity Purification (TAP)

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

2.11.1. 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 $5x10^9$ 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).

2.11.2. 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 tranferred into the column containing the washed beads and rotated for 2hrs at 4°C. Elution is done by gravity flow. 25µl of flowthrough (IgG flowthrough, #2) is taken for subsequent analysis. The beads are washed three tiems 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 $CaCl_2$, 0.1% IGEPAL). Three mililiters of IPP150 calmodulin binding buffer and 3 µl of 1M $CaCl_2$ 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 calmoduling 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 $CaCl_2$, 0.1% IGEPAL, 2mM EGTA). The eluate is concentrated by TCA precipitation.

2.11.3. 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, $1x10^9$ 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 RNaseIn and 5µl Vanadyl Ribonucleoside complexes. 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 precitpitation. 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.

2.12. Tethered-functional analysis

The tethered-functional analysis is based on RNA-protein interaction. The binding of the RNA-binding domain of the λ phage antiterminator protein N (referred to here as λN) to a specific λ-N binding site (boxB) in an mRNA containing this boxB was used in this study (Fig.2.2.). An asset of this approach is that analysis of function is independent of knowledge of the natural RNA target. To this end, a chimeric protein consisting of λN linked to TbPUF1 (or TbPUF9) was expressed in *T. brucei* procyclic cells; the same strain also carried a second plasmid expressing the CAT reporter mRNA with six λN recognition sites in its 3'-UTR.

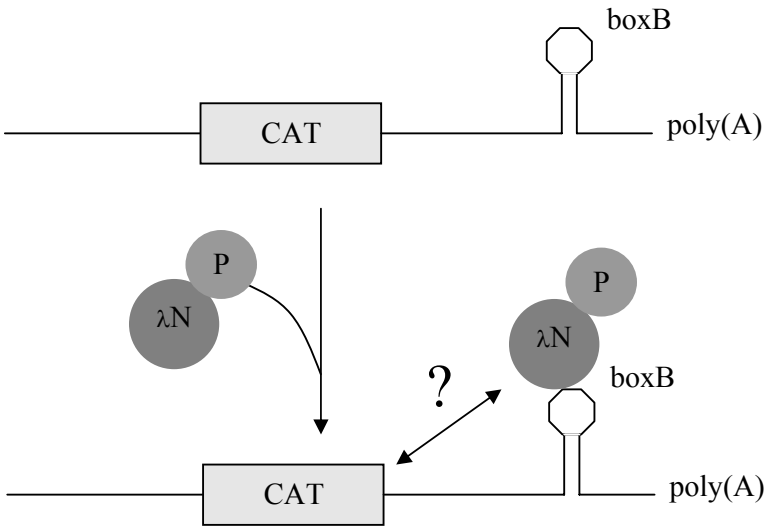


Fig.2.2. Tethered function assays using the 3'-UTR. PUF (P) is brought to the CAT reporter mRNA through binding of λN to the boxB in the 3'-UTR. The function of the tethered PUF protein in any aspect of the mRNA's metabolism or function can be assayed.

2.13. Microarray

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

2.13.1. 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 microtiter plates using primers d(TTGTAAAACGACGGCCAGTG) 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, Grahlmann et al. 2001). 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).

2.13.2. Sample preparation, labeling and hybridisation

Total RNA from $4x10^8$ exponentially growing cells (below $1x10^6$ cells/ml for bloodstream forms and $2x10^6$ cells/ml for procyclics) was isolated using Qiagen RNeasy Midi Kit. 15µg of total RNA was reverse transcribed using SuperScript™ III (Invitrogen). The following protocol for a 40-µl reaction volume was used:

1. Add the following components to a nuclease-free microcentrifuge tube:
   - 5µl of oligo$(dT)_{12-18}$ (100ng/µl) (500ng)
   - 15µg of total RNA
   - 3µl dAGT-mix (10mM)
   - 2µl dCTP (1mM)
   - 2µl Cy3/Cy5-dCTP (1mM)

   RNase free water to 27µl
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:
   - 8µl 5x First-Strand Buffer
   - 2µl 0.1 M DTT
   - 1µl RNAseIn™ (Promega)
   - 2µl SuperScript™ III RT (200 units/µl)
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 1µl 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 dH_20, and 3 min in 95°C dH_20.
15. Place slide into Atlas Glass Hybridization chamber (DNA-Array side up!) and fill with 2ml Prehybridization Buffer (5x SSC, 0.1% SDS, 1% BSA)
16. Incubate for 45 min at 55°C.
17. Wash the slide by dipping 3x in dH_20 at RT.
18. Dip the slide in Isopropanol (abs.) and dry. Slides should be used immediately after prehybrization.
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). Dip the slide in Isopropanol (abs.) and dry.

2.13.3. 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. 2001) was used for data quality assessment and normalisation. Differentially regulated transcripts were confirmed by Northern blot analyses.

2.14. Ettan DIGE system (Amersham Biosciences)

The DIGE work was performed under the supervision of Richard Burchmore at the Sir Henry Wellcome Functional Genomics Facility in the Institute of Biomedical and Life Sciences (IBLS) at the University of Glasgow, Scotland.

Ettan™ DIGE system is based on the technique of two-dimensional difference gel electrophoreis (2-D DIGE). In this approach, protein samples are labeled with up to three spectrally distinct, mass and charge matched, fluorescent dyes (Cy2, Cy3, and Cy5). Labeled proteins are then mixed and resolved simultaneously on the same 2-D gel (Fig.2.3.). Sample multiplexing greatly defines the detection of changes between samples at the protein level. Spot maps can be overlaid and compared directly for samples resolved on the same 2-D gel. In addition, variation in spot intensities due to experimental factors will be the same for each sample on the 2-D gel. Thus the relative concentrations of the samples in a gel will be effectively unchanged. This increases the confidence with which protein differences (due to changes in protein expression) can be both detected and quantified using Ettan DIGE system. Following protocol is optimized for *T. brucei* sample preparation. For a more detailed description of the system refer to Ettan DIGE System User Manual 18-1173-17 Edition AA.

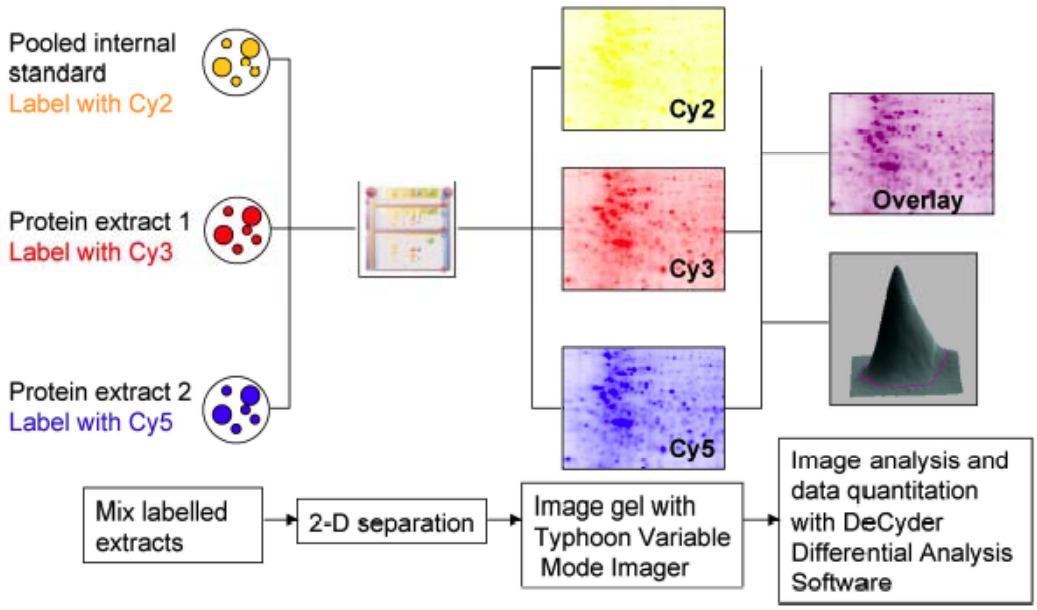


Fig.2.3. Outline of Ettan DIGE system. Work in this thesis was performed using only two labels (Cy3 and Cy5). Figure is taken from Ettan DIGE System User Manual 18-1173-17 Edition AA.

2.14.1. Protein sample preparation for DIGE

For each sample ~$2x10^8$ cells were collected at 2000 rpm at 4°C for 10 min and washed twice with cold PBS. Cell pellet was lysed in 500ml DIGE lysis buffer (7M urea, 2M thiourea, 4% CHAPS, 25mM Tris-base pH 8.5) and cell lysate was cleared twice at 13'000rpm at 4°C for 10 min. The protein concentration of the supernatant was measured using BRADFORD assay (2.8.2.). One volume of ice-cold 20% TCA is added and incubation was done on ice for 30 min (or overnight). Protein was pelleted at 13'000 rpm for 10 min 4°C. The supernatant was discarded and pellet washed three times with cold acetone. After drying for 10 min at RT the pellet was dissolved in DIGE lysis buffer at protein concentration >5mg/ml. Adjust pH to 8.5. Note, protein pellet is somewhat difficult to dissolve. Vortexing, grinding and passing through a 21-25 gauge needle might be necessary. Protein sample was adjusted to a concentration of 5mg/ml and aliquoted in 10µl.

2.14.2. CyDye labeling of protein samples

On ice, 400 pmol CyDye was added to the 10ml, mixed immediately by pipette strokes and incubated on ice for 30 min in the dark. One microliter of 10mM lysine was added to stop the reaction. Labeled samples can be stored at –80°C until use. The Cy3 and Cy5-labeled proteins samples were mixed and 40µl of each unlabelled protein sample (5mg/ml) was added to give a final volume of 102µl.

2.14.3. Running the first dimension

370µl of rehydration buffer was added to the 102µl and mixed by a brief vortex. The Immobiline DryStrips were rehydrated in the Immobiline DryStrip Reswelling Tray in the presence of the 450µl protein sample. The protein samples were then focused on the Ettan IPGphor IEF unit with following settings:

Immobiline DryStrip		Rehydration loading			
Length	pH range	Step and voltage mode	Voltage (V)	Step duration (h:min)	Volt-hours (kVh)
24 cm	3-10 NL	1 Step and Hold	500	1:00	0.5
		2 Step and Hold	1 000	1:00	1.0
		3 Step and Hold	8 000	8:20	62.5
		Total		10:20	64

2.14.4. Equilibration and running second dimension

After isoelectrifocusing the IPG strips were placed in individual tubes and equilibrated 15 min in 10ml SDS equilibration solution supplemented with 100mg DTT and then 15 min in 10ml of the same buffer without DTT but with 250mg iodacetamide with gentle agitation. The strip was placed on to a 12.5% SDS gel and run until the bromphenolblue has left the bottom.

2.14.5. Scanning and Image acquisition

The gel was scanned subsequently with the Cy3- and Cy5-channel at 100 microns resolution using a Typhoon Imaging scanner 9400 (Amersham Biosciences). Representative spots were picked for normalisation over the two channels using Typhoon Scanner Control Software, version 3.0 and ImageQuant Tools software, version 3.0.

2.14.6. DeCyder Differential Analysis Software

DeCyder Differential Analysis Software is a fully automated image analysis software suite for detection, quantitiation, positional matching and differential protein abundance analysis. A description of the algorithms and workflow would go beyond the scope of this thesis. The reader is referred to the DeCyder Differential Analysis User Manual 18-1173-16 Edition AA.

3. Results

The experimental goal of this thesis was to look for PUF proteins in *Trypanosoma brucei*, to characterize them, and to analyze their role in global transcript modulation and translational control. This was done using a combination of transcriptomics (microarray analysis) and proteomics (two-dimensional gel electrophoresis) approaches. Furthermore, attempts to look for PUF interaction partners were undertaken.

3.1. *T. brucei* has ten members of the PUF protein family

Since most organisms in which PUF proteins have been characterized have multiple PUF members, I tried to determine whether this was also true for *T. brucei*. With the recent completion of the *T. brucei* genome (Berriman, Ghedin et al. 2005) and the help of database mining the presence of nine additional PUF proteins in *T. brucei* became apparent (Fig.3.1.). All ten *T. brucei* PUF proteins bear at least four of the eight characteristic Puf repeats. Some PUF repeat sequences are somewhat divergent from the consensus, for example in *Tb*PUF5, and are therefore not recognised by Pfam model (Pfam accession number PF00806). The high number of PUF protein family members in *T. brucei* is consistent with the observation that more primitive organisms, including *C. elegans* and *S. cerevisiae* possess multiple PUF proteins, whereas only one PUF protein (with two isoforms) is present in *Drosophila*. Vertebrates also appear to have a smaller number of PUF proteins - humans and mice each have two (Barker, Wang et al. 1992; Zamore, Williamson et al. 1997; Zhang, Gallegos et al. 1997; Wickens, Bernstein et al. 2002).

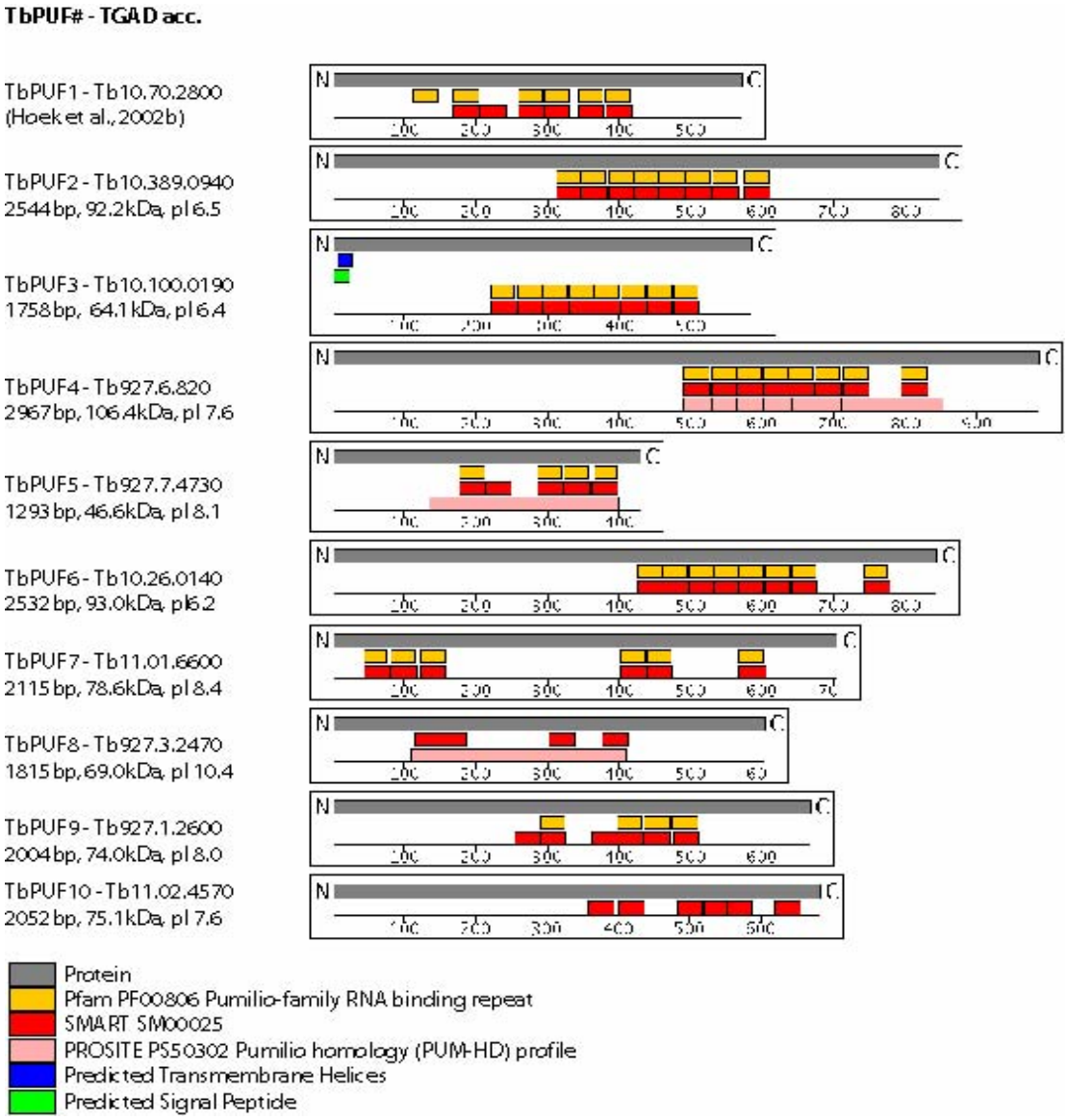


Fig.3.1. Schematic representation of the ten *PUF* genes in *Trypanosoma brucei*. Orange boxes show Pfam PF00806 Pumilio-family RNA binding repeats. Pfam model does not necessarily recognize all eight repeats in all sequences; some sequences appear to have only 4 or 5 repeats on initial analysis, but further analysis suggests the presence of additional divergent repeats. Puf repeats together form the Pumilio homology domain (PUM-HD), which is predominantly located in the middle or at the C-terminus of the protein. The N-terminus of the *Tb*PUF proteins is more divergent. Diagrams are taken from the homepage www.genedb.org/genedb/tryp, and *Tb*PUFs are identified by their TIGR Genome Annotation Database (TGAD) number.

In overall organization, as is true for other PUF proteins, the Puf repeats of *Tb*PUFs are located in the middle or near the C-terminus of the protein; typically the N-terminal regions are more divergent. The smaller PUF proteins in *T. brucei* (e.g. *Tb*PUF5) appear to consist of only the PUF domain. Since this domain is necessary but also sufficient to exert

the proteins functions, it is expected, that *T. brucei* PUF proteins are indeed functional. Except for the conserved PUF domains, the *Tb*PUF proteins differ considerably. For example *Tb*PUF4 is a relatively large protein with 988 amino acids and a predicted molecular weight of 106.4 kDa, whereas *Tb*PUF5 has only 430 residues and a predicted molecular mass of 46.6 kDa. The calculated pI of *Tb*PUFs range from pH 6.1 (*Tb*PUF1) to basic pH 10.4. (*Tb*PUF8). With the exception of a predicted transmembrane helix in *Tb*PUF3 and a putative mitochondrial localization signal in *Tb*PUF5 none of the *Tb*PUFs bears a targeting signal.

