Elongator Protein 3b Negatively Regulates Ribosomal DNA Transcription in African Trypanosomes

Sam Alsford and David Horn*

London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, United Kingdom

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Eukaryotic cells limit ribosomal DNA (rDNA) transcription by RNA polymerase I (RNAP-I) to maintain genome integrity. African trypanosomes present an excellent model for studies on RNAP-I regulation because they possess a bifunctional RNAP-I and because RNAP-II transcription appears unregulated. Since Elp3, the catalytic component of Elongator, controls RNAP-II transcription in yeast and human cells, we predicted a role for a trypanosome Elp3-related protein, ELp3a or ELp3b, in RNAP-I regulation. elp3b null and conditional strains specifically exhibited resistance to a transcription elongation inhibitor, suggesting that ELp3b negatively impacts elongation. Nascent RNA analysis and expression of integrated reporter cassettes supported this interpretation and revealed negative control of rDNA transcription. ELp3b specifically localized to the nucleolus, and ELp3b loss rendered cells hypersensitive to DNA damage and to translation inhibition, suggesting that anti-Elongator function was important to maintain genome integrity rather than to modulate ribosome production. Finally, ELp3b displayed discrimination between RNAP-I compartments in the same cell. Our results establish ELp3b as a major negative regulator of rDNA transcription and extend the roles of the Elp3-related proteins to RNAP-I transcription units. ELp3b is also the first trypanosome protein shown to distinguish between rDNA and variant surface glycoprotein transcription within different RNAP-I compartments.

In eukaryotic cells, RNA polymerase II (RNAP-II) transcription regulation appears to operate predominantly at the level of elongation (18), and several factors that influence this process have been described in human cells and in model organisms (43). Much less is known about RNAP-I control, but cells do limit ribosomal DNA (rDNA) transcription, and this is important for the maintenance of genome integrity (22); extra copies of rDNA allow for reduced transcription, which facilitates repair.

Trypanosomatids are protozoa that branched early from the eukaryotic lineage and are important human and animal pathogens which are emerging as model organisms for the study of epigenetic regulation (13). In trypanosomatids, RNAP-II transcription of protein-coding genes is polycistronic and apparently constitutive (10). In the bloodstream-form African trypanosome Trypanosoma brucei, RNAP-I is bifunctional, directing transcription of rDNA in the nucleolus and monocistronic transcription of a variant surface glycoprotein (VSG) gene in an expression site body (ESB) that is distinct from the nucleolus (32). RNAP-I is readily able to contribute to mRNA production in trypanosomatids (45) because all mature mRNAs are fused to an RNAP-II-transcribed, transspliced leader sequence (19). The high rate of transcription achieved by RNAP-I may be important for VSG expression since T. brucei bloodstream-form cells derive >10% of total cellular protein from a single VSG gene. These features lead us to predict that conserved factors involved in RNAP-II transcription elongation control in other eukaryotes, such as Elongator, might function in RNAP-I control in trypanosomes.

Saccharomyces cerevisiae Elongator (49) associates with elongating RNA polymerase II (RNAP-II) with a hyperphosphorylated carboxyl-terminal domain (37). The catalytic subunit of the six-subunit Elongator complex (26), Elp3 (Elongator protein 3, also called KAT9), appears to provide a direct link between histone acetylation and transcription by facilitating RNAP-II elongation in a chromatin- and acetyl coenzyme A (acyetyl-CoA)-dependent manner (56, 57). Human Elongator also facilitates RNAP-II elongation through chromatin and displays histone acetyltransferase activity with specificity for lysine residues in the N-terminal tail of histone H3 (25). More recently, S. cerevisiae Elp3 was shown to modulate transcriptional silencing at telomeres and to modulate DNA repair through an interaction with proliferating cell nuclear antigen (28). As well as a GNAT-type acetyltransferase domain, Elp3 contains a radical S-adenosylmethionine (SAM) domain with an iron-sulfur (FeS) cluster. Recent evidence points to a role for the mouse Elp3 radical SAM domain in DNA demethylation (36). Surprisingly, S. cerevisiae Elongator (40, 41) and human Elongator (25) localize predominantly to the cytoplasm, and roles have also been reported in exocytosis (41) and tRNA metabolism in S. cerevisiae (21) and in tubulin acetylation in mouse neurons (11).

There are two Elp3-related proteins in trypanosomatids, and we predicted a role for one or both of these proteins in RNAP-I regulation. Here, we demonstrate that T. brucei ELp3b negatively regulates rDNA transcription. This conclusion is supported by elongation inhibitor resistance, increased nascent rDNA transcription, and increased rDNA-integrated
reporter expression in elp3b-depleted strains and ELP3b nuclear localization. Remarkably, ELP3b selectively controls rDNA transcription, indicating an ability to distinguish between different RNASI transcription units.

MATERIALS AