Published in final edited form as:

Mol Biochem Parasitol. 2011 November ; 180(1): 35–42. doi:10.1016/j.molbiopara.2011.06.008.

Transcription by the multifunctional RNA polymerase I in *Trypanosoma brucei* functions independently of RPB7

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Abstract

Trypanosoma brucei has a multifunctional RNA polymerase (pol) I that transcribes ribosomal gene units (*RRNA*) and units encoding its major cell surface proteins variant surface glycoprotein (*VSG*) and procyclin. Previous analysis of tandem affinity-purified, transcriptionally active RNA pol I identified ten subunits including an apparently trypanosomatid-specific protein termed RPA31. Another ortholog was identified *in silico*. No orthologs of the yeast subunit doublet RPA43/RPA14 have been identified yet. Instead, a recent report presented evidence that RPB7, the RNA pol II paralog of RPA43, is an RNA pol I subunit and essential for *RRNA* and *VSG* transcription in bloodstream form trypanosomes (Penate *et al.*, 2009, EMBO Rep. 10:252–257). Revisiting this attractive hypothesis, we were unable to detect a stable interaction between RPB7 and RNA pol I in either reciprocal co-immunoprecipitation or tandem affinity purification. Furthermore, immunodepletion of RPB7 from extract virtually abolished RNA pol II transcription *in vitro* but had no effect on *RRNA* or *VSG* ES promoter transcription in the same reactions. Accordingly, chromatin immunoprecipitation analysis revealed cross-linking of RPB7 to known RNA pol II transcription units but not to the *VSG* ES promoter or to the 18S rRNA coding region. Interestingly, RPB7 did crosslink to the *RRNA* promoter but so did the RNA pol II-specific subunit RPB9 suggesting that RNA pol II is recruited to this promoter. Overall, our data led to the conclusion that RNA pol I transcription in *T. brucei* does not require the RNA pol II subunit RPB7.

1. Introduction

Trypanosoma brucei has a multifunctional RNA polymerase (pol) I that transcribes the large ribosomal (*RRNA*) gene unit in all life cycle stages, and, uniquely, the telomeric expression site encoding the active variant surface glycoprotein (*VSG*) gene in its bloodstream form (BF) as well as gene units encoding procyclin in its procyclic form (PF). BFs evade the mammalian immune response by antigenic variation of their cell surface coat consisting of ~10 million identical *VSG* molecules expressed from a single gene. The variation occurs when parasites switch to the expression of a different *VSG* gene drawn from a large *VSG*

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gene repertoire (recently reviewed in [1]). Expression of the whole VSG coat from a single gene requires extremely high expression levels and it has been determined that the transcription rate of the active VSG gene is approximately 50 times higher than that of a β tubulin gene [2]. Such high rates are the hallmark of RNA pol I transcription which in general accounts for more than 50% of the transcriptional activity in a eukaryotic cell [3,4]. However, utilization of RNA pol I for protein coding gene expression requires a deviating mode of gene expression. In the mouse, strong *RRNA* promoter-driven transcription of a reporter construct, led to reporter enzyme activities which were 20–50 fold lower than in a control experiment with an RNA pol II promoter [5]. Conversely, *RRNA* promoter-driven expression of a selectable marker gene in *T. brucei* increased parasite resistance around 30fold over RNA pol II-mediated expression of the same gene demonstrating that, in trypanosomes, RNA pol I can efficiently synthesize functional mRNA [6,7]. The opposite outcomes in these studies are most likely due to different modes of mRNA capping. In the budding yeast *Saccharomyces cerevisiae* and higher eukaryotes, RNA capping is co-transcriptional and specifically linked to RNA pol II because the capping enzymes bind to the phosphorylated carboxy-terminal domain (CTD) of the largest RNA pol II subunit RPB1 [8]. Conversely, in trypanosomes and related organisms, protein-coding gene transcription is polycistronic and individual mRNAs are processed from precursors by spliced leader (SL) *trans* splicing and polyadenylation. Since in *trans* splicing the capped SL, comprising the 5'-terminal part of the small nuclear SL RNA, is transferred to the 5' end of each mRNA, this process constitutes a post-transcriptional capping mechanism that decouples capping from RNA pol II transcription [9].