The *TbPUF* genes are located on chromosome 1, 3, 6, 7 and 10. Interestingly, the genes share high conservation of synteny between the *T. brucei*, *T. cruzi*, and *L. major* (referred to here as the "Tritryps"). For example the downstream gene of *PUF9* in all three pathogens codes for an exosome associated protein EAP1, which is an Rrp42 homologue similar to RNasePH proteins in bacteria. The upstream gene of *PUF7* in the Tritryps genome codes for a zinc finger protein 2. *TbPUF1* is a single copy-gene (Hoek, Zanders et al. 2002). To investigate the copy number of *TbPUF9* Southern blot analysis was performed. This analysis indicated that *TPUF9* is also a single copy gene (Fig.3.2.). It should be noted, that the strains used in our laboratory are different from the strain sequenced in the course of the genome project. Southern blot analyses have not been done for the other *TbPUF* genes.

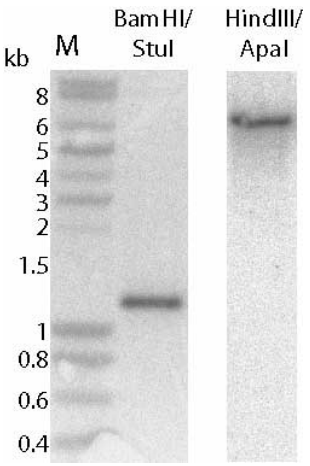


Fig.3.2. *TbPUF9* is a single copy gene. Southern blot analysis with 10µg of genomic DNA from *T. brucei* 427 pc cut with indicated restriction enzymes and hybridzed with a ^{32}P labeled probe recognizing *TbPUF9*.

PUF proteins contain eight consecutive repeats of ~40 amino acids, called Puf repeats. Each Puf repeat contains a diagnostic central consensus sequence (Zamore, Williamson et al. 1997; Zhang, Gallegos et al. 1997). When the eight Puf repeats of the ten *Tb*PUF

proteins were aligned with those of the *Drosophila* Pumilio protein, the highest conservation can be seen in regions corresponding to the core consensus sequences (Barker, Wang et al. 1992; Macdonald 1992) (Fig.3.3.). The flanking regions around the trypanosome Puf repeats are also shorter compared with other family members. The RNA-binding domain of *Tb*PUF6 is most similar to that of Pumilio (45% identity) whereas *Tb*PUF8 Puf-domain only shares 24% identity with that of Pumilio. The conserved aromatic and basic amino acids in the core consensus confer base stacking and backbone interactions with RNA (Edwards, Pyle et al. 2001; Wang, Zamore et al. 2001; Wang, McLachlan et al. 2002; Edwards, Wilkinson et al. 2003). The conservation of the repeats of the Puf domain suggests that *Tb*PUFs share a similar 3-dimensional structure and possess similar RNA binding activity. On the other hand the divergence between the Puf domains within *Tb*PUFs may imply that they bind related but distinct mRNA targets and have different roles during parasite development.

```
                                   Repeat 1                                Repeat 2

PUF1                      NGLREAIRYVDALKALKECTSPDEVNRKKHLSRNTFVHAMIAR..S.GELMVDANGSPLIQH.ALGMLKSGGKVAPVLYSTDYIAA
PUF2                      ~~~~~~~~~~EFRLQVVSLCKD......QDGSR..CVQRLLN.....NPENIEPIFNPVFPR.THELIID...VFGNYVLQKLLDM
PUF3                      ~~~~~~~~~~NLRGNVYELAKD......QHGCR..FLQRLLCDPEA.DCEIPRTIMSEIVPH.VAELMTD...QYANFLVQKLFDI
PUF4                      ~~~~~~~~~~LHGGRIVKLAAD......QQGCR..MLQSVLERFPF.HSSEVQKVISELLPV.LTDVMKD...PYGNFLVQKLLEV
PUF5                      ~~~~~~~DNDGADPVCVSGSGDG....NCEAPRHVYNSAVLDS.GN.LSNGLVERFRAIVGE.LPRAACT...SAGRNLLVSVLRL
PUF6                      ~~~~~~~~~~HILNHAVEFAQD......QEGSR..FIQRAVES..A.THDFVDALFREILFES.PLELVVD...VFGNYVLQKLLEV
PUF7                      LDKCDGLIRVQVLYQLRRKLVDLS....RSNVGNIIVREMLEKLPAKQKKEIAEVFVLNAEEDEFKRLCT..HRIGNYVAQKIIEY
PUF8                      ~~~~~~~~~~KIEPKFDTYVRT......PRTSR..VIQSMIKYG...STEQLGKIVGLISSG.YANYATD...AYGHFVVVALLRH
PUF9                      ~~~~~~~LYAQPRPSQQPPPSQ.....APIYSKAVTAPTALDVH.P.LIDTTSEFLRKCAGR.VVELACT...PDGRSMLINALSS
PUM                       NQPYPNLQLRDLANHIVEFSQD......QHGSR..FIQQKLER..A.TAAEKQMVFSEILAA.AYSLMTD...VFGNYVIQKFFEF

                                                Repeat 3                Repeat 4

PUF1                                   SESDLSEFLLLLEVLKDKIPQICCDTNGSRAMQKVFDSLKSL.EEVEFSAQCFSECIIELCKDIDGN.HAVSRLLAA
PUF2                                   LPTESDMCKRLIKQVSGRLKEYSFQMYGCRVIQKMLEKASP..EKREEVLFELKDCLVECIFDQNAN.HVAQKLIEV
PUF3                                   MPKD..VRYSVACVAAPKIAAIALTPHGTFSVQKMIETISSR.EELVIIREALSKDVVRLVKDANGN.HAIQKVLQL
PUF4                                   APDE.ERMRLLDYHISASLCDVAISPHGNYAVQKLIDSLRSS.QEVQVVCRALQRGTLQLMTDLNGG.HVIQKLLQC
PUF5                                   HHVE..MTRTVVDEFRPVLPAVALDSNGCHVVRALVEFIPTG.LMATLVPHFTPSLVRDLAVSSPYTRRVLQSVFER
PUF6                                   GNAR..QLAYAATRLQNNVVNLTLQTYGCRVIQKCIEVMPP..EGLDIILSELRGNVAKCIQDQNGN.HVVQKCVEV
PUF7                                   PSSC....EVVEERFLPYLGQLALHEFGQRVVAKYVGVTSDG.WKQVCKALFGLDDDVTLKKGKTGDDTLQERISQV
PUF8                                   APHD..LFDKILSLTIPAVPTLISHRFGIEVIHSAYSSNLCTATNRNLLILAVFKDNIALMKRWKGY.PILEDVLAQ
PUF9                                   QDAA..LVDTMVREIADDLERVALDVHGCHVLRALQSYASAE.HTRILVSCFTETLVLNLCTATQHTRHILESLFQR
PUM                                    GTPE..QKNTLGMQVKGHVLQLALQMYGCRVIQKALESISP..EQQQEIVHELDGHVLKCVKDQNGN.HVVQKCIEC

                                       Repeat 5                                Repeat 6

PUF1                ARGTPLWESGAAGDDSSSKLAHIHQLLYGKFPESCVDVCGNRHG....CCVIQKCLQWAPE....PYFSTLMDTIVHDTIKLVHDP.FGNYVLQFILDH
PUF2                I...PEKT................QLLVDSFMPHLKALSRHPYG....CRVLQCVFFRCSTA.HGVNIRPMLEAVLENVHEYVMDQ.YGNYVVQYALLN
PUF3                FE..PDDK................EFVYAAVAVDCITIAKNKQG....CCVLQRCLEYASP....AQRSTLVRHILECCLQIAEDP.YGNYVLQYVISA
PUF4                IS..PKDI................TFLYDVIVKDTVDVCNDKHG....CCVVQKCMDHAIN....VHLQRTQKAILRHMLQLSLNP.YGNYVVTHLISM
PUF5                HK..SDAL................TPIVEAIAQDSQLLAQRQQG....CITIIRTIENTLP....HQQRFIISRLLPALPALTMNC.YGNYVVQCVLHH
PUF6                I...PQRC................GFIVSAFSGRVMELATHAYG....CRVIQCIMDHCP.....DQEEAIFSELLDCVGTLATDQ.YGNYVIQHVLQH
PUF7                IKTTNDNMTLSALLKHP....LVPSRVKDALCAHLSEYAGEYLNPQSVCTGAQKGEKVAEDD...EFAAPEFGDLPLTKASRGHDQXYGDFAQRKELWD
PUF8                EV..EQR.................KRLLPKLFELCEKLVSQKSAVD..FPFVQRLAAAFIRNGTKHEVSELCDTLRPHLAVLCTIR.EGAPLASLAFSL
PUF9                R...LIDL................QPIIDVLASHSRYLAATQQG....CISFMHILEFCNE....AQKKQLISPLVPYFAHVALDP.FGNYVVQRIIQS
PUM                 VD..PVAL................QFIINAFKGQVYSLSTHPYG....CRVIQRILEHCTA....EQTTPILDELHEHTEQLIQDQ.YGNYVIQHVLEH

                                   Repeat 7                                Repeat 8

PUF1   EQELSQRSSTGSEGADYTNRIIRQMLHNVAAL..SC....NKFCSNVIEKCLKSAT..PDVRQLIVDELTDPQ.............ILPKLLTDSFANYVIQTAIVT
PUF2   AP...EELRQ.........RFVTQLIPHVYAL..SC....SKFASNVAEKTIIKAN..AEELQQVVETLTHPLG......ASEDGNYLVLMMQDQYANYVVQRLLQQ
PUF3   GD...SKTID.........TIAIAFLPHLVQL..CM....NKFSSNVMEKVLCRVS..PLVQEMYVDTMCTPE.............VAARLIQDDFGNYVLQTALTI
PUF4   QCNSSQRHVVN........EAAHCAGPALELL..CA....NKFASNVVEKIVQGCA..PSAKLEICRFLFNRST......IN.TGMSARLMGMDYVLGEQDPQQRLM
PUF5   MD...PEAVT........VVVCHAFAGHWVAL..SC....NKFASNVVEKVVRVLEG.PARRALIAETVCDPV.............NLRRLMNDCFGNFVLQAIIDS
PUF6   MK...DDEKVG........RIFDALKGNFYES..SK....QKFASNVMEKLFVRAD..PQQRMEIVNMMCSPIG..........DDSGEPVEVLSFKRSSAPKKENV
PUF7   CIVAAPGLVDHIVS...HKFVINVAVAAFKSYPESQDALWNALVGNEGRDVVEVSQD.PVGTMLLRAAMEVDNKRFTAAHRRHLAESTLTLSQDPVSSPVIQKLLEC
PUF8   .TE..PKKRK.........VILRAFSENLGVL..VV....SKYSAPVIARLFDIVY...DVQLLCKYVVNDVVS............HITQLINSPFGHQILLHLLTP
PUF9   IG...LDASE.........YITSCFAGELLNM..SC....NKFGSNVVEETIKVCGGVPAVRRLLMEELISKPG............ALQRLVQDSFGNFVVQTFIGS
PUM    GK...QEDKS.........ILINSVRGKVLVL..SQ....HKFASNVVEKCVTHAT..RGERTGLIDEVCTFN...........DNALHVMMKDQYANYVVQKMIDV
```

Fig.3.3. ClustalW alignment of the Puf domains of the nine *Tb*PUFs and *Drosophila* Pumilio. Eight imperfect Puf repeats are indicated. Conserved residues (at least 6 out of 10 cases) are colored black. Less conserved amino acids are highlighted grey (at least 4 out of 10). Dots (.) are introduced to optimize alignment.

To investigate the conservation of PUF proteins among *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania major*, each *Tb*PUF protein sequence was used to blast against the database of *T. cruzi* and *L. major* using the BlastP algorithm (www.genedb.org/genedb/tryp/blast.jsp). Interestingly, each PUF protein has an orthologue in all three pathogens (Fig.3.4.). Highest homology exists within the PUF8 group with 62% and 52% identity between *Tb*PUF8 and *Tc*PUF8, and *Tb*PUF8 and *Lm*PUF8, respectively.

To investigate the distribution of Tritryp PUF proteins among eukaryotes a phylogenetic tree was generated using only the PUF domains from 93 Puf members (Fig.3.5.).

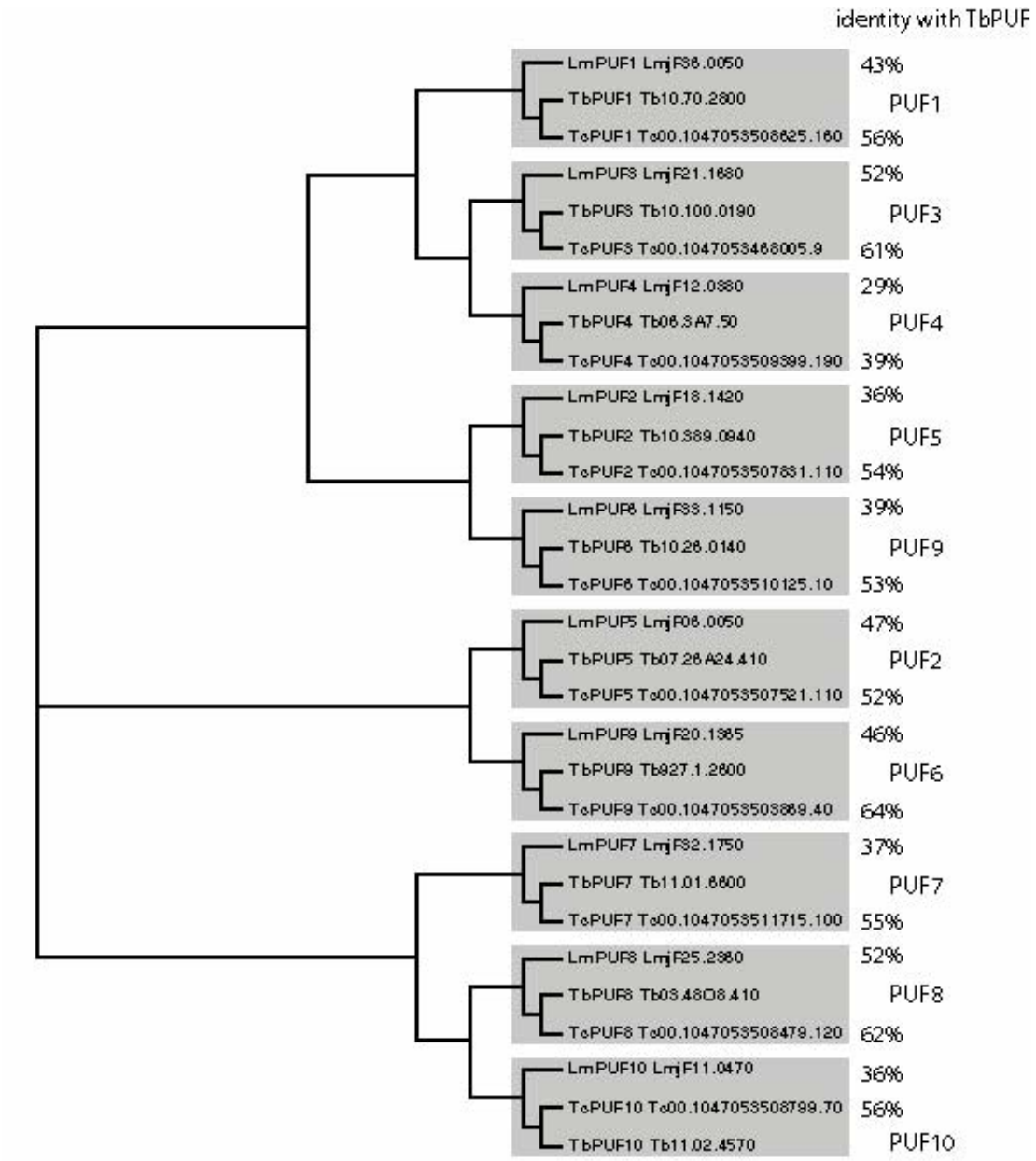


Fig.3.4. Conservation of PUF proteins among kinetoplastids. A phylogenetic tree was derived by aligning the amino acid sequences of the PUF proteins of *Trypanosoma brucei* (Tb), *Trypanosoma cruzi* (Tc), and *Leishmania major* (Lm) with Clustal W. Each member of the PUF family has an orthologue in the three species.

