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The *Trypanosoma brucei* spliced leader RNA and rRNA gene promoters have interchangeable TbSNAP50-binding elements

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ABSTRACT

In the protist parasite *Trypanosoma brucei*, the small nuclear spliced leader (SL) RNA and the large rRNAs are key molecules for mRNA maturation and protein synthesis, respectively. The SL RNA gene (*SLRNA*) promoter recruits RNA polymerase II and consists of a bipartite upstream sequence element (USE) and an element close to the transcription initiation site. Here, we analyzed the distal part of the ribosomal (*RRNA*) promoter and identified two sequence blocks which, in reverse orientation, closely resemble the *SLRNA* USE by both sequence and spacing. A detailed mutational analysis revealed that the ribosomal (r)USE is essential for efficient *RRNA* transcription *in vivo* and that it functions in an orientation-dependent manner. Moreover, we showed that USE and rUSE are functionally interchangeable and that rUSE stably interacted with an essential factor of *SLRNA* transcription. Finally, we demonstrated that the *T.brucei* homolog of the recently characterized transcription factor p57 of the related organism *Leptomonas seymouri* specifically bound to USE and rUSE. Since p57 and its *T.brucei* counterpart are homologous to SNAP50, a component of the human small nuclear RNA gene activation protein complex (SNAPc), both *SLRNA* and *RRNA* transcription in *T.brucei* may depend on a SNAPc-like transcription factor.

INTRODUCTION

Eukaryotic RNA polymerase (pol) I exclusively transcribes the large ribosomal gene unit (*RRNA*), denoted as class I transcription, and is specifically recruited solely to the *RRNA* promoter. The parasite *Trypanosoma brucei*, causative pathogen of African trypanosomiasis, belongs to the protist family Trypanosomatidae and is the only known species which utilizes RNA pol I for both rRNA synthesis and transcription of some of its protein-coding genes, namely those encoding its major surface antigens procyclin and variant surface glycoprotein (VSG). Four different types of class I promoters have been characterized in *T.brucei*. Besides the *RRNA* promoter, these are the metacyclic and bloodstream form *VSG* gene expression site promoters and the promoters of the procyclin transcription units [reviewed in Günzl (1)]. There is no obvious sequence homology among these promoter types, and structurally they fall into two classes. The two expression site promoters are very short, extending only to position –67 relative to the transcription initiation site (2–4), whereas the procyclin gene and *RRNA* promoters have a four-domain structure extending approximately to position –250 (5–7). The details known about the latter two promoters closely resemble the structure of the *RRNA* promoter of *Saccharomyces cerevisiae*. The yeast promoter has been characterized in detail and consists of a proximal core promoter, denoted as domain I, and a bipartite upstream element (USE) comprising domains II and III (8,9). The distal domain IV corresponds to the Reb1p-binding element (Reb1) centered at position –215. In *T.brucei*, procyclin gene promoter domains I–IV and *RRNA* promoter domains I and II have been exactly mapped by block substitution analyses (5–7,10) and the presence of *RRNA* promoter domains III and IV has been indicated by progressive 5′ deletions (7,11). The elements in these two promoters are similar in size to their yeast counterparts and are located at corresponding positions, suggesting that they may be functionally analogous.

Like rRNA, spliced leader (SL) RNA is an essential structural RNA which trypanosomes need continuously in large amounts for protein-coding gene expression. *Trypanosoma brucei* and related organisms transcribe their protein-coding genes polycistronically, and individual mRNAs are processed from large precursors by *trans* splicing and polyadenylation. In *trans* splicing, the 39 nt long SL is cleaved from the 5′ terminus of the SL RNA and fused to the 5′ end of each mRNA. This SL addition *trans* splicing is an obligatory mRNA processing step in trypanosomes and requires the consumption of one SL RNA molecule for the maturation of one mRNA molecule. Hence, the pathogen crucially depends on strong constitutive SL RNA gene (*SLRNA*) expression throughout its life cycle. Each trypanosome contains approximately 200 *SLRNA*s which are organized in tandem repeats of 1.35 kb and which accommodate the

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high rate of SL RNA synthesis. As has been demonstrated in the related organism *Leptomonas seymouri*, *SLRNA* transcription is mediated by RNA pol II (12). The structure of the *SLRNA* promoter has been meticulously characterized in the three trypanosomatid species: *T.brucei* (13), *L.seymouri* (14,15) and *Leishmania tarentolae* (16). In all three cases, two USEs, here denoted as USE1 and USE2, were essential for *SLRNA* transcription. The two sequence blocks form a bipartite USE because minimal changes of the distance between the two blocks severely affected transcription efficiency (16,17). In *L.seymouri*, a transcription factor which binds specifically to the USE has been characterized (18). The factor was termed promoter-binding protein 1 (PBP-1) and shown to be part of a functional *SLRNA* transcription initiation complex (19). PBP-1 consists of three subunits with apparent M_rs of 57, 46 and 36 kDa. Purification of PBP-1 led to the identification and cloning of two subunits (19). Whereas p46 has no homology to any known transcription factor or to sequences of other trypanosomatid genome databases, p57 is homologous to the SNAP50 subunit of the human small nuclear RNA (snRNA)-activating protein complex (SNAPc). Human SNAPc is an essential factor for RNA pol II- or III-mediated transcription of genes encoding spliceosomal uridylic acid-rich (U) snRNAs [reviewed in Hernandez (20)]. No other function has been reported yet. Appropriately, the trypanosome SL RNA resembles a spliceosomal U snRNA because it has the same size, it is predominantly located in the nucleus (21) and it assembles in a corresponding ribonucleoprotein particle by binding a set of common proteins (22).

In this study, we discovered that *T.brucei RRNA* promoter domain IV harbors two sequence elements which closely resembled the bipartite *SLRNA* USE. Astonishingly, this ribosomal (r)USE was essential for efficient *RRNA* transcription in transiently transfected cells and could be functionally replaced by the *SLRNA* USE. Furthermore, it specifically bound the *T.brucei* homolog of SNAP50 (TbSNAP50), suggesting that a SNAPc-like complex is involved in *T.brucei* class I transcription.

MATERIALS AND METHODS

Plasmid construction

Transcription template constructs SLins19, Rib-trm and GPEET-trm have been described in detail previously (23) as well as SLins19 linker scanner mutations LS –71/–62 and LS –53/–42 (13). Construct RibCAT was made for transient transfection analysis and is a derivative of pJP44, a *T.brucei* transfection vector, in which the procyclin gene promoter *GPEET* and flanking regions drive the expression of the chloramphenicol acetyltransferase gene [*CAT* (5)]. RibCAT was constructed by replacing the *GPEET* promoter in pJP44 by the *RRNA* promoter from construct Rib-trm using KpnI and SmaI restriction sites. For the block substitution constructs RibCAT1–6 and constructs RibCAT +4, +11, REV and USE, mutated *RRNA* promoter sequences were generated by single-step or overlapping PCR and cloned into KpnI–SmaI sites of RibCAT replacing the wild-type promoter. Plasmid TbSNAP50-TAP was used to epitope-tag TbSNAP50 and contains two sequence units. The first unit consists of 462 bp of the 3′-terminal TbSNAP50-coding region followed by the tandem affinity purification (TAP) tag sequence (24) and 482 bp of the 3′-flanking region of *TbRPA1*, the gene encoding the largest subunit of RNA pol I. An engineered NotI site connects the TbSNAP50-coding region and the TAP tag. As a selectable marker, the second unit harbors the neomycin phosphotransferase gene flanked 5′ and 3′ by the intergenic regions of heat shock protein 70 (*HSP70*) genes 2 and 3, and of β- and α-tubulin genes, respectively.

Cell culture, cell lines and extract preparation

Cultivation of procyclic forms of *T.brucei brucei* strain 427 and preparation of transcription-competent extracts were carried out as described (23). For the generation of cell line TbC8, 10 μg of pTbSNAP50-TAP were linearized with BstBI inside the TbSNAP50-coding region and transfected by electroporation into 10^8 trypanosomes (25,26). Transfected cells were cloned by limiting dilution and selected at 40 μg/ml of the antibiotic G418. Correct integration of the plasmid at the TbSNAP50 locus was confirmed by Southern blotting of BstNI- and HincII-digested genomic DNA of TbC8 and non-transfected cells (data not shown). Cell line TbD11 was generated in an analogous way expressing the TAP tag at the homolog of the human U1 snRNA-specific 70K protein.

Transient transfection assays

Transient transfection, RNA preparation and RNA detection were essentially conducted as described previously (27). In brief, cells were co-transfected with 50 μg of plasmid RibCAT and 20 μg of construct TU2∇81 containing a tagged U2 snRNA gene (28). At 16 h after transfection, total RNA was isolated and CAT and TU2∇81 RNAs were detected by primer extension of ^{32}P-end-labeled oligonucleotides CAT5 (5′-GCCATTGGGATATATCAACGG-3′) and U2-Btag (5′-GATCCTTGCGGGATCCCG-3′), respectively. Primer extension products were separated on 6% polyacrylamide–50% urea gels, visualized by autoradiography and quantified by densitometry. A minimum of three independent experiments was conducted for each construct.

In vitro transcription assays

In vitro transcription assays have been described in detail elsewhere (10,23). A standard *in vitro* transcription reaction was carried out in a volume of 40 μl for 60 min at 27°C and contained 8 μl of extract, 20 mM potassium L-glutamate, 20 mM KCl, 3 mM $MgCl_2$, 20 mM HEPES-KOH, pH 7.7, 0.5 mM of each NTP, 20 mM creatine phosphate, 0.48 mg ml^{-1} of creatine kinase, 2.5% polyethylene glycol, 0.2 mM EDTA, 0.5 mM EGTA, 4 mM dithiothreitol (DTT), 10 mg ml^{-1} of leupeptin, 10 mg ml^{-1} of aprotinin and 40 μg ml^{-1} of template DNA. For competition experiments, linear competitor DNAs were produced by PCR using the High Fidelity Expand System (Roche). For wild-type *RRNA* and *SLRNA* competitors, RibCAT and SLins19 served as templates, respectively, while rUSE1-mut was generated with RibCAT4, rUSE2-mut with RibCAT2, USE1mut with SLins19 LS –71/–62, and USE2-mut with SLins19 LS –53/–42. The competitor fragments were separated by agarose gel electrophoresis, and purified from gel slices with the QIAquick gel extraction kit (Qiagen) according to the manufacturer's protocol. The transcription competition

experiments were carried out by pre-incubating competitor DNA, cell extract and reaction components on ice for 15 min before transcription was started by transferring reactions to 27°C and adding template DNA and nucleoside triphosphates. Transcription was terminated after 60 min by addition of guanidinium thiocyanate solution. Subsequently, total RNA was prepared and Rib-trm and SLins19 RNAs were specifically detected by primer extension of 5′-end-labeled oligonucleotides Tag_PE (23) and SLtag (13), respectively. In control reactions, endogenous U2 snRNA was detected with either oligonucleotide U2f or oligonucleotide U2k (29).

Promoter pull-down assay

Biotinylated promoter DNA fragments were generated by PCR using a 5′-biotinylated sense oligonucleotide. For the generation of fragments *GPEET* –246/–162, *RRNA* –257/–162 and rUSE1-mut, the 5′-biotinylated T7 sense oligonucleotide was used which added 41 bp of vector sequence 5′ to the promoter region, whereas for fragments *SLRNA* –126/–18 and USE1-mut, the biotinylated oligonucleotide SL14 was used which is sense to *SLRNA* promoter positions 126 to 107 and did not add extra base pairs to the fragment. For each reaction, 500 ng of biotinylated DNA fragments were coupled to 10 µl (100 µg) of RNase-free, paramagnetic M-280 Streptavidin Dynabeads (Dynal) according to the manufacturer's protocol. Consistently, we observed a DNA binding efficiency of >90% (data not shown). After binding, the beads were equilibrated and blocked for 30 min at room temperature in TK_{20} buffer (150 mM sucrose, 20 mM HEPES-KOH, pH 7.7, 20 mM potassium L-glutamate, 20 mM KCl, 3 mM $MgCl_2$, 2.5% (w/v) polyethylene glycol, 0.2 mM EDTA, 0.5 mM EGTA, 4 mM DTT, 10 µg ml^{-1} leupeptin, 10 µg ml^{-1} aprotinin) containing 5 mg ml^{-1} bovine serum albumin and 5 mg ml^{-1} polyvinylpyrrolidone. The beads were washed twice with 0.5 ml of TK_{20} buffer. For TbSNAP50 binding, the beads were incubated in a 40 µl *in vitro* transcription reaction first on ice for 15 min and then at 27°C for 15 min. Subsequently, the beads were washed three times with 0.5 ml of TK_{20} buffer and once with 0.5 ml of TN_{40} buffer (150 mM sucrose, 20 mM Tris pH 8.0, 40 mM NaCl, 3 mM $MgCl_2$, 0.5 mM DTT, 10 µg ml^{-1} leupeptin, 10 µg ml^{-1} aprotinin). For protein elution, beads were resuspended in standard SDS gel loading buffer and incubated for 5 min at 70°C. Half of the eluate was separated on an SDS–8% polyacrylamide gel and electroblotted onto a PVDF membrane. TbSNAP50-TAP was detected by the PAP reagent (Sigma) in combination with the BM Chemiluminescence Blotting substrate (Roche).

