

# Multifunctional class I transcription in *Trypanosoma brucei* depends on a novel protein complex

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The vector-borne, protistan parasite *Trypanosoma brucei* is the only known eukaryote with a multifunctional RNA polymerase I that, in addition to ribosomal genes, transcribes genes encoding the parasite's major cell-surface proteins—the variant surface glycoprotein (VSG) and procyclin. In the mammalian bloodstream, antigenic variation of the VSG coat is the parasite's means to evade the immune response, while procyclin is necessary for effective establishment of trypanosome infection in the fly. Moreover, the exceptionally high efficiency of mono-allelic VSG expression is essential to bloodstream trypanosomes since its silencing caused rapid cell-cycle arrest *in vitro* and clearance of parasites from infected mice. Here we describe a novel protein complex that recognizes class I promoters and is indispensable for class I transcription; it consists of a dynein light chain and six polypeptides that are conserved only among trypanosomatid parasites. In accordance with an essential transcriptional function of the complex, silencing the expression of a key subunit was lethal to bloodstream trypanosomes and specifically affected the abundance of rRNA and VSG mRNA. The complex was dubbed class I transcription factor A.

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## Introduction

The protistan parasite *Trypanosoma brucei* is transmitted by *Glossina* spp. and lives freely in the bloodstream and extracellular tissue spaces of its mammalian host, causing the lethal disease known as African Sleeping Sickness in humans. Key factors for successful *T. brucei* infection of mammalian and insect hosts are the parasite's variant surface glycoprotein (VSG) and procyclin, respectively. In the mammalian bloodstream, antigenic variation of the dense VSG coat is the parasite's means to evade the immune system (reviewed by Pays *et al*, 2007), whereas procyclins are important to establish an infection in the fly midgut (Ruepp *et al*, 1997).

VSG and *procyclin* genes are expressed with an extremely high efficiency in bloodstream and procyclic stages, respectively. The cell coat of a bloodstream trypanosome consists of 10<sup>7</sup> identical VSG molecules, which are derived from a single gene drawn from a large VSG repertoire. Accordingly, *in vivo* RNA labeling experiments indicated that VSG mRNA synthesis is approximately 100 times higher than that of a single  $\beta$ -tubulin gene (Ehlers *et al*, 1987). This level of VSG expression is essential for parasite growth and persistence of infection: RNAi-mediated VSG silencing caused rapid cell-cycle arrest *in vitro* and efficient clearance of trypanosomes from the bloodstream of infected mice (Sheader *et al*, 2005). The requirement for this extraordinarily high expression is probably the reason why *T. brucei* has evolved the unique capability of utilizing the high efficiency of RNA polymerase (pol) I-mediated transcription for VSG and procyclin genes (Kooter and Borst, 1984; Rudenko *et al*, 1989; Clayton *et al*, 1990; Günzl *et al*, 2003). In other eukaryotes, RNA pol I exclusively transcribes ribosomal RNA genes (*RRNA*), as is also the case in trypanosomes, whereas mRNA is invariably synthesized by RNA pol II. This clear division of function is most likely due to the specific association of the mRNA capping enzyme with RNA pol II and the co-transcriptional mode of mRNA capping (McCracken *et al*, 1997; Yue *et al*, 1997). In trypanosomes, however, protein-coding genes are arranged in long tandem arrays that are transcribed in a polycistronic manner. Individual mRNAs are resolved from their precursors by *trans* splicing a 39-nt 5' leader sequence, derived from a capped RNA pol II-synthesized spliced leader donor RNA (SL RNA), and by polyadenylation. This post-transcriptional capping process uncouples capping from mRNA synthesis and enables *T. brucei* to efficiently express protein-coding genes by RNA pol I (Rudenko *et al*, 1991; Zomerdijk *et al*, 1991) or even by bacteriophage RNA polymerases (Wirtz *et al*, 1994).

The active VSG gene is always located in a telomeric expression site (ES), whereas there are two chromosome-internal loci of procyclin genes, termed *GPEET* and *EPI*. While the VSG promoter is very short with two distinct sequence boxes upstream of the transcription initiation site (TIS; Vanhamme *et al*, 1995; Pham *et al*, 1996), the *RRNA*, *GPEET*, and *EPI* promoters are larger and consist of four

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distinct domains (Sherman *et al*, 1991; Brown *et al*, 1992; Janz and Clayton, 1994; Laufer and Günzl, 2001). Although there are no obvious sequence homologies between these promoters, an *in vitro* transcription competition study indicated that they bind a common *trans*-activating factor (Laufer and Günzl, 2001). In addition to the structural variation among *T. brucei* class I promoters, the nuclear distribution of RNA pol I makes class I transcription in this parasite even more complex. While in other eukaryotes, RNA pol I transcribes *RRNA* genes exclusively in the nucleolus, in bloodstream *T. brucei* RNA pol I is sequestered in the nucleolus and in a novel DNase I-resistant compartment dubbed the ES body (Navarro and Gull, 2001). It was hypothesized that this compartment accommodates only a single ES, ensuring mono-allelic *VSG* expression.

Despite the importance and multifaceted nature of class I transcription in *T. brucei*, a class I transcription factor has not been identified and our knowledge of the transcription machinery has been restricted to conserved subunits of RNA pol I (Walgraffe *et al*, 2005; Nguyen *et al*, 2006). Only very recently, a novel RNA pol I subunit with an essential transcriptional function was characterized (Nguyen *et al*, 2007). Our knowledge about class I transcription factors stems from research on mammals and the budding yeast *Saccharomyces cerevisiae* (recently reviewed in Grummt, 2003; Russell and Zomerdijk, 2005). In mammals, the basal *RRNA* promoter-binding factor is termed selectivity factor 1 (SL-1) in humans and TIF-1B in the mouse. It consists of the TATA-binding protein (TBP) and four TBP-associated proteins (TAFs). The interaction between RNA pol I and SL1 is mediated by a single polypeptide-dubbed RRN3 in humans or TIF-1A in the mouse, and activated *RRNA* transcription requires a dimer of the 94 kDa-large upstream binding factor UBF. In yeast, RRN3 is conserved and TBP stimulates RNA pol I transcription in the absence of TAFs, whereas the three subunits of the core factor, which is the functional equivalent of SL1, and the six subunits of the upstream activation factor, share no sequence similarity with the mammalian proteins.

Here, we have purified and characterized a protein that recognizes the *VSG* ES promoter with sequence specificity. We show by independent criteria that this protein is indispensable for class I transcription *in vitro* and for *VSG* and *RRNA* expression *in vivo*. Tandem affinity purification of the protein revealed a complex of seven subunits that contained five additional parasite-specific polypeptides and, surprisingly, a dynein light chain. We demonstrated that the protein complex is the functional entity that binds to the *VSG* ES promoter and conclude that we have characterized a novel multi-subunit transcription factor which we named class I transcription factor A (CITFA).