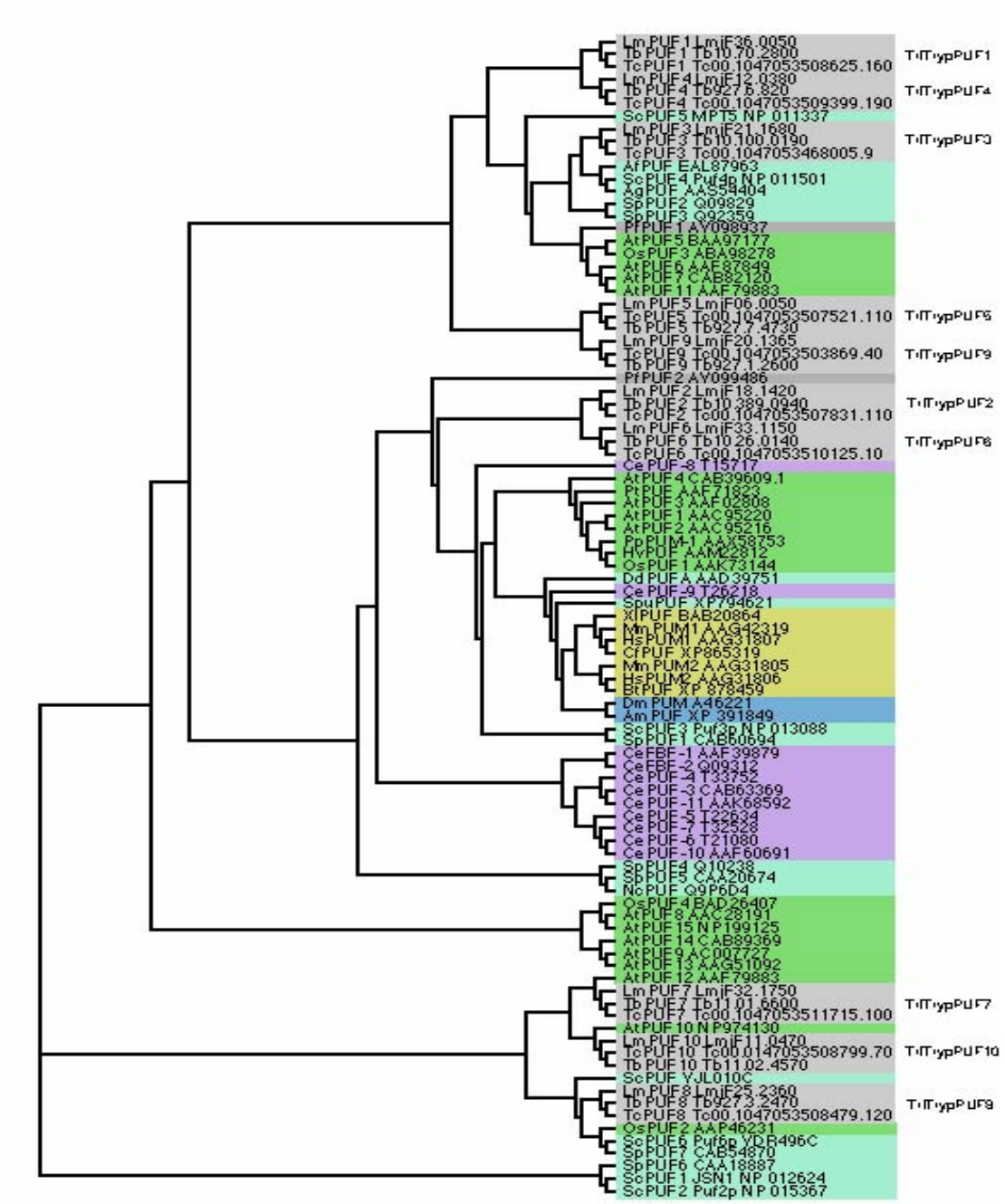


Fig.3.5. PUF proteins troughout eukaryotes. A phylogenetic tree was derived by aligning only the PUF domains from 93 Puf members. Af, *Aspergillus fumigatus*; Ag, *Ashbya gossypii*; Am, *Apis mellifera*; At, *Arabidopsis thaliana*; Bt, *Bos taurus*; Ce, *Caenorhabditis elegans*; Cf, *Canis familiaris*; Dd, *Dictyostelium discoideum*; Dj, *Dugesia Japonica*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Hv, *Hordeum vulgare*; Lm, *Leishmania major*; Mm, *Mus musculus*; Nc, *Neurospora crassa*; Os, *Oryza sativa*; Pf, *Plasmodium falciparum*; Pp, *Physcomitrella patens*; Pt, *Populus tremula x Populus tremuloides*; Rn, *Ratus norvegicus*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosachharomyces pombe*; Spu, *Strongylocentrotus purpuratus*; Tb, *Trypanosoma brucei*; Tc, *Trypanosoma cruzi*; Xl, *Xenopus laevis*. GenBank accession numbers identify each entry.

Some clustering can be observed when looking at the phylogenetic tree representing the distribution of 90 Puf members. PUF proteins of vertebrates (yellow) seem to form a subfamily, and they are closest related to PUF members of dipterans (blue). In contrast to a previous phylogenetic analysis using only 34 Puf domain amino acid sequences (Cui, Fan et al. 2002) no obvious separation between plant and animal PUF members can be drawn. Interestingly, kinetoplastid PUF members are spread throughout the eukaryotic lineage, as is true for yeast PUF proteins. A hypothesis, based on this observation, is that PUF proteins lost their diversity in the course of evolution.

3.2. Depletion of PUF proteins or their RNAs does not affect growth of *Trypanosoma brucei* in vitro

3.2.1. RNA interference of each PUF protein in *Trypanosoma brucei*

The use of double-stranded RNA (dsRNA) to disrupt gene expression has become a powerful method in a wide variety of organisms ranging from Kinetoplastida, fungi, green plants, planaria, dipterans, teleosts to most recently mammals, including humans (Cogoni and Macino 2000). This tool, called RNA interference (or RNAi), has become the method of choice to study gene function in *T. brucei*. As has been shown in multicellular organisms, the RNAi mechanism in *T. brucei* involves processing of double-stranded RNA to 24- to 26-nt RNAs, termed small interfering RNAs (siRNAs), which guide degradation of the target mRNA (LaCount, Bruse et al. 2000; Shi, Djikeng et al. 2000; Wang, Morris et al. 2000; Inoue, Otsu et al. 2002). Very recently it was demonstrated that chemically as well as enzymatically synthesized siRNAs are also functional in the parasite (Best, Handoko et al. 2005).

To investigate the role of PUF proteins in *T. brucei*, RNAi knockdown was performed for each PUF protein in bloodstream and procyclic cells. To ensure high specificity of RNAi knockdown we used a software tool (RNAit, (Redmond, Vadivelu et al. 2003) for the selection of RNAi targets. Figure (Fig.3.6.) shows the RNAi-targets of the *Tb*PUFs. Primers are selected utilising the MIT's primer3 program (http://wwwgenome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and NCBI blast (http://www.ncbi.nlm.nih.gov/BLAST/). This combination allows theoretical PCR

products to be checked against the available *T. brucei* genome sequence. Only those with sufficiently low homology to the rest of the genome are selected. Therefore unspecific knockdown of more than one PUF gene is not expected, despite the fact that nearly all regions for RNAi lie within the PUF-domain (Fig.3.6).

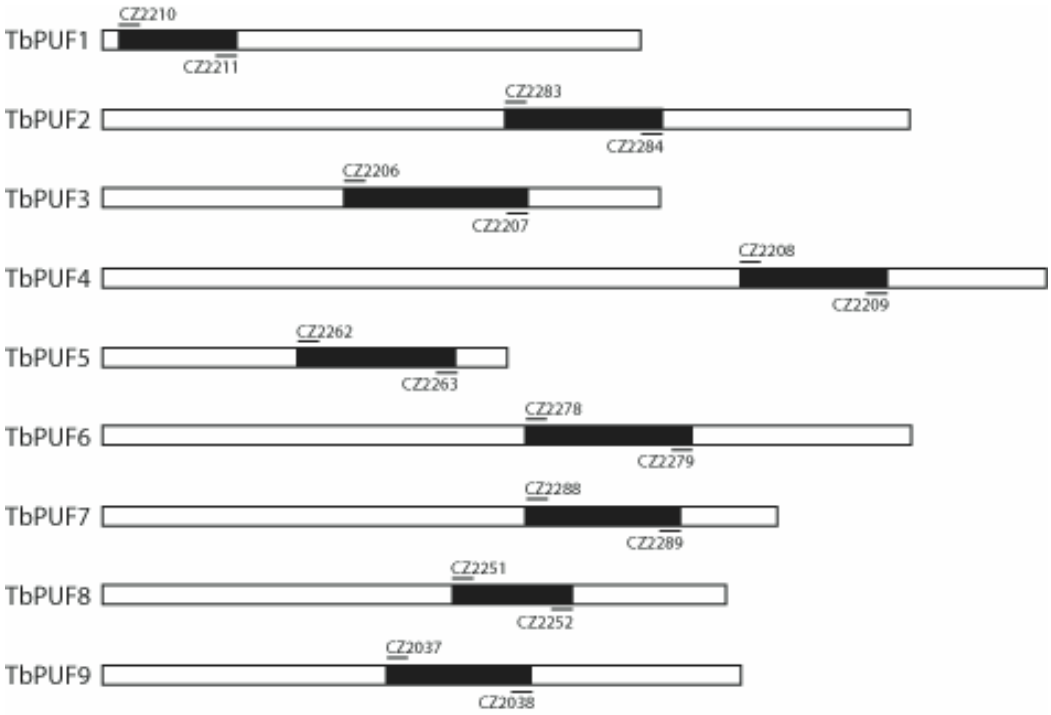


Fig.3.6. Primers used to amplify the region of RNAi-target within the open reading frame. A combination of the MIT's primer3 program and NCBI blast was used to select suitable primers and to minimise off-target knockdown. With the exception of *Tb*PUF1, all regions for RNAi lie within the PUF-domain.

*Tb*PUF-RNAi PCR products were cloned between two opposing T7 promoters in p2T7-177 (LaCount, Bruse et al. 2000) (PUF1,3,4, and 9) or in p2T7^{TA}-blue (Clayton, Esteacutevez et al. 2005) (PUF2,5,6,7, and 8). The resulting *Tb*PUF-RNAi plasmids were transfected into *T. brucei* 427 1313 514 bloodstream (bf) and procyclics (pc) cell lines. After selection with hygromycin, RNAi was induced by the addition of tetracycline and total RNA was prepared after 48 hours. To check depletion of *Tb*PUF transcripts Northern blot analyses were performed. As can be seen in Fig.3.7. the transcripts were down-regulated by the addition of tetracycline. RNAi efficiency ranged from 82% (*Tb*PUF3) to only 25% (*Tb*PUF6) knockdown. Increasing the tetracycline concentration did not lead to

higher RNAi efficiency (data not shown). *Tb*PUF1 transcript downregulation was most efficient already 24 hrs after induction for bf and pc cells (Fig.3.9). Knockdown of *Tb*PUF1 was also verified by Western blot analysis (Fig.3.9). However, *Tb*PUF1 protein levels (compared to transcript levels) seemed to be totally unaffected in procyclic cells as shown by Western blot analysis. RNAi induced cells were grown in the presence of tetraycline for five days and growth was monitored every 24 hours (Fig.3.8.). Silencing of *Tb*PUFs did not affect growth of bloodstream or procyclic cells.

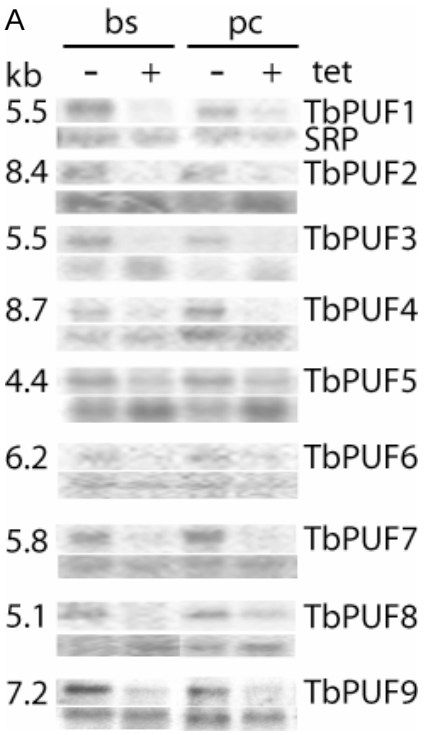
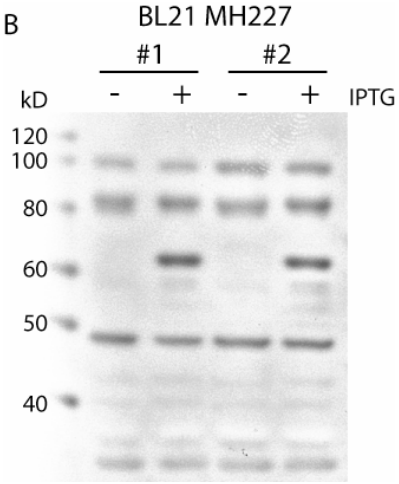


Fig.3.7. A) Northern blot showing downregulation of *Tb*PUF transcripts. Equal amounts of RNA (10µg) from RNAi uninduced (-) and induced (+) bloodstream (bs) and procyclics (pc) cells were loaded on each lane. After probing for *Tb*PUF transcripts, the same filter was hybridized with srp (signal recognition particle) serving as a loading control. *Tb*PUF transcript sizes are indicated. B) Western blot of *E. coli* expresssing *Tb*PUF1 (MH227, (Hoek, Zanders et al. 2002) using α-*Tb*PUF1. Expression was induced by the addition of IPTG. C) Western blot using α-*Tb*PUF1 to assess PUF1 protein expression level in wild-type procyclic (pc) and bloodstream form (bf) cells, as well as in *Tb*PUF1 RNAi (1538) uninduced (-) and induced (+) cell lines. Cytosolic marker (CSM) was used to assess for loading variation.



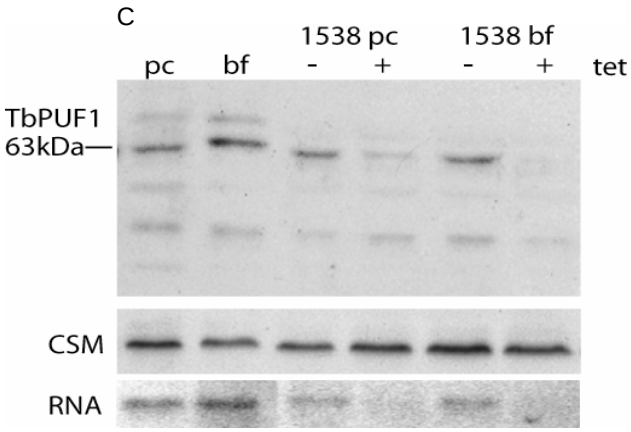
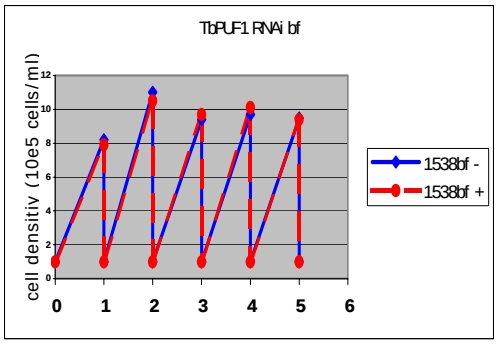
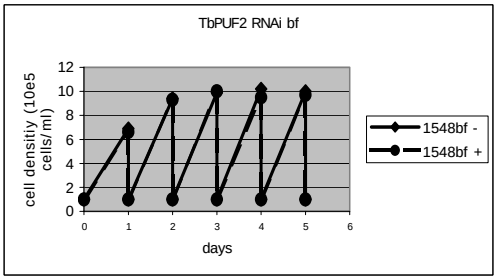
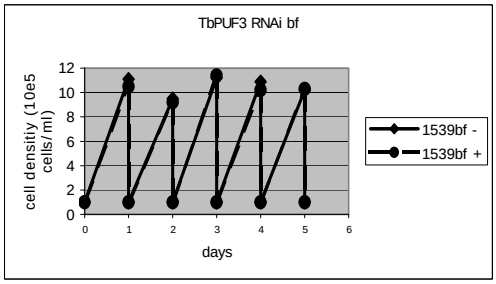
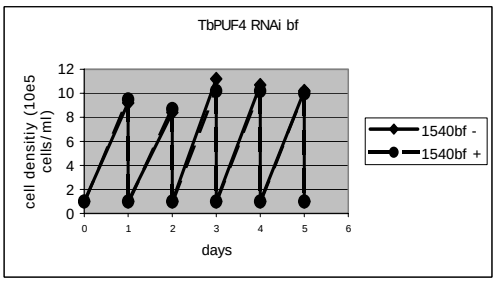


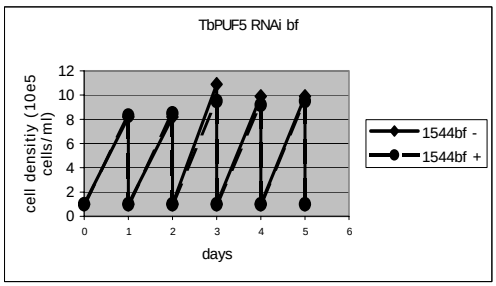
Fig.3.8. Effects of depletion of single *Tb*PUFs on cell growth. RNAi bloodstream form cell lines were grown in the absence (-) or presence (+) of tetracycline and growth monitored for 5 days. Cells were diluted all 24 hours to a density of $1x10^5$ cells/ml. Similar results were obtained for RNAi procyclic form cell lines (data not shown).

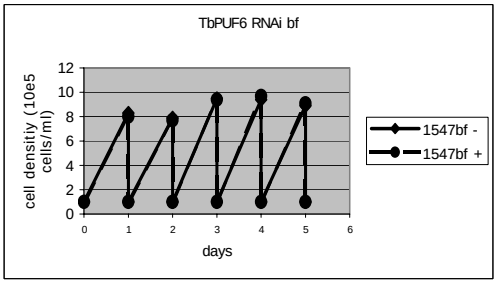


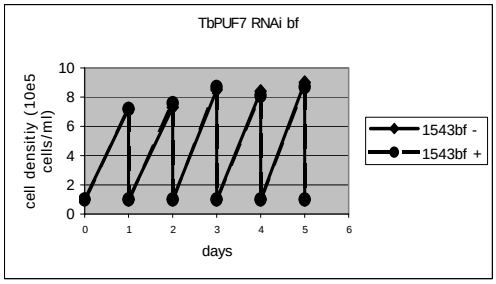


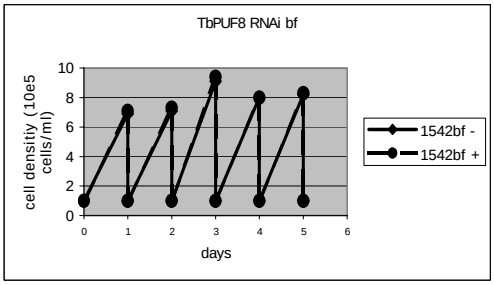


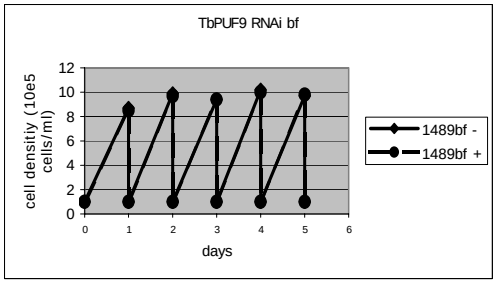












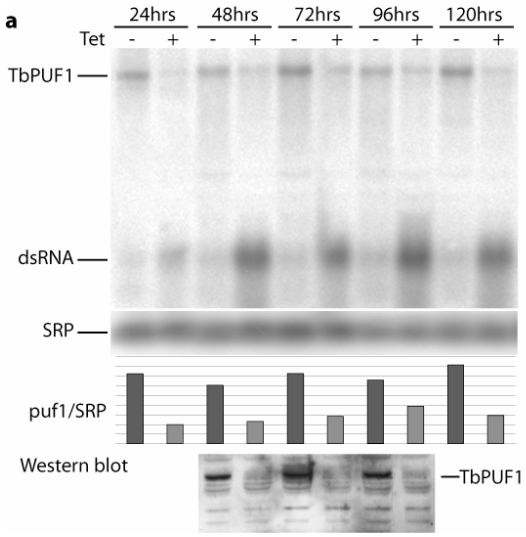
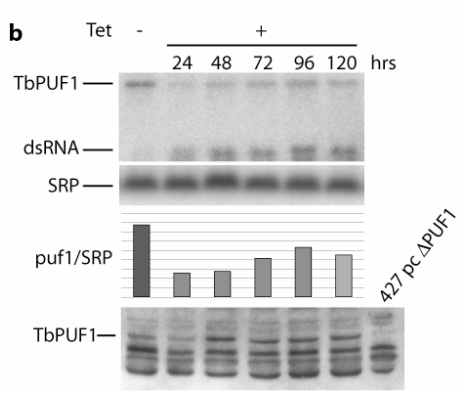


Fig.3.9. Northern and Western blot analysis to assess *Tb*PUF1 RNAi efficiency for a prolonged time of incubation for bf (a) and pc cells (b).



