RNA pol II subunit RPB7 is required for RNA pol I-mediated transcription in *Trypanosoma brucei*

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In the protozoan parasite *Trypanosoma brucei*, the two main surface glycoprotein genes are transcribed by RNA polymerase I (pol I) instead of RNA pol II, the polymerase committed to the production of mRNA in eukaryotes. This unusual feature might be accomplished by the recruitment of specific subunits or cofactors that allow pol I to transcribe protein-coding RNAs. Here, we report that transcription mediated by pol I requires TbRPB7, a dissociable subunit of the pol II complex. TbRPB7 was found to interact with two pol I-specific subunits, TbRPA1 and TbRPB6z. Pol I-specific transcription was affected on depletion of TbRPB7 in run-on assays, whereas recombinant TbRPB7 increased transcription driven by a pol I promoter. These results represent a unique example of a functional RNA polymerase chimera consisting of a core pol I complex that recruits a specific pol II subunit.

Keywords: antigenic variation; VSG; transcription; RNA polymerase I; RPB7

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INTRODUCTION

Trypanosoma brucei, located in a distal position in the eukaryotic cell lineage, is the aetiological agent of African trypanosomiasis. Changes in the type of variant surface glycoprotein (VSG) on the surface of the parasite allow it to elude the host's immune antibody response and ensure a persistent infection (Borst & Ulbert, 2001; Pays, 2005). In the procyclic or insect mid-gut stage of the parasite, the procyclin family of glycoproteins covers the surface. Transcription of *VSG* and *procyclin* by RNA polymerase I (pol I) is an exceptional feature among eukaryotes, as pol I does not normally transcribe protein-coding genes. For other genes, the transcriptional apparatus of *T. brucei* includes the three characteristic eukaryotic RNA polymerases.

The evolutionary specialization of the eukaryotic RNA polymerases results in a complex situation whereby subunits are either shared between polymerases or are specific for each of the three classes (I, II and III). There are various RNA polymerase subunits that are conserved in function but encoded by three genes, an example being the largest subunit: RPA1 (I), RPB1 (II) and RPC1 (III). Other subunits are shared by the three polymerases such as RPB5, RPB6, RPB8, RPB10 and RPB12. However, in *T. brucei*, three of the shared subunits, RPB5, RPB6 and RPB10, have other isoforms, known as TbRPB5z, TbRPB6z and TbRPB10z, which are included in the RNA pol I complex (Walgraffe *et al*, 2005; Nguyen *et al*, 2006), whereas TbRPB5, TbRPB6 and TbRPB10 are used in the pol II complex (Das *et al*, 2006; Devaux *et al*, 2006). Other specific subunits for each type of polymerase are related in sequence and/or function with an archaeal ancestor; for example, the RNA polymerase subunit E (RpoE) subunit family, which in eukaryotes includes RPA43 (I), RPB7 (II) and RPC25 (III). In trypanosomes, the lack of an orthologue of RPA43/RPA14 in the genome, together with the absence of a charged amino terminus in the variant TbRPB6z, suggested that the incorporation of TbRPB6z into the pol I complex impedes the recruitment of RPB4/RPB7 counterparts (Devaux *et al*, 2007). A new parasite-specific pol I subunit called TbRPA31 was recently described, but no sequence or structural similarity with RPA43 was detected (Nguyen *et al*, 2007). Thus, the main question is whether the RNA pol I complex recruits any additional subunits related to archaeal RpoE/RpoF family members. Our results indicate that TbRPB7, a subunit present exclusively in the RNA pol II complex of all eukaryotes, is also associated with RNA pol I in *T. brucei* and in this manner might contribute to a multifunctional polymerase that is able to transcribe not only ribosomal DNA (rDNA) but also messenger RNA (mRNA).