## Results

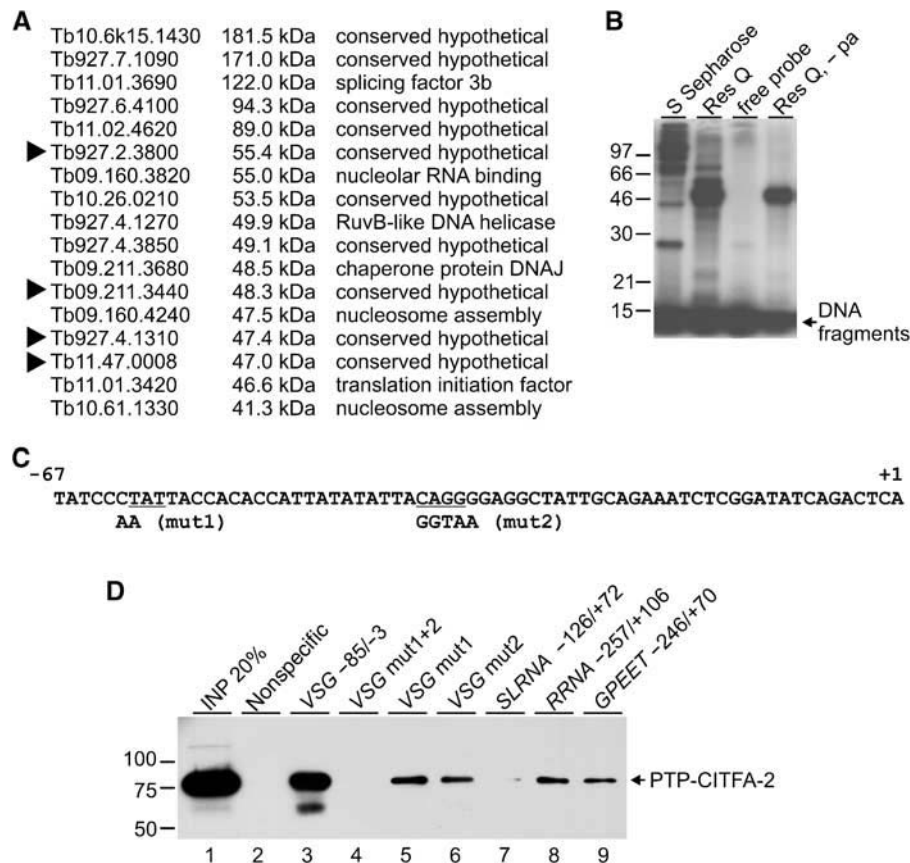
### Identification of two *VSG* ES promoter-binding proteins

In a previous study, an electrophoretic mobility shift assay (EMSA) was used to convincingly demonstrate specific protein binding to the *VSG* ES promoter in crude trypanosome extracts (Pham *et al*, 1997). These extracts were prepared from procyclic trypanosomes because, in contrast to bloodstream forms, procyclics can easily be grown in large numbers *in vitro* and because the *VSG* ES promoter can direct accurate transcription in procyclic cells (Lee and Van der

Ploeg, 1997, and references therein) and in procyclic extracts (Laufer *et al*, 1999). To identify factors that specifically interact with the *VSG* ES promoter, we grew 301 of procyclic forms, prepared a crude extract and, by testing individual fractions with EMSA, purified the *VSG* ES promoter-binding activity by a combination of ion exchange, heparin affinity, and DNA affinity chromatography (data not shown). Mass spectrometric analysis of the promoter-binding activity revealed 17 proteins with calculated masses ranging from 41.3 to 181.5 kDa (Figure 1A). While some of these proteins probably co-purified due to nonspecific DNA-protein interactions, a protein with an apparent size of ~50 kDa could be specifically UV-crosslinked to *VSG* ES promoter DNA (Figure 1B). For further analysis, we therefore chose four proteins (Figure 1A arrowheads) with a predicted size in the 50 kDa range that had been annotated as 'conserved hypothetical proteins' in the *T. brucei* genome database (<http://www.genedb.org/genedb/tryp/index.jsp>). The four proteins were C-terminally tagged in individual cell lines either with the HA epitope or the 19-kDa PTP tag (Schimanski *et al*, 2005b), which is suitable for tandem affinity purification. The DNA-binding activity of these proteins was tested by promoter pull-down and immunoblot experiments, using a *VSG* ES promoter containing the wild-type sequence or a few point mutations in either or both of the two essential sequence elements (Figure 1C), and a nonspecific control DNA. In addition, DNAs comprising the complete *SLRNA*, *RRNA* and *GPEET* promoters were co-analyzed. The proteins encoded by *Tb927.2.3800* and *Tb927.4.1310* bound to DNA in a nonspecific manner (data not shown), whereas the wild-type *VSG* ES promoter bound the *Tb09.211.3440* protein very efficiently, and the nonspecific control and the class II *SLRNA* promoter DNA did not (Figure 1D: compare lane 3 with lanes 2 and 7). Binding of this protein to the *VSG* ES promoter required the integrity of both promoter elements, because mutation of either element strongly diminished the binding activity, and mutation of both elements abolished it completely (lanes 4–6). Albeit to a lesser extent, *Tb09.211.3440* reproducibly bound to the other two class I promoters, which raised the possibility that this protein is a general class I transcription factor in *T. brucei* (compare lane 3 with lanes 8 and 9). The pull-down results with protein *Tb11.47.0008* were similar but the binding specificity was not as clear (data not shown). We therefore continued our analysis with *Tb09.211.3440*, which from now on we refer to as CITFA-2 (as will become apparent below, CITFA-2 is the second largest subunit of the complex).

### Silencing of CITFA-2 expression is lethal

We silenced the expression of CITFA-2 in procyclic and bloodstream *T. brucei* by using an inducible RNA interference system that is based on stable transfection of cells expressing both the tetracycline repressor and T7 RNA polymerase (Wirtz *et al*, 1999). We first cloned 530 bp of the CITFA-2-coding region into the inducible construct pZJM, which harbors T7 but no class I promoters (Wang *et al*, 2000). While this strategy led to inducible procyclic cell lines, it failed to generate corresponding bloodstream forms. We therefore modified the available and more tightly regulated stem-loop vector for dsRNA synthesis (Shi *et al*, 2000) by replacing the inducible class I promoter with an inducible T7 promoter and by inserting two T7 terminators downstream of



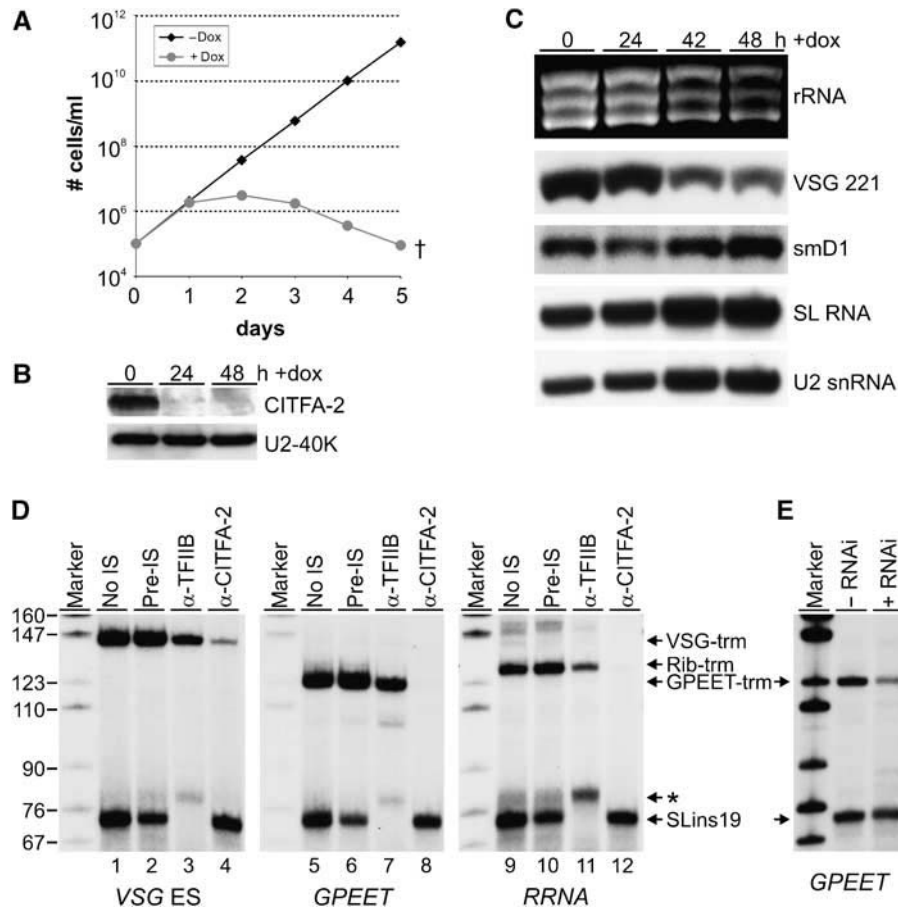
**Figure 1** Identification of VSG ES promoter-binding proteins. (A) List of identified proteins with their GeneDB gene accession numbers, calculated molecular weights, and current GeneDB annotations. Arrowheads indicate proteins that were further analyzed. (B) Autoradiograph of proteins from S-Sepharose or Resource Q (Res Q) fractions that were UV crosslinked to radio-labeled VSG ES promoter, DNase-digested, and separated by SDS-PAGE. -pa, no polyamines added. (C) Sequence of the VSG ES 118 promoter from position -67 to +1 relative to the TIS. The important residues of boxes 1 and 2 are underlined and the point mutations are indicated below the wild-type sequence. (D) Immunoblot of PTP-CITFA-2 subsequent to promoter pull-downs carried out with a nonspecific DNA, with VSG ES promoter DNAs as indicated above, and with wild-type *SLRNa*, *RRNa*, and *GPEET* promoter DNAs. The numbers indicate the end positions of the DNA fragments relative to the TIS.