3.2.2. Knockout of *Tb*puf1

Double-stranded RNA based gene silencing of *Tb*PUF1 did not result in a growth phenotype for bloodstream and procyclic cells. It is possible that downregulation by RNAi was not sufficient and that residual (~20% in bf and ~25% in pc) *Tb*PUF1 is still able to exert its physiological function. To check this, a *Tb*PUF1 knockout was performed in procyclic cells using classical homologous recombination as described in the Materials & Methods section. Southern, Northern and Western blot analyses showed that *TbPUF1* was successfully knocked out (Fig.3.10.). Surprisingly, deletion of the *TbPUF1* gene was not lethal for procyclic cells. This contradicts the results of (Hoek, Zanders et al. 2002) who claimed, based on a failure to delete the gene, that *TbPUF1* was an essential gene in *T. brucei* procyclic and bloodstream forms.

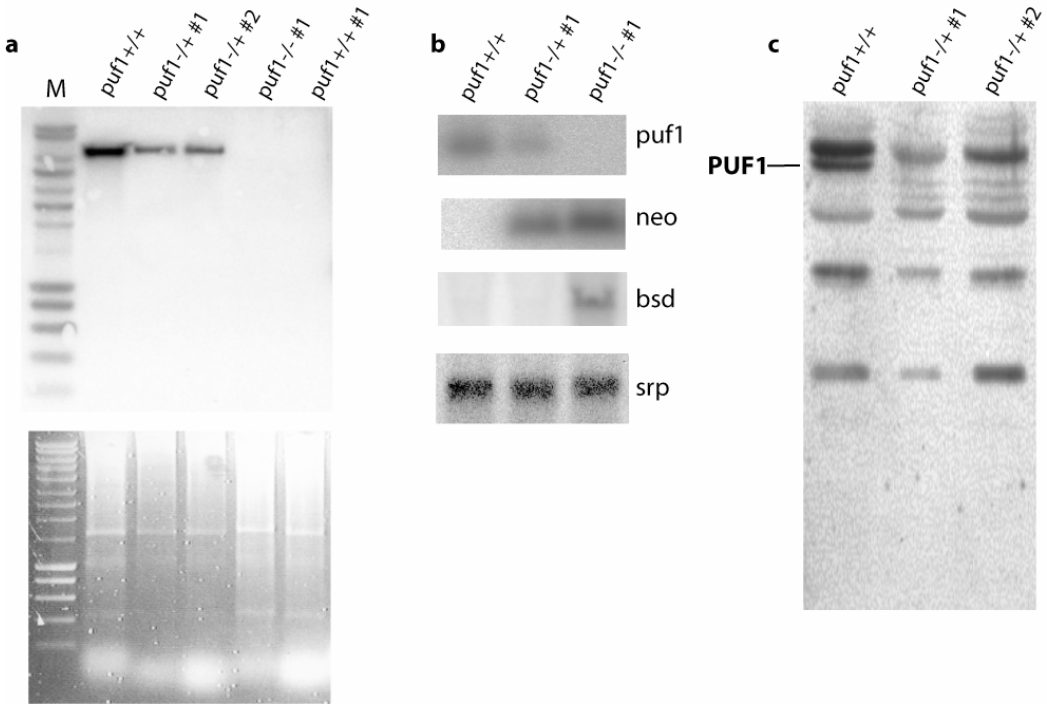


Fig.3.10. Deletion of *TbPUF1* in procyclic cells. Southern (a), Northern (b), and Western (c) blot confirming *TbPUF1* deletion. a) 10µg of genomic DNA were cut with BamH1 and loaded onto each lane. A probe for *PUF1* (recognizing bp 206-692 of *PUF1*-cds) was labeled with ^{32}P. Ethidium bromide staining of gel serves as loading control. b) 10µg of total RNA was loaded onto each lane. Probes are for puf1, neomycin (neo), blasticidin (bsd), and SRP transcript. c) $3x10^6$ cells were loaded on to each lane, and the blot was probed with α-*Tb*PUF1 antibody.

3.2.3. RNAi double knockdown of PUF proteins

Since *T. brucei* possesses multiple PUF proteins, it is possible that they share redundant functions. To assess the question which PUF-pairs would be most likely to have redundant functions, a phylogenetic tree using only the *Tb*PUF-domains was generated. PUF1, PUF3, and PUF4 together form a branch. Within the Puf repeats, the amino acid sequence of PUF1 is 33% identical to PUF3 (and 31% to PUF4), whereas PUF3 is 36% identical to PUF4 within that same domain. To perform double RNAi experiments, two different strategies were theoretically possible. RNAi fragments for both targets can either be cloned into the same RNAi plasmid (p2T7^{TA}-blue) or a second transfection is performed, using a plasmid with a different antibiotic resistance. We decided to follow latter strategy. To this

end, the hygromycin cassette of p2T7-177 was replaced by a blasticidin resistance cassette, generating plasmid pHD1621 (done by Corinna Benz). Subsequent transfections of bloodstream cells with the two different RNAi plasmids resulted in a double RNAi cell line that can be simultaneously depleted of two distinct PUF transcripts (Fig.3.11.). However, these double RNAi cell lines also exhibited normal growth (data not shown).

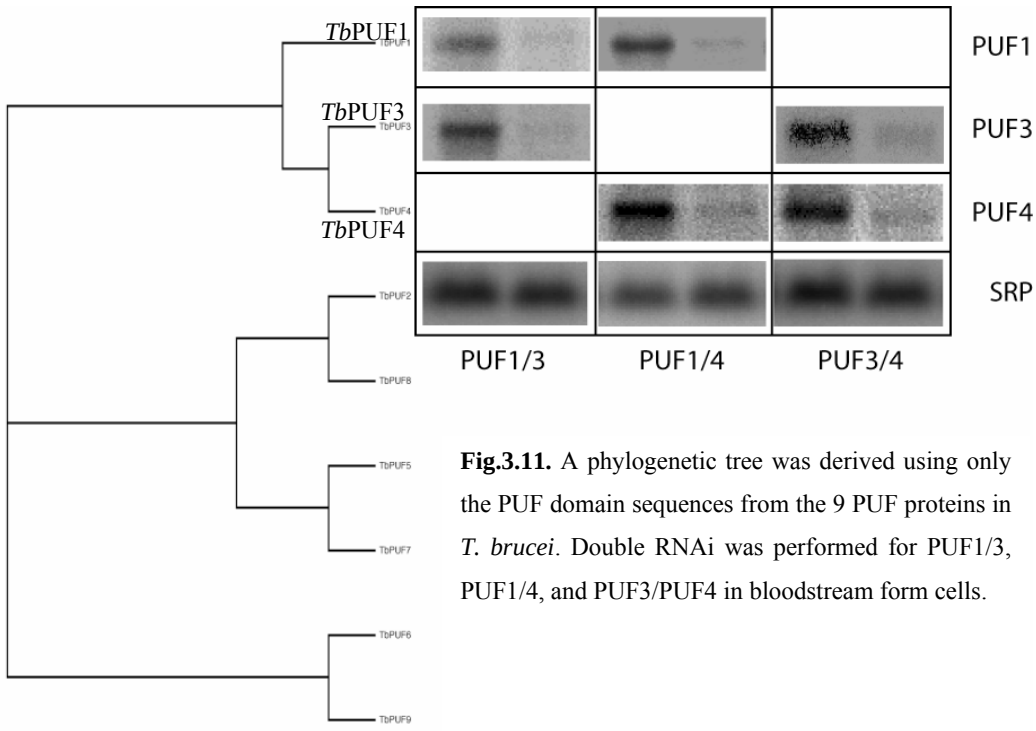


Fig.3.11. A phylogenetic tree was derived using only the PUF domain sequences from the 9 PUF proteins in *T. brucei*. Double RNAi was performed for PUF1/3, PUF1/4, and PUF3/PUF4 in bloodstream form cells.

3.3. Microarray analysis of PUF strains

3.3.1. Microarray analysis of PUF RNAi and overexpression strains

Based on the functions of *Drosophila* Pumilio and *C. elegans* FBF, I reasoned that *Tb*PUFs would bind to and regulate specific mRNAs, either by altering mRNA stability or interfering with translation. Using mRNA degradation assays (Hoek, Zanders et al. 2002) suggested that perturbation of *Tb*PUF1 levels affects the stability of several ES-derived mRNAs (e.g. VSG221 and ESAG6). These results were, however, somewhat preliminary, since loading controls were not applied. Furthermore, the authors emphasized, that the ES-associated mRNAs need not be the primary targets of *Tb*PUF1. To confirm the results of (Hoek, Zanders et al. 2002) and to examine the hypothesis of *Tb*PUF1 being a modulator of mRNA stability, I asked if perturbation of PUF protein levels in *T. brucei* would

influence steady-state levels of specific mRNAs. Here I compared global transcript levels of *T. brucei* wild-type cells with cells where PUF-levels had been perturbed (either by RNAi or overexpression) using *T. brucei* specific genomic DNA microarrays (Diehl, Diehl et al. 2002; Brems, Guilbride et al. 2005). In brief, total RNA was isolated from cells uninduced and induced for RNAi (or overexpression) and reverse transcribed with Cy3 and Cy5-labeled dCTP. To prevent bias caused by preferential label incorporation, the two CyDyes were swapped between the two types of RNA (uninduced or induced). Six independent hybridisations were performed for each PUF RNAi or overexpression cell line. Figure 3.12. shows a section of the DNA chip of *Tb*PUF1 RNAi microarray analysis in bloodstream and procyclic cells. Software analysis (MCHIPS, (Fellenberg, Hauser et al. 2001)) showed only the RNAi-downregulated *Tb*PUF1 transcript to be regulated (Table 3.1.). Similar results were obtained in microarray analysis with *Tb*PUF9 RNAi cells (Table 3.1.). *Tb*PUF1 and *Tb*PUF9 contain only six and four, respectively, Puf repeats that are recognized by Pfam model. I speculated that the presence of all eight Puf repeats might necessary for fulfilling physiological function. To this end, I performed microarray hybridisations with *Tb*PUF2 and *Tb*PUF6 RNAi procyclic cells. Both proteins bear eight Puf repeats that are recognized by Pfam model. However, overall transcript levels also remained unaffected in these RNAi strains.

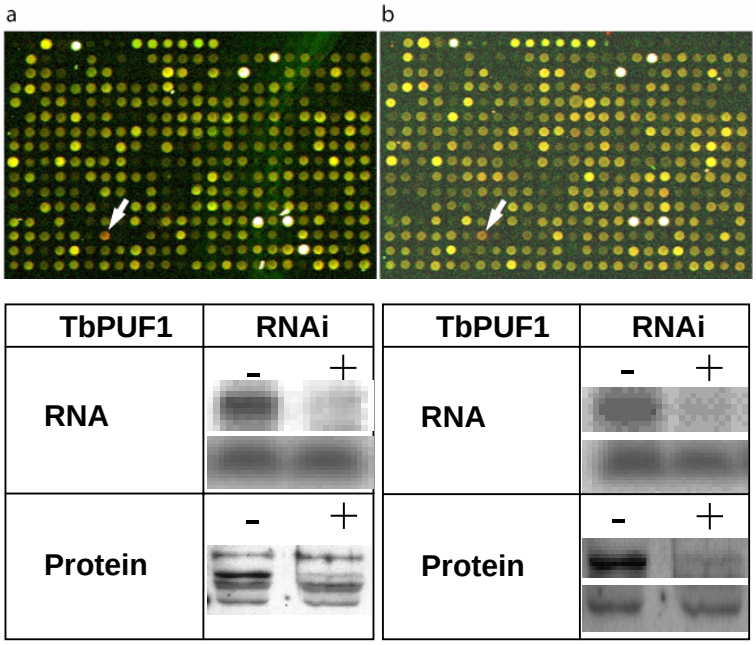


Fig. 3.12. DNA chip spotted with 24,567 PCR products (Brems, Guilbride et al. 2005). Total RNA of TbPUF1 RNAi-induced (Cy5) and uninduced (Cy3) cells were compared. A section with the down-regulated PUF1-transcript (35H11, white arrow) in *T. brucei* bloodstream (a, 2.50 fold down regulation) and procyclics form (b, 2.01) is shown. Northern and Western blot confirming *Tb*PUF1 knockdown are shown below.

Since PUF proteins do generally suppress mRNA either by promoting mRNA degradation or inhibiting translation we hypothesized that overexpression of PUF proteins in *T. brucei* (Fig.3.13a.) might lead to downregulation of target mRNAs. As observed with the RNAi

analysis, upregulation of the overexpressed PUF transcript was detected in the microarray analysis, again serving as an internal positive control. Notably, transcript levels of a specific rRNA spacer region (marked with * in the Table 3.1.) also increased upon overexpression of *Tb*PUF1, *Tb*PUF9 (both bf and pc) and *Tb*PUF5 (pc). Subsequent analysis revealed that the overexpression plasmid integrated into these rRNA spacer regions. However, Northern blot analysis failed to show the differential expression of these rRNA transcripts. Interestingly, overexpression of *Tb*PUF5 in procyclic cells resulted in a growth defect (Fig.3.13c.) and differential regulation of 14 transcripts (Table 3.1.); nearly all upregulated transcripts derived from a locus complementary to the rRNA spacer region in the overexpression plasmid. We suspect that the overexpression plasmid integrated stably in this region of the genome. Two downregulated spots (clone 08B07 and 50B19) corresponded to Tb11.01.7880, a gene encoding CAP17 (corset-associated protein 17). Notably, Tb11.01.7880 was already found to be upregulated in procyclics in microarray analysis (Diehl, Diehl et al. 2002). To confirm these differentially regulated hits, Northern blot analyses were performed. As can appreciated from Fig.3.14., *CAP17* mRNA is indeed downregulated upon overexpression of *Tb*PUF5 in procyclic cells. However, further analysis confirming the putative interaction between *Tb*PUF5 and Tb*CAP17*-mRNA need to be done, for example by gel mobility shift assay.

Tab.3.1.

	gene array	ratio	Northern	false positive	identity
PUF1-o.e. bf					
	13_P_24*	2,55		x	rRNA_region, Tb927.1.3720, hyp. protein, unlikely
o.e.					
	21_O_07	2,17		x	Tb927.3.3421 |||rRNA small subunit
	21_P_03	2,36		x	
	33_K_24*	2,23		x	rRNA_region, Tb927.1.3720, hyp. protein, unlikely
	40_P_15*	2,24		x	rRNA_region, Tb927.1.3720, hyp. protein, unlikely
	44_A_08	2,06		x	
	47_E_01	2,39		x	
PUF1-o.e. pc	10_A_18*	2,61		x	rRNA_region, Tb927.1.3720, hyp. protein, unlikely
	13_P_24*	2,67		x	rRNA_region, Tb927.1.3720, hyp. protein, unlikely
	14_L_17*	2,25		x	rRNA_region, Tb927.1.3720, hyp. protein, unlikely
	33_H_13*	2,21			rRNA_region, Tb927.1.3720, hyp. protein, unlikely
	33_K_24*	2,22		x	rRNA_region, Tb927.1.3720, hyp. protein, unlikely
	35_H_11	1,55	4,83		TbPUF1, Tb10.70.2800
	40_P_15*	2,24		x	rRNA_region, Tb927.1.3720, hyp. protein, unlikely
PUF1-RNAi bf	35_H_11	-2,50	-3,42		TbPUF1, Tb10.70.2800
PUF1-RNAi pc	35_H_11	-2,1	-3,09		TbPUF1, Tb10.70.2800
PUF9-o.e. bf	35_N_23	3,26	4,27		TbPUF9, Tb927.1.2600
PUF9-o.e. pc	35_N_23	3,77	4,58		TbPUF9, Tb927.1.2600
	40_P_15*	1,49		x	rRNA_region, Tb927.1.3720, hyp. protein, unlikely
PUF9-RNAi bf	05_P_19	-2,21		x	
	35_N_23	-2,43	-3,09		TbPUF9, Tb927.1.2600
	none				