Eukaryotic RNA pols I–III consist of twelve subunits which are either shared or paralogous to each other. In addition, yeast RNA pol I contains two RNA pol I-specific subunits, RPA49 and RPA34, which are not essential for yeast proliferation. The multifunctional nature of trypanosome RNA pol I has spurred the investigation of this enzyme. While the largest two subunits, RPA1 and RPA2, were discovered first [10–12], eight of the remaining ten core subunits could be identified bioinformatically after the *T. brucei* genome was completed [13]. The missing subunits were the orthologs of yeast RPA43 and RPA14. Since these two subunits form a functional doublet in yeast and humans, it was proposed that trypanosome RNA pol I may assemble the paralogous RNA pol II subunits RPB7 (GeneDB/TritrypDB accession number Tb11.01.6090) and RPB4 (Tb927.3.5270) instead [13], possibly to aid this polymerase in the synthesis of functional mRNA. This was an attractive hypothesis because the respective genes were readily detected in trypanosomatid genomes and because RPB4/7 analysis in other systems indicated specific functions of this protein doublet in RNA synthesis such as binding of an RNA processing factor [14], direct RNA interaction [15], and linking mRNA synthesis to mRNA decay [16]. Moreover and most recently, the RPB4/7 doublet was characterized as a potential “mRNA coordinator” in yeast that can be deposited on mRNA facilitating efficient translation and thereby linking expression outcomes from gene transcription to translation [17].

Based on co-immunoprecipitation assays, *RPB7* expression silencing experiments, co-localization of RPB7 and the RNA pol I-specific subunit RPB6z (Tb11.03.0935), and *in vitro* transcription assays in BFs, Penate *et al.* recently concluded in their publication title that “RNA pol II subunit RPB7 is required for RNA pol I-mediated transcription in *Trypanosoma brucei*” [18]. However, tandem affinity purification of trypanosome RNA pol I in two different laboratories did not identify RPB7 as a co-purifying subunit [19–21]. Moreover, isolation of RNA pol I from PF extract that was active in both non-specific and promoter-dependent transcription assays did not reveal a protein band of ~20 kDa which is the apparent size of *T. brucei* RPB7 [21]. This was of particular concern because RPB7 and its RNA pol I and III paralogues RPA43 and RPC25 were shown in yeast to be essential for promoter-dependent transcription initiation [22–24]. We therefore revisited the role of *T.*

brucei RPB7 in RNA pol I transcription omitting *RPB7* expression silencing which may affect gene expression independently of RNA pol function or may rapidly lead to secondary defects through a general shut-down of RNA pol II transcription.

2. Methods

2.1. DNAs

For the generation of BF cell lines that exclusively expressed RPB7 or RPB9 with a C-terminal fusion of the composite PTP tag sequence, encoding tandem protein A (ProtA) domains, a tobacco etch virus (TEV) protease site and the protein C epitope (ProtC), plasmids RPB7-PTP-NEO and RPB9-PTP-NEO were first integrated into endogenous alleles. The plasmids were generated by inserting, respectively, 549 bp and 385 bp of the *RPB7* and *RPB9* (Tb11.02.5180) C-terminal coding regions into pC-PTP-NEO [25] using *ApaI* and *NotI* restriction sites. For pRPB9-PTP-NEO linearization, an *AflIII* restriction site was engineered into the RPB9 sequence creating a silent mutation in *RPB9* codon 58. In a second round of stable transfections, the remaining wild-type *RPB7* and *RPB9* alleles were knocked out by transfection of 10 µg of a chimeric linear DNA in which 100 bp-long gene flanks were fused to the coding region of the hygromycin phosphotransferase by standard PCR as described previously [26]. pRPB7-HA-BLA was created by pasting the *RPB7* target sequence from pRPB7-PTP-NEO into the published plasmid pRPA31-HA-BLA [21]. For transfections, pRPB7-PTP-NEO/pRPB7-HA-BLA, pRPB9-PTP-NEO, and pPTP-RPB6z-PURO were linearized with restriction enzymes *PmeI*, *AflIII*, and *BmgBI*, respectively. pPURO-PTP-RPB6z and transcription templates Rib-trm, VSG-trm, and SLins19 were described previously [21,27].

