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Development of an efficient *in vitro* transcription system for bloodstream form *Trypanosoma brucei* reveals life cycle-independent functionality of class I transcription factor A

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Abstract

Trypanosomatid parasites possess extremely divergent transcription factors whose identification typically relied on biochemical, structural and functional analyses because they could not be identified by standard sequence analysis. For example, subunits of the *Trypanosoma brucei* mediator and class I transcription factor A (CITFA) have no sequence resemblance to putative counterparts in higher eukaryotes. Therefore, homologous *in vitro* transcription systems have been crucial in evaluating the transcriptional roles of *T. brucei* proteins but so far such systems have been restricted to the insect-stage, procyclic form (PF) of the parasite. Here, we report the development of a homologous system for the mammalian-infective, bloodstream form (BF) of *T. brucei* which supports accurately initiated transcription from three different RNA polymerase (pol) I promoters as well as from the RNA pol II-recruiting spliced leader RNA gene promoter. The system is based on a small scale extract preparation procedure which accommodates the low cell densities obtainable in BF culture. BF and PF systems behave surprisingly similar and we show that the CITFA complex purified from procyclic extract is fully functional in the BF system indicating that the transcriptional machinery in general is equivalent in both life cycle stages. A notable difference, however, was observed with the RNA pol I-recruiting *GPEET* procyclin promoter whose reduced promoter strength and increased sensitivity to manganese ions in the BF system suggests the specific presence of a transcriptional activator in the PF system.

1. Introduction

In trypanosomatid parasites transcriptional processes deviate substantially from those of their mammalian hosts [1]. Protein coding genes in these protistan organisms are arranged in long tandem arrays that are transcribed polycistronically, a mechanism which requires spliced leader (SL) *trans* splicing and polyadenylation for the processing of individual mRNAs from polycistronic precursors [2]. A key molecule in this gene expression mode is the small nuclear SL RNA which donates its 5' terminal sequence for the maturation of each

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mRNA. All trypanosomatids depend on continuously strong SL RNA synthesis to maintain a vital level of gene expression and, therefore, harbor up to 100 tandem copies of the SL RNA gene (*SLRNA*) that are transcribed monocistronically by RNA polymerase (pol) II [3]. Since transcription initiation of RNA pol II at protein coding gene arrays is still not understood, the *SLRNA* promoter remains the only characterized RNA pol II promoter in trypanosomatids.

Another unique deviation from other eukaryotes is the multifunctional RNA pol I of *Trypanosoma brucei* which not only transcribes the repeats of the large ribosomal gene unit as in all other eukaryotes but, in addition, units encoding its major cell surface antigens variant surface glycoprotein (VSG) and procyclin [4,5]. This expansion of RNA pol I function is absolutely crucial for parasite survival because VSG and procyclin gene expression are necessities for successful infections of mammals and tsetse flies, respectively and, in case of VSG expression, for cell viability per se: RNAi-mediated silencing of VSG expression rapidly ceased proliferation of bloodstream form (BF) trypanosomes in culture and caused clearance of parasites from infected mice [6]. Moreover, BFs use antigenic variation of the VSG coat as their main strategy to evade the mammalian immune system [7]. This strategy, however, depends on the restriction of RNA pol I-mediated VSG expression to a single gene [8]. This active VSG, which is drawn from a large VSG repertoire, is transcribed from a telomeric expression site (ES) in a dedicated, RNA pol I-packed nuclear compartment termed expression site body or ESB [9].

A prerequisite to understand these deviating processes at the biochemical level is the identification of proteins involved in transcription. Unfortunately, annotation of the genomes of *T. brucei*, *Trypanosoma cruzi* and *Leishmania major* resulted in the unambiguous identification of very few transcription factors including the TATA-binding protein (TBP) homolog TRF4 (TBP-related factor 4) [10] and the helicases *Xeroderma pigmentosum* B (XPB) and XPD of the basal transcription factor TFIIH [11]. However, in subsequent studies, which were carried out in *T. brucei* and primarily based on factor purification, mass spectrometric protein identification and functional analyses, several basal transcription initiation factors were characterized. These factors included a complex of small nuclear RNA-activating proteins (SNAPs), TRF4 and TFIIA [12–14], TFIIB [15,16], TFIIH [17–19] and mediator [20] for RNA pol II transcription and the class I transcription factor A (CITFA) for RNA pol I transcription [21]. The amino acid sequences of these transcription factors exhibit an unprecedented divergence from those of their eukaryotic orthologs and despite the typically well-conserved nature of these factors, among the new identifications, only the single polypeptide TFIIB was found *in silico* whereas the identification of all other factors and factor subunits depended on the co-purification in a complex. Moreover, subunits of the mediator and CITFA complexes lack sequence similarity to their putative counterparts in higher eukaryotes altogether and the identity of the trypanosome mediator complex was revealed exclusively by its low resolution structure and functional properties and not by analysis of amino acid sequences of its subunits [20]. This situation extends even to the strongly conserved RNA pol subunits because an essential RNA pol I subunit was recently characterized that lacks sequence similarity to the well-characterized subunits of yeast and human RNA pols [5].