RESULTS AND DISCUSSION

Development of a dual-reporter cell line

Most of the RNA pol I complexes in a trypanosome cell are dedicated to the transcription of rRNA in the nucleolus, whereas a smaller proportion is involved in the transcription of *VSG* and *procyclin* mRNA. Thus, the functional analysis of candidate subunits needs to be evaluated on the basis of their specific

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Fig 1 | *In vivo* analysis of RNA pol I and pol II transcription in a dual-reporter cell line (SALR). (A) Schematic outline of the genomic positioning of the reporter cassettes. The upper portion represents the active VSG (variant surface glycoprotein) locus, known as VSG-ES. Black boxes indicate associated genes polycistronically transcribed with the VSG (open box); the telomere is represented by a circle. The reporter gene *FLuc* (firefly luciferase) is 400 bp downstream from the active VSG-ES promoter (arrow) and the *RLuc* gene (*Renilla* luciferase) was inserted within the cluster of α - β tubulin repeats. (B) Effect of TbRPA1 (8 h), TbRPB5z (18 h), TbRPB5 (16 h), TbRPB9 and TbRPC19 (24 h) depletion on the reporter activities. Relative reporter activities are represented as a percentage of the non-induced controls; mean and standard deviation (error bars) of three independent experiments are shown. (C) Effect of TbRPB7 depletion on VSG-ES pol I- and pol II-mediated transcription. Reporter activities during a time course of RNAi induction. FLuc (VSG-ES promoter transcription) and RLuc (*tubulin* read-through) reporter activities are presented as percent activity relative to the uninduced culture. Mean and standard deviation of three independent experiments are shown. (D) Kinetics of TbRPB7 protein depletion at several time points after induction, analysed using anti-TbRPB7 antibody. As a loading control, we used an anti-TbFKBP12 antibody. (E) Effect on the activities of FLuc and RLuc reporters after 18 h of depletion of TbRPB4, as represented in (C). ES, expression site; pol, polymerase; RNAi, RNA interference; Tb, *Trypanosoma brucei*.

effects on VSG expression site (ES) promoter activity *in vivo*. Initially, we decided to test the function of the various RNA polymerase subunits by evaluating their function in a cell line containing a firefly luciferase (FLuc) reporter gene inserted downstream from the active VSG-ES promoter. To monitor the transcription of pol II simultaneously, a *Renilla* luciferase (RLuc) reporter gene was inserted within the tubulin chromosomal locus (Fig 1A). In this cell line, we were able to obtain reporter measurements of both pol I- and pol II-mediated transcription on depletion by inducible RNA interference (RNAi; Wang *et al*, 2000) of a candidate gene using a dual-reporter assay (Promega, Madison, WI, USA). All measurements of reporter activities were obtained when no significant reduction in cell growth was detected. To validate this cell line, we first carried out a tetracycline-inducible depletion of the largest subunit of pol I. Activity of FLuc on TbRPA1 RNAi (8 h) was effectively reduced, whereas pol II-driven RLuc did not change significantly (Fig 1B). Analysis of TbRPA1 protein levels using TbRPA1 antibodies showed a clear depletion (supplementary Fig S1 online).

Functional analysis of polymerase subunits

Next, we analysed the requirements for the transcription of VSG-ES by specific pol I subunits that, although present in the complex,

might not necessarily be involved in VSG-ES transcription. Recent data show that TbRPB5z is required for the transcription of rRNA, but no information on a direct function in VSG-ES transcription was addressed (Devaux *et al*, 2007). Fig 1B shows that pol I-driven FLuc activity decreased on RNAi depletion of TbRPB5z, indicating that TbRPB5z is indeed involved in the transcription of VSG-ES. Conversely, depletion of TbRPB5 resulted in a 50% reduction of pol II-mediated RLuc reporter activity, whereas the activity of FLuc was altered only slightly. To assess the efficiency of our depletion experiments, we simultaneously analysed parasite growth and mRNA levels (supplementary Fig S2A and S2B online).

Rpb9 is a non-essential subunit of the yeast pol II that regulates transcription elongation and has been shown to be essential in *T. brucei* (Devaux *et al* 2006). Reporter activity on depletion of TbRPB9 at 24 h showed that RLuc activity at the *tubulin* locus decreased, in contrast to FLuc activity at the VSG-ES locus (Fig 1B). Another pol I subunit that Günzl's group has co-purified in a complex together with TbRPA1 is TbRPC19 (Nguyen *et al*, 2006). In yeast, Rpc19 is essential for the transcription of pol I and pol III. We measured reporter activities 24 h after TbRPC19 RNAi and observed a decrease in FLuc reporter activity, confirming its requirement for the transcription of VSG-ES (Fig 1B). These results

show the utility of this dual-reporter cell line to assess the functions of essential polymerase subunits *in vivo*.

Transcription of VSG-ES involves TbRPB7

Previous studies in *T. brucei* were unable to co-purify the pol I-specific subunits RPA43 and RPA14, counterparts of Rpb4/Rpb7 in the pol II complex (Walgraffe et al, 2005; Nguyen et al, 2006). Furthermore, in the *T. brucei* genome database, we failed to identify an orthologue of RPA43, whereas TbRPB7 and TbRPC25, counterpart subunits of pol II and pol III, were clearly identified through homology searches. Thus, we hypothesized that the trypanosome pol I complex might use subunits from the canonical mRNA production machinery during the transcription of VSG. As expected, pol II-driven RLuc reporter activity was lower on TbRPB7 depletion (16 h). Surprisingly, we also detected a reduction in the VSG promoter-driven FLuc reporter activity in a time course TbRPB7 depletion experiment (Fig 1C), when no significant reduction in cell growth was detected in three independent clones (supplementary Fig S2 online). We also developed an anti-TbRPB7 antiserum (supplementary Fig S3 online) and conducted a Western analysis on RNAi, which confirmed the reduction in TbRPB7 protein levels (Fig 1D).

Rpb7 in yeast forms a heterodimer with Rpb4 and adopts a similar structure to the archaeal RpoE/RpoF counterpart when binding to the 10-subunit core pol II complex (Bushnell & Kornberg, 2003; Armache et al, 2005). On the basis of this observation, we decided to investigate a possible function of TbRPB4 in the transcription of VSG. Several independent TbRPB4-RNAi clones showed no significant effect on the transcriptional reporter activity of VSG, although they did show a reduction of pol II-driven RLuc reporter activity in the *tubulin* locus (Fig 1E). Thus, we found that the transcription of VSG in *T. brucei* seems to be TbRPB4 independent. It has been shown that yeast Rpb7 can interact with pol II in the absence of Rpb4 (Sheffer et al, 1999); thus, Rpb7 alone is sufficient to carry out transcription under non-stress conditions (Choder, 2004).

RNA pol I transcription in trypanosomes requires TbRPB7

To investigate whether TbRPB7 is involved directly in the transcription of pol I, we performed a series of run-on experiments in permeabilized bloodstream-form trypanosomes after the depletion of TbRPB7 in a Tet-inducible manner. Nascent RNA labelled with α -³²P UTP was isolated 24 h after the induction of TbRPB7 depletion and hybridized to Southern-blotted DNA fragments from the 18S rRNA, VSG, *tubulin* and transfer RNA (tRNA) genes (Fig 2). Quantification of the hybridization signal from pol I-mediated transcription of VSG mRNA showed a significant decrease on TbRPB7 knockdown, with a concurrent reduction of 18S rRNA levels. Nascent *tubulin* mRNA showed a similar reduction, consistent with the role of RPB7 in pol II transcription in eukaryotes. As a control, the transcription of tRNA by pol III was not significantly affected. Taken together, these data indicate that TbRPB7 is involved not only in pol II transcription as in other eukaryotes, but is also required for the transcription of pol I in *T. brucei*. These results raise questions about whether TbRPB4 is required for RNA pol I transcription. To investigate this question, we carried out run-on experiments on TbRPB4 depletion. Fig 2 shows that transcriptional activities from the VSG and ribosomal promoters were not affected on TbRPB4 depletion,

Fig 2 | Run-on analysis of nascent RNAs in bloodstream-form trypanosomes. After induction of TbRPB7 (24 h), TbRPB4 (16 h) or TbRPC25 (24 h) depletion, saponin-permeabilized cells were incubated in the presence of [α -³²P]UTP for 15 min at 35 °C. Total RNA was extracted and hybridized with Southern blots of DNA fragments from the indicated genes. Hybridization was quantified and is represented on the histogram as a percentage signal obtained from non-induced cells. Mean and standard error of three independent experiments are shown. RNAi, RNA interference; Tb, *Trypanosoma brucei*; tRNA, transfer RNA; VSG, variant surface glycoprotein.