the dsRNA cassette before cloning the *CITFA-2* sequence in opposite orientations into this T7-stl vector (Supplementary Figure S1). While *T. brucei* growth is not affected by the doxycycline concentrations used (Luu *et al* (2006) and data not shown), procyclic and bloodstream cell lines that were stably transfected with these plasmids rapidly ceased growth when *CITFA-2* dsRNA expression was induced (Figure 2A and data not shown). Typically, cell growth was inhibited after 24 h and cell cultures died out after day 5, indicating that *CITFA-2* is essential for both life-cycle stages. Semiquantitative RT-PCR revealed that *CITFA-2* mRNA abundance was specifically reduced in induced cells within the first 24 h (data not shown). To evaluate the *CITFA-2* protein levels in these cells, we raised a rat polyclonal antiserum against the N-terminal half of *CITFA-2*, which recognizes the endogenous protein with high specificity (Supplementary Figure S2). On immunoblots, *CITFA-2* levels were seen to be strongly reduced, 24 and 48 h after RNAi induction, which confirmed effective silencing of *CITFA-2* expression in these cells (Figure 2B). Analysis of total RNA prepared from non-induced and induced bloodstream cells showed that the abundance of RNA pol I-synthesized 18S, 28S $\alpha$  and 28S $\beta$  rRNAs and of VSG 221 mRNA was clearly reduced 42 and 48 h after induction (Figure 2C). Concomitant with the decrease of these major RNAs, the RNA pol II transcripts smD1 mRNA

and SL RNA and the RNA pol III transcript U2 snRNA increased in these samples of equal amounts. When standardized against smD1 signals, rRNA/VSG mRNA decreased to 56/44% and 30/29% 42 and 48 h after induction, respectively. The latter numbers are in close accordance with the 73% reduction of rRNA found upon silencing an essential RNA pol I subunit in procyclic trypanosomes (Nguyen *et al*, 2007). We therefore concluded that silencing of *CITFA-2* specifically affected RNA pol I transcripts. Together with our finding that *CITFA-2* specifically bound to class I promoters, these results suggested that this protein is involved in class I transcription.

#### ***CITFA-2* is essential for class I transcription *in vitro***

To directly evaluate the role of *CITFA-2* in class I transcription, we employed a homologous *in vitro* transcription system. This system, which is based on a crude mix of cytoplasmic and extracted nuclear components of procyclic trypanosomes, is active for both class I and class II *SLRNa* transcription (Laufer *et al*, 1999). The template plasmids VSG-trm, GPEET-trm and Rib-trm, which contain a VSG ES, the *GPEET* procyclin and an *RRNa* promoter, respectively, were co-transcribed with the *SLRNa* template SLins19. These templates contain insertions of unrelated oligonucleotide sequences downstream of the transcription initiation site,



**Figure 2** CITFA-2 is an essential class I transcription factor. (A) Growth curve of a bloodstream-form RNAi cell line in the presence (circles, gray) or absence (diamonds, black) of doxycycline, which induces the expression of *CITFA-2* dsRNA. (B) Immunoblot of whole-cell lysates prepared from bloodstream RNAi cells before and 24 and 48 h after induction of *CITFA-2* dsRNA synthesis. Detection of the nuclear protein U2-40K served as a loading control. (C) Analysis of total RNA prepared from noninduced cells and from cells 24, 42, and 48 h after induction. The rRNAs were visualized by ethidium bromide staining, VSG 221 and smD1 mRNAs by hybridization with appropriate probes, and the small SL and U2 RNAs by a primer extension assay. (D) *In vitro* transcription of the class I templates VSG-trm, GPEET-trm, and Rib-trm and the class II template SLins19 in the absence of rat serum (no IS) or in the presence of anti-CITFA-2 pre-immune serum (pre-IS), TFIIB antiserum ( $\alpha$ -TFIIB), or CITFA-2 antiserum ( $\alpha$ -CITFA-2). The indicated transcription signals were obtained by primer extension assays and extension products were separated by denaturing PAGE and visualized by autoradiography. The asterisk designates an aberrantly initiated SLins19 transcript caused by the TFIIB antiserum. Marker, pBR322-*MspI*. (E) Co-transcription of GPEET-trm and SLins19 in extracts of procyclic 29-13 cells in which *CITFA-2* silencing was not induced (–RNAi) or induced for 36 h (+RNAi).

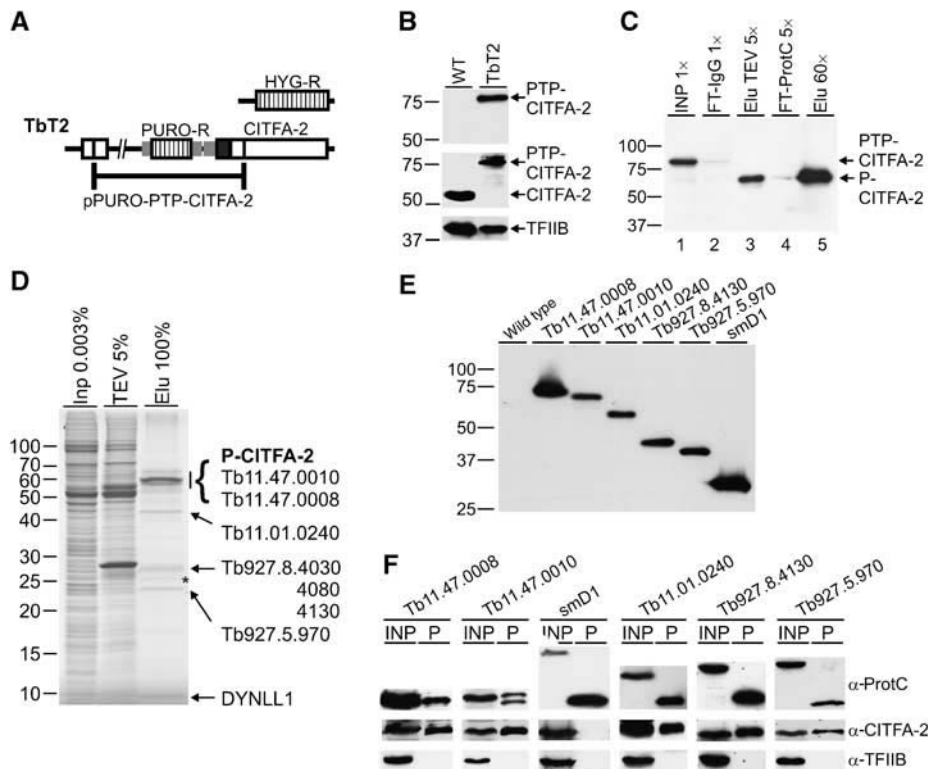
which allow specific detection of transcripts by primer extension assays. In a first analysis, we tested whether the addition of CITFA-2 antiserum affected RNA pol I-mediated transcription *in vitro*. In these reactions, pre-immune serum did not significantly affect transcription of either the class I templates or of SLins19 (Figure 2D, compare lanes 2, 6, and 10 with lanes 1, 5, and 9, respectively). When we added, as another control, a similarly derived antiserum directed against the general class II transcription factor TFIIB of *T. brucei* (Schimanski *et al*, 2006), correctly initiated SLins19 transcription was abolished whereas class I transcription was only mildly affected, most likely due to a nonspecific effect from this particular serum (lanes 3, 7, and 11; Nguyen *et al*, 2007). In contrast, anti-CITFA-2 serum specifically interfered with class I transcription. While VSG-trm transcription was strongly reduced (lane 4), GPEET-trm and Rib-trm transcription was abolished (lanes 8 and 12). This differential and reproducible effect may be a consequence of higher affinity binding of CITFA-2 to the VSG ES promoter (see Figure 1D).