In analyzing these extremely divergent proteins, *in vitro* transcription systems have been an invaluable tool to determine and dissect their functional roles. *In vivo* analyses of a putative transcription factor can be ambiguous because a transcription defect in conditional gene silencing or knockout may be indirect and could also be due to a rapidly occurring secondary effect, the consequence of a particular stress [22], or a general phenotype of death. In contrast, *in vitro* transcription systems have enabled the unambiguous demonstration of a protein's transcriptional role because it allows direct assessment of the

consequence of depleting/adding back a protein or inhibiting the protein by a small molecule or a specific antibody (for examples see references [16,21]).

Although *in vitro* systems for accurate transcription were established for eukaryotes as early as 1978 [23,24], a homologous transcription system for a trypanosomatid organism was not available until 1995 when a G-less cassette approach was successfully used to establish accurate transcription of the *T. brucei* U2 snRNA gene by RNA pol III [25]. The two major problems with trypanosome extracts were the presence of a strong glycerol kinase activity which rapidly depleted ATP pools even in the presence of ATP regeneration machinery and a high non-specific labeling activity due to genomic DNA contaminations of extract. Thus, replacing the important viscosity component glycerol with sucrose in extract, suppressing nonspecific transcription with the chain-terminating nucleotide 3'/OMeGTP, and degrading all labeled RNA with G-specific RNase T1 allowed the specific detection of newly synthesized, G-less U2 RNA in this system [25]. Subsequent optimizations led to systems in *T. brucei* and the related organism *Leptomonas seymouri* which also supported the accurate synthesis of the essential SL RNA [26,27]. Importantly, in these systems, transcription signals did not rely on a G-less approach any longer but were obtained through primer extension assays with oligonucleotides complementary to oligonucleotide tags that were introduced into template DNAs downstream of transcription initiation sites (TISs). Finally, switching from a nuclear extract to a cruder extract that consisted of cytoplasmic and nuclear extracted components created a system that was active for accurate transcription from all RNA pol I promoters, namely those of the *RRNA* gene unit, the procyclin gene unit, the *VSG* expression site (ES) and the metacyclic *VSG* ES [28–30].

All these systems were established for the insect-stage, procyclic form (PF) of *T. brucei* but only a comparable system for the mammalian-infective BF would enable the analysis of a BF-specific protein or the detection of BF-specific function of a constitutively expressed factor. In addition, a BF system would be a promising starting point for the future development of high throughput screening assays of trypanosome transcriptional processes. Here, we report the development of such a system. It is based on a straightforward small scale procedure for the preparation of PF and BF extracts that are highly active in accurately initiated RNA pol I and *SLRNA* promoter-dependent RNA pol II transcription. The small scale procedure accommodates the low BF densities obtainable in *in vitro* culture.

2. Methods

2.1. Cell culture and DNAs

T. brucei brucei strain 427 PFs were cultured as described previously [28] except that the fetal bovine serum concentration was raised from 10% to 15%. BFs from the same strain were cultured at 37 °C, 5% CO₂, and >90% humidity in HMI-9 medium [31] supplemented with 20% (vol/vol) fetal bovine serum, 20 µg/ml kanamycin sulfate (Invitrogen), 10 µg/ml gentamycin (Sigma), 30 µg/ml penicillin (Sigma) and 50 µg/ml streptomycin (Sigma). For depletion of the CITFA transcription factor complex from extract, a clonal BF cell line was generated that exclusively expressed the CITFA7 subunit with a C-terminally fused PTP tag consisting of a protein C epitope (ProtC), a tobacco etch virus protease site and tandem protein A (ProtA) domains [32]. The cell line was generated by knocking out one *CITFA7* allele with a PCR product in which the *CITFA7* gene flanks were fused onto the coding region of hygromycin phosphotransferase according to a published procedure [33] and by integrating the plasmid CITFA7-PTP-NEO into the second *CITFA7* allele thereby fusing the PTP sequence to the 3' end of the *CITFA7* coding region.