The following oligonucleotide pairs were used in semi-quantitative and quantitative (q)PCR analyses of DNA obtained in chromatin immunoprecipitation (ChIP) assays: consensus VSG ES promoter, 5'-TCTAAAAGAATCATATCC-3'/5'-AAGCGTAGATGAGATTAAAGTC-3'; consensus *RRNA* promoter, 5'-AATACAACACACAATAGG-3'/5'-GTCTGAGAGCGGTCAGTTGC-3'; 18S rRNA coding region, 5'-TCATCAAACGTGCCGATTAC-3'/5'-CTATTGAAGCAATATCGG-3'; *SLRNA* promoter, 5'-CTACCGACACATTTCTGGC-3'/5'-GCTGCTACTGGGAGCTTCTCATACC-3'; β – α tubulin intergenic region, 5'-GCTGATTTCTGACAGATCTTCAAAC-3'/5'-GTGGATGCAGATAGCCTCACGCATG-3'.

2.2. Cells

PF and BF of *Trypanosoma brucei brucei* strain 427 were cultured as described previously [28,29]. Transfected cells were cloned by limiting dilution immediately after electroporation. BFs were selected with 2.5 µg/ml of G418, 1 µg/ml of hygromycin, and 2 µg/ml of blasticidin whereas PFs were selected with increasing antibiotic concentrations ranging from 15 to 40 µg/ml of G418 and from 10 to 20 µg/ml of hygromycin. DNA integrations were confirmed in each clonal cell line by PCR of total DNA using oligonucleotides that hybridize outside the cloned region. Protein tagging for each clonal cell line was confirmed by immunoblotting.

2.3. Protein Analysis

Tandem affinity purification of PTP-tagged RPB7 (RPB7-PTP) was carried out exactly as specified in the standard protocol [25]. Purified proteins were separated on a 10–20 % SDS–polyacrylamide gradient gel and detected with Pierce Gelcode Coomassie blue stain. The same protocol was used for the preparation of active RNA pol II, with the PTP tag fused C-terminally to subunit RPB9. However, in this purification the enzyme was kept on the anti-

ProtC beads and not eluted. The beads were stored at -20°C until used in *in vitro* transcription assays.

Co-immunoprecipitation assays were carried out with 100 μl of crude cell extract corresponding to 4×10^8 BF cells. PTP-RPB6z was precipitated with 40 μl settled volume of human IgG beads (GE Healthcare) which bind the ProtA domains of the PTP tag. RPB7-HA was precipitated with a rat monoclonal anti-HA antibody (Roche) that we bound to paramagnetic protein G beads (GE Healthcare) to avoid a non-specific interaction of this antibody with the ProtA domains. After one hour on ice, the supernatant was taken off and the precipitate washed seven times with 700 μl of TET100 buffer (100 mM NaCl, 20 mM Tris pH 8.0, 3 mM MgCl_2 , 0.1% Tween 20). Precipitated PTP-RPB6z was eluted by resuspending the beads in 45 μl of TEV protease buffer (150 mM KCl, 20 mM Tris pH 7.7, 3 mM 1M MgCl_2 , 0.5 mM EDTA, 1 mM dithiothreitol [DTT], 0.1 % Tween 20) containing 40 units of AcTEV protease (Invitrogen) and by an incubation of the protease digest at 28°C for 30 min. Eluted proteins were mixed with 15 μl of 4x standard SDS loading buffer and boiled for 10 min. Anti-RPB7-HA precipitates were directly released into SDS loading buffer.

In immunoblots, PTP-tagged proteins were probed with the mouse monoclonal anti-ProtC antibody HPC4 (Roche) in the presence of 1 mM CaCl_2 and HA-tagged proteins with a rat monoclonal anti-HA antibody (Roche). Protein detection was achieved with peroxidase-labeled anti-mouse or anti-rat IgG secondary antibody (Vector Laboratories) in combination with the BM chemiluminescence blotting substrate (Roche) according to the manufacturer's protocol. Previously published antisera were used to detect the two largest RNA pol I subunits RPA1 and RPA2 [12] whereas for RPB1 detection a polyclonal anti-RPB1 immune serum was raised in rat against an *Escherichia coli*-expressed GST fusion protein comprising RPB1 residues 1424 to 1766 according to a published procedure [30].