whereas pol II transcription of *tubulin* decreased. By contrast, depletion of TbRPC25, the counterpart subunit of TbRPB7 in pol III, showed a 40% reduction of tRNA transcription without a decrease in the transcription of VSG or *tubulin*, showing that TbRPC25 functions exclusively in the transcription of pol III (Fig 2). We simultaneously analysed TbRPB4 and TbRPC25 mRNA levels to confirm depletion (supplementary Fig S2B online). These data suggest that depletion of either a pol II or a pol III subunit does not necessarily affect the transcriptional activity of VSG-ES at early depletion times.

The RNA pol I complex recruits TbRPB7

Functional reporter assays, together with nascent RNA labelling experiments on TbRPB7 depletion, suggest that TbRPB7 is involved in pol I-driven transcription. However, previous attempts have failed to detect TbRPB7 in partially purified pol I complexes analysed using proteomics approaches (Walgraffe et al, 2005; Nguyen et al, 2006, 2007). In yeast, recruitment of Rpb7 to the pol II complex occurs in a dissociable manner in just 20% of complexes (Sheffer et al, 1999). The X-ray structure of a 12-subunit complex from *Saccharomyces cerevisiae*, including a Rpb4–Rpb7 heterodimer not present in previous pol II structures, has been described (Armache et al, 2003; Bushnell & Kornberg, 2003). These structural data have shown a direct interaction of Rpb6 with Rpb1 and Rpb7 in the pol II complex. Thus, we decided to investigate whether TbRPB7 interacts with TbRPB6z, a trypanosome variant of TbRPB6 present in pol I but not in pol II complexes (Nguyen et al, 2006). We expressed TbRPB6z fused to the tandem affinity purification (TAP) epitope (TAP-TbRPB6z) in a procyclic cell line, where the transcription of pol I of the surface protein procyclin also occurs. After purification of the complex containing TAP-TbRPB6z, proteins were eluted by cleavage with

Fig 3 | Co-immunoprecipitation of TbRPB7 and RNA pol I subunits. (A) Extracts from procyclic cell lines expressing either TAP-TbRPB6z or TAP-control were precipitated with IgG sepharose, eluted with TEV protease (EluTEV) and analysed by Western blots (WB) with the indicated antibodies. PAP antibody (peroxidase anti-peroxidase) was used to detect protein A of TAP-TbRPB6z or the TAP-control. (B) Co-IP of TbRPB7 with TAP-TbRPB6z. Extracts from the procyclic cell line expressing TAP-TbRPB6z were incubated with protein G sepharose previously bound to anti-TbRPB7 antiserum (α -RPB7) or anti-VSG121 (α -121) as an unrelated control. Samples were analysed by Western blot with the indicated antibodies. PAP antibody was used to detect protein A of TAP-TbRPB6z. (C) Interaction between endogenous TbRPB7 and TbRPA1 subunits. Extracts from wild-type procyclic trypanosomes were incubated with protein G sepharose previously bound to anti-TbRPA1 (α -RPA1), anti-TbRPB7 antiserum (α -RPB7) or anti-EP as a control. The samples were eluted in SDS loading buffer and analysed by Western blots with the indicated antibodies. Loading information of (A, B) is available in the supplementary information online. For comparison in (C): IP α -RPA1, WB RPB7 input 1%, IP 85%; WB RPA1: input 1%, IP 5%. WB EP input 1%, IP 10%, was loaded, in IP α -RPB7: input 5%, IP 100%. EP, EP procyclin (procyclin protein rich in Glu-Pro repeats); FT, flow-through; Inp, input; IP, immunoprecipitate; TAP, tandem affinity purification; Tb, *Trypanosoma brucei*; TEV, tobacco etch virus protease.

tobacco etch virus (TEV) protease and analysed by Western blot, which revealed that TAP-TbRPB6z efficiently co-immunoprecipitates with both TbRPB7 and TbRPA1 (Fig 3A). As a negative control, an extract expressing only the TAP tag did not co-immunoprecipitate with either of the two pol I subunits. This result was confirmed by reciprocal co-immunoprecipitation experiments using anti-TbRPB7 antiserum (Fig 3B). To confirm this interaction

with pol I in bloodstream-form trypanosomes, we expressed TbRPB7-TAP (supplementary Fig S3 online) and were able to coimmunoprecipitate TbRPA1 (supplementary Fig S4A online). Conversely, co-immunoprecipitation experiments using antibodies against TbRPA1 showed that TbRPB7 is associated with the pol I complex (supplementary Fig S4B online). Furthermore, we investigated this interaction with endogenous subunits, avoiding any possibility of nonspecific interactions by the over-expression of the tagged proteins. Co-immunoprecipitation experiments using wild-type trypanosome extracts with TbRPA1 antibodies detected co-precipitated TbRPB7 by Western blot analysis using anti-TbRPB7 antiserum (Fig 3C). These data indicate that TbRPB7 associates with the pol I complex defined by the core subunits TbRPA1 and TbRPB6z. Given the overall structural conservation between the three eukaryotic RNA polymerases (Cramer *et al*, 2008), it is tempting to speculate that TbRPB7, TbRPB6z and TbRPA1 in the trypanosome pol I complex might be interacting in a similar manner to Rpb7, Rpb6 and Rpb1 in the yeast pol II complex (Armache *et al*, 2003).

TbRPB7 associates with the pol I complex in the nucleus

To investigate further whether TbRPB7 and the pol I complex interact in the trypanosome nucleus, we carried out double immunofluorescence experiments by three-dimensional microscopy using affinity-purified anti-TbRPA1 and anti-TbRPB7 antisera. TbRPA1 antibodies recognized the pol I complex located in the nucleolus and in the extra-nucleolar expression-site body associated with VSG expression (Fig 4A; Navarro & Gull, 2001). TbRPB7 partly colocalized to the extra-nucleolar expression-site body (Fig 4). Furthermore, TbRPB7 localized to the nucleoplasm (presumably with pol II); however, it was also found to colocalize partly with TbRPA1 in the nucleolar periphery (Fig 4B). Similarly, Br-UTP-labelled nascent RNA resistant to α -amanitin was restricted to TbRPA1 protein located at the nucleolar periphery (supplementary Fig S5 online), where ribosomal promoter sequences are also located (Landeira & Navarro, 2007). This nucleolar periphery localization of TbRPA1, TbRPB7 and nascent RNA suggests that the dissociable TbRPB7 subunit is recruited into the active pol I but not the remaining inactive pol I complex, defined by the lack of Br-UTP labelling. This situation is similar to that observed in yeast where Rpb7 is recruited to a small fraction of pol II complexes only on the initiation of transcription (Sheffer *et al*, 1999 reviewed in Choder, 2004).

TbRPB7 promotes the transcription of RNA pol I *in vitro*

Next, we wanted to address whether purified TbRPB7 was able to enhance the activity of a VSG-ES promoter by *in vitro* transcription using cytoplasmic protein extracts that contain low amounts of TbRPB7 (data not show). To measure the transcription efficiencies, we used an *in vitro* assay that uses a 377 bp G-less cassette template driven by the VSG-ES promoter (see Methods; Fig 5A). Transcription from the VSG-ES promoter was not very efficient; however, the addition of exogenous recombinant TbRPB7 to the reaction increased the amount of G-less transcripts threefold in proportion to the amount of cell extract used (Fig 5B). As a control, we carried out transcription using the same template but without the promoter, showing that transcription was promoter dependent. In addition, pol I *in vitro* transcription was abolished when

Fig 4 | TbRPB7 nuclear localization by three-dimensional microscopy. Analysis of bloodstream-form trypanosomes was carried out with double indirect immunofluorescence and three-dimensional deconvolution microscopy. Rabbit anti-TbRPA1 and mouse anti-TbRPB7 antisera were used. Nuclear (N) and mitochondrial DNA (kinetoplast, K) were stained with DAPI (blue). (A) Anti-TbRPA1 shows the pol I-containing nucleolus (arrow) and the expression site body (arrowhead). Anti-TbRPB7 shows a nucleoplasmic distribution; maximum intensity projections of three-dimensional stacks are shown. (B) Colocalization analysis of three-dimensional deconvolved data sets. A colocalization mask (white) was calculated for each non-equalized 8-byte slice, and merged with both TbRPA1 (red) and TbRPB7 (green) labels. The slice animation of a three-channel three-dimensional (Z step) data set through the nucleus can be found as supplementary Video online. Scale bars, 1 μ m. DAPI, 4,6-diamidino-2-phenylindole; pol, polymerase; Tb, *Trypanosoma brucei*.