PUF9-RNAi pc					
PUF5 o.e. pc	02_O_17	-3,16			
	05_P_03	-2,54		x	Tb927.8.8300, amino acid transporter, putative
	08_B_07	-3,89	-1,82		CAP17_microtubule-associated_protein, Tb11.01.7880
	08_I_03	2,58		x	rRNA_region, Tb927.1.3720, hyp. protein, unlikely
	12_K_21*	10,42	3,50		rRNA_region, Tb927.1.3720, hyp. protein, unlikely
	13_N_02	3,94		x	Tb08.27P2.50_|__||hyp._protein|
	13_P_24*	10,66		x	rRNA_region, Tb927.1.3720, hyp. protein, unlikely
	14_L_17	4,38		x	rRNA_region, Tb927.1.3720, hyp. protein, unlikely
	16_G_3	-1,88			Tb09_SLRNA_0005 SL RNA
	18_K_16*	6,87		x	Tb927.1.3580 |||hyp. protein, unlikely
	33_A_24	3,08		x	Tb10.70.7820 hyp. protein
	33_K_24*	2,31		x	
	43_H_11*	3,45		x	rRNA_region, Tb927.1.3720, hyp. protein, unlikely
	50_B_19	-2,89	-1,73		CAP17_microtubule-associated_protein, Tb11.01.7880
	58_C_14	2,74			Tb02_rRNA_17||18S_ribosomal_RNA

abbreviation: o.e., overexpression; hyp., hypothetical

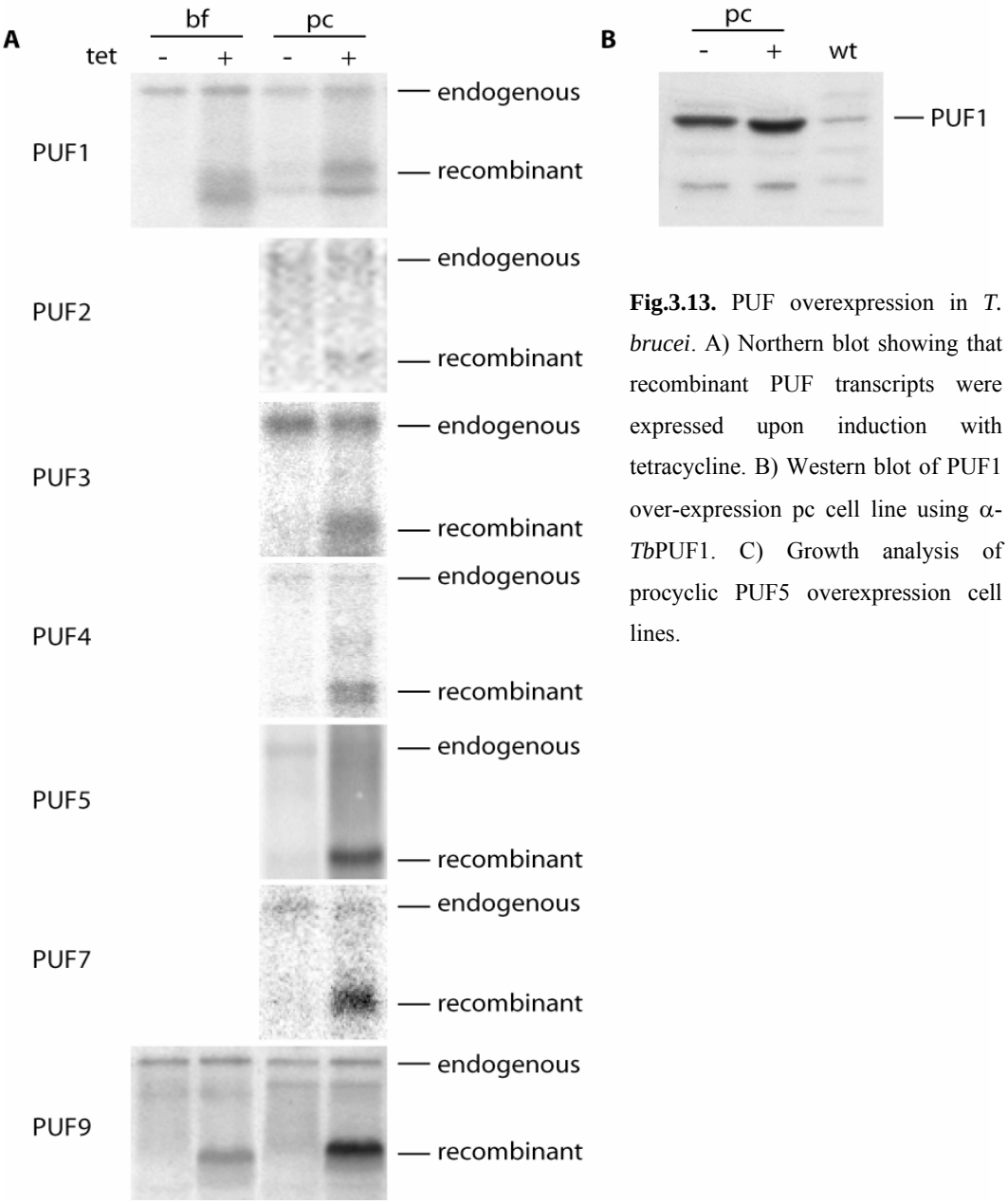
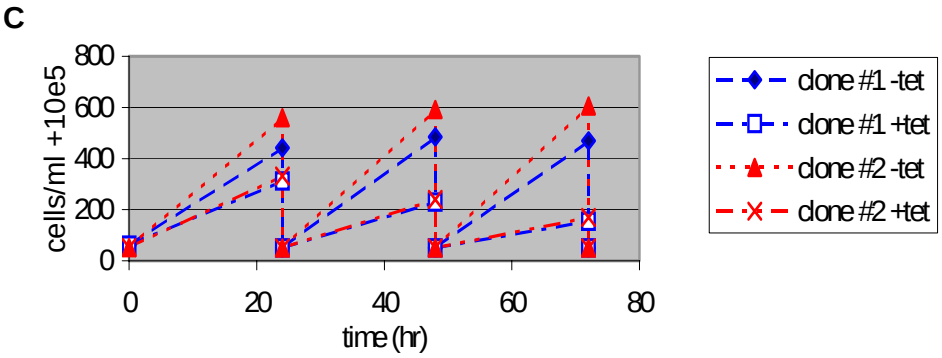


Fig.3.13. PUF overexpression in *T. brucei*. A) Northern blot showing that recombinant PUF transcripts were expressed upon induction with tetracycline. B) Western blot of PUF1 over-expression pc cell line using α-*Tb*PUF1. C) Growth analysis of procyclic PUF5 overexpression cell lines.



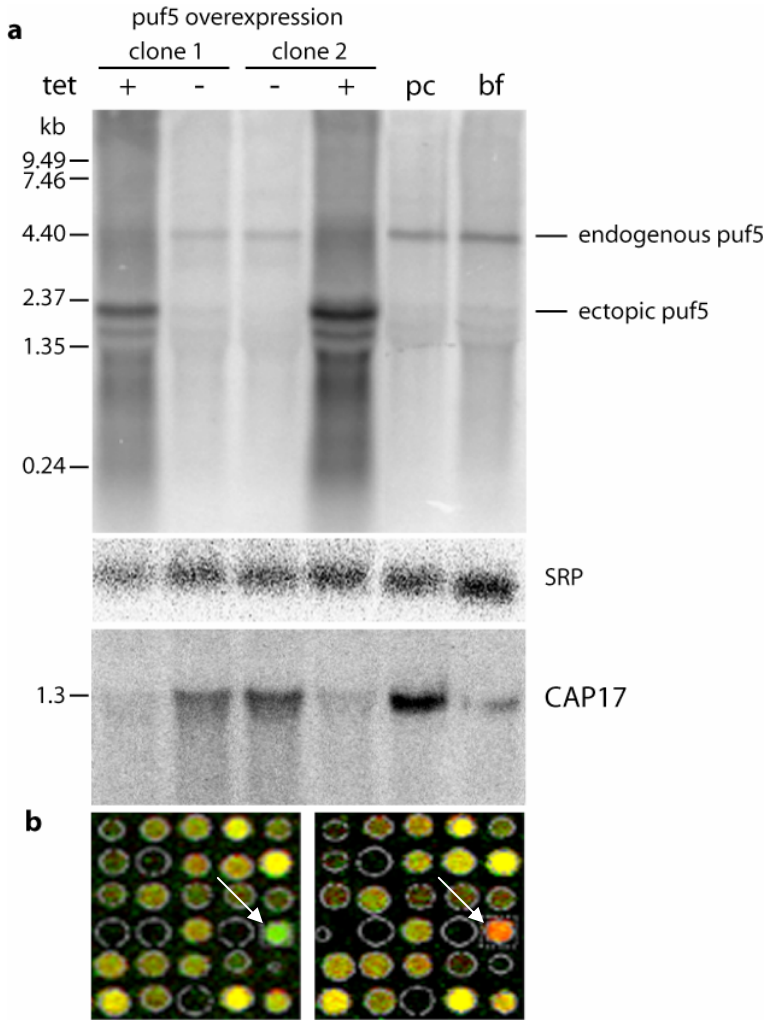


Fig.3.14. Overexpression of *Tb*PUF5 in procyclics cells lead to downregulation of Tb*CAP17* (corset-associated protein 17) mRNA. a) Overexpression of *Tb*PUF5 and concomitant downregulation of TbCAP17 was assessed by Northern blot analysis 48 hrs after tetracycline addition. SRP was used as a loading control. b) A section of the DNA chip with the downregulated *CAP17* transcript (white arrow) is shown. Left: Cy3 –Tet/Cy5 +Tet; right Cy3 +Tet/Cy5 –Tet.

3.3.2. Looking for PUF targets by a combination of affinity purification and microarray analysis

Although members of the PUF protein family are widely distributed among eukaryotes, only a few of their mRNA targets have been identified so far. Recently, it was demonstrated that yeast PUF proteins all interact selectively with distinct groups of functionally related mRNAs (Gerber, Herschlag et al. 2004). We have used a similar approach, namely affinity purification of PUF proteins and analysis of associated mRNAs

by microarray hybridisations. To this end, I C-terminally TAP-tagged *Tb*PUF1, *Tb*PUF5, and *Tb*PUF9. The TAP tag (Rigaut, Shevchenko et al. 1999) consists of two IgG-binding units of Protein A, a specific protease recognition site, and a calmodulin-binding domain. Cell extracts of TAP-tagged strains were prepared, and ribonucleoprotein complexes were affinity purified on IgG beads and recovered by subsequent cleavage with tobacco etch virus (TEV) protease. The same procedure was performed using cells expressing only the TAP tag, serving as a control for non-specifically enriched mRNAs. RNA was isolated from the purified protein samples (PUF-TAP and TAP only strains). The two different RNA samples were used to prepare cDNA probes labeled with different fluorescent dyes, which were mixed and hybridized to *T. brucei* genomic microarrays (Diehl, Diehl et al. 2002; Brems, Guilbride et al. 2005). Three microarray hybridisations for two independent Puf affinity isolations were performed for PUF9 bf cells and two hybridisations from one isolation for PUF1 pc, PUF5 bf and PUF9 pc cells. Preliminary results are shown in Table 3.2. Microarray analyses with RNA from additional PUF affinity purifications to confirm these data will be done.

3.4. Looking for PUF binding partners in *T. brucei*

To date, PUF proteins were shown to physically interact with members of three protein families (Nanos, CPEB, and Brat). Currently, no homologues of these PUF interactors are found in the genome of protozoa. (Hoek, Zanders et al. 2002) suggested that *Tb*PUF1 interacts with *Tb*ESAG8. However, the results showing this interaction were somewhat preliminary (see Introduction). Therefore, I used two different methods to look for interaction partners, namely the TAP approach and co-immunoprecipitation as described in the following sections.

3.4.1. Tandem Affinity Purification of putative PUF interacting partners

To search for interacting partners I used the tandem affinity purification (TAP) strategy introduced by (Rigaut, Shevchenko et al. 1999; Puig, Caspary et al. 2001). Briefly, the TAP-tag containing two IgG binding units of Protein A, a tobacco etch virus (TEV) protease cleavage site, and a calmodulin binding domain is fused C-terminally to *Tb*PUF1 and *Tb*PUF9, and TAP-tagged PUF1 (PUF9) expression is induced by the the addition of

Table 3.2.

TAP/RNA

PUF1-TAP pc

	Isolation #1 slide #1	Isolation#1 slide#2	
01K10	x	x	repeat unit
02D11	x	x	INGI
02L04	x	x	nucleobase/nucleoside transporter 8.1, Tb11.02.1105
03A12	x	x	
11C13	x	x	ATP-dependent phosphofructokinase, Tb927.3.3270
14C01	x	x	
16D17	x	x	MLH1, mismatch repair protein, Tb08.30K1.770
17M19	x	x	
18O19	x	x	ESAG like
21P09	x	x	HK1, hexokinase, Tb10.70.5820
22L01	x	x	
26O18	x	x	
28C12	x	x	Tb927.3.4080, hyp. protein, 14 predicted TM domains
28H10	x	x	
32H10	x	x	INGI
32H14	x	x	
33J14	x	x	
35B20	x	x	
41D22	x	x	
42K02	x	x	
44O19	x	x	INGI
45N01	x	x	
45N09	x	x	
46G04	x	x	
47A12	x	x	rRNA region; Tb927.1.3720, hyp. protein, unlikely
48K23	x	x	
	x	x	INGI

55D23				
55H03	x	x		ESAG, putative, Tb927.2.2020
56D07	x	x		
56O4	x	x		

PUF9-TAP bf

	Isolation #1 slide #1	Isolation #2 slide #2	Isolation #2 slide #3		comments in PUF9-RNAi bf
05P19	x	X	X		-2.21
16L12		X	X		
21N07		X	X	TbPUF9	
22D07	x				
22P10		X	X		-1.26
28H19					
31G22		X	X		
32E16	x	x	x		-1.87
32E16		X	X		
32F10	x	X	X	DNA ligase, Tb07.29K4.760	-1.45
32L04					
35F01	x				
39O15		X	X		
40D05	x				
48G14		X	X		
51M11					
52L23		X	X		
55O18		X	X		
58C08	x	X	X		

PUF5-TAP bf

	Isolation #1	Isolation #1		
			slide #1	slide #2
01B03	x	x		
02L12	x	x		

05P03	x	x	XUK.....NIGDB
08L22	x	x	
13L21	X	X	
17P17	x	x	amino acid transporter, putative, Tb927.4.3990
18M03	x	x	hypothetical protein, unlikely, Tb927.1.2840
24C06	x	x	
24O07	x	x	
28L21	x	x	Best matches to amino acid transporters
29P10	x	x	SLACS reverse transcriptase, putative, Tb09.211.5015
31J08	x	x	
35H11	X	X	TbPUF1, Tb10.70.2800
39L01	x	x	
40K15	x	x	AATP11, aa transporter, putative, Tb927.4.4730
41C09	x	x	
42G06	x	x	
48E04	x	x	AATP11, aa transporter, putative, Tb927.4.4730
57F07	X	X	
58J07	x	x	

PUF9-TAP pc

44F20	x	x

tetracycline (Fig.3.15.). However, attempts to purify binding partners of *Tb*PUF1 and *Tb*PUF9 using this method failed. It is possible that binding with the interactors occurs only transiently and is therefore impossible to detect by the TAP approach. Another possibility is that the TAP tag interferes with binding to the interacting partners. Mutational and structural analyses indicate that the C-terminal region of Drosophila Pumilio confers binding to two other proteins, Nos and BRAT. Both endogenous *PUF1* (*PUF9*) alleles have to be deleted to show that TAP-tagged PUF1 (PUF9) is functional. This has not been done so far.

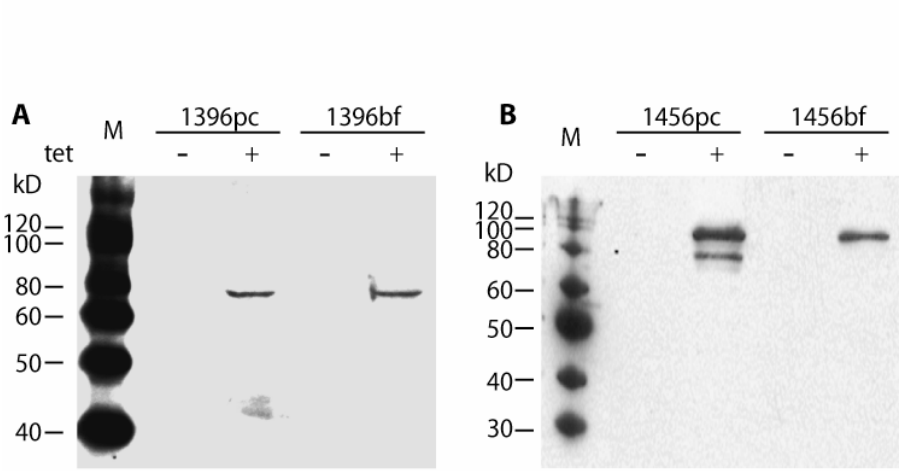


Fig.3.15. Figure PUF-TAP. Western blot using PAP antibody which recognizes the TAP-tag (1:1000 dilution in 5% milk). Induction with tetracycline leads to expression of TAP-tagged PUF1 (1396) in pc and bf (A) and TAP-tagged PUF9 (1456) in pc and bf (B).

3.4.2. Co-immunoprecipitation using α-*Tb*PUF1 antibody

α-*Tb*PUF1 antibody was coupled to Protein A Sepharose beads (Amersham Biosciences). Protein extracts prepared from in vivo ^{35}S methionine radiolabeled cells were incubated with α-*Tb*PUF1 coupled beads. Co-immunoprecipitation was performed as described in the Materials and Methods section. As a negative control we either used pre-immune serum or PUF1-RNAi strain cell extracts (Fig.3.16.). A few bands which were not present in the negative control suggested the presence of binding partners. However, attempts to upscale the co-immunoprecipitation using more unlabeled cell extracts to obtain suitable amounts for mass spectrometry sequencing were not successful.