2.4. Chromatin Immunoprecipitation

For each ChIP experiment, 1×10^8 bloodstream trypanosomes were fixed with formaldehyde and washed as published [31]. Cells were sonicated in a Bioruptor UCD-200 (Diagenode) for a total of 25 min (30 s on/30 s off) at 4°C , with pulse setting on "high". Following a 10-min centrifugation at 25,000g and 4°C to pellet cell debris, the supernatant was pooled and chromatin pre-cleared with antibody-free, bovine serum albumin-blocked ProtA Dynabeads (Invitrogen) for 1 hour at 4°C while rotating. For immunoprecipitation of PTP-tagged proteins, 0.75 ml of chromatin solution was incubated overnight at 4°C with either a rabbit polyclonal anti-ProtA antibody (Sigma) or a nonspecific rabbit immune serum as negative control. Sodium chloride was then added to a final concentration of 350 mM and the chromatin was captured with ProtA Dynabeads for 40 min at 4°C . Subsequent washing of the beads and DNA preparation through RNase and proteinase K treatments were carried out as detailed previously [32]. In a final step, the DNA was purified using a MinElute kit (Qiagen) and eluted in 20 μl of water.

The DNA was analyzed by standard qPCR assays using the SsoFast EvaGreen Supermix (BioRad) on a CFX96 cycler (BioRad) according to the manufacturer's recommendations. Between two and five independent ChIP experiments were conducted for each cell line. For each amplification and ChIP experiment, triplicate qPCR samples were analyzed using the Bio-Rad CFX Manager software package. Each amplification product was analyzed for specificity by both agarose gel electrophoresis and melt curve analysis. Standard curves for oligonucleotide pairs were derived from input DNA dilution series and ranged in their r^2 value from 0.98 to 1.0. Fold enrichment values were calculated as the ratio between starting quantities of positive and control precipitations.

2.5. In vitro transcription

The bloodstream *in vitro* transcription system will be described elsewhere in detail (manuscript in preparation). Briefly, BF cells were harvested from 6 liter of culture that was grown to a cell density of 2×10^6 cells/ml. Cells were washed twice with Tryp wash solution (100 mM NaCl, 3 mM MgCl₂, 20 mM Tris-HCl, pH 7.5), equilibrated in transcription buffer (150 mM sucrose, 20 mM potassium l-glutamate, 3 mM MgCl₂, 20 mM HEPES-KOH, pH 7.7), and resuspended in 1.5 times the packed cell volume of transcription buffer containing 1 mM DTT, 10 µg/ml leupeptin, and 10 µg/ml aprotinin. After adding 20 µl of phosphatase inhibitor cocktail (Sigma) and ~200 µl settled volume of 800 µm low binding silica beads (OPS Diagnostics), cells were broken by five cycles of shock freezing in liquid nitrogen, thawing and vortexing for 2 min in a 4°C cold room. Broken cells were extracted by mixing the sample quickly with one tenth volume of buffer C (1500 mM KCl, 20 mM HEPES-KOH pH 7.7, 3mM MgCl₂) and by incubating the mix for 20 min on ice. After centrifugation at 25,000g and 2 °C for 10 min, the extract was separated from insoluble cell debris, diluted with 0.5 volumes of ice-cold transcription buffer, concentrated ~5x in a centricon-10 centrifugal filter device (Millipore), aliquoted, shock-frozen in liquid nitrogen, and stored at -80°C. For mock- or immuno-depletion of RPB7-PTP, 50 µl of BF transcription extract was mixed with transcription buffer-equilibrated protein G beads or IgG beads (settled volume of 30 µl), respectively, and incubated on ice for 1.5 hours. Beads were pelleted for 2 min. at 3000 g and 2 °C and extract was separated from beads, shock-frozen, and stored at -80 °C.