the extracts were TbRPA1-depleted. VSG-ES promoter activity was also affected by incubating the reactions with any of our anti-TbRPB7 antisera, but not with unrelated antisera, suggesting that TbRPB7 is essential for VSG-ES promoter-dependent transcription of pol I (Fig 5C).

RPB7, a conserved subunit of RNA pol II, is the functional counterpart of the RPA43 in pol I (Peyroche *et al*, 2002), with a structure similar to that of archaeal RpoE (Siaut *et al*, 2003). However, our results represent the first eukaryotic example in which a pol I complex recruits the pol II-specific subunit, RPB7. Previous studies have suggested that RPB7 recruits processing factors and localizes in the exit path for nascent RNA in the pol II complex (Mitsuzawa *et al*, 2003; Ujvari & Luse, 2006). Thus, it is tempting to speculate that the recruitment of TbRPB7 to the trypanosome RNA pol I complex underlies the unusual ability of this polymerase to transcribe protein-coding genes.

METHODS

Cell lines. Details regarding the *T. brucei* cell lines, transfection, plasmids and RNAi experiments can be found in the supplementary information online.

Run-on experiments. Transcription in saponin-permeabilized bloodstream parasites was performed with [α - 32]UTP for 15 min at 35 $^{\circ}$ C, as described in the supplementary information online.

Co-immunoprecipitation and immunofluorescence. Generation of anti-TbRPB7 mice antisera is detailed in the supplementary information online. Protein extracts were prepared from TbRPB7-TAP, TAP-TbRPB6z or TAP-expressing cells, and co-immunoprecipitation was performed with anti-TbRPA1, anti-TbRPB7 or IgG sepharose, and developed with the appropriate antibodies as detailed in the supplementary information online. Suspension immunofluorescence and deconvolution of multichannel three-dimensional data sets were carried out as described by Landeira & Navarro (2007); for details see the supplementary information online.

In vitro transcription assays. Cell extract preparation and transcription reactions were performed following the protocols of Günzl lab (Laufer *et al*, 1999). Reactions with pVSG-Gless, which contains the VSG-ES promoter region (from -729 to +145 bp) and a Gless cassette (377 bp) at 1.962 kb, were incubated at 28 $^{\circ}$ C for 1 h and then digested with RNase T1, as described in the supplementary information online.

Fig 5 | TbRBP7 increases VSG-ES promoter transcription. (A) Schematic representation of DNA templates; pVSG-Gless containing the VSG121 promoter region and a Gless cassette (377 bp) inserted approximately 2 kb downstream (top), or a pVSG-Gless-Δpromoter, the same construct without the core promoter (bottom). (B) TbRBP7 enhances *in vitro* transcription in a dose-dependent manner. Transcription reactions were performed with 4–8 μl of concentrated cell extract and 200 ng of DNA template. Increasing amounts of TbRBP7 recombinant protein were added as indicated. Depletion of extracts with anti-TbRPA1 antiserum (α-TbRPA1) completely abolishes transcription, contrary to mock depletion performed with protein G (Prot G). As a negative control, pVSG-Gless without promoter (Δpromoter) yielded no detectable transcription. Fold increase of transcription was calculated as the ratio of transcript in the presence of recombinant TbRBP7 to the transcript in the absence of the protein. (C) Antibody-depleted extracts using anti-TbRPA1 or anti-TbRBP7 antisera S1 or S2 abolished *in vitro* transcription, whereas anti-EP or anti-TOR2 antisera controls did not. Ab, antibody; EP, EP procyclin (procyclin protein rich in Glu-Pro repeats); ES, expression site; Tb, *Trypanosoma brucei*; TOR2, target of rapamycin 2; VSG, variant surface glycoprotein.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>)

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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