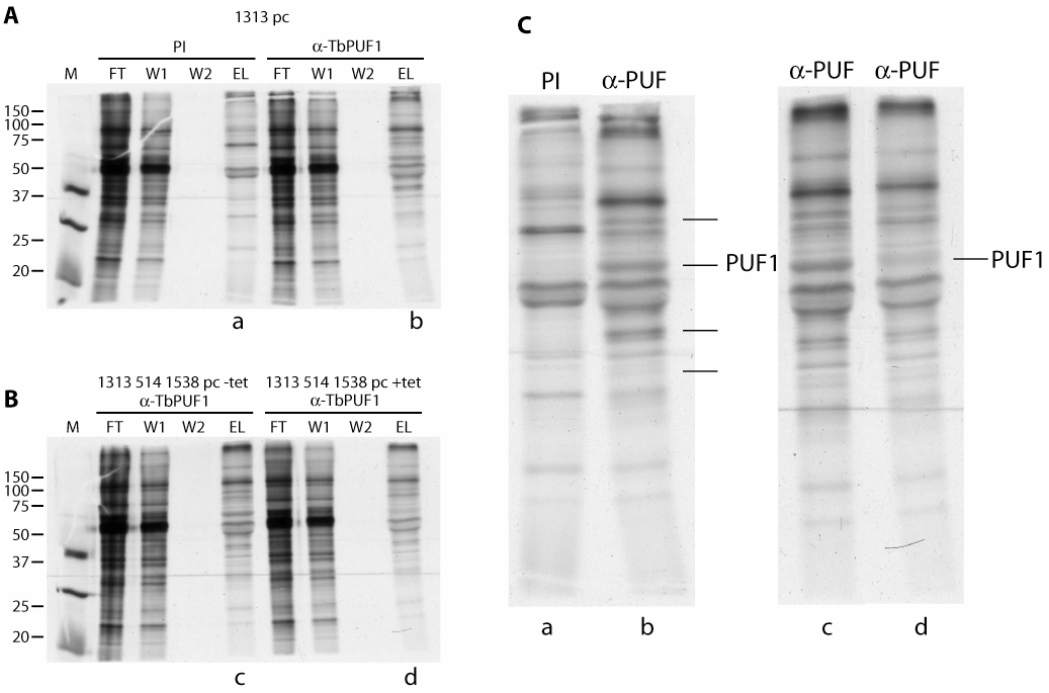


Fig.3.16. Co-immunoprecipitation using α-*Tb*PUF1 coupled sepharose beads. A) Co-immunoprecitpiation with 35S metabolically labeled procyclic 427 1313 cells. Pre-immune (PI) serum was used as a negative control to assess for non-specific binding to the beads. B) Co-immunoprecipiation with cells uninduced (-tet) and induced (+tet) for *Tb*PUF1-RNAi. C) An enlargement of elutions a, b, c, and d. Bands which are only present in α-*Tb*PUF1 co-IP compared to PI co-IP are indicated with arrows.

3.5. Localization of *Tb*PUF9 and *Tb*PUF5

To date all characterized PUF proteins reside in the cytoplasm, this being consistent with their role in translational control. To investigate the localization of *Tb*PUF9, immunofluorescence assays were performed. TAP tagged *Tb*PUF9 is predominantly localized to the cytosol in both bloodstream and procyclic stage with a higher concentration at the perinuclear space (Fig.3.17.). Despite a mitochondrial localization signal as predicted by the program ESLpred (Bhasin and Raghava 2004), *Tb*PUF5 is also localized to the cytosol, even though interestingly the protein is concentrated at discrete foci, a pattern which was also observed for HA-tagged PUF1 in *T. brucei* bloodstream form (Hoek, Zanders et al. 2002) and His-tagged *Tc*PUF6 in *T. cruzi* (Dallagiovanna, Perez et al. 2005). In yeast each of the five members of the PUF-family is associated with specific mRNAs at discrete locations in the cytoplasm (Gerber, Herschlag et al. 2004). It is possible that in *T. brucei*, too, PUF proteins act at discrete foci. However, testing this hypothesis would go beyond the scope of this work.

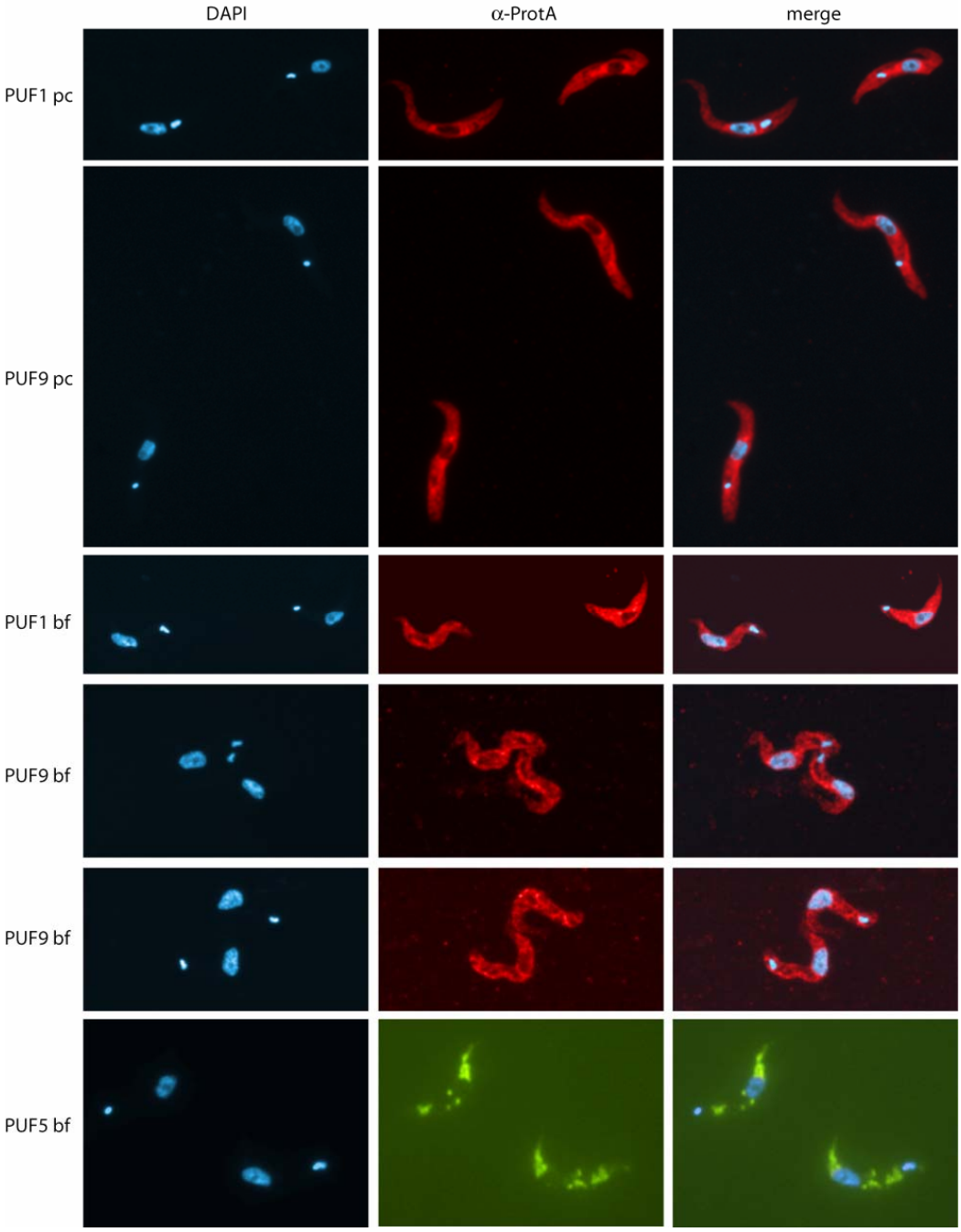


Fig.3.17. Subcellular localization of TAP-tagged *Tb*PUF1 and *Tb*PUF9 in procyclic (pc) and bloodstream (bf) form cells, and of TAP-tagged *Tb*PUF5 in bf cells. DAPI was used to stain the nucleus and the kinetoplast. Anti ProteinA antibody was used to detect the TAP-tagged PUF proteins. An overlay of both images is shown on the right panel.

*Tb*PUF5 bears a mitochondrial localization signal. To assess the possibility of a mitochondrial localization of *Tb*PUF5, immunofluorescene analysis was performed (Fig.3.18.). To this end, bloodstream form cells expressing TAP-tagged *Tb*PUF5 were incubated with MitoTracker Red CMXRos (Molecular Probes) at a final concentration of

0.5µM. The cells were incubated for 10 min. in an open tube in a gassed incubator (5% CO_2), then centrifuged and washed with HMI-9 and reincubated without dye for 20 min. The cells were then fixed for indirect immunofluorescence as described in the Materials & Methods section (2.9.).

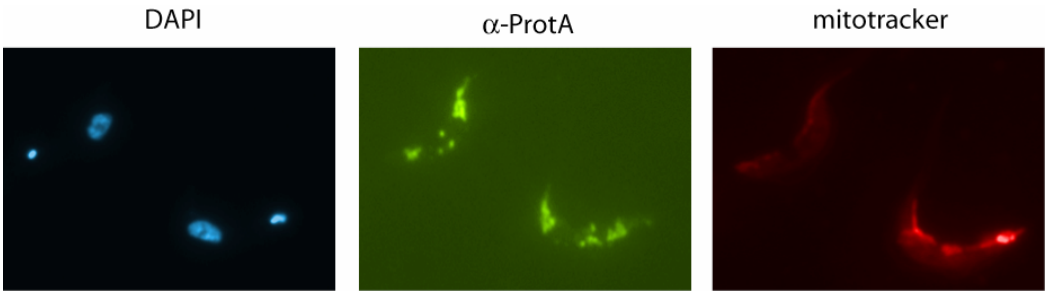


Fig.3.18. No co-localization can be observed for the mitochondrium (stained with mitotracker) and TAP-tagged *Tb*PUF5.

3.6. Testing PUF protein function without knowing the target

To investigate a putative role for PUF protein in gene expression in *T. brucei* independent of target identity we have adopted the method of tethered function analysis developed by (Coller, Gray et al. 1998) and discussed in more detail by (Coller and Wickens 2002). To this end, a chimeric protein consisting of λN linked to *Tb*PUF1 (or *Tb*PUF9) was expressed in *T. brucei* procyclic cells; the same strain also carried a second plasmid expressing the CAT (chloramphenicol acetyl transferase) reporter mRNA with six λN recognition sites in its 3'-UTR. Binding of λN to the boxB element brings the PUF protein in close proximity of the reporter mRNA (Fig.3.19.). Having such a system in our hands, we were then able to assay the biological functions of the tethered PUF protein in terms of mRNA stability modulation (using Northern blot analysis) and control of translation (Western blot and CAT assay) (Fig.3.19.). Northern blot analysis showed that PUF1-λN and PUF9-λN transcripts were expressed - although to somewhat lower level in the PUF1 strain - and that CAT mRNA level stayed constant. However, when looking at the protein level, we were surprised to see that CAT protein amount had increased by about 2-fold in cells where PUF9-λN was expressed, whereas CAT protein expression was not influenced by the expression of PUF1-λN. Furthermore, these results from the Western blot analysis were confirmed by measurement of the CAT activity (Fig.3.20.). We do not know why PUF9 seems to promote translation, since all PUF proteins studied so far have a repressing

function. Obviously, a lack of results in a tethered function assay does not lead to any conclusions. At the same time, results acquired in such an assay should be treated with care, since a tethered function assay system represents a rather artificial situation.

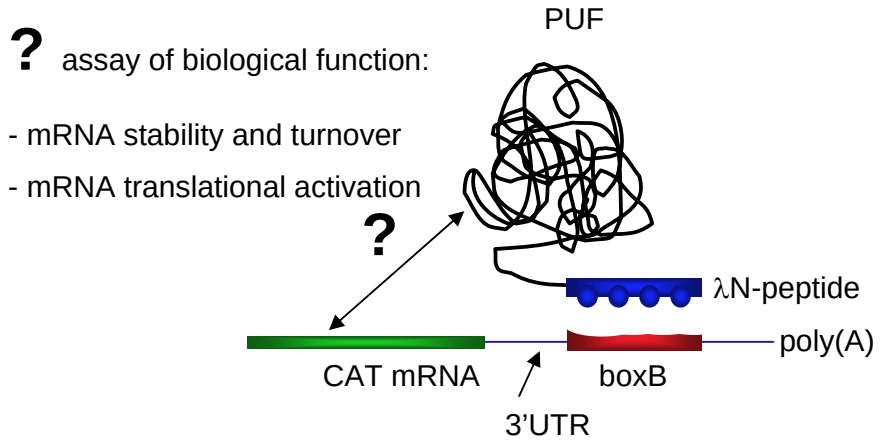


Fig.3.19. Binding of the λN-peptide to the boxB element in the 3'-UTR of the chloramphenicol acetyl transferase mRNA brings the PUF protein in close proximity of the reporter mRNA.

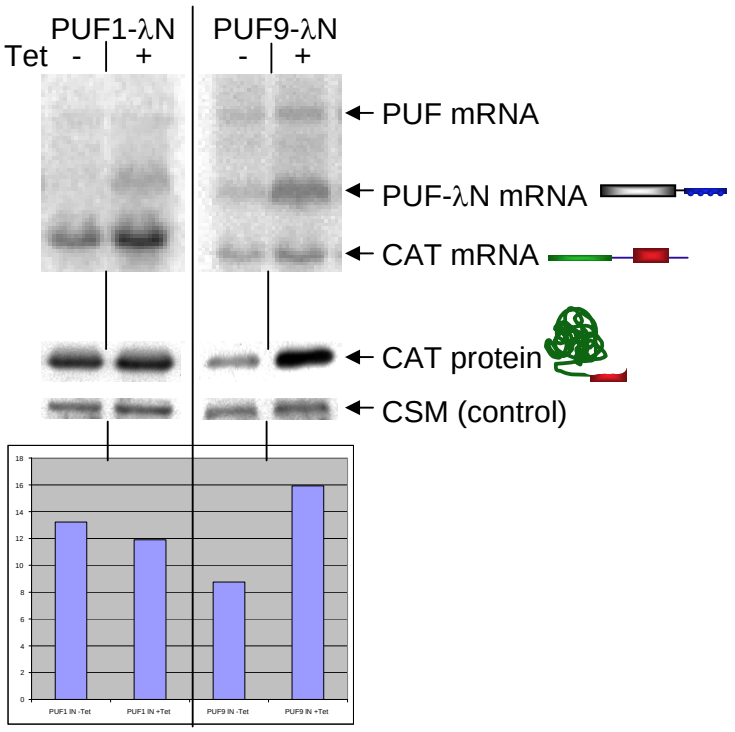


Fig.3.20. Tethered function analysis using tethered PUF protein. a) Northern blot showing that a chimeric puf1-λN (puf9-λN) transcript was expressed upon induction with tetracycline. Endogenous puf transcript remained constant. CAT mRNA is slightly increased in puf1-λN induced cells. b) Western blot analysis showing that CAT protein level remains unchanged for PUF1 but increased for PUF9. CSM (cytosolic marker is used as loading control. c) CAT activity assay confirming the results from b).

3.7. A role for PUF proteins in translational control in *Trypanosoma brucei*

PUF proteins have been shown to be involved in mRNA stability modulation as well as in translational control. To test the latter function in *T. brucei* we asked whether perturbation of PUF protein level would result in altered translational control and therefore differential protein expression of target mRNAs. Two-dimensional gel electrophoresis has become a powerful method to study global gene expression on the protein level. However, comparing different proteomes by classical two-dimensional electrophoresis is challenging and often complicated by substantial gel-to-gel variation. Separating two or more protein samples labeled with different fluorescent dyes in one single gel, as in two-dimensional difference gel electrophoresis (DIGE), reduces this variability considerably. To this end, we have applied the DIGE system (Amersham Biosciences) to compare the global protein levels of *T. brucei* wild-type cells with *Tb*PUF1 knockout cells, and cells uninduced versus induced for PUF RNAi. In addition we performed DIGE analysis with 427 1313 514 bf and pc cells treated and untreated with tetracycline to assess the possibility that the drug per se is controlling translation. Two independent DIGE analyses were performed for each cell line. As can be seen in the tetracycline control, incubation of the cell culture with this drug for 48 hours does not lead to changes in global protein expression level, nor up- or downregulation of specific proteins (Fig.3.21a.). Only one protein was differentially expressed upon depletion of *Tb*PUF1 in bloodstream form cells (Fig.3.21b.), whereas four proteins were downregulated in the ΔPUF1 procyclic cell line compared to wild-type cells (Fig.3.21c.). No differences on protein levels were apparent when comparing *Tb*PUF9 RNAi uninduced and induced procyclic cells (Fig.3.21d.), and one protein (tryparedoxin) was downregulated when depleting *Tb*PUF9 in bloodstream form cells (Fig.3.21d.). However, Western blot analysis of PUF9-RNAi cells using α-tryparedoxin antibody did not confirm DIGE results.

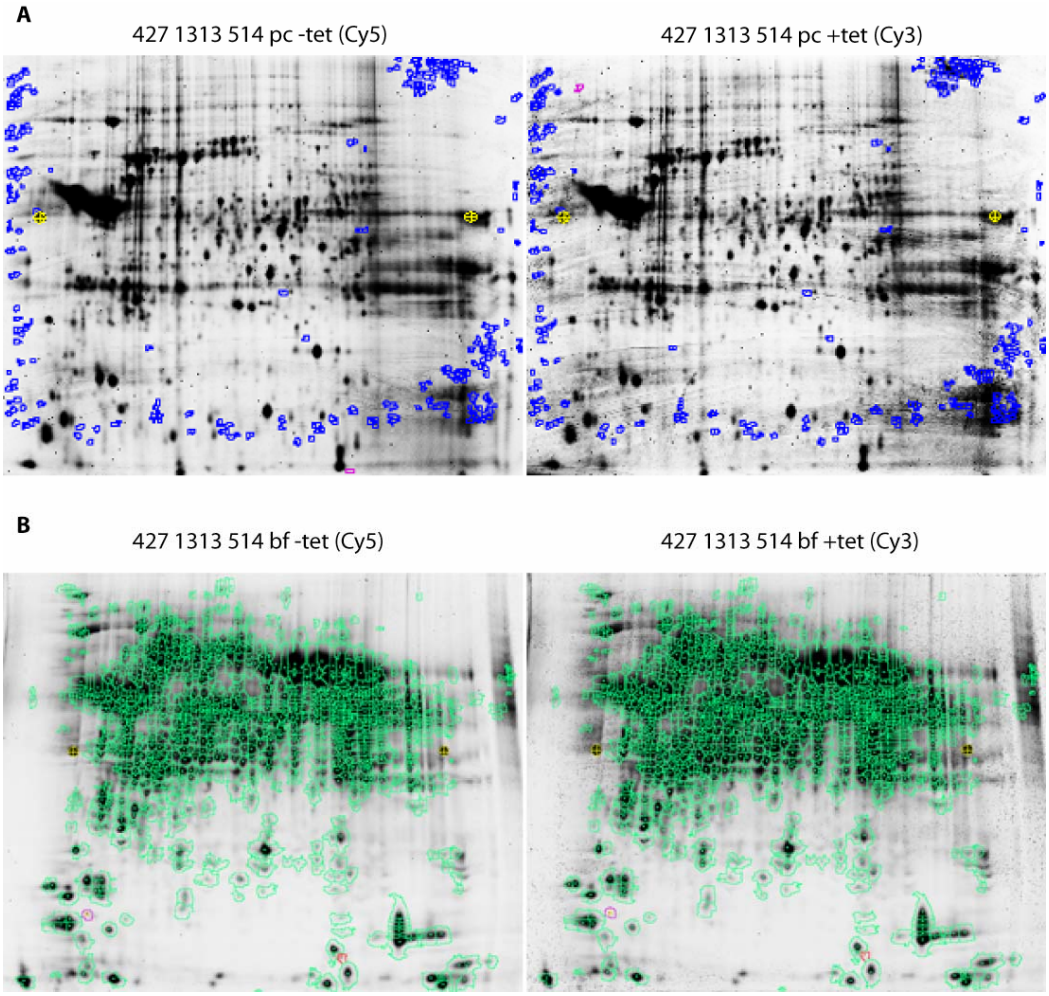


Fig.3.21a. DIGE analysis with tetracycline induced 427 procyclic (A) and bloodstream form (B) cells bearing the tet repressor (1313) and a T7-polymerase (514). Protein extract from uninduced (-tet) and induced (+tet) cells were labelled with Cy5 and Cy3, respectively. Both protein samples were mixed and loaded onto the same 2-dimensional gel.