Standard transcription reactions were carried out in a volume of 20 µl containing 4 µl of cell-free extract, 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% polyethylene glycol, 0.2 mM EDTA, 0.5 mM EGTA, 4 mM DTT, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 20 µg/ml RNA pol I promoter template (Rib-trm or VSG-trm), 7.5 µg/ml SLins19 template, 12.5 µg/ml unrelated plasmid DNA, 10 µg/ml leupeptin, and 10 µg/ml aprotinin. The reactions were pre-incubated on ice for 10 min in the absence of nucleoside triphosphates (NTPs). After adding the NTPs to a final concentration of 0.5 mM, the reaction was incubated for 1 h at 27°C and stopped by adding Trizol solution (Invitrogen). Total RNA was prepared as described previously [27]. Newly synthesized RNAs were detected by primer extension of ³²P-end-labeled oligonucleotides Tag-PE and SLtag which hybridize to unrelated oligonucleotide tags of VSG-trm/Rib-trm and SLins19 RNAs, respectively. Primer extension products were resolved on 6% polyacrylamide-50% urea gels and visualized by autoradiography. In RNA pol II reconstitution assays, tandem affinity-purified RNA pol II, bound to anti-protein C matrix and equilibrated in transcription buffer, was added back to the reactions. The amounts corresponded to 0.35% and 0.7% of a standard PTP tandem affinity purification [21,25].

2.6. Indirect immunofluorescence light microscopy

Immunolocalizations of procyclic cells were carried out as described previously [33].

3. Results and Discussion

3.1. Interaction of RPB7 with RNA polymerases I and II in extract

In a first step, we wanted to confirm an interaction between RPB7 and the RNA pol I subunit RPB6z in a BF extract that was active in both accurate RNA pol I and II transcription [27,34]. To avoid any cross-reactivity of polyclonal antisera, we generated a cell line in which RPB6z was N-terminally tagged with the composite PTP tag (PTP-RPB6z; [25]) and RPB7 C-terminally with the HA tag (RPB7-HA). These proteins were then precipitated with antibodies directed against their tags. Since we have previously shown that

PTP-RPB6z is fully functional [21] and since BF and PF cell lines which exclusively expressed C-terminally tagged RPB7 did not exhibit any growth defect (data not shown), we concluded that tagging of these two subunits did not interfere with their function. When we immunoprecipitated PTP-RPB6z from extract at low stringency conditions, the RNA pol I subunits RPA1 and RPA2 were efficiently co-precipitated whereas RPB7-HA, RPB1 and a non-specific control were not (Figure 1A, left panels). Since there was the possibility that only a small amount of RNA pol I was active and bound to RPB7, we increased the relative amount of precipitate from 4x to 50x but still could not detect a signal above the background found with the spliceosomal U2-40K protein (Figure 1A, lower left panels). Similarly, when we precipitated RPB7-HA with a monoclonal anti-HA antibody, we saw efficient co-precipitation of RPB1 but not of the RNA pol I subunits RPA1, RPA2 and PTP-RPB6z (Figure 1A, right panels). Only when the precipitate was increased to 50x, we detected a faint PTP-RPB6z signal but a band of similar strength was detected with the U2-40K control suggesting that this was the background level due to the low stringency conditions applied. But even when the background signal was not taken into account, densitometry revealed that RPB7 is bound to RNA pol I in transcriptionally active extract to less than 1%.

In a different approach investigating the interaction of RPB7 with RNA pols I and II, we tandem affinity-purified RPB7 complexes from extract prepared from PFs that exclusively expressed RPB7 as a C-terminal PTP tag fusion. Although protein tags can change the affinities of protein-protein interactions, the rationale here was that if RPB7 is an essential subunit for RNA pol I transcription then it should interact in a similar manner with this enzyme as it does with RNA pol II. The tandem affinity purification of RPB7-PTP by IgG chromatography and by anti-ProtC affinity chromatography efficiently concentrated RPB7 complexes in the final eluate (Figure 1B, top panel). RPB1 was enriched throughout the purification whereas RPA1 was only detected in crude extract and the flow-through of the IgG column demonstrating that RPB7 is a *bona fide* RNA pol II subunit but not assembled into a stable RNA pol I complex to a detectable extent (Figure 1B, middle and bottom panels, respectively). Accordingly, when the final eluate was separated by SDS-PAGE and stained with Coomassie blue, an RNA pol II-specific band pattern [35,36] was detected and not bands characteristic for RNA pol I (Figure 1C). We therefore concluded that in both BF and PF extracts that are active in RNA pol I and II transcription, tagged RPB7 was quantitatively and stably associated with RNA pol II but not with RNA pol I at our detection level.