To confirm this observation, we prepared transcription extracts from procyclic cells in which *CITFA-2* expression

was silenced for 36 h, and from non-RNAi-induced cells. *CITFA-2* silencing was less effective in procyclic than in bloodstream RNAi cells and reduced the CITFA-2 protein level by only 64% (Supplementary Figure S3). Nevertheless, when GPEET-trm, which drives the most efficient class I transcription in this *in vitro* system (Laufer *et al*, 1999), was co-transcribed with SLins19, GPEET-trm transcription was decreased by ~73% when *CITFA-2* was silenced, whereas the same extract supported SLins19 transcription nearly as well (~13% reduction) as the extract prepared from noninduced cells (Figure 2E). Moreover, as a third criterion, depletion of CITFA-2 from the extract by IgG affinity chromatography specifically abolished class I transcription (see below, Figure 6). We therefore concluded that CITFA-2 is a general class I transcription factor in *T. brucei*.

#### Six proteins specifically co-purify with CITFA-2

While a single CITFA-2 ortholog is encoded in the genomes of the trypanosomatids *Trypanosoma vivax*, *Trypanosoma cruzi*, *Leishmania major* and *Leishmania infantum* (for a sequence alignment, see Supplementary Figure S4), we were unable to



**Figure 3** Six proteins co-purify with CITFA-2. (A) Schematic depiction (not to scale) of the *CITFA-2* gene locus in cell line TbT2. In one allele, the *CITFA-2*-coding region was replaced by a hygromycin-resistance gene (HYG-R) and in the second allele the PTP sequence was fused to the 5' end of the coding region by targeted insertion of pPURO-PTP-CITFA-2. Coding regions are represented by open boxes, the PTP tag by a black box, and introduced gene flanks by small gray boxes. (B) Immunoblot analysis of PTP-CITFA-2 in extracts of wild-type (WT) and TbT2 cells. The tagged protein was detected with the protein A-specific PAP reagent (top panel) or with the polyclonal anti-CITFA-2 serum (middle panel). Protein loading was controlled by reprobing the same blot with an antibody against *T. brucei* TFIIB. (C) Immunoblot monitoring of PTP-CITFA-2 purification. Aliquots of the input material (INP), the flow-through of the IgG affinity chromatography (FT-IgG), the TEV protease elution (Elu TEV), the flow-through of the anti-ProtC affinity chromatography (FT-ProtC), and the final EGTA eluate (Elu) were separated on a 10% SDS/polyacrylamide gel, blotted, and probed with anti-ProtC antibody. The relative amount of each sample to the input material is specified. It should be noted that the size of the tagged protein (PTP-CITFA-2) was reduced by ~15 kDa after protease cleavage (P-CITFA-2). (D) Coomassie staining of purified proteins. The total eluate of a standard PTP-CITFA-2 purification was separated on a 15% SDS/polyacrylamide gel and stained with Coomassie. For comparison, 0.003% of the input material (Inp) and 5% of the TEV protease eluate (TEV) were loaded. On the right, proteins identified by mass spectrometry are specified by their GeneDB accession numbers or protein name. The asterisk marks a minor IgG kappa light chain contamination of the anti-ProtC matrix. (E) Immunoblot of whole-cell lysates derived from cell lines that express the proteins of the indicated genes as C-terminal PTP fusions. In a control cell line, the spliceosomal smD1 protein was PTP-tagged. (F) Co-precipitation of CITFA-2 with PTP-tagged proteins. Proteins were eluted from IgG beads either with glycine (Tb11.47.0008 and Tb11.47.0010) or by TEV protease digest (smD1, Tb11.01.0240, Tb927.8.4130, Tb927.5.970) that reduced the protein sizes. In the precipitates (P), the tagged proteins were detected with the anti-ProtC antibody, and CITFA-2 and TFIIB with polyclonal antisera. For comparison, 20% of input material (INP) was co-analyzed.

identify homologous sequences outside of this taxonomic family or to detect a characteristic sequence motif. Hence, by primary structure, CITFA-2 and its orthologs appear to be trypanosomatid-specific proteins. In the next step, we therefore asked whether this protein is associated with more conserved proteins resembling known transcription factors. We generated the procyclic cell line TbT2 which exclusively expressed CITFA-2 with an N-terminal PTP tag at a level corresponding to wild-type CITFA-2 expression (PTP-CITFA-2; Figure 3A and B). Since we had shown that CITFA-2 is encoded by an essential gene and TbT2 cells grew normally, we concluded that the tag did not interfere with protein function. The PTP tag is a modified tandem affinity purification tag consisting of a protein A tandem domain and a protein C (ProtC) epitope separated by a tobacco etch virus (TEV) protease cleavage site. PTP fusion proteins can be sequentially purified by IgG chromatography, TEV protease elution, anti-ProtC immunoaffinity chromatography, and

elution with an EGTA-containing buffer (Schimanski *et al*, 2005b). According to the anti-ProtC immunoblot analysis, both PTP-CITFA-2 purification steps were highly efficient and resulted in the near depletion of the protein from input material (Figure 3C). In the final eluate, 13.7% (360 ng) of the CITFA-2 protein was recovered from the input material. Since approximately 81% of CITFA-2 was extracted from cells (data not shown), we estimate that a single trypanosome harbors ~750 CITFA-2 molecules. Coomassie staining of the final eluate detected several distinct protein bands (Figure 3D), whose excision and analysis by liquid chromatography–tandem mass spectrometry (LC–MS/MS) led to the identification of P-CITFA-2 and six co-purified proteins (identified peptides are listed in Supplementary Table S1). The mix of bands around 55 kDa contained tagged CITFA-2 and proteins encoded by genes *Tb11.47.0008* and *Tb11.47.0010*. While Tb11.47.0008 had been purified by our initial chromatography approach, Tb11.47.0010 was a new identification. Similarly,

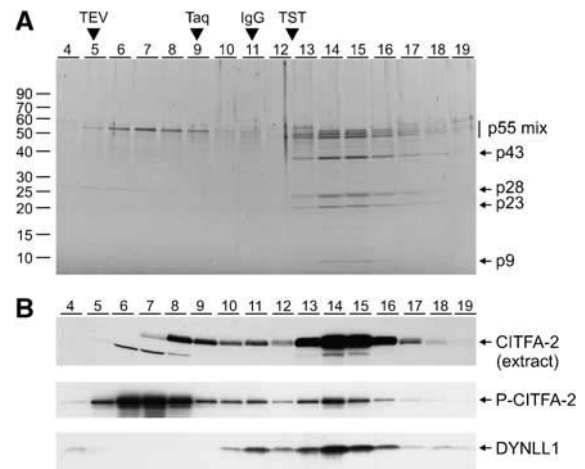
the bands with apparent sizes of 43 and 23 kDa were the products of genes *Tb11.01.0240* and *Tb927.5.970*, respectively, and were not identified before. The band with an apparent size of 28 kDa reproducibly appeared to be fuzzy, suggesting that it contained more than one protein or differently modified versions of the same protein. Interestingly, the identified protein is encoded by three genes, which are part of a tandem repeat of five genes on chromosome 8. *Tb927.8.4030* and *Tb927.8.4080* encode the same protein, whereas the product of gene *Tb927.8.4130* has a different C-terminal region. According to four diagnostic peptide identifications (Supplementary Table S1), both protein versions co-purified with P-CITFA-2.

These six identified proteins are conserved among trypanosomatids (Supplementary Figures S4–S9), but they exhibit no similarity to proteins of other organisms. Nor could we identify a conserved sequence motif using the InterPro (<http://www.ebi.ac.uk/interpro>), Motif scan ([http://myhits.isb-sib.ch/cgi-bin/motif\\_scan](http://myhits.isb-sib.ch/cgi-bin/motif_scan)), SMART (<http://smart.embl-heidelberg.de/>), and Minimotif miner ([http://sms.engr.uconn.edu/servlet/SMS\\_SearchServlet](http://sms.engr.uconn.edu/servlet/SMS_SearchServlet)) search engines. Hence, based on primary structure, these six proteins appear to be specific to trypanosomatid parasites. In contrast, the smallest co-purified protein with an apparent size of 9 kDa was identified by a single peptide as the motor protein subunit dynein light chain DYNLL, which is also known as LC8 (Pfister *et al*, 2006). In *T. brucei*, this protein is encoded by the genes *Tb11.50.0007* and *Tb11.0845*. Among several *T. brucei* DYNLL paralogs, this dynein light chain is most closely related to human DYNLL1 and 2 (data not shown). Accordingly, we propose to name the identified protein TbDYNLL1.

Previous PTP purifications of several nuclear protein complexes, which included RNA pol I (Nguyen *et al*, 2006, 2007) and the transcription factors TRF4/SNAPc/TFIIA (Schimanski *et al*, 2005a) and TFIIF (Lee *et al*, 2007) did not reveal any of the proteins that co-purified in this study. This strongly suggested that these proteins are specifically associated with CITFA-2. Nevertheless, we independently confirmed the interaction of each protein with CITFA-2 by co-immunoprecipitation. Except for DYNLL1, we C-terminally PTP-tagged each co-purified protein and, as a control, smD1 in individual cell lines (Figure 3E). As expected, precipitation of each PTP-tagged protein by IgG beads co-precipitated CITFA-2, whereas the control precipitation of smD1-PTP exhibited no detectable amounts of CITFA-2 (Figure 3F). Hence, we had correctly identified five proteins that co-purified with CITFA-2 and, at this stage, had obtained evidence that the sixth co-purified protein is DYNLL1.

#### CITFA-2 and the co-purified proteins form a complex

Next, we asked whether these proteins purified independently with CITFA-2 or as a complex. The final eluate of the PTP-CITFA-2 purification was therefore sedimented in a linear 10–40% sucrose gradient by ultracentrifugation and the proteins from individual fractions taken from top to bottom were separated by SDS-PAGE and visualized by Sypro Ruby staining (Figure 4A). A protein with the expected size of P-CITFA-2 sedimented by itself in fractions 6–8 and together with the other six co-purified proteins in fractions 14 and 15, indicating that CITFA-2 is part of a protein complex of seven subunits. It is unlikely that two independent complexes



**Figure 4** Sedimentation analysis of the CITFA complex. (A) Sucrose gradient sedimentation of purified P-CITFA-2. Gradients were fractionated from top (fraction 4) to bottom (fraction 19) and the proteins of each fraction were separated on a 15% SDS/polyacrylamide gradient gel and stained with SYPRO Ruby. For comparison, sedimentations of TEV protease (29 kDa), *Taq* DNA polymerase (95 kDa), IgG (150 kDa), and TRF4/SNAPc/TFIIA (TST, ~230 kDa) were co-analyzed. (B) Immunoblot analysis of sucrose gradient fractions. CITFA-2 was detected by anti-CITFA-2 serum in gradient fractions in which extract from wild-type cells (top panel) or purified CITFA complex (middle panel) was sedimented. The latter blot was re-probed with polyclonal anti-*Chlamydomonas reinhardtii* LC8 antibody to detect DYNLL1 in the purified material (bottom panel).

co-sedimented in the higher fractions because, under identical conditions, the ~230 kDa transcription factor complex TRF4/SNAPc/TFIIA (Schimanski *et al*, 2005a) sedimented slower than the CITFA complex, which, assuming a stoichiometry of one for all of its component proteins, has an expected size of ~250 kDa. When extract from wild-type cells was sedimented in an equivalent gradient and fractions were analyzed by immunoblotting, CITFA-2 sedimentation peaked in fractions 14 and 15 virtually identical to the sedimentation of the purified complex (Figure 4B). Furthermore, CITFA-2 sedimentation remained the same when the potassium chloride concentration of the extract was increased from 150 to 400 mM, suggesting that the protein complex is stable (data not shown). Although we cannot exclude the possibility that an important subunit of the complex was lost during purification, these results strongly indicated that we had purified an intact protein complex.

We used the fractionated material and a polyclonal antibody directed against the *Chlamydomonas reinhardtii* LC8 to verify that DYNLL1 is part of this complex. DYNLL1 is highly conserved among all eukaryotes and the heterologous antibody detected a protein of the correct size in unfractionated extract and only p9 in the purified CITFA complex (data not shown). In immunoblots of sucrose gradient fractions of purified P-CITFA-2, nearly all DYNLL1 co-sedimented with P-CITFA-2 in higher fractions and only a minor amount of free DYNLL1 was detected at the top of the gradient (Figure 4B and data not shown). Furthermore, we confirmed by immunoblotting that DYNLL1 did not co-purify in PTP purifications of the transcription factor TFIIF and of a cytoplasmic kinase (data not shown). These findings make it very unlikely that

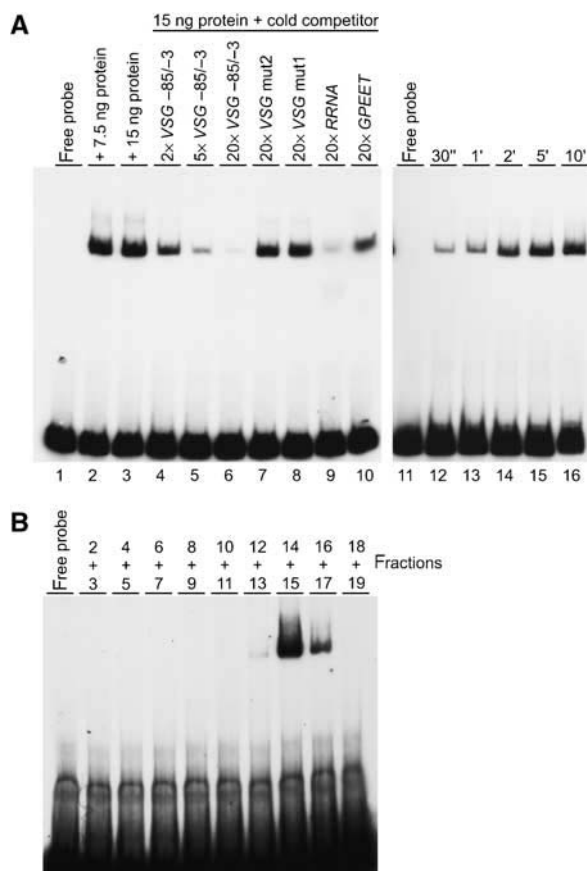
DYNLL1 was a contaminating protein in the CITFA-2 purification and therefore confirmed our identification of DYNLL1 as a subunit of the CITFA complex.