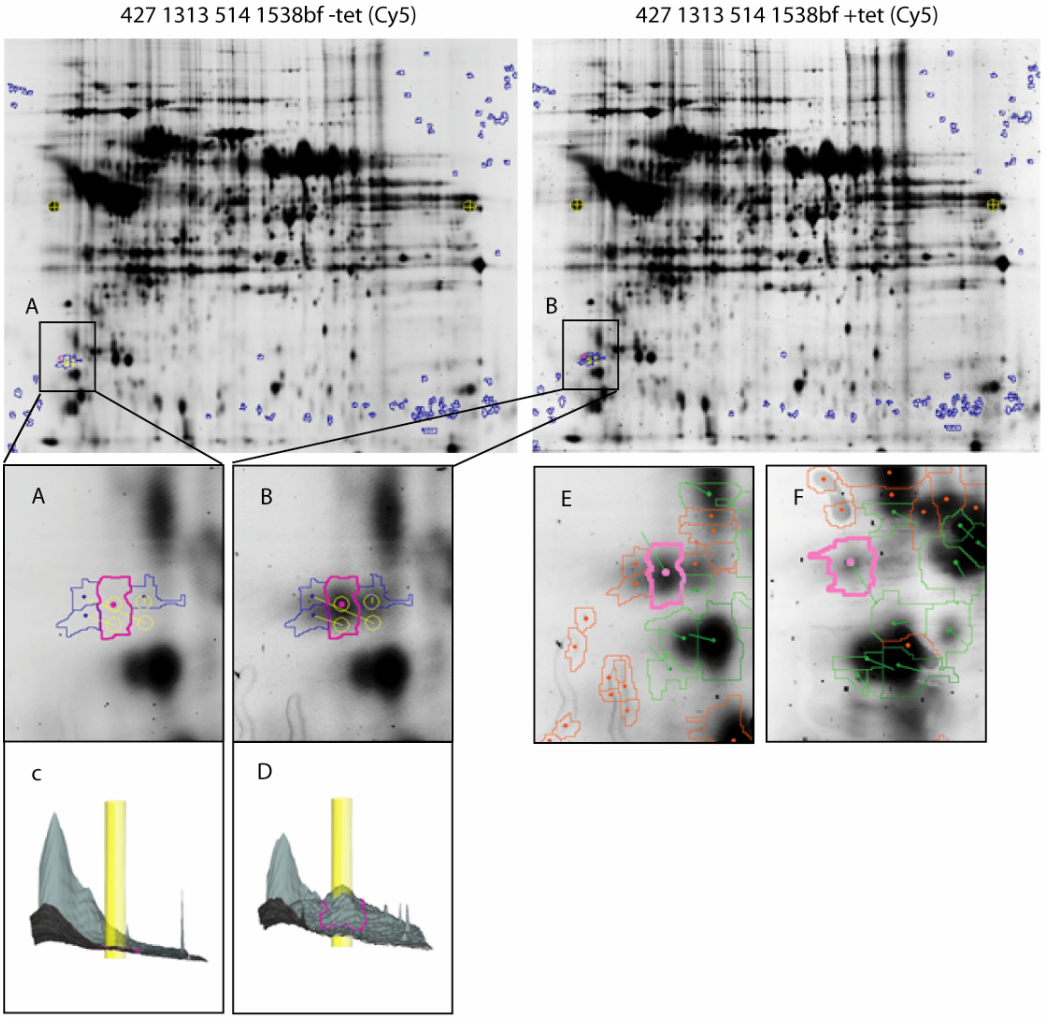


Fig.3.21b. DIGE analysis of *Tb*PUF1-RNAi (1538) bloodstream form cell lines. Cell extracts from uninduced (-tet) and induced (+tet) cells were labeled with Cy5 and Cy3, respectively. Enlargments of A and B are shown. C and D are 3D representations of the spot-intensity. E and F show comparison between the analytical gel (Cy3) and the preparative gel (stained with Sypro Orange). The spot of interest is highlighted in red and after mass spectrometry was identified as a putative phosphatidyl inositol kinase domain protein.

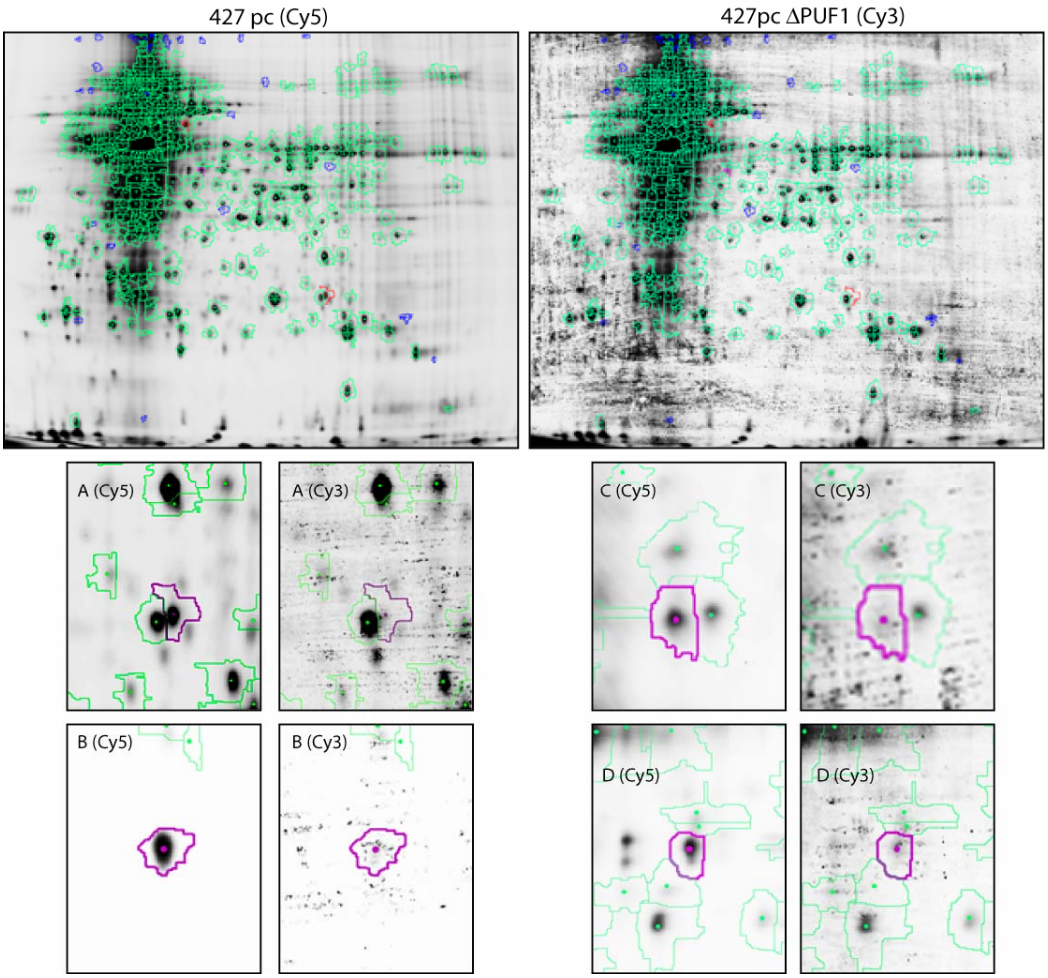
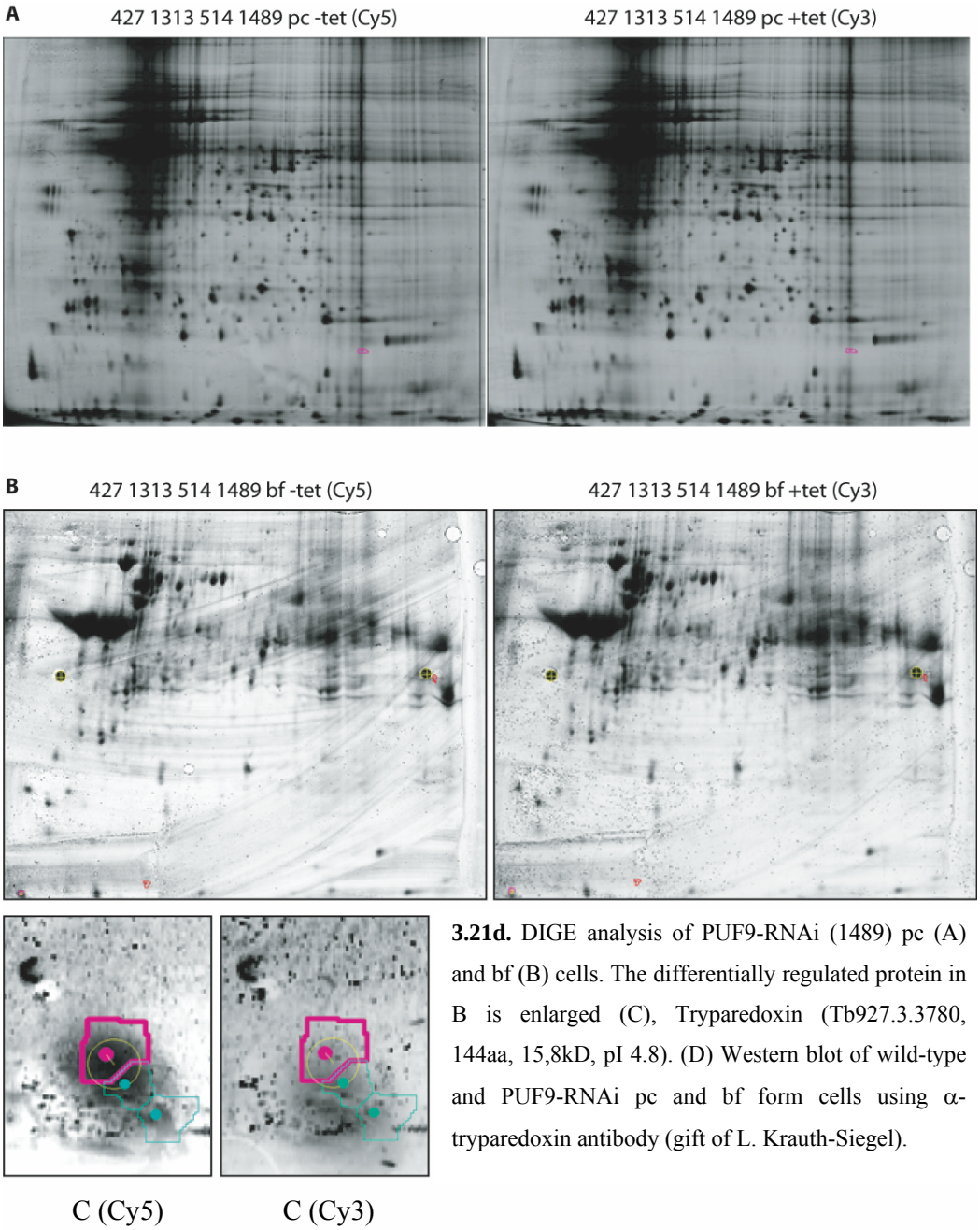
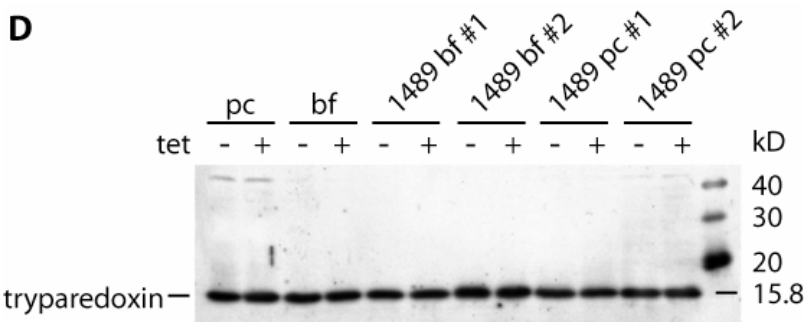


Fig.3.21c. DIGE analysis comparing wild-type procyclic cells (Cy5) with 427 ΔPUF1 pc cells (Cy3). The identity of the differentially regulated proteins are: A) tryparedoxin peroxidase (Tb09.160.4250 199aa, 22.4kD, pI 6.4); B) heat-shock protein 70 (Tb11.01.3110, 690aa, 75.3 kD, pI 6.3); C) ribonucleoprotein, p18, mitochondrial precursor, putative, Tb927.5.1710, 188aa, 21.2kDa, pI 6.7); D) S-adenosylmethionine synthetase, Tb927.6.4840, 397aa, 43.5kD, pI 5.9).



3.21d. DIGE analysis of PUF9-RNAi (1489) pc (A) and bf (B) cells. The differentially regulated protein in B is enlarged (C), Tryparedoxin (Tb927.3.3780, 144aa, 15,8kD, pI 4.8). (D) Western blot of wild-type and PUF9-RNAi pc and bf form cells using α-tryparedoxin antibody (gift of L. Krauth-Siegel).



3.8. *Tb*PUF1 is not associated with polyribosomes

Since preliminary DIGE analysis suggested a role for PUF proteins in translational control, I wanted to investigate whether *Tb*PUF1 might be associated with polysomes. To this end, polysomal fractionation of bloodstream form cells was performed. As can be appreciated from the A_{254} profile (Fig.3.22a.) polysomes were separated from the 80S monosomes and soluble material. Western blot analysis was performed using α-PABP1 antibody to assess for the presence of polysomes (Fig.3.22b.). *Tb*PUF1 did not cosediment with polyribosomes.

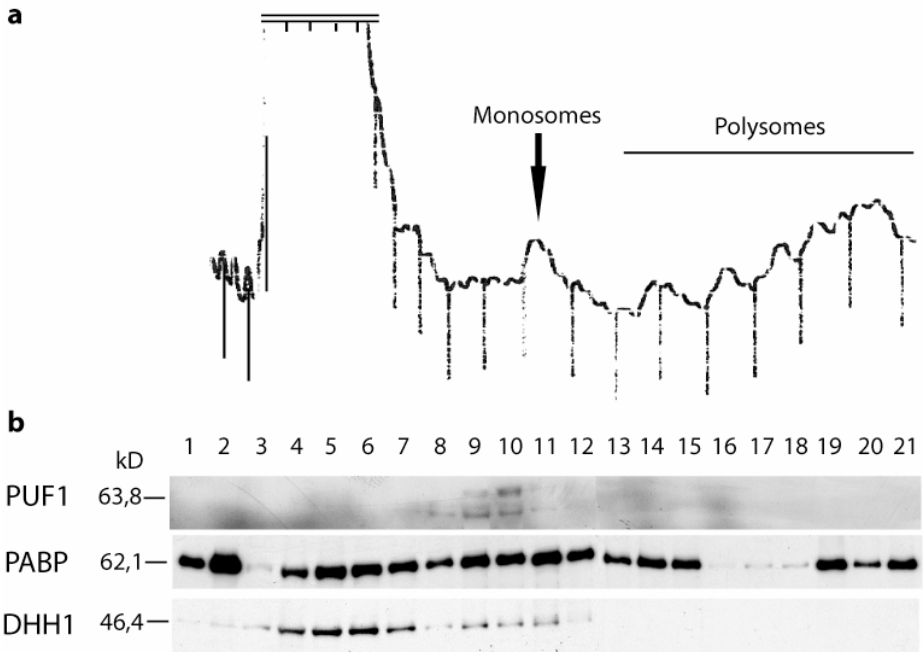


Fig.3.22. *Tb*PUF1 does not cosediment with polyribosomes. Sucrose density gradient analysis of cytoplasmic extracts from *Trypanosoma brucei* bloodstream form cells. (a) Absorbance profile at 254 nm; the positions of the 80S monosome and polyribosomes are indicated. (b) Western blot analysis using α-*Tb*PUF1, α-PABP, and α-DHH1 antibody.

4. Discussion

Two lines of evidence form the basis of this work. First, the phenomenon that gene expression in *Trypanosoma brucei* almost entirely depends on post-transcriptional control. Second, the existence of a family of RNA-binding proteins, which exactly perform this role, namely post-transcriptional gene regulation by promoting mRNA degradation and interfering with translation.

From a naive and somewhat unbiased point of view, PUF proteins could have any possible biological functions in *T. brucei*, e.g. control of life-cycle, stress response, and cell cycle; they could regulate cytokinesis or certain signal transduction pathways, and they could even be implicated in antigenic variation and editing, two processes characteristic to Kinetoplastids. However, based on the biological functions of PUF proteins that have been studied so far, it was found that all of them are involved in regulation of gene expression, and many of them are implicated in developmental control. The first two members of this protein family to be characterized in detail were *Drosophila* Pumilio and *C. elegans* FBF. PUF proteins are found in animals, plants, and fungi. The high degree of sequence conservation of the PUM RNA-binding domain in other far-flung species such as kinetoplastids suggests that the domain is an ancient protein motif, and generally conservation of sequence reflects conservation of function. Most of the discoveries concerning PUF protein functions have been acquired in the model organisms yeast, *C. elegans* and *Drosophila*. So far, nothing is known about the presence of PUF proteins in Archaea or eubacteria.