3.2. Chromatin immunoprecipitation of RPB7

Although the RPB4/7 doublet can be deposited on mRNA [17], the genome-wide occupancy profile of RPB7 in yeast was congruent with that of the RNA pol II subunit RPB3 [37]. Hence, If RPB7 is an essential component of RNA pol I, it should be found with RPB6z at RNA pol I promoters in ChIP experiments because it is unlikely that major amounts of inactive polymerase that lack RPB7 are recruited to these promoters. Since we had previously shown that a polyclonal anti-ProtA antibody is of ChIP grade that can enrich DNA-bound transcription factors more than 50fold over control precipitations with a non-specific immune serum [32] and since we wanted to directly compare occupancies of RPB6z, RPB7 and the essential, RNA pol II-specific subunit RPB9 with each other, we generated clonal BF cell lines that exclusively expressed these three essential proteins as PTP fusions (data not shown). As expected, RPB6z was clearly enriched at *VSG* ES and *RRNA* promoters that recruit RNA pol I (Figure 2). The weaker enrichment of RPB6z at *VSG* ES promoters in comparison to *RRNA* promoters is most likely due to the fact that *RRNA* transcription is spread over several repeat units whereas only one of fifteen *VSG* ESs is actively transcribed in an extranucleolar compartment [38]. RPB7 and RPB9 did not significantly cross-link to *VSG* ES promoters. In contrast, occupancy of RPB7 was clearly

established at *RRNA* promoters albeit to a lesser extent than for RPB6z. Since the *RRNA* promoter was also occupied by RPB9 (Fig. 2), this finding suggests that RNA pol II is recruited to this site rather than RPB7 being a subunit of RNA pol I. This notion was strengthened by high RPB6z occupancy and concomitant absence of RPB7 and RPB9 in the 18S rRNA coding region downstream of the *RRNA* promoter. The presence of RNA pol II at the *RRNA* promoter correlates well with the previously reported finding that the most distal domain of the *T. brucei* *RRNA* promoter resembles the bipartite upstream sequence element (USE) of the *SLRNA* promoter in opposite orientation and that this ribosomal USE, as its *SLRNA* counterpart, bound the small nuclear RNA-activating protein (SNAP)50 *in vitro* [39]. Therefore, it is possible that binding of the SNAP complex to the distal domain of the *RRNA* promoter nucleates an RNA pol II transcription pre-initiation complex that recruits RNA pol II. This recruitment may be of functional significance because it was recently shown in the human system that noncoding RNA, derived from the *RRNA* promoter sequence, induces epigenetic regulation of *RRNA* transcription [40].

The absence or low occupancies observed with RPB7 and RPB9 at RNA pol I transcription units cannot be due to inefficient cross-linking to DNA because both proteins were nearly 60fold enriched at *SLRNA* promoters and clearly detectable in the $\beta - \alpha$ tubulin intergenic region (Figure 2). In sum, these ChIP experiments demonstrated that RPB7 occupancy closely paralleled the occupancy of the RNA pol II subunit RPB9 at both RNA pol I and II transcription units whereas it did not correlate with the occupancy of RPB6z at these sites. These findings do not support an essential role of RPB7 in RNA pol I transcription.

3.3. RPB7 depletion from extracts specifically affects RNA pol II transcription

Previously, we have developed a homologous *in vitro* transcription system in procyclic *T. brucei* that was active in RNA pol I transcription and in RNA pol II-mediated *SLRNA* transcription [27,34]. However, to compare our results with those of Penate et al. [18], we needed to establish the system in BF extract. We have been able to develop a small scale extract procedure that was applicable to BFs. We prepared extract from cells that exclusively expressed RPB7-PTP and depleted the extract of ~90% of RPB7 using PTP-binding IgG beads (Figure 3A). We then analyzed RNA pol I and II activity in assays in which the *SLRNA* promoter template SLins19 was co-transcribed either with the *VSG* ES promoter template VSG-trm or the *RRNA* promoter template Rib-trm. In previous work, we have unambiguously shown that SLins19 transcription is mediated by RNA pol II and VSG-trm and Rib-trm transcription by RNA pol I [41]. In comparison to mock-depleted extract, RPB7 depletion virtually abolished *SLRNA* transcription whereas it had no effect on VSG-trm and Rib-trm transcription (Figure 3B, compare lanes 1 and 2 and lanes 5 and 6). The defect on *SLRNA* transcription was not a non-specific artifact because adding back tandem affinity-purified and active RNA pol II [36] partially restored transcriptional activity from the *SLRNA* promoter in a dose-dependent manner (lanes 3 and 4, and lanes 7 and 8). We therefore concluded that RPB7 cannot have an indispensable role in promoter-dependent transcription of RNA pol I.