**The purified CITFA complex is active in VSG ES promoter binding and class I transcription**

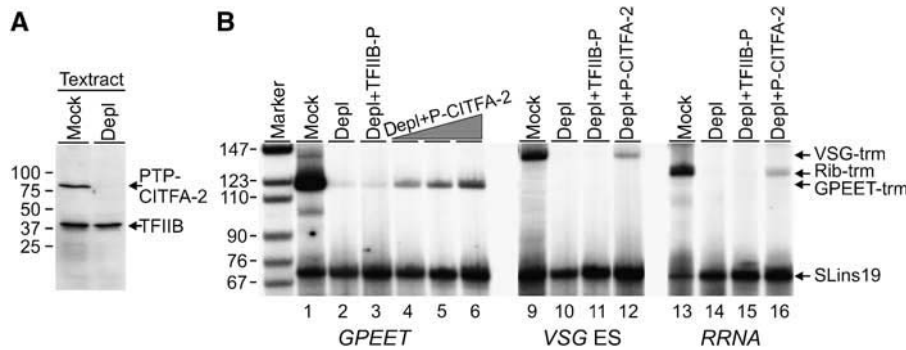
To test the activity of the purified proteins, we first conducted EMSA with a radio-labeled VSG ES promoter probe (Figure 5A). The purified proteins efficiently shifted the probe to a single position, and the shift was effectively competed by adding unlabeled VSG ES promoter DNA (compare lanes 3 with lanes 4–6). In contrast, mutation of either upstream sequence element drastically diminished the competitive effect, which confirmed the promoter pull-down result that both elements are important for the interaction with CITFA-2 (compare lane 6 with lanes 7 and 8). Unlabeled DNA fragments comprising the *RRNA* and *GPEET* promoters were also able to compete, but with different efficiencies. Whereas the *RRNA* promoter competed for complex formation almost as efficiently as the VSG ES promoter (lane 9), the competitive effect of the *GPEET* promoter was much weaker (lane 10), suggesting that purified CITFA-2 in the absence of other factors binds to the *T. brucei* class I promoters, which have distinct sequences, with different affinities.

Furthermore, in a time-course experiment, the DNA–protein complex was rapidly formed without an intermediate state, which indicated that the gel shift was caused either by CITFA-2 or by the protein complex (lanes 11–16). To discriminate between these two possibilities, EMSA was conducted with sucrose gradient fractions of the purified protein. While the lower fractions harboring CITFA-2 alone did not detectably shift the VSG probe, incubation of this probe with the complex containing fractions 14–17 reproduced the shift (Figure 5B). Hence, CITFA-2 does not bind to the DNA by itself but requires other subunits of the protein complex to achieve this task.

In a final step, we analyzed whether the purified proteins are functional in class I transcription. For this, we prepared extract from TbT2 cells (described in Figure 3A) and depleted PTP-CITFA-2 from the extract by IgG chromatography (Figure 6A). As expected, transcription of *GPEET*-trm, *VSG*-trm, and *Rib*-trm was nearly abolished in the depleted extract while *SLRNA* transcription was not affected (Figure 6B, compare lanes 1, 9, and 13 with lanes 2, 10, and 14, respectively). In previous studies, we have used the same technique to deplete the essential class II transcription factors SNAP2 (Schimanski *et al*, 2005a) and TFIIB (Schimanski *et al*, 2006), and found that depletion inhibited *SLRNA* transcription but not *GPEET*-trm transcription. In contrast, depletion of an essential RNA pol I subunit from the extract resulted in the specific inhibition of class I promoter transcription in the system (Nguyen *et al*, 2007). Hence, it is very unlikely that IgG or the PTP tag caused a nonspecific depletion effect on class I template transcription. In agreement with these results, the addition of PTP-purified TFIIB, which had previously been shown to be functional in reconstituting *SLRNA* transcription (Schimanski *et al*, 2006), had no effect on the transcription of the three class I templates (lanes 3, 11, and 15). In contrast, class I transcription in the depleted extract was partially reconstituted by adding back the tandem affinity-purified P-CITFA-2 (Figure 3D, lanes labeled *depl* + *P-CITFA-2*). As we showed for the efficient *GPEET*-trm transcription, this reconstitution was dose-dependent (lanes 4–6). The reconstitution signals for *GPEET*, *VSG*, and *RRNA* promoter transcription were 21, 17, and 13%, respectively (Figure 6B and data not shown). Since we added purified CITFA in an amount comparable to that present in the cell extract, the purified complex was not fully active, perhaps because elution of the complex from anti-ProtC beads by chelating divalent cations caused an inactivating conformational change in the complex that was partially rescued in the subsequent dialysis against transcription buffer. This is a likely explanation because we have observed EGTA-mediated deactivation of a *T. brucei* class II transcription factor before (Schimanski *et al*, 2005a). Alternatively, it is possible that an essential component of the complex was partially lost during the purification and not identified in our biochemical characterization. Interestingly, the P-CITFA-2 eluate did not reconstitute *GPEET*-trm transcription in extract prepared from cells in which *CITFA-2* expression was silenced by RNAi (data not shown), suggesting that *CITFA-2* silencing caused a downstream effect that could not be rescued by adding back a functional CITFA complex. In summary, these data showed that tandem affinity-purified CITFA bound to the VSG ES promoter in a specific manner and was partially active in reconstituting class I transcription in a depleted extract.



**Figure 5** The purified CITFA complex binds to the VSG ES promoter. (A) EMSA with radio-labeled VSG -85/-3 DNA which was incubated in the absence (free probe) or presence of P-CITFA-2 eluate. The gel shift was competed with the indicated molar excess of specified unlabeled promoter DNAs. For the time course, the binding reaction was upscaled five-fold and aliquots were taken from the reaction at specified time points. (B) Equivalent gel shift assays with proteins from specified sucrose gradient fractions.



**Figure 6** The purified CITFA complex is transcriptionally active. (A) Immunoblot of mock-treated and PTP-CITFA-2-depleted (depl) transcription extract (Textract). PTP-CITFA-2 and TFIIB were co-detected with the PAP reagent and a polyclonal antibody, respectively. (B) Co-transcription of class I templates and SLins19 in mock-treated or CITFA-2-depleted extract. The depleted extract was complemented with final eluate of TFIIB (depl + TFIIB-P) or CITFA-2 (depl + P-CITFA-2) PTP purifications. Marker, pBR322-*MspI*.

## Discussion

In this study, we have characterized a protein complex that is indispensable for multifunctional class I transcription in *T. brucei*. The complex specifically bound to *VSG* ES, *RRNA*, and *GPEET* promoters and as shown for the *VSG* ES promoter, this binding depended on the integrity of the two upstream promoter elements. Furthermore, transcription from all three class I promoters was strongly affected or even abolished when anti-CITFA-2 serum was added to the extract, or when CITFA-2 was depleted from extract either through *CITFA-2* expression silencing or through antibody affinity chromatography. In accordance with these strong effects observed *in vitro*, *CITFA-2* silencing led to a rapid cessation of procyclic and bloodstream cell growth and to reduced abundance of class I transcripts in bloodstream forms. The complex consists of at least seven proteins, six of which are conserved within trypanosomatids but have no resemblance to proteins of other organisms. The seventh subunit is the dynein light chain TbDYNLL1, which has not previously been identified as part of the class I transcription machinery in other organisms. Hence, we have characterized a novel protein complex, which we propose to name *T. brucei* class I transcription factor A (TbCITFA). We also propose to number individual subunits of this complex according to their predicted molecular weight, because a terminology based on apparent sizes is ambiguous for the three largest subunits (Table 1).