The template DNAs for transcription reactions *VSG*-trm, *Rib*-trm, *GPEET*-trm (originally *PARP*-trm), and *SLins19* were described previously [28]; the second version of these

plasmids [5] were used here. pCITFA7-PTP-NEO was generated by inserting 353 bp of the C-terminal *CITFA7* coding region (nucleotide positions 56 to 408) at the *ApaI* and *NotI* sites of the vector pC-PTP-NEO [32] and was linearized prior to transfection with restriction enzyme *PshAI*.

2.2 Preparation of transcriptionally active extract

Two liters of BF culture was grown in eleven to thirteen 600-ml culture flasks to a cell density of 2×10^6 cells/ml. The cells were harvested by centrifugation for 8 min at 2,700 g and 2 °C. Cell pellets were resuspended in a total of 15 ml ice-cold Tryp wash solution (100 mM NaCl, 3 mM MgCl₂, 20 mM Tris-HCl pH 7.5), transferred to a pre-cooled 15-ml Falcon tube and spun again for 5 min at 2,700g and 2 °C. This wash was repeated twice with Tryp wash solution and once with 10 ml of ice-cold transcription buffer (150 mM sucrose, 20 mM potassium L-glutamate, 3 mM MgCl₂, 20 mM HEPES-KOH pH 7.7, 1 mM DTT, 10 µg/ml leupeptin, 10 µg/ml aprotinin). The cells were finally resuspended in 1.5 times the packed cell volume (pcv; 200–250 µl) of transcription buffer, kept on ice for 20 min (the transcription buffer is slightly hypotonic and causes cells to swell), shock-frozen in liquid nitrogen, and stored at –80 °C.

For extract preparation, the cells were thawed and mixed with ~200 µl settled volume of 800 µm low binding silica beads (OPS Diagnostics) which had been equilibrated four times with 0.8 ml of ice-cold transcription buffer. After adding 10 µl of the phosphatase inhibitor cocktail 2 (Sigma) to the tube, cells were broken by five cycles of strong vortexing for 1 min in a 4 °C cold room, shock freezing the sample in liquid nitrogen, and thawing the material for the next round of vortexing. The broken cell suspension was then transferred into a new pre-cooled tube containing a tenth volume of ice-cold extraction buffer (1500 mM KCl, 20 mM HEPES-KOH pH 7.7, 3 mM MgCl₂), and nuclear proteins were extracted by incubating the samples on ice for 20 min with three rounds of inverting and strong tapping the tube this period. It should be noted that it was important to quickly mix the broken cell suspension with extraction buffer to avoid high local salt concentrations which can lead to nuclear lysis and genomic DNA contamination of the extract. Following the incubation, extract was separated from insoluble cell debris and remaining glass beads by centrifugation at 25,000 g and 2 °C for 10 min. To reduce the salt concentration, the extract was diluted with 0.8 volumes of ice-cold transcription buffer and concentrated about five times to a volume of 200–250 µl in a centricon-10 centrifugal filter device (Millipore) which was pre-washed with 2 ml of RNase-free water to remove trace amounts of glycerol from the filter and used at 2 °C according to the manufacturer's specifications. The extract was split into 50 µl-aliquots, shock frozen in liquid nitrogen, and stored at –80 °C. For the small scale preparation of PF extract, 200 ml of a $\sim 1.5 \times 10^7$ cells/ml culture was harvested and processed as described above.

For immunodepletion of CITFA7-PTP, 50 µl of BF transcription extract was mixed with 30 µl settled volume of transcription buffer-equilibrated IgG beads (GE Healthcare) and incubated on ice for 1.5 hours, tapping the tubes several times during this period. After spinning the sample at 3,000 g and 2 °C for 2 min, the extract was carefully separated from beads and transferred into a new, pre-cooled tube. Mock depletion of extract was carried out identically except that protein G beads were used.

2.3 In vitro transcription reactions

Standard transcription reactions were performed in a volume of 20 µl containing 4 µl of BF cell-free extract and a final concentration of 60 mM sucrose, 20 mM potassium L-glutamate, 20 mM KCl, 3 mM MgCl₂, 20 mM HEPES-KOH pH 7.7, 20 mM creatine phosphate, 0.48 mg/ml of creatine kinase, 2.5% (vol/vol) polyethylene glycol, 0.2 mM EDTA, 0.5 mM

EGTA, 4 mM DTT, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 40 µg/ml total plasmid DNA. In a standard co-transcription reaction, 7.5 µg/ml of RNA pol I promoter template (GPEET-trm, Rib-trm, or VSG-trm), 7.5 µg/ml of SLins19 template, and 25 µg/ml of the vector pUC19 were used. For each experiment, creatine kinase was freshly prepared by dissolving the enzyme in transcription buffer. The reactions were incubated for 10 min on ice after which all four nucleoside triphosphates were added to a final concentration of 0.5 mM and the reactions carried out for one hour at 27 °C. The reactions were stopped by adding 80 µl of RNase-free H₂O, 300 µl of TRIZOL LS reagent (Invitrogen) and 60 µl of chloroform. Total RNA was then prepared as described previously [28]. Transcription signals were obtained by primer extension reactions using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol and the ³²P-5' end-labeled oligonucleotides Tag-PE [28] and SLtag [26] which hybridize to unrelated oligonucleotide tags of the RNA pol I promoter template-derived RNAs and the SLins19 RNA, respectively. Primer extension products were separated on 6% polyacrylamide–50% urea gels, visualized by autoradiography, and quantified by phosphorimaging on a Personal Molecular Imager System (Biorad). In all experiments, ³²P-radiolabeled fragments of *MspI*-digested pBR322 served as markers.

A detailed step-by-step protocol which includes extract preparation and *in vitro* transcription reactions is available upon request.

3. Results and Discussion

3.1. A small scale procedure for the preparation of transcription extract

In the established PF *in vitro* transcription systems cells are broken by a glass douncer under slightly hypotonic conditions [26,28]. This method of cell breakage requires a pcv of at least 4 ml which corresponds to $\sim 5 \times 10^{10}$ procyclic cells or 2.5 liter of a healthy PF culture. These cell numbers are too high for BFs grown *in vitro* because they can reach only about one tenth of the density of PFs in culture. BFs can be obtained in large quantities by infecting rat hosts, in which the 427 strain of *T. brucei brucei* grows up to a density of 10^9 cells/ml blood. However, we noticed that trypanosomes which had been grown up to this high density and harvested from rat blood according to a standard protocol [34], had deactivated most of their RNA pol I and were not suitable for the preparation of transcription extract (Sung Hee Park and Arthur Günzl, unpublished data). Thus, for establishing a BF *in vitro* system, a small scale extract procedure needed to be developed.

This was first achieved with 200 ml of PF culture that was grown up to a density of $\sim 1.5 \times 10^7$ cells/ml and corresponded to a pcv of 200–250 µl. Since trypanosomes are difficult to disrupt, we incubated the cells in the slightly hypotonic transcription buffer for 20 min on ice, mixed them with low binding silica beads, and performed a repeated freeze-thaw-vortex cycle for cell disruption (see methods). A similar procedure had been successfully used to efficiently break prokaryotic cells [35]. Salt extraction of nuclear proteins, extract dilution to reduce salt concentration, and concentrating the extract in a centrifugal filter device were done as in the established large scale extraction procedure [28]. Testing the extract in co-transcription reactions with the *SLRNA* promoter template SLins19 and one of the three RNA pol I promoter templates GPEET-trm, Rib-trm and VSG-trm which harbor the *GPEET* procyclin promoter, the *RRNA* promoter and a *VSG* ES promoter, respectively, showed that extract prepared at the small scale was only slightly less active than extract from a large scale preparation (Fig. 1A). Importantly, the relative signal strengths for each template corresponded well between the two extracts with the *GPEET* signals being the strongest among the RNA pol I signals as was observed previously [28]. Moreover, since the transcription signals were generated by primer extension assays, the co-migration of the bands showed that accurate transcription initiation was maintained for all templates in the

small scale extract preparation. It should be noted that in all Rib-trm reactions the SLins19 signal was generally reduced due to a competitive effect: we have previously shown that the most distal domain of the *RRNA* promoter harbors an element that corresponds to the upstream sequence element of the *SLRNA* promoter and, *in vitro*, binds the SNAP complex thereby competing *SLRNA* promoter transcription [29,36].