RESULTS

The *T.brucei RRNA* promoter contains sequences similar to the *SLRNA* promoter USE

In a previous study, we unexpectedly observed that *SLRNA* transcription *in vitro* was efficiently competed by a linear *RRNA* promoter fragment extending from position –257 to –3 (–257/–3), suggesting that the *RRNA* promoter sequence was able to stably bind a *trans*-activator of *SLRNA* transcription (10). This competitive effect was specific for the *RRNA* promoter fragment and not seen with corresponding fragments of other *T.brucei* class I promoters (10). When we compared

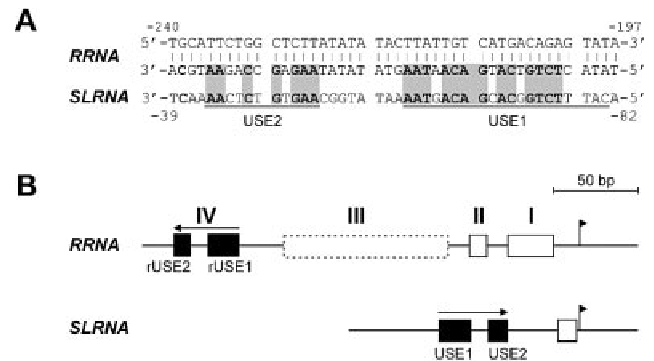

Figure 1. Similar sequence elements are present in *T.brucei RRNA* and *SLRNA* promoters. (**A**) The double-stranded sequence of the *RRNA* promoter from position –240 to position –197 relative to the transcription initiation site is aligned with the reversed sense strand of the *SLRNA* promoter spanning positions –39 to –82. Identical nucleotides in the antisense strand of the *RRNA* promoter and the sense strand of the *SLRNA* promoter are drawn in bold letters and shaded in gray. The two essential *SLRNA* promoter elements USE1 and USE2 are underlined. (**B**) Schematic outline to scale of *RRNA* and *SLRNA* promoters. Promoter elements are represented as boxes and the transcription initiation sites by flags. *RRNA* promoter domain IV consists of two sequence blocks which were designated as rUSE1 and rUSE2 and, as their *SLRNA* counterparts USE1 and USE2, are drawn as black boxes. The arrows indicate the opposite orientation of USE and rUSE in their respective promoters. The stippled line of the box representing *RRNA* promoter domain III indicates that promoter sequences have not been mapped in this domain.

the sequences of *RRNA* and *SLRNA* promoters, we found two sequence blocks in the distal part of the *RRNA* promoter which closely resembled USE1 and USE2 of the *SLRNA* promoter by both sequence and spacing (Fig. 1). However, in the *RRNA* promoter, these sequence elements are in opposite orientation to the direction of transcription. In the ribosomal sequence, 13 bp out of 16 bp are identical to USE1 and 6 bp out of 10 bp are identical to USE2. The first 4 bp of USE1 do not match the ribosomal sequence, indicating that they may not be relevant to transcription. The spacing between the two ribosomal sequence blocks is 8 bp and identical to that of USE1 and USE2 in the *SLRNA* promoter. Hence, we named these two elements according to their *SLRNA* counterparts as rUSE1 and rUSE2 (Fig. 1). The sequence consensus is confined to these two blocks and not present in the spacer or flanking regions. We could not detect any similarity to the SL RNA sequence in the ribosomal spacer, indicating that it does not contain an *SLRNA*-like gene.

rUSE1 and rUSE2 sequester an essential factor of *SLRNA* transcription

To determine whether rUSE1 and rUSE2 are responsible for the competitive effect on *SLRNA* transcription, we first dissected the *RRNA* –257/–3 competitor fragment and analyzed its effect on both *RRNA* and *SLRNA* transcription. The *in vitro* transcription system we employed is based on a crude cytoplasmic extract with high non-specific labeling activity. To avoid excessive background labeling, we inserted an unrelated tag sequence into our template constructs downstream of the transcription initiation site and detected transcripts from these templates specifically by primer extension of a 5′-end-labeled oligonucleotide complementary to the

tag (23). For *RRNA* promoter transcription, we used the construct Rib-trm and for *SLRNA* promoter transcription the construct SLins19 (23). Rib-trm and SLins19 RNAs with correct 5′ ends gave rise to 127 and 71 nt primer extension products, respectively (Fig. 2). As previously observed, competition of Rib-trm transcription with a 10-fold molar excess of the linear DNA promoter fragment *RRNA* –257/–3 strongly reduced the Rib-trm transcription signal (Fig. 2, compare lanes 1 and 2). The competitor fragment was then split in two. Fragment *RRNA* –162/–3 spanning promoter domains I–III competed nearly as efficiently as the full-length competitor, whereas fragment *RRNA* –257/–162, containing the bipartite rUSE, had no detectable effect on *RRNA* transcription (Fig. 2, lanes 3 and 4). The latter result was expected because we had shown that *RRNA* promoter domain IV is dispensable for transcription *in vitro* [(23) and data not shown]. When *SLRNA* transcription was competed with these DNA fragments, the opposite result was obtained. Whereas fragment *RRNA* –257/–162 retained the competitive effect of the full-length competitor, *RRNA* –162/–3 had no influence on *SLRNA* transcription, demonstrating that an essential *SLRNA* *trans*-activator interacted with *RRNA* promoter domain IV (Fig. 2, lanes 6–8).

In a second set of experiments, we mutated rUSE1 and rUSE2 in the *RRNA* –257/–162 competitor fragment and competed SLins19 transcription *in vitro* (Fig. 3). Mutation of rUSE1 completely abolished the competitive effect of *RRNA* –257/–167, whereas mutation of rUSE2 retained some of the DNA fragment's competitive ability, suggesting that rUSE1 is the main sequence element binding to the transcription factor (Fig. 3B, compare lanes 1–4). We prepared corresponding competitor fragments from the *SLRNA* promoter to analyze whether USE1 and USE2 were capable of competing *SLRNA* transcription in a similar fashion. The wild-type competitor fragment *SLRNA* –126/–18 competed SLins19 transcription as efficiently as the wild-type ribosomal fragment (Fig. 3B, compare lanes 1, 2 and 5), whereas SLRNA –126/–18 competitors with mutations in either USE1 or USE2 did not compete at all (Fig. 3B, lanes 6 and 7). We have reproducibly seen that mutation of USE2 had a stronger effect on *SLRNA* transcription competition than mutation in rUSE2, indicating that factor binding is not completely equivalent in the two different promoters. Taken together, these data demonstrated that USE and rUSE stably interacted with and sequestered a *trans*-activating factor of *SLRNA* transcription.

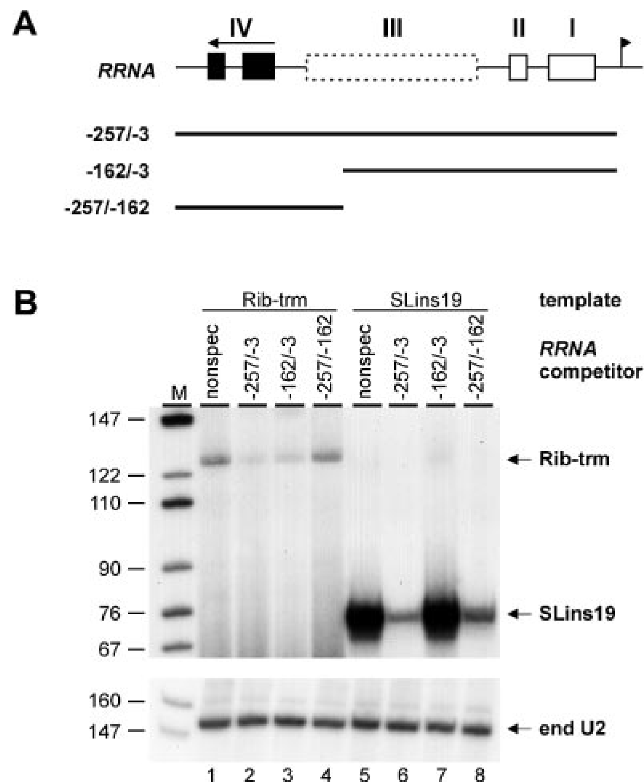

Figure 2. *In vitro* competition of Rib-trm and SLins19 transcription with linear *RRNA* promoter fragments. (**A**) Schematic outline to scale of the *RRNA* promoter (see legend of Fig. 1) and of the competitor fragments *RRNA* –257/–3, *RRNA* –162/–3 and *RRNA* –257/–162. (**B**) Transcription competition analysis. Template constructs Rib-trm or SLins19 were transcribed *in vitro* in the presence of linear DNA competitor fragments. As competitors, a 222 bp non-specific linear DNA fragment (nonspec) or *RRNA* promoter fragments were used in a 10-fold molar excess to template DNA. Rib-trm and SLins19 transcripts were detected by primer extension of total RNA prepared from transcription reactions with 5′-end-labeled oligonucleotides Tag_PE and SLtag, respectively. In control reactions, endogenous U2 snRNA was detected in the same RNA preparations by primer extension using the 5′-end-labeled oligonucleotide U2k. Primer extension products were separated on 6% polyacrylamide–50% urea gels and visualized by autoradiography. Arrows on the right point to primer extension signals of Rib-trm, SLins19 and endogenous U2 (end U2) RNAs. M, marker (MspI-digested pBR322); lengths of marker fragments are indicated on the left.

rUSE1 and rUSE2 are essential sequence determinants for efficient *RRNA* transcription *in vivo*

Next, we asked whether rUSE1 and rUSE2 function in *RRNA* transcription. Mutation of these sequence elements, however, did not detectably affect *in vitro* transcription of the construct Rib-trm (data not shown). This result was in accordance with our previous finding that deletion of the distal *RRNA* promoter portion did not significantly affect *in vitro* transcription efficiency (23). In contrast, deletion of *RRNA* promoter domain IV to position –181 reduced transient reporter gene expression to 14% in a different study (30). Accordingly, we investigated whether rUSE is responsible for this effect. Since in trypanosomes expression of protein-coding genes requires a splice site and a polyadenylation signal we made the construct RibCAT in which the *RRNA* promoter drives *CAT* expression and the procyclin gene-flanking regions provide the RNA processing signals. To analyze the complete domain IV of the *RRNA* promoter, we mutated the region from position –257 to –182 in six adjacent blocks (Fig. 4A). The constructs were transiently transfected into procyclic trypanosomes and *CAT* expression was analyzed at the level of mRNA by primer extension assays using a 5′-end-labeled, *CAT*-specific oligonucleotide. To control transfection, RNA preparation and primer extension efficiencies in our experiments, we co-transfected a tagged version of the U2 snRNA gene and analyzed its expression correspondingly. Mutation of rUSE2 in plasmid RibCAT2 reduced the *CAT* mRNA level in comparison with experiments with the wild-type construct to 23% and the two block mutations of rUSE1 in constructs RibCAT4 and RibCAT5 diminished the level to 10 and 9%, respectively (Fig. 4B and C). These results clearly

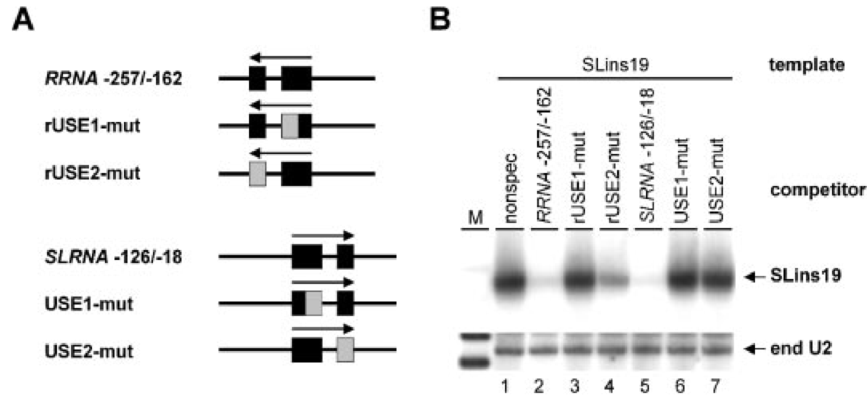

Figure 3. rUSE and USE stably interact with a *trans*-activating *SLRNA* transcription factor. (**A**) Schematic outline of *RRNA* –257/–162 and *SLRNA* –126/–18 competitor fragments and their mutated versions (r)USE1-mut and (r)USE2-mut. (r)USE1 and (r)USE2 are represented by black boxes and mutated regions by gray boxes. Mutation of rUSE1 and USE1 comprised the substitution of the inner 10 bp by the sequence 5′-TGACATATGA-3′, whereas rUSE2 and USE2 were completely replaced by the sequence 5′-CTTGACATATGC-3′. These sequences relate to the orientation of rUSE and USE as indicated by arrows. (**B**) Competition of SLins19 transcription. *In vitro* transcription reactions were carried out in the presence of a 10-fold molar excess of linear DNA competitor fragments. SLins19 transcription signals and the endogenous U2 snRNA control signals (end U2) were obtained by primer extension assays of total RNA with 5′-end-labeled oligonucleotides. The primer extension products were separated by denaturing gel electrophoresis and visualized by autoradiography. M, marker MspI-digested pBR322.

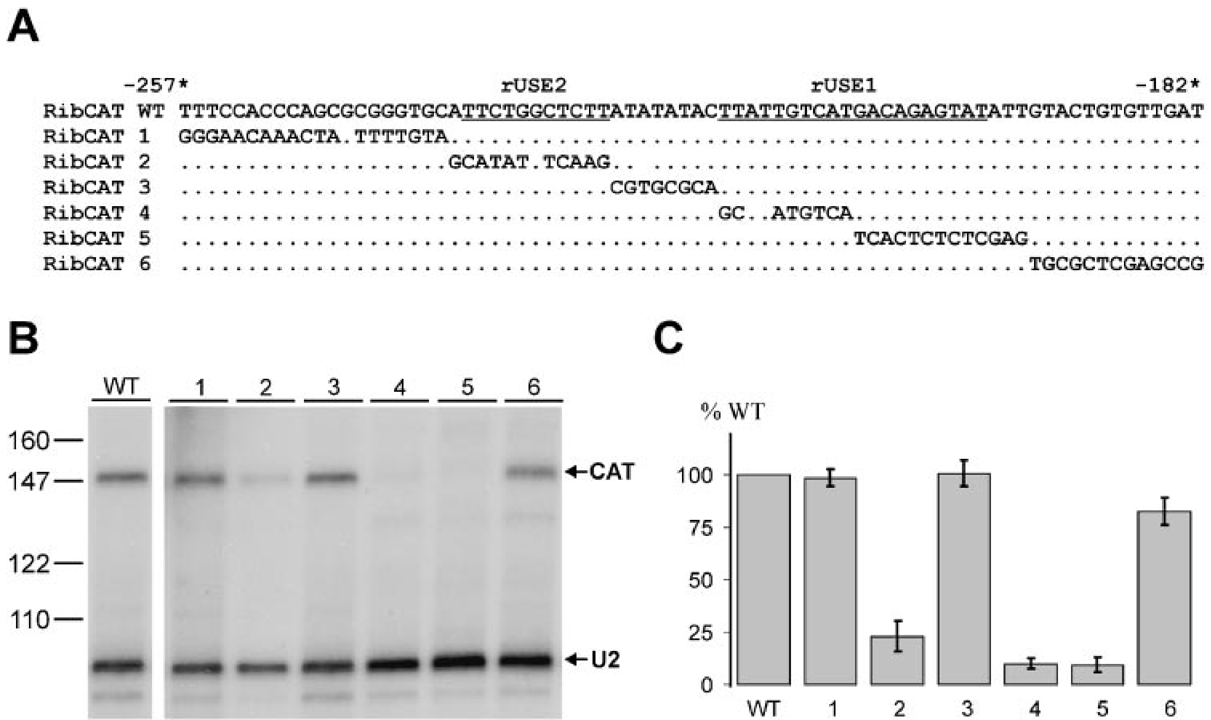

Figure 4. Block substitution analysis of *RRNA* promoter domain IV. (**A**) Depicted are sequences of the wild-type *RRNA* promoter domain IV in the construct RibCAT and of the block substitutions in the mutational constructs RibCAT1–6. Numbering is relative to the transcription initiation site, and the sequences of rUSE1 and rUSE2 are underlined. Unchanged nucleotides are indicated by dots. (**B**) Transient transfection analysis of promoter mutations. The wild-type RibCAT (WT) and the mutational constructs RibCAT1–6 were transfected into procyclic *T.brucei* cells together with the control plasmid TU2∇81 carrying an oligonucleotide-tagged U2 snRNA gene. At 16 h after transfection, total RNA was prepared from transfected cells and analyzed by primer extension with the 5′-end-labeled oligonucleotides CAT5 and U2-BTAG which are complementary to the CAT-coding region and the TU2∇81 tag sequence, respectively. Primer extension products were separated on a 6% polyacrylamide–50% urea gel and visualized by autoradiography. On the left, positions of MspI-digested pBR322 marker fragments are indicated, and on the right, arrows point to the primer extension products of CAT mRNA and TU2∇81 snRNA (U2). (**C**) Primer extension signals of three independent experiments were quantified by densitometry. The CAT signal strengths were standardized with TU2∇81 signals and the signal strength of wild-type RibCAT was set to 100%. Means and standard deviations are graphically depicted.

demonstrated that both rUSE1 and rUSE2 were essential for efficient transcription of the ribosomal gene unit inside the cells. In contrast, mutation of the rUSE spacer and flanking regions did not strongly affect *CAT* expression. Hence, the promoter-relevant sequences in *RRNA* promoter domain IV are confined to rUSE1 and rUSE2.

The function of rUSE is orientation dependent and it can be functionally replaced by the USE

Having identified the bipartite rUSE as an important *RRNA* promoter element, we altered promoter domain IV in construct RibCAT in several ways to learn more about its operating mode (Fig. 5A). First, we increased the distance between rUSE and the transcription initiation site by 4 and 11 bp. In transient transfection experiments, these manipulations did not interfere with *RRNA* promoter function and even increased *RRNA* promoter-driven *CAT* expression to some extent, suggesting that there is some flexibility in the position of rUSE (Fig. 5B, lanes WT, +4 and +11). Furthermore, we made construct RibCAT-REV in which rUSE was replaced by its reverse complement. This manipulation reduced *CAT* expression to 26% of the wild-type level (Fig. 5B and C, lane REV). Since this reduction is in the range of what was observed with mutating rUSE2 (Fig. 4), we concluded that rUSE functions in an orientation-dependent manner. Finally, the sequence homology between rUSE and USE, and the capability of rUSE to sequester an essential *SLRNA* transcription factor *in vitro* suggested that the two bipartite promoter elements are functionally equivalent. To test this hypothesis in the *RRNA* promoter, we substituted rUSE by USE in the construct RibCAT-USE (Fig. 5A). Astonishingly, the *SLRNA* promoter element was able to functionally replace rUSE in the *RRNA* promoter and even increased *CAT* expression significantly above the wild-type level (Fig. 5B and C, lane USE). We therefore concluded that USE has the functional property of rUSE. To the best of our knowledge, this is the first report of a small RNA gene promoter element functioning in eukaryotic class I transcription.

We also conducted the reciprocal experiment and investigated whether rUSE can functionally replace USE in the *SLRNA* promoter. In Figure 6, *in vitro* transcription results of the unaltered SLins19 construct and three derivatives are shown. The latter comprised linker scanner mutations of USE1 and USE2, and the construct SLins19-rUSE in which USE was replaced by its ribosomal counterpart. The two linker scanner mutations had been analyzed before in a nuclear extract and dramatically reduced *SLRNA* transcription efficiency, with LS –53/–42, the construct harboring the USE2 mutation, having an even stronger effect than the USE1 mutation LS –71/–62 (13). For comparison, these constructs were re-tested in our cytoplasmic transcription extract and revealed results similar to those in the previous study. Compared with the wild-type signal, mutation of USE1 caused a reduction of the transcription signal by 82%, whereas mutation of USE2 nearly abolished transcription (Fig. 6, compare lane 1 with lanes 2 and 3). In construct SLins19-rUSE, the ribosomal sequence was able to promote significantly more *SLRNA* transcription than either USE mutation ($P < 0.005$, *t*-test), but 66.3% less than the wild-type promoter, revealing that rUSE can only partially replace USE in the *SLRNA* promoter, again indicating that the interaction of the *trans*-activating factor with the *RRNA* and the *SLRNA* promoter is not equivalent.

rUSE and USE bind TbSNAP50 *in vitro*

The data obtained thus far suggested that the same factor interacted with both *SLRNA* promoter and *RRNA* promoter. Characterization of the *L.seymouri* SNAP50 homolog as a component of an *SLRNA* transcription factor binding to USE1 (19) enabled us to directly test this hypothesis. As a prerequisite to study the *T.brucei* SNAP50 homolog (TbSNAP50), we isolated, cloned and sequenced its complete cDNA (GenBank accession no. AJ581666). The sequence consists of the SL, a 94 bp 5′-untranslated region (UTR), 1347 bp of coding region, 484 bp of 3′ UTR and the poly(A) tail. The encoded protein comprises 448 amino acids with a predicted M_r of 51 kDa and a theoretical pI of 5.58. A pairwise alignment using the program ClustalW (31) revealed that TbSNAP50 is 38% identical and 57% similar to its homolog in *L.seymouri* and 19% identical and 40% similar to its human counterpart [(19) and data not shown]. Furthermore, based on eight different digests of genomic DNA, Southern analysis showed that TbSNAP50 is encoded by a single copy gene

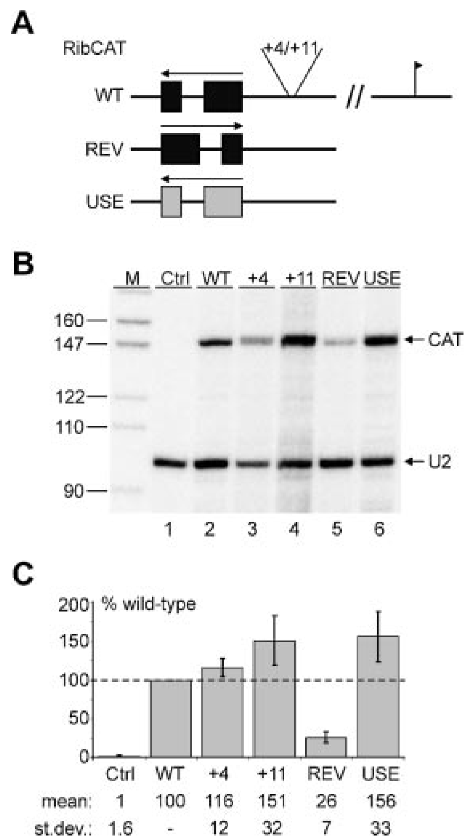

Figure 5. Manipulation of *RRNA* promoter domain IV. (**A**) Schematic outline of mutant constructs. In plasmids RibCAT +4 and RibCAT +11, rUSE was moved further upstream of the transcription initiation site by 4 and 11 bp, respectively. In construct RibCAT-REV, rUSE was replaced by its reverse complement and in construct RibCAT-USE by the corresponding sequence of the *SLRNA* promoter. (**B**) In transient expression experiments, RibCAT constructs were co-transfected with the control plasmid TU2∇81 into procyclic cells. In a control experiment (Ctrl), only the TU2∇81 plasmid was transfected. CAT and TU2∇81 expression was analyzed as described in Figure 4 by primer extension of total RNA prepared from transfected cells. M, marker MspI-digested pBR322; lengths of marker fragments are indicated on the left. Arrows on the right point to primer extension products of CAT mRNA and TU2∇81 snRNA (U2). (**C**) Densitometric analysis of CAT and TU2∇81 signal strengths in three independent experiments. The standardized RibCAT (WT) signal strength was set to 100. Means and standard deviations (st.dev.) obtained with mutant constructs are given in graphic and numeric form.

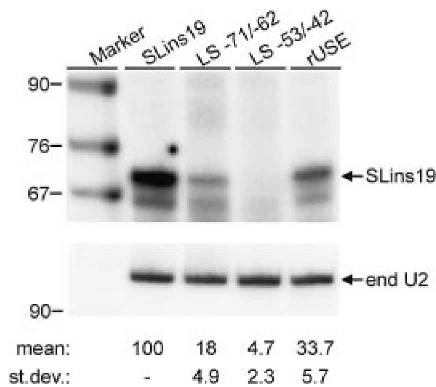

Figure 6. rUSE can partially replace USE function in the *SLRNA* promoter. Standard *in vitro* transcription reactions were carried out with SLins19 constructs carrying the *SLRNA* wild-type promoter or derivatives with linker scanner mutations in USE1 (LS –71/–62) or USE2 (LS –53/–42). In construct SLins19-rUSE (rUSE), the complete USE sequence from position –71 to position –42 was replaced by the corresponding sequence of rUSE. As a control, endogenous U2 snRNA (end U2) was detected in each reaction by primer extension of oligonucleotide U2f. Below each lane, means and standard deviations of standardized transcription signal strengths derived from six independent experiments are given relative to the wild-type SLins19 signal which was set to 100. The arrow on the right points to the specific primer extension signal of SLins19 RNA. The band below the main signal appears in some transcription extracts and may be caused by *in vitro* methylation of 5′-terminal SLins19 RNA nucleotides which is known to terminate primer extension signals prematurely. On the left, sizes of pBR322 MspI marker fragments are indicated.