*T. brucei* CITFA is the first characterized class I transcription factor of a protistan organism. To the best of our knowledge, partially characterized factors are only known from *Acanthamoeba castellanii*, where, with the exception of TBP, the proteins have not been identified (Al Khouri and Paule, 2002). The general lack of sequence conservation among class I transcription factors of distantly related species may explain the parasite-specific sequences of trypanosomatid CITFA subunits and it is possible that functional and structural analyses of CITFA subunits will reveal orthologs or functional equivalents of known class I transcription factors. On the other hand, the number of subunits, the absence of a TBP homolog, and the presence of DYNLL1 clearly distinguish *T. brucei* CITFA from known class I transcription factors.

While we have shown that DYNLL1 is a CITFA subunit, we do not yet understand its function in this complex. In other organisms, DYNLL interacts with many cytoplasmic proteins, probably linking these interactors to the dynein or myosin V

**Table 1** *T. brucei* CITFA subunits

Subunit	GeneDB acc no.	MW (kDa)	No. aa	Th. pI	Apparent size (kDa)
CITFA-1	Tb11.47.0010	52.9	465	8.9	~ 55
CITFA-2	Tb09.211.3440	48.2	421	4.7	~ 55
CITFA-3	Tb11.47.0008	47.0	416	6.1	~ 55
CITFA-4	Tb11.01.0240	42.8	388	8.6	43
CITFA-5a	Tb927.8.4030	25.8	232	5.5	28
	Tb927.8.4080				
CITFA-5b	Tb927.8.4130	25.7	231	5.3	28
CITFA-6	Tb927.5.970	21.7	190	6.7	23
DYNLL1	Tb11.50.0007	10.4	90	6.3	9
	Tb11.0845				

Th. pI, theoretical pI.

motors (Pfister *et al* (2006) and references therein). DYNLL has also been implicated in a number of regulatory roles such as inhibitor of nitric oxide synthase (Jaffrey and Snyder, 1996), regulator of apoptotic signaling (Puthalakath *et al*, 1999), or suppressor of the human transcription repressor TRPS1 (Kaiser *et al*, 2003). While in most cases, the mode of DYNLL-mediated regulation is unknown, some results suggest that it is by sequestering an interacting protein to cytoskeleton-associated motor complexes (Puthalakath *et al*, 1999). As the active *VSG* in bloodstream *T. brucei* is localized to the expression site body (Navarro and Gull, 2001) and procyclin gene units in procyclic cells are arranged at the nucleolar periphery (Ladeira and Navarro, 2007), it is an intriguing possibility that TbDYNLL1 facilitates the positioning of these transcription units. Alternatively, the dynein light chain may directly promote efficient transcription as has been recently implicated for LC8 that binds to the phosphoprotein of rabies virus (Tan *et al*, 2007).

In conclusion, the identification and characterization of CITFA in *T. brucei* represents a major step towards deciphering the unique and essential properties of the multifunctional RNA pol I transcription machinery of both an early diverged eukaryote and a lethal human parasite.

## Materials and methods

### DNA sequences

The DNA sequence of the *VSG* ES 118 promoter spanning the region from position -3 to -85 relative to the TIS was amplified by PCR



from plasmid MIG35 (Navarro M, Wirtz LE, Cross GAM, unpublished) with oligonucleotides 5'-GCCCTCGAGAACCGTCTAAAAGA-3' and 5'-GCCAAGCTTGTCTGATAT CCGAGA-3', which incorporate a *Xho*I and a *Hind*III site, respectively. The amplification product was cloned into the corresponding sites of plasmid pBluescript SKII+ (Stratagene), generating the plasmid pESP3. Similarly, plasmids pESP4 and pESP5, which contain the VSG ES promoter with mutations in box 2 (-62 CT -61 was mutated to AA) and box 1 (-38 CAGGG -34 was mutated to GGTA), respectively, were amplified and cloned from plasmids pLEW115 and pLEW116, respectively (Navarro M, Wirtz LE, Cross GAM, unpublished). For N-terminal PTP tagging of CITFA-2, 387 bp of the 5' terminal coding region, which excluded the initiation codon, were amplified from genomic DNA and inserted into the *Not*I and *Ap*aI sites of plasmid pN-PURO-PTP (Schimanski *et al*, 2005b). For C-terminal PTP tagging of CITFA subunits, the following 3' terminal coding regions were cloned into the *Not*I and *Ap*aI sites of plasmid pC-PTP-NEO: 702 bp of *Tb11.47.0010*, 585 bp of *Tb11.47.0008*, 600 bp of *Tb11.01.0240*, 336 bp of *Tb927.8.4130*, and 313 bp of *Tb927.5.970*. pT7-stl is a derivative of pLew100 (Wirtz *et al*, 1999) and was derived by replacing the sequence between *B*siWI and *M*luI restriction sites with two T7 transcription terminators and by inserting the sequence 5'-CTAATACCACTACTATAGG GATCTCCCT ATCAGTGATAGAGATCCCTATCAGTGATAGAGA-3', which contains the T7 promoter and two tandem tetracycline operators, in the form of two hybridized oligonucleotides into the *K*pnl and *H*indIII sites. For CITFA-2 RNAi, the CITFA-2-coding sequence from position 121 to position 650 was inserted into pZJM (Wang *et al*, 2000) or the stem-loop cassette of pT7-stl following the established cloning strategy (Shi *et al*, 2000).

### Cells

*T. brucei* cell culture, targeted integration of linear DNAs into cells by electroporation, and the generation of stable cell lines by selection and limiting dilution were described in detail previously (Wirtz *et al*, 1999; Günzl *et al*, 2000). Depending on the selectable markers present in procyclic lines, cells were kept in medium with 40 µg/ml of G418, 20 µg/ml of hygromycin, and/or 4 µg/ml of puromycin. Procyclic 29-13 cells (Wirtz *et al*, 1999) transfected with the CITFA-2 RNAi construct were cultured in the presence of 15 µg/ml of G418, 50 µg/ml of hygromycin, and 2.5 µg/ml of phleomycin. The corresponding 'single marker' bloodstream cell line (Wirtz *et al*, 1999) was maintained in medium containing 2.5 µg/ml of G418 and 0.25 µg/ml of phleomycin. In RNAi experiments, dsRNA synthesis was induced with 2 µg/ml of doxycycline. Cells were counted and diluted to specific densities daily.

### Initial purification and analysis of the promoter-binding complex

A nuclear extract was prepared from 301 of procyclic cells and contained 20 mM HEPES, pH 7.6, 420 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 25% glycerol, and protease inhibitors. The extract was subjected to chromatographic purification by a protocol that was devised by extensive empirical testing. All steps were carried out at 4°C and purification was monitored by EMSA. Purification was achieved consecutively by a 25–50% saturation ammonium sulfate precipitation, *S*-Sepharose cation exchange chromatography, heparin affinity chromatography, DNA affinity chromatography in which an 83-bp biotinylated VSG ES promoter, PCR-amplified from pESP3, was coupled to a Streptavidin Sepharose High Performance column (Amersham), and Resource Q ion exchange chromatography (Amersham). In a final step, the active fractions were loaded onto 4 ml 15–45% (v/v) linear glycerol gradients (10 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, 1 mM DTT) and centrifuged at 50 000 rev/min in a Beckman SW 60 rotor for 16 h at 7°C. Purified proteins were resolved by 10% SDS-PAGE and silver-stained. Each visible band was excised, destained, dehydrated and treated with 100 ng of modified TPCK-trypsin for 8 h at 37°C. Peptides were subjected to LC-ESI-MS/MS analysis using an HPLC system (Smart System, Amersham). Protein searches were performed with SonarMSMS (Genomic Solutions) using locally collated versions of the available *T. brucei* genome data in 2001, with a re-analysis in May 2005 (a detailed protocol for this subheading is included in the Supplementary data).