In the next step we prepared extract from BFs. To obtain a pcv of 200–250 μ l, we grew up 2 liters of BF culture to a density of $\sim 2 \times 10^6$ cells/ml. Breakage of BFs with the silica beads was as efficient as it was for PFs and the resulting extract supported efficient and accurate transcription from all four template DNAs (Fig. 1B). However, from this initial result it appeared that the relative GPEET-trm signal strength was reduced in the BF extract.

3.2. Optimizing the BF system

The initial transcription reaction conditions used with the BF extract were those of the optimized PF system. However, since BFs represent a different life cycle stage of the parasite and are grown in a different medium and under different conditions, it was possible that changing reaction parameters would increase transcriptional activity. We used the VSG-trm template for these analyses because the active VSG ES promoter is particularly well transcribed in BFs [37]. Since BFs are grown at 37 °C, the temperature of the mammalian bloodstream, and procyclics at 27 °C, we first varied the temperature of the reaction (Fig. 2A). Transcriptional activity from SLins19 and VSG-trm templates was nearly abolished at 37 °C and highest at 27 °C, the optimal temperature for PF extract activity [28]. While this result may suggest that there is no major difference in the transcription machinery between the two life cycle stages, it is not completely unexpected because *in vitro* transcriptions in mammalian systems are most efficient at temperatures well below 37 °C (see reference [38] for example).

Next, we varied the concentration of magnesium, an important co-factor in enzymatic reactions. High efficiency transcription of VSG-trm required at least 3 mM MgCl_2 and peaked at 5 mM (Fig. 2B and data not shown) whereas SLins19 transcription was already highly active at 2 mM MgCl_2 (Fig. 2B). At higher magnesium concentrations, the SLins19 RNA became partially degraded which suggested that RNases, present in crude trypanosome extracts ([26,28] and data not shown), become activated at these conditions. Thus, for co-transcription reactions, the optimal Mg^{2+} concentration is 3 mM, the same concentration as in reactions with PF extract [28].

Finally, we analyzed the template concentrations in the reactions. In the PF system, GPEET-trm transcription was most efficient at a concentration of 40 μ g/ml whereas the other templates were optimal at 7.5 μ g/ml [28]. In the BF system, we analyzed the GPEET-trm and the VSG-trm templates and found that both had optimal concentrations of 7.5–12.5 μ g/ml (data not shown). Hence, except for the GPEET-trm template concentration, PF and BF systems exhibited their highest activity at the same reaction temperature, Mg^{2+} concentration and template concentrations.

3.3. Confirming the validity of the BF system

Since the primer extension-derived transcription signals of the BF and PF small scale systems had exactly the same sizes as those of the PF large scale system (Fig. 1) for which we had determined accurate transcription initiation by comparing the primer extension products to sequencing ladders [26,28], we concluded that accurate transcription initiation was maintained in the small scale systems.

To further confirm the validity of the BF system, we carried out co-transcription reactions in the absence and presence of the RNA pol II inhibitor α amanitin. As expected and shown in

Fig. 3A, a high concentration of the inhibitor abolished *SLins19* transcription but did not affect *Rib-trm* or *VSG-trm* transcription. This result demonstrated that the two RNA pol I promoters which direct gene expression in BFs do recruit RNA pol I in the extract and it was in agreement with *SLRNA* promoter transcription being mediated by RNA pol II.

We then tested the activity of *VSG* ES promoter mutations. The *VSG* ES promoter consists of two small sequence elements located within 67 bp upstream of the TIS (Fig. 3B). While single nucleotide mutations identified only a few important nucleotides [39], we based our mutations on a published block substitution analysis [40]. Mutation of either promoter element dramatically reduced the *VSG-trm* signal whereas mutating both elements together completely abolished *VSG* ES promoter transcription (Fig. 3C). This result was in close agreement with results obtained in gel shift assays conducted with the *VSG* ES promoter-binding factor CITFA where mutation of either promoter element strongly reduced the interaction between CITFA and the *VSG* ES promoter and mutation of both elements abolished the interaction [21]. In addition to the decreased transcription signal, we observed two additional signals in single mutations that were not detectable in the reaction with wild-type template (Fig. 3C, asterisks). Most likely, these signals stem from aberrant TISs upstream of the correct TIS due to improper binding of CITFA to the promoter.