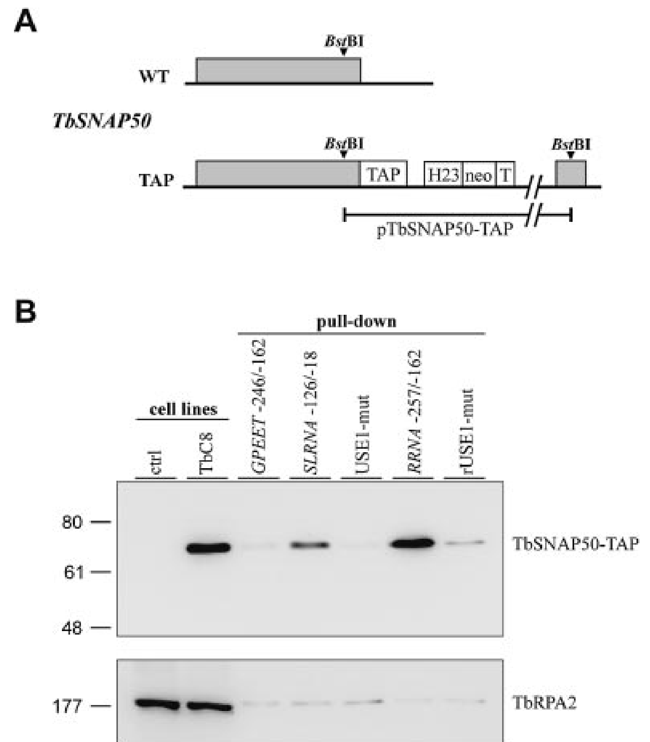

Figure 7. TbSNAP50 binds to USE and rUSE. (**A**) Illustration of the two TbSNAP50 alleles (not to scale) in the procyclic cell line TbC8. Shown are the unaltered wild-type allele (WT) and the modified allele in which the TAP sequence was fused 3′ terminally to the *TbSNAP50* coding region by targeted insertion of the BstBI-linearized construct pTbSNAP50-TAP (TAP). As a selection marker, the construct contained the neomycin phosphotransferase gene (neo) flanked by *HSP70* genes 2 and 3 (H23) and βα tubulin (T) intergenic regions. (**B**) Immunoblot analysis of transcription extract prepared from TbC8 or control cells (ctrl), and of proteins bound to immobilized DNA fragments (pull-down). The latter comprised procyclin *GPEET* promoter domain IV (*GPEET* –246/–162), the wild-type *SLRNA* promoter upstream region (*SLRNA* –126/–18) and a corresponding fragment carrying a mutation in USE1 (USE1-mut) as well as the wild-type *RRNA* promoter domain IV (*RRNA* –257/–162) and an equivalent fragment with a mutation in rUSE1 (rUSE1-mut). The same blot was analyzed with the PAP reagent specific for the protein A epitopes within the TAP tag (TbSNAP50-TAP) and with a polyclonal antibody directed against TbRPA2, the second largest subunit of RNA pol I.

(data not shown). As a tool to investigate binding of TbSNAP50 to *SLRNA* and *RRNA* promoters, we epitope-tagged TbSNAP50 C-terminally with the TAP tag (24). Tagging was achieved by targeted insertion of construct pTbSNAP50-TAP into one *TbSNAP50* allele (Fig. 7A). The calculated mass of TAP-tagged TbSNAP50 is 72 kDa, and immunoblot analysis with a peroxidase-labeled IgG domain recognizing the protein A epitopes within the TAP tag specifically detected a polypeptide of this size in cell line TbC8 (Fig. 7B, compare lanes 1 and 2). We then prepared a transcription extract from TbC8 cells and employed a pull-down assay using immobilized promoter DNA fragments to analyze binding of TbSNAP50 to USE and rUSE. In a negative control, we showed that procyclin gene promoter domain IV which did not compete *SLRNA* transcription in a previous study (10) was unable to bind TbSNAP50 in the pull-down assay (Fig. 7B, lane 3). In contrast, tagged TbSNAP50 specifically bound to the upstream region of the *SLRNA* promoter and domain IV of the *RRNA* promoter (Fig. 7B, lanes 4 and 6). In *L.seymouri*, the transcription factor harboring the SNAP50 homolog p57 binds to USE1. In accordance with this observation, mutation of USE1 and rUSE1 abolished binding of TbSNAP50 to the *SLRNA* and *RRNA* promoter fragments, respectively (Fig. 7B, lanes 5 and 7). In a control experiment, cell line TbD11 was generated in which the TAP tag was fused to an snRNP-specific protein with no known transcriptional function. Pull-down assays with TbD11 extract revealed that the tagged protein did not bind to *RRNA* and *SLRNA* promoter DNA, excluding the possibility that the TAP tag is responsible for the observed TbSNAP50-binding phenotype (data not shown). Furthermore, we have reproducibly seen that the ribosomal DNA bound TbSNAP50 more efficiently than the *SLRNA* fragment, which is in contrast to the transcription competition assays where both promoter DNAs competed *SLRNA* transcription equally well. An explanation for this discrepancy may be that in the *SLRNA* fragment, the distance between the immobilizing biotin group and USE is shorter and that in this fragment USE1 is located towards the biotin group, possibly causing a steric problem for efficient binding of a multi-subunit transcription complex. Nevertheless, our results clearly showed that TbSNAP50 specifically binds to the USE of the *SLRNA* promoter and, in addition, to the rUSE of the *RRNA* promoter. Taking into account that *RRNA* promoter domain IV is capable of efficiently competing *SLRNA* transcription, this finding strongly indicates that in *T.brucei* synthesis of both rRNA and SL RNA depends on the same SNAPc-like transcription factor.