Penate *et al.* [18] presented *in vitro* transcription results which indicated that RPB7 is important for *VSG* ES promoter-dependent transcription. However, they used a G-less cassette approach which did not discriminate between accurately initiated transcription and non-specific read-through transcription raising the possibility that their transcription signals were generated in a promoter-independent manner. On the other hand, they did show that *VSG* ES promoter deletion in the template construct resulted in a loss of transcription signal, a finding which cannot be resolved in the light of our results.

3.4. RPB7 does not detectably localize to the nucleolus

RNA pol I has been localized either throughout the nucleolus [38] or to the nucleolar periphery [18,42]. However, in the latter case the signal was clearly inside the spherical structure of low DNA density that marks the nucleolus in a DAPI stain. Penate *et al.* showed a co-localization mask of RPB7 and RPA1, and argued that in the nucleolar periphery, which is the site of RNA pol I transcription [42], there is significant co-localization. However, their RPB7 localization did not reveal a clear nucleolar or ESB signal and it is possible that the co-localization of RPB7 and RPA1 was derived from adjacent signals that could not be resolved in their analysis. To assess whether RPB7 can be found in the nucleolus we transiently transfected procyclic cells that expressed RPB7-PTP with a construct harboring a NOP10-GFP fusion gene [43]. NOP10 is a component of small nucleolar ribonucleoprotein particles and localized in the central part of the nucleolus [44]. In the representative cell shown in Figure 4, DAPI staining revealed a large and clear nucleolus. RPB7-PTP was found in most parts of the nucleus closely following the perimeter of the nucleolus but not detectably extending into the nucleolus. As expected, in the same cell NOP10-GFP was found in a central nucleolar position. Importantly, the co-localization demonstrates a clear gap between Nop10 and RPB7 representing the nucleolar periphery. The gap suggests that RPB7, in contrast to previous localizations of RNA pol I, does not localize to the nucleolus in detectable amounts and is present only at the nucleolar perimeter. This, however, is not surprising because *RRNA* repeats are tightly flanked by RNA pol II transcribed loci, and in procyclic cells, an apparent RNA pol II transcription unit partially overlaps with a procyclin gene unit [45] suggesting that on DNA, RNA pol II-based RPB7 should be present in close proximity to RNA pol I.

4. Conclusion

In this study, we have revisited the role of RPB7 in *T. brucei* RNA pol I transcription. We did not find detectable interactions of RPB7 with RNA pol I in reciprocal co-immunoprecipitations or in tandem affinity purifications with RPB7 as bait. Moreover, RBP7 did not cross-link to RNA pol I transcription units independent of the RNA pol II subunit RPB9, and immunodepletion of RPB7 from BF extracts did not affect *VSG* ES and *RRNA* promoter transcription whereas it did abolish *SLRNA* transcription by RNA pol II. Unlike RNAi-mediated expression silencing of *RPB7*, these approaches avoid potential secondary effects of an RNA pol II shut-down. While our data do not exclude a potential role of RPB7 in *VSG* expression, they show that RPB7 is not a *bona fide* subunit of RNA pol I and has no indispensable role in RNA pol I transcription in *T. brucei*.

It should be noted here that RPA31 could be the missing RNA pol I paralog of RPB7 because it was essential for promoter-dependent RNA pol I transcription and found to be associated with RNA pol I only in the presence of RPB6z [20,21], the subunit ortholog that recruits RPA43 in yeast [46]. Although RPA31 was described as a “novel” subunit because its sequence is only conserved among trypanosomatids [21], recent characterizations of *T. brucei* TFIIB [47,48], TFIIF [49] and, in particular, mediator [32] revealed that trypanosomatid transcription factor sequences are extremely divergent to those of other eukaryotes and, therefore, may not reveal the identity of a protein.