3.4. Employing the BF system

Early on, it was observed that in BF nuclear run-on assays, *VSG* ES and *SLRNA* transcription, in contrast to *RRNA* transcription, was resistant to Mn^{2+} concentrations up to 4 mM [41]. Similarly, we observed in the PF *in vitro* system that GPEET-trm transcription was resistant to 4 mM Mn^{2+} whereas *VSG-trm* and *Rib-trm* transcription was not [28]. Since it was unequivocally demonstrated that these promoters recruit and initiate RNA pol I transcription [4,5], it is likely that transcription factor differences account for this discrepancy in Mn^{2+} sensitivities. When we tested the effect of increasing Mn^{2+} concentrations in the BF system, we found that GPEET-trm transcription was no longer resistant to 4 mM Mn^{2+} and behaved similarly to *VSG-trm* transcription (Fig. 4A). The difference in Mn^{2+} resistance of GPEET-trm transcription in BF and PF systems raises the possibility that PFs express or activate a *trans*-activating factor that promotes the formation of a more stable transcription pre-initiation complex at procyclin gene promoters in this life cycle stage. This notion was further supported by quantifying transcription signal strengths from all three RNA pol I promoters (Fig. 4B). To avoid the *RRNA* / *SLRNA* promoter competition effect, transcription reactions were independently repeated without the *SLins19* template. The average signal strength for the three promoters differed by only 30% with GPEET-trm and *Rib-trm* providing the strongest and weakest signals, respectively. This was in contrast to the PF system in which the GPEET-trm signal was approximately four times stronger than the signals from the other two RNA pol I promoters ([28], see also Fig. 1). This difference corresponds well to *in vivo* studies which showed that the procyclin promoter was about fivefold reduced in BFs compared to PFs independent of its chromosomal location and led to the conclusion that this difference is regulated at the level of transcription initiation [42].

According to published nuclear run-on data [41], we had expected that *VSG-trm* transcription in the BF system would be resistant to 4 mM Mn^{2+} but this was not the case (Fig. 4A). A possible explanation for this discrepancy is that a dedicated activator of *VSG* ES transcription was not extracted or inactivated in the crude extract. Alternatively, concentration of transcription factors and RNA pol I in the ESB may enable the extremely strong transcription of the active *VSG* ES observed in BFs [37] that cannot be reproduced in an extract.

Higher Mn^{2+} concentrations stimulated SLins19 transcription (Figure 4A) and corresponding effects were observed in BF nuclear run-ons [41] and the PF system [28] suggesting that RNA pol II transcription of *SLRNAs* is equivalent in both life cycle stages. In accordance with this notion, we recently showed that tandem affinity-purified PF RNA pol II was fully functional in the BF system: the PF enzyme effectively restored SLins19 transcription in BF extract that had been immunodepleted of the RNA pol II subunit RPB7 and therefore was inactive in *SLRNA* transcription [43].

Since our results thus far suggested that there are differences in RNA pol I transcription between the PF and BF systems, we wanted to test whether CITFA, the only known RNA pol I transcription factor, was able to function across the two life cycle stages. CITFA is a multi-subunit protein complex that interacts with all three RNA pol I promoters, and the subunit CITFA-2 was shown to be essential for RNA pol I transcription in both life cycle stages [21]. To assess whether PF CITFA is functional in the BF system for *VSG* ES and *RRNA* promoter transcription, we first generated a clonal BF cell line that expressed the essential subunit CITFA7 (accession number Tb927.7.2600) exclusively as a C-terminal fusion with the composite PTP tag (Tu N. Nguyen et al., unpublished data). Extract from this cell line was depleted of CITFA7-PTP by virtue of IgG beads that tightly bind to the ProtA domains of the PTP tag (Fig. 5A). As expected, depletion of CITFA7 caused a dramatic decrease of *VSG*-trm and *Rib*-trm transcription whereas *SLins19* transcription remained unaffected (Figs. 5B and 5C). Adding back the CITFA complex, which was purified from a procyclic cell line [21] clearly restored RNA pol I transcription in a dose dependent manner from both *VSG* ES and *RRNA* promoters. These results demonstrated that, in regard to their essential transcription function, the CITFA complexes of BFs and PFs are equivalent. However and importantly, these results do not exclude the possibility that, *in vivo*, CITFA plays a role in RNA pol I-regulated expression of *VSG* ESs and procyclin gene units, for example by undergoing life cycle-specific interactions with other proteins, e.g. an activator of procyclin gene transcription in PFs or protein deposited in the ESB.