DISCUSSION

We have found that the distal part of the *T.brucei RRNA* promoter contains the bipartite sequence element rUSE which closely resembles the USE of the *SLRNA* promoter and which binds an essential *SLRNA* transcription factor *in vitro*. Although rUSE and USE apparently bind the same transcription factor, these elements serve different functions in their respective promoters. In the *SLRNA* promoter, USE is required for efficient *SLRNA* transcription initiation *in vivo* and *in vitro*, indicating that this bipartite element is directly involved in the formation of a transcription initiation complex (13). This hypothesis is supported by the finding that changing the USE location affected the site of transcription initiation (16,17). Conversely, in the *RRNA* promoter, rUSE is dispensable for *in vitro* transcription, suggesting that this element and its binding factor facilitate transcription initiation indirectly. At a similar distance from the transcription initiation site, the *RRNA* promoters of yeast and vertebrates harbor an element which has the same property as rUSE. In yeast, the corresponding element Reb1 is only necessary for efficient transcription within its chromosomal context (32). Investigation of Reb1p binding revealed that the protein protects only 15–20 nt from enzymatic or chemical degradation but clears ~200 bp from nucleosomes (33), indicating that Reb1/Reb1p is involved in chromatin remodeling. In vertebrate *RRNA* promoters, an element termed the proximal terminator T_0 is located between positions –150 and –200. The function of T_0 has been meticulously investigated in the mouse system where it has been shown to bind the transcription termination factor I [TTF-1 (34)] By analyzing transcription of naked DNA and pre-assembled chromatin templates *in vitro*, it was elegantly demonstrated that binding of TTF-1 to T_0 induced chromatin remodeling and relieved transcriptional repression (35). Analogously, chromatin remodeling may be the predominant function of *RRNA* promoter domain IV in *T.brucei*.

Strikingly, both the yeast Reb1 and the mouse T_0 sequences are RNA pol I transcription termination signals also present at the 3′ end of the ribosomal transcription unit. It has been shown that RNA pol I-mediated read-through transcription initiated upstream of an *RRNA* promoter at so-called spacer promoters can displace assembled transcription factors from the actual promoter (36). In vertebrate systems, T_0 protects the promoter by terminating transcription of incoming RNA pol I (37). However, in trypanosomes, the region upstream of the *RRNA* promoter is transcriptionally silent and it remains to be determined whether rUSE functions in RNA pol I transcription termination. The presence of the terminator sequences at both promoter and 3′ end regions has led to the hypothesis that they functionally link transcription initiation and termination possibly through DNA looping. This DNA configuration should then facilitate efficient recycling of RNA pol I from the termination to the initiation site. This model is supported by micrographs of chromatin spreads in which active *RRNA* transcription units have been visualized as loops separated by intergenic spacers (38). Moreover, TTF-1 has the property to induce DNA looping because it can oligomerize and simultaneously interact with two separate DNA fragments containing its binding site (39). To function in a similar manner, the *T.brucei* rUSE would have to be located at the 3′ end of the *RRNA* transcription unit which has been mapped by nuclear run-on assays and an S1 nuclease protection analysis just downstream of the 3′-terminal coding sequences (40). Thus far, we were unable to identify a motif similar to the rUSE/USE consensus sequence at the putative termination region or within the whole *RRNA* repeat. The information about the nucleotides required for binding the TbSNAP50 complex is limited and the binding motif may be too degenerate for detection by sequence comparison. However, it will be possible to employ *SLRNA* transcription competition assays using putative *RRNA* terminator sequences as competitors to determine whether the TbSNAP50 factor binds downstream of the *RRNA* transcription unit.

rUSE is in opposite orientation to the transcription direction of USE in the *SLRNA* promoter and its function is orientation dependent. This arrangement of rUSE is reminiscent of the conserved head-to-head organization of tRNA and small RNA genes in trypanosomatids [(41), reviewed in Günzl (1) and Nakaar *et al*. (42)]. In these gene associations, the tRNA gene-internal A and B box promoter elements are important for both tRNA and small RNA gene transcription. A detailed study of the associated U6 snRNA and threonine tRNA genes in *T.brucei* revealed that the A box which binds TFIIIB and recruits RNA pol III to the tRNA gene is essential for U6 snRNA gene transcription *in vivo* and *in vitro*, and that the position of this element relative to the U6 transcription initiation site is critical (43). Conversely, the B box, which in other systems was shown to be involved in chromatin remodeling, was only essential *in vivo* and its position relative to the transcription initiation site was flexible. In comparison, rUSE has the properties of the B box and not of the A box, because its function did not depend on its exact location and became apparent only *in vivo*. At the threonine tRNA/U6 snRNA gene locus, transcription is initiated in both directions, suggesting that this may also be the case in the *RRNA* promoter. However, it was shown previously that the *RRNA* spacer region is transcriptionally silent (40) and, accordingly, we were unable to detect a specific transcript from this region by employing northern blotting and RT–PCR (data not shown).

Unlike the two types of *VSG* expression site promoters which are very short and do not contain a promoter domain IV, the procyclin gene promoter has a four-domain structure (1). Procyclin gene promoter domain IV appears to be functionally equivalent to its *RRNA* counterpart because it is required *in vivo* but not *in vitro* and it resides at a similar position (6,10). Moreover, procyclin–*RRNA* hybrid promoters were fully functional in transient reporter gene assays, indicating that procyclin gene promoter domain IV can functionally substitute its *RRNA* counterpart (7). However, the procyclin gene promoter does not interact detectably with TbSNAP50 in our assays and most probably binds a different *trans*-acting factor. It is thus possible that the parasite utilizes domain IV of the *RRNA* and procyclin gene promoters to differently regulate transcription of these gene units. For example, while rUSE may promote constitutive *RRNA* expression in different life cycle stages, its procyclin counterpart may be responsible for the known up- and downregulation of procyclin gene transcription in procyclic and bloodstream form trypanosomes, respectively (44).

Finally, the question arises of how conserved is rUSE among trypanosomatid organisms. The *RRNA* promoter of

Leishmania donovani has been investigated in detail and, surprisingly, it structurally resembles the bloodstream form *VSG* expression site promoter and not the *RRNA* promoter of *T.brucei* (45). It has the same small size and two-domain structure upstream of the transcription initiation site, and there is no indication of an rUSE element. In other trypanosomatid species with known promoter sequences, there is no convincing sequence conservation between *SLRNA* and *RRNA* promoters, and it remains to be determined experimentally whether rUSE is a common feature among trypanosomatids. Alternatively, rUSE may be a *T.brucei* invention to facilitate differential regulation of multi-functional class I transcription.

In conclusion, our data strongly suggest that in *T.brucei,* a SNAPc-like transcription factor is essential for both *RRNA* and *SLRNA* transcription. Intriguingly, such a factor would enable the parasite to regulate global gene expression simultaneously at the level of protein synthesis and RNA maturation. We have initiated *in vivo* experiments to investigate these possibilities and begun to purify and functionally characterize the TbSNAP50 complex.

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