Acknowledgments

We are grateful to Dr. Christian Tschudi (Yale University) for providing plasmid NOP10-GFP. This work was funded by NIH grant R01 AI059377 to A.G.

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Highlights

- RPB7 is not a stable component of *T. brucei* RNA pol I
- DNA occupancies of RPB7 and RPB9 appear to be congruent with each other, and different from that of the RNA pol I subunit RPB6z
- RPB7 depletion from extract affects RNA pol II but not RNA pol I transcription
- The nuclear localization of RPB7 does not appear to extend into the nucleolus

Fig. 1.

RPB7 does not detectably interact with RNA pol I in transcriptionally active extract. (A) Immunoblot analysis of PTP-RPB6z immunoprecipitation (PTP-RPB6z IP; left panels) and of RPB7-HA immunoprecipitation (RPB7-HA IP, right panels). For each immunoprecipitation, equivalent amounts of crude extract (Inp) and of supernatant (S) were analyzed and four or fifty times the relative amount of the precipitate (P). Asterisks indicate IgG heavy and light chain signals. Note the reduction in size of PTP-RPB6z to P-RPB6z upon TEV protease digest in the PTP-RPB6z immunoprecipitation. (B) Immunoblot analysis of TbRPB7-PTP purification. Crude extract (Inp), flow-through of the IgG sepharose column (FT-IgG), TEV protease eluate (TEV), flow-through of the anti-protein C matrix (FT-ProtC), and final eluate (Elu) were analyzed in relative amounts as indicated. Tagged RPB7 and endogenous RPB1 and RPA1 were successively detected on the same blot. (C) RPB7 co-purified proteins. The complete final eluate (Elu) of a standard RPB7-PTP purification was separated on a 10–20% SDS/polyacrylamide gradient gel and stained with Coomassie blue. For comparison, small aliquots of crude extract (Inp) and TEV protease eluate were co-analyzed. The band assignments on the right were according to previous RNA pol II purifications [35,36,47] and our own immunoblot results. RPB1 occurs in two bands, the upper one being phosphorylated (ph. RPB1). The question mark indicates several co-purified bands which have not been identified yet. The band pattern of RPB12 and RPB10 has not been determined yet.

Fig. 2.

ChIP analysis of RBP6z, RBP7 and RBP9. (A) Chromatin of clonal BF cells which exclusively express either PTP-RBP6z, RBP7-PTP or RBP9-PTP was precipitated with a rabbit polyclonal anti-ProtA antibody (IP) and analyzed by semi-quantitative PCR. In negative control reactions, chromatin was precipitated with a non-specific rabbit immune serum and for a positive PCR control, a small aliquot of DNA isolated from total chromatin (Inp) was amplified. The occupancy of these subunits were determined for the consensus *VSG* ES and *RRNA* promoters, the 18S rRNA coding region, the SL RNA promoter and the β - α tubulin intergenic region. (B) Corresponding qPCR analysis shown as the fold enrichment over the negative immunoprecipitation. These results are based on at least two independent ChIP experiments each of which was analyzed by three qPCR reactions. Note the different scales on the y axes.

Fig. 3.

In vitro transcription analysis of RPB7-depleted extracts. (A) Immunoblot of BF extract prepared from cells exclusively expressing RPB7-PTP that was either mock-depleted or RPB7-depleted by means of ProtA-interacting IgG beads. Detection of U2-40k served as a loading control. (B) Co-transcription reactions of the SLRNA promoter template SLins19 with the VSG ES promoter template VSG-trm or the *RRNA* promoter template Rib-trm. Reactions were carried out with either mock or RPB7-depleted (depl) extracts. Extracts were reconstituted with tandem affinity-purified RNA pol II. Transcription signals were obtained through primer extension of radio-labeled oligonucleotides that hybridize to unique tag insertions in the SLins19, VSG-trm and Rib-trm RNAs. Primer extension signals were separated on a 6% polyacrylamide-50% urea gel and visualized by autoradiography. On the right, signals for correctly initiated transcription from VSG ES, *RRNA* and *SLRNA* promoters are indicated. Marker, MspI-digested pBR322.

Fig. 4. Immunofluorescence light microscopy of procyclic trypanosomes showing constitutively expressed RPB7-PTP in red, transiently expressed Nop10-GFP in green, and DNA in blue. Scale bar is 6.5 μ m.