### Analysis of the protein complex by tandem affinity purification

Preparation of crude extract from  $5 \times 10^{10}$  procyclic cells and tandem affinity purification of PTP-tagged CITFA-2 was carried out according to the standard PTP purification protocol (Schimanski *et al*, 2005b). For immunoblot analyses, proteins were separated by SDS-PAGE, electroblotted onto a polyvinylidene difluoride membrane, and detected with specified antibodies and the BM Chemiluminescence Blotting substrate (Roche). PTP-tagged proteins were either detected with the PAP reagent (Sigma) or the monoclonal anti-ProtC antibody HPC4 (Roche). The antiserum against CITFA-2 was raised against recombinant protein. For this, the coding sequence of the 208 amino acid-long N-terminal half was fused to the ProtC epitope, a thrombin cleavage site, and a 6xHis tag in the expression vector pET100/D-TOPO (Invitrogen). The vector was transformed into *Escherichia coli* strain BL21 and protein expression was induced for 4 h at 37°C by adding 1 mM IPTG. Recombinant protein was tandem affinity-purified in subsequent steps by TALON metal affinity chromatography (BD Biosciences), imidazole elution, and anti-ProtC immunoaffinity chromatography. Recombinant protein was finally eluted in phosphate-buffered saline (PBS) containing 10 mM EGTA and 5 mM EDTA and dialyzed against PBS. The protein was used for immunizing rats as detailed elsewhere (Schimanski *et al*, 2006).

For identification, purified proteins were collected by StrataClean resin (Stratagene), released into SDS loading buffer by boiling the samples for 5 min, separated on a 15% SDS/polyacrylamide gel, and stained with Gelcode Coomassie stain (Pierce). Individual bands were excised from the gel, proteins were digested with trypsin or subtilisin, and peptides were identified by LC-MS/MS.

Sedimentation analysis was carried out in 4 ml 10–40% linear sucrose gradients (20 mM HEPES-KOH, pH 7.7, 150 mM potassium chloride, 20 mM potassium L-glutamate, 3 mM MgCl<sub>2</sub>, 0.1% Tween 20), which were centrifuged in a Beckman SW55 rotor for 19 h at 42 000 rev/min and 4°C. Twenty fractions were collected from top to bottom and protein was precipitated using StrataClean resin as described above. Proteins were separated on a 15% SDS/polyacrylamide gels and stained with SYPRO Ruby (Invitrogen) according to the manufacturer's protocol.

For co-immunoprecipitations of various PTP-tagged proteins, 100 µl of crude cell extract, corresponding to  $8 \times 10^8$  cells, was mixed with 20 µl settled volume of IgG beads and incubated on ice for 1 h. The beads were washed four times with TET150 buffer (150 mM NaCl, 20 mM Tris-HCl, pH 8.0, 3 mM MgCl<sub>2</sub>, 0.1% Tween 20). For glycine elution of proteins, the beads were incubated twice in 150 µl of 100 mM glycine-HCl (pH 2) for 2 min. The two fractions were pooled and mixed with 1 ml of TET150 buffer. Alternatively, proteins were eluted by adding 4 units of AcTEV protease (Invitrogen) to the beads and by incubating the reactions at 28°C for 30 min. Immunoprecipitates were collected using StrataClean resin and analyzed by immunoblotting as described above.

### DNA-protein interaction

Promoter pull-downs were carried out as described previously (Schimanski *et al*, 2004) except that the KCl concentration was increased to 80 mM. For EMSA, the 93 bp-long DNA (ESP3) generated from the digestion of plasmids pESP3 with *Xho*I and *Hind*III was 3'-end-labeled using the Klenow fragment of DNA polymerase and [ $\alpha$ -<sup>32</sup>P]dCTP. The probe was purified on a NucTrap column (Stratagene) followed by phenol-chloroform extraction and ethanol precipitation. EMSA conditions were based on those described by Pham *et al* (1997) and carried out in 15 µl containing 6 mM HEPES, pH 7.6, 40 mM DTT, 4% Ficoll 400, 1 µg poly-dI/dC, 0.17 mM spermine, and 1.7 mM spermidine. Unless in exceptional cases, the final concentration of sodium or potassium chloride was kept between 50 and 150 mM. Protein extract was added to the binding reaction and the mixture incubated for 10 min at 25°C. Then, 1 ng of radio-labeled DNA probe was added and the reaction incubated for another 10 min at 25°C, before separation in a 4% polyacrylamide/0.5 × TBE gel at 150 V and 4°C for 3 h. Gels were dried onto filter paper and exposed for autoradiography. EMSAs with PTP-purified CITFA-2 were slightly modified and conducted with 15 ng of total protein and 25 ng of poly-dI/dC in the absence of spermine and spermidine. Competition assays were carried out in the same way, with the competitor being added before the protein. The unlabeled competitors ESP4 and ESP5 were excised as ESP3 from correspond-

ing plasmids, whereas the *RRNA* and *GPEET* competitor DNAs, which extended from positions –257 to +106 and from –246 to +70 relative to the TIS, respectively, were generated by PCR.

For UV crosslinking, the VSG ES 118 sequence from position –85 to –3 was PCR amplified from plasmid ESP3 in the presence of [ $\alpha$ - $^{32}$ P]dCTP and 5'-bromodeoxy uridine triphosphate. The binding reaction was carried out under the same conditions as for EMSA, and then irradiated with UV at 3000  $\mu$ W/cm<sup>2</sup> in a Stratelinker (Stratagene) for 30 min on ice. CaCl<sub>2</sub> was added to final concentration of 15 mM and the DNA digested for 30 min at 37°C using 5 units of DNase I and 5 units of S7 micrococcal nuclease (Roche). The proteins were resolved on a 10% SDS-PAGE and visualized by autoradiography.

### **In vitro transcription and RNA analysis**

The *in vitro* transcription system has been previously described in detail (Laufer *et al*, 1999; Laufer and Günzl, 2001). In brief, a standard reaction was conducted in a volume of 40  $\mu$ l and contained 8  $\mu$ l of extract, 20 mM potassium L-glutamate, 20 mM KCl, 3 mM MgCl<sub>2</sub>, 20 mM HEPES-KOH, pH 7.7, 0.5 mM of each nucleoside triphosphate, 20 mM creatine phosphate, 0.48 mg/ml of creatine kinase, 2.5% polyethylene glycol, 0.2 mM EDTA, 0.5 mM EGTA, 4 mM dithiothreitol, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, 12.5  $\mu$ g/ml vector DNA, 20  $\mu$ g/ml class I template, and 7.5  $\mu$ g/ml SLins19 template. In reactions with extract from 29-13 cells, modified template versions lacking T7 promoters were used (Lee *et al*, 2007). In reactions with antisera, the volume of the extract was reduced to 4  $\mu$ l, and extract was pre-incubated with antiserum

for 30 min on ice before reactions were started by adding templates and nucleotides. Reactions were incubated for 1 h at 27°C and stopped by the addition of 300  $\mu$ l of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% *N*-lauroylsarcosine). In total RNA preparations, specific transcripts were detected by Superscript II (Invitrogen)-mediated extension of  $^{32}$ P-end-labeled primers Tag\_PE and SLtag which hybridize to unrelated oligonucleotide tags of the class I and SLins19 RNAs, respectively (Laufer *et al*, 1999).

Northern analysis and U2 and SL RNA primer extension assays of total RNA prepared from RNAi-induced cells were conducted exactly as recently described (Nguyen *et al*, 2007).

### **Supplementary data**

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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