*Tb*PUF1 was the first member of this family described in an early branching eukaryote (Hoek, Zanders et al. 2002). The observation that most organisms in which PUF proteins have been studied have multiple PUF members lead us to look for other PUF proteins in *T. brucei*. With the recently completed predicted proteome of *T. brucei* (Berriman, Ghedin et al. 2005) and the help of database mining the presence of eight additional PUF proteins became apparent. The high number of PUF protein members in *T. brucei* is in line with the observation that more primitive organisms, such as lower eukaryotes, including *C. elegans* and *S. cerevisiae* possess mutliple PUF proteins, whereas higher organisms (e.g. vertebrates) have only one or two. Only four *T. brucei* PUF proteins (PUF2, PUF3, PUF4, and PUF6) have a full set of eight characteristic PUF repeats. Some PUF repeat sequences

are somewhat divergent from the consensus, and are therefore not recognized by Pfam model. For example *Tb*PUF7 and *Tb*PUF8 are most divergent within *T. brucei*. Their Puf repeats are most divergent from the consensus sequence with major insertions between and even within the Puf repeats. On the other hand the divergence between the Puf domains within *Tb*PUFs may imply that they bind related but distinct mRNA targets and have different roles during parasite development. Recent studies showed that two *C. elegans* PUF proteins, FBF and PUF-8, differ in their RNA-binding specificity which is due to the presence or absence, respectively, of only a single nucleotide in their binding sites (Opperman, Hook et al. 2005). In this context, it is intriguing to think, that deviations from the consensus repeat sequence (e.g. in *Tb*PUF7 and *Tb*PUF8) might lead to disortion of the Puf repeats and altered RNA binding specifity. The smaller PUF proteins in *T. brucei* (e.g. *Tb*PUF5) appear to consist exclusively of the PUF domain. Since this domain was demonstrated to be necessary and sufficient to exert the proteins functions, it is expected that all PUF proteins in *T. brucei* are indeed functional. For example, the PUF domain of yeast Puf1p and Puf2p contains only six Puf repeats recognized by Pfam model (Tadauchi, Matsumoto et al. 2001). In the *T. brucei* PUF proteins, as with PUFs in general, the Puf repeat region is near the carboxyl terminus and the proteins diverge toward the amino terminus. Except for the conserved PUF domains, the *Tb*PUF proteins differ considerably. So far, nothing is known about the role of the N-terminal region. It has been reported that PUF proteins of different species, human, mouse, and Xenopus bind to the *Drosophila hunchback* Nanos Response Element (NRE) *in vitro*. So far, RNA binding activity of kinetoplastid PUF proteins has only been shown for *Tc*PUF1 (previously named *Tc*PUF6), the *Tb*PUF1 homologue in *T. cruzi* (Dallagiovanna, Perez et al. 2005). *Plasmodium falciparum* also has two PUF proteins (*Pf*PUF1 and *Pf*PUF2), which were demonstrated to bind the NRE of *hunchback in vitro* (Cui, Fan et al. 2002; Fan, Li et al. 2004).

Interestingly, each of the *Tb*PUFs has a homologue in *T. cruzi* and *L. major*. Highest homology exists within the PUF8 group with 62% and 52% identity between *Tb*PUF8 and *Tc*PUF8, and *Tb*PUF8 and *Lm*PUF8, respectively. The protein sequence of PUF4 is least conserved among the three species. Overall, *T. brucei* PUF proteins are more similar to their *T. cruzi* than to their *L. major* orthologue. Notably, the PUF proteins are not only highly conserved among Tritryps, but also their genes share high conservation of synteny. For example the downstream gene of *PUF9* in all three pathogens codes for an exosome associated protein EAP1, which is an Rrp42 homologue similar to RNasePH proteins in

bacteria. The upstream gene of *PUF7* in the Tritryp genome codes for a zinc finger protein 2. The high degree of sequence conservation and synteny among Tritryp PUF proteins suggest that they not only share a similar protein structure but also exert similar functions in each of the three pathogens. PUF proteins might have evolved very early in the eukaryotic evolution, possibly at the time when kinetoplastids branched from the eukaryotic lineage. A phylogenetic tree showing the relationship between 90 PUF proteins shows that no clear clustering can be drawn for kinetoplastid PUF protein family members, as is true for yeast PUF proteins. Notably, the few vertebrate PUF proteins that have been identified so far form a distinct group which is most related to dipteran PUF proteins. With the exception of PUF-8 and PUF-9, all *C. elegans* PUF proteins belong to one group. No clear separation between plant and animal PUF proteins can not be drawn, a was previously done comparing only 34 PUF proteins (Cui, Fan et al. 2002). Overall, one could speculate that PUF proteins might have lost their diversity in the course of the evolution.

To investigate the role of PUF proteins in *T. brucei*, RNAi knockdown was performed for each PUF protein in bloodstream and procyclic form cells. It should be noted, that RNAi in this case did not lead to a 100% depletion of the PUF transcripts. Residual PUF transcript (and concomitantly residual protein) was present in all RNAi induced cell lines, even after a prolonged induction of RNAi or incubation with higher tetracycline concentration. With this caveat, none of the nine PUF proteins appears to be essential for the *in vitro* growth of *T. brucei*. It is possible, that they do play a role in differentiation, as this holds true for many organisms where PUF proteins have been studied. For example, Pumilio prevents female *Drosophila* germline stem cells from differentiating prematurely as cystoblasts (Lin and Spradling 1997; Forbes and Lehmann 1998), and *C. elegans* FBF regulates the switch from spermatogenesis to oogenesis and also the maintenance of germline stem cells (Zhang, Gallegos et al. 1997; Crittenden, Bernstein et al. 2002). In this sense, it should be noted that the *T. brucei* cell lines used in this work are monomorphic, i.e. they have lost the ability to differentiate from one life stage (bloodstream) to the other (procyclic form) or vice versa. On the other hand most of the reverse genetics (e.g. tetracycline inducible RNAi) can only be performed in these monomorphic strains. A circumvention of this dilemma could be the use of synthetic siRNAs, which were recently shown to be functional in the parasite (Best, Handoko et al. 2005). Thus, pleiomorphic cell lines could be depleted of PUF proteins using this technique.

It is possible that residual protein due to incomplete efficiency of RNAi could still be sufficient to exert normal cellular function. To check this possibility, a *TbPUF1* knockout was performed in procyclic cells. Surprisingly, deletion of the *TbPUF1* gene was not lethal for procyclic cells, and growth was not affected. This contradicts the results of (Hoek, Zanders et al. 2002). The authors of this work concluded, based on a failure to generate *PUF1* knockout cell lines, that this gene is essential. Furthermore, they showed, that either over- or under-expression of PUF1 reduces growth in culture (Hoek, Zanders et al. 2002), a phenotype which was not observed with our *PUF1* RNAi and overexpression cell lines. To test the hypothesis of PUF1 being involved in differentiation, I tried to knock out *PUF1* in a pleiomorphic (Antat1.1) bloodstream form cell line. However, several attempts to do this failed.

Since *T. brucei* possesses multiple PUF proteins, it is possible that they share redundant functions. This hypothesis is based on several lines of evidence. In *C. elegans*, the two nearly identical (>90%) PUF proteins, FBF-1 and FBF-2, which together are called FBF ('fem-3 mRNA binding factor') share redundant functions as regulators of germline stem cell maintainenance (Crittenden, Bernstein et al. 2002; Lamont, Crittenden et al. 2004). Furthermore, a very recent work by (Bachorik and Kimble 2005) demonstrated that FBF-1 and PUF-8 are redundant in their capacities to control the hermaphrodite sperm/oocyte switch in *C. elegans*. Since most work so far has been done for *Tb*PUF1, the idea was to simultaneously knock-down another PUF protein which is most related to PUF1. Phylogenetic analysis revealed that PUF3 and PUF4 are most likely to share redundant function with PUF1 and with each other. Simultaneous depletion of PUF1/PUF3, PUF1/PUF4, and PUF3/PUF4 pairs in bloodstream form cells were performed, but these cell lines also exhibited normal growth. Of course, RNAi double knockdown of other PUF pairs could be done, and even a triple knockdown would be theoretically feasible. However, the possibility that residual protein due to incomplete efficiency of RNAi could still be sufficient to exert normal cellular function led me to abandon the RNAi approach and instead look for mRNA targets using the RNAi strains which were already generated.

Drosophila Pumilio and *C. elegans* FBF bind to and regulate specific mRNAs, either by promoting mRNA degradation or inhibiting translation. Furthermore, using microarray approaches it was demonstrated that yeast strains which lack all five PUF proteins showed differential expression of several yeast mRNAs (Olivas and Parker 2000). The same work

also showed that one of these five yeast PUF proteins, Puf3p, promotes deadenylation and concomitant degradation of *COX17* mRNA. Preliminary results in *T. brucei* suggested that *Tb*PUF1 is involved in mRNA-stability regulation of several ES-derived mRNAs (e.g. *VSG221* and *ESAG6*). However, the authors emphasized, that the ES-associated mRNAs need not be the primary targets of *Tb*PUF1 (Hoek, Zanders et al. 2002). Based on these observations, the question was whether *Tb*PUF1 is indeed a modulator of mRNA stability and whether perturbation of PUF protein levels in *T. brucei* would influence steady-state levels of specific mRNAs. The method of choice to investigate this question was the use of *T. brucei* genomic DNA microarrays which have been used in other transcriptome studies (Diehl, Diehl et al. 2002; Brems, Guilbride et al. 2005). To this end, I employed DNA microarrays to compare global transcript levels of *T. brucei* wild-type cells with cells where PUF-levels had been perturbed (either by RNAi or overexpression). Notably, only the RNAi-downregulated *Tb*PUF1 transcript was shown to be differentially regulated in the microarray analysis, again contradicting the results from (Hoek, Zanders et al. 2002) mentioned above. Similar results were obtained when studying the transcriptome level of *Tb*PUF9 RNAi cell lines. On the one hand, it is somewhat remarkable, that one single transcript could be detected emphasizing the high integrity of these DNA chips. On the other hand, we were surprised not to see additional differentially regulated transcripts that could be targets of *Tb*PUF1 or *Tb*PUF9.

Since PUF proteins do generally suppress mRNA we hypothesized that overexpression of PUF proteins in *T. brucei* might lead to higher turnover of target mRNAs. As observed with the RNAi analysis, upregulation of the overexpressed PUF transcript was detected in the microarray analysis, again serving as an internal positive control. Notably, transcript levels of specific rRNA spacer region also increased upon overexpression of *Tb*PUF1, *Tb*PUF5, and *Tb*PUF9. Subsequent analysis revealed that the overexpression plasmid integrated into these rRNA spacer regions. However, Northern blot analysis failed to show the differential expression of these rRNA transcripts. It is not understood, why differential expression cannot be observed in the Northern blot analyses. One explanation could be that these rRNA transcripts are too small to be detected on a Northern blot analysis. Notably, when overexpressing *Tb*PUF5 in procyclic cells, *CAP17* mRNA was downregulated. *CAP17* transcript was already found to be upregulated in procyclics in microarray analysis (Diehl, Diehl et al. 2002). CAP17 (corset-associated protein 17) is differentially expressed in the life cycle of *T. brucei* and when overexpressed it induces morphological disorders

and disruptions of processes of the cell cycle (Vedrenne, Giroud et al. 2002). It is not clear, whether the growth phenotype of procyclic PUF5 overexpression cell lines is caused by downregulation of *CAP17*. Unfortunately, attempts to generate *T. brucei* procyclic cell lines that express a tagged version of PUF5 have failed so far. Once such a cell line is established, it will be possible to investigate whether *CAP17* mRNA is associated with *Tb*PUF5 in a messenger ribonucleoprotein (mRNP) complex. Additionally, electrophoretic gel mobility shift assay with recombinant *Tb*PUF5 protein and radioactively labeled *CAP17* mRNA would confirm this putative interaction.

Although members of the PUF protein family are widely spread throughout eukaryotes, only a few of their targets have been identified so far. Examples of specific interactions between PUF protein and mRNA are Drosophila PUM and *hunchback* mRNA, FBFs and *fem-3* and *gld-1* mRNA, respectively, and Puf3p and *COX17* mRNA (Wickens, Bernstein et al. 2002). In vitro RNA binding activity has been shown for three parasite PUF proteins, *Trypanosoma cruzi Tc*PUF6 (Dallagiovanna, Perez et al. 2005), *Plasmodium falciparum Pf*PUF1 (Cui, Fan et al. 2002) and *Pf*PUF2 (Fan, Li et al. 2004). However, it has to be emphasized that in all three cases, the bound mRNA (*hunchback*) was not the endogenous target. In yeast, each of the five PUF proteins associates specifically with distinct groups of functionally and cytotopically related mRNAs (Gerber, Herschlag et al. 2004). To investigate whether this also holds true for *T. brucei* PUF proteins, I have adopted a similar approach, namely a combination of affinity purification of tagged PUF proteins and microarray analysis of RNA that are associated with the purified mRNP complexes. Preliminary results indicated that PUF5 selectively associates with amino acid transporter mRNAs in bloodstream form cells. Interestingly, PUF1 mRNA was also enriched during this mRNP purification. It is intriguing to think, that PUF proteins might regulate themselves. But much more work has to be done, to underlie this hypothesis. However, the vast majority of bound mRNAs has not yet been identified by sequencing of the genomic clones. This will be done together with additional mRNP purifications confirming specific PUF-mRNA interaction. However, a drawback of this large-scale approach, the possibility that PUF proteins bind their physiological targets only transiently and under certain conditions, has to be taken into account.

So far, PUF proteins were shown to interact with members of three protein families (Nanos, CPEB, and Brat). No homologues of these PUF interactors are found in the

genome of protozoa. Interaction of *Tb*PUF1 and *Tb*ESAG8 was demonstrated by (Hoek, Zanders et al. 2002), however this result were somewhat preliminary. To look for *Tb*PUF1 and *Tb*PUF9 binding partners I have used the tandem affinity purification (TAP) approach. This method, as well as co-immunoprecipitation strategies, have failed to identify *Tb*PUF1 interaction partners. It is possible that binding with the interactors is only transient and therefore not possible to detect by the TAP approach. Another possibility is that the TAP tag interferes with binding to the interacting partners. Mutational and structural analyses indicate that the carboxy-terminal region of *Drosophila* Pumilio confers binding to two other proteins, Nos and BRAT. Both endogenous *PUF1* (*PUF9*) alleles have to be deleted to show that TAP-tagged PUF1 (PUF9) is functional. This has not been done so far.

*Tb*PUF1 is localized to the cytosol (Hoek, Zanders et al. 2002). TAP-tagged *Tb*PUF5 in bloodstream form also resides in the cytoplasm, with a somewhat uneven distribution. This pattern can also be observed for *Tb*PUF9-TAP in both bloodstream and procyclic forms. Obviously, the considerably large tag could interfere with the physiological localization. It should be noted, that all tagged PUF proteins are overexpressed, and that unphysiological protein level can also cause protein mislocalization. In yeast each of the five members of the PUF-familiy is associated with specific mRNAs at discrete locations in the cytoplasm (Gerber, Herschlag et al. 2004). It is possible that in *T. brucei*, too, PUF proteins act at distinct locations. Further studies to underlie this hypothesis have to be performed.

So far, only a few mRNA targets for *T. brucei* PUF1, PUF5, and PUF9 were found using microarray analysis and mRNP affinity purification approaches. However, these results are somewhat preliminary, and the possibility of missed mRNA targets should not be underestimated. To study the role of PUF proteins in *T. brucei* without the identity of an mRNA target, we have adopted the method of tethered function analysis developed by (Coller and Wickens 2002). In this approach the PUF protein is brought into close proximity of a reporter (CAT, chloramphenicol acetyl transferase) mRNA, which is then assayed for mRNA stability and translatability. Northern blot analysis showed that neither PUF1 nor PUF9 influenced the stability of the reporter mRNA. However, Western blot analysis and CAT activity assays indicated that PUF9 increases translation, something which was not observed for PUF1. These results were surprising because all PUF proteins studied so far have a repressing function, i.e. promoting mRNA degradation or interfering with translation.

The possibility that PUF proteins are involved in translational control in *T. brucei* was further investigated by two-dimensional (2-D) gel electrophoresis approaches. Due to substantial gel-to-gel variation when using classical 2-D gel electrophoresis systems, I took advantage of the new DIGE system (described in section 2.14.). Preliminary results showed that incubation of *T. brucei* culture with tetracycline had no effect on global protein expression levels nor differential regulation of specific proteins. One protein, a putative phosphatidyl inositol kinase domain protein, was upregulated when depleting PUF1 in bloodstream form cells, and four proteins (tryparedoxin peroxidase, heat-shock protein 70, ribonucleoprotein p18, and S-adenosylmethionine synthetase) were downregulated in the procyclic PUF1 knockout cell line.

The work of this thesis, the characterization of PUF proteins and their role in gene expression in *T. brucei* has set the foundation for future work concerning this family of RNA-binding proteins. The unique position of trypanosomes in the eukaryotic evolution means that the study of this –or other protein families- is vital in determining its evolutionary conservation, and hence its importance.

5. General abbreviations

aa	amino acid
Amp	ampicillin
APS	ammonium persulfate
ATP	adenosine-5-triosephosphate
bf	bloodstream form
bp	base pairs
BSA	Bovine Serum Albumin
bsd	blasticidin
CAT	chloramphenicol acetyl tranferase
DAPI	4’, 6’-diamidino-2-phenylindole
dATP	2’-deoxyadenosine 5’-triphosphate
dCTP	2’-deoxycytidine 5’-triphosphate
DEPC	diethyl pyrocarbonate
dGTP	2’-deoxyguanosine 5’-triphosphate
DIGE	differential gel electrophoresis
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphates
dsRNA	double-stranded RNA
DTT	1,4-Dithiothreitol
dTTP	2’-deoxythymidine 5’-triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenedinitrilo tetraacetic acid
et al.	and others
ESAG	expression site associated gene
FCS	fetal calf serum
fig.	figure
hyg	hyrgomycin
IPTG	isopropyl-β-D-thiogalactopyranoside
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
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
pBS	plasmid Bluescript
pc	procyclic
PCR	polymerase chain reaction
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
U, u	unit
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-galactosidas

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