4. Conclusion

We have developed a straightforward, easy-to-establish method of extract preparation for a relatively small number of trypanosomes that accommodates the low cell densities of BFs in culture and thus may be applicable to other trypanosomatids. The extract is highly active and supports accurate RNA pol I transcription from three different promoters as well as RNA pol II transcription from the *SLRNA* promoter. The system will allow the functional characterization of trypanosome transcription factors or RNA pol subunits of which some may be specific to trypanosomes and others that are too divergent in sequence to be identified bioinformatically. An initial exploration of the BF system revealed that PF and BF systems behave very similarly for most reaction parameters suggesting that overall the basal transcription machineries in these two life cycle stages are equivalent. Accordingly, we could show that RNA pol II [43] and CITFA (Fig. 5), that were isolated from PFs, were fully functional in the BF system. On the other hand, we found clear differences in regard to GPEET promoter transcription. In the BF system, the optimal GPEET-trm template concentration was the same as those of the other two RNA pol I templates and distinctly lower than in the PF system. Accordingly, the about fourfold stronger GPEET-trm transcription signal relative to *Rib*-trm and *VSG*-trm signals in PF extract ([28] and Fig. 1) was lost in the BF system in which the signals of all three RNA pol I templates were of comparable strength (Fig. 4B). Furthermore, GPEET-trm transcription was sensitive to 4 mM Mn^{2+} in the BF system (Fig. 4A) whereas it was completely resistant to this manganese concentration in PFs [28]. These findings strongly indicate that PFs possess a procyclin promoter-specific transcriptional activator that is either not expressed or inactive in BFs.

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Fig. 1.

Establishing small scale preparation of transcriptionally active extract. (A) Comparison of co-transcription reactions conducted with procyclic extract that was prepared either in large scale (lsc) or in small scale (ssc). The reactions contained the template SLins19 (*SLRNA* promoter) and one of the RNA pol I templates GPEET-trm (*GPEET* promoter), Rib-trm (*RRNA* promoter) or VSG-trm (*VSG ES* promoter). Arrowheads on the right point to the correct transcriptional signals which were obtained by primer extension assays, separated by denaturing PAGE, and visualized by autoradiography. DNA marker sizes are specified on the left. (B) Corresponding experiments conducted with PF and BF extracts prepared in

small scale. Note that the relative *SLRNA* signal strengths can vary between different extracts and independent experiments.

Fig. 2. BF system optimizations. Co-transcriptions of VSG-trm and SLins19 at 27 °C, 30 °C, 33 °C and 37 °C (A) and in 2, 3, 4 and 5 mM MgCl₂ (B).

Fig. 3. Validation of the BF system. (A) Co-transcription of VSG-trm or Rib-trm with SLins19 in the presence and absence of 500 $\mu\text{g/ml}$ α amanitin (α ama). (B) VSG ES promoter sequence of VSG-trm from position -1 to position -70 relative to the TIS. The region of the block substitutions Mut1 and Mut2 are marked by asterisks and their sequences are provided under the wild-type sequence. For comparison, the residues which were found to be most important for promoter activity in a mutational analysis at single base pair resolution are underlined. (C) Co-transcription of SLins19 and VSG-trm constructs that harbor the wild-type promoter or a mutated promoter with the Mut1, the Mut2 or both (Mut1+2) block substitutions. Asterisks on the right indicate transcription signals of aberrant TISs.

Fig. 4.

GPEET promoter transcription differs in the BF system. (A) Co-transcriptions of SLins19 and GPEET-trm or VSG-trm in the absence (ctrl) or the presence of 2, 3 and 4 mM manganese chloride (MnCl₂). (B) Single transcription reactions were carried out with each of the three RNA pol I templates (left panel). On the right, the diagram shows the relative signal strengths from three independent experiments which were quantified by phosphor-imaging. The average of the *GPEET* signals was arbitrarily set to 100. Error bars represent standard deviations.

Fig. 5. CITFA purified from PFs is functional in the BF system. (A) Anti-ProtC immunoblot of CITFA7-PTP mock and immuno (depl)-depleted BF extract. Detection of the unrelated protein U2-40K served as loading control. (B,C) SLins19 and either VSG-trm (B) or Rib-trm (C) were co-transcribed in mock-depleted or CITFA7-depleted (depl) BF extract. The latter reaction was reconstituted with increasing amounts of the CITFA complex purified from PFs (depl + PF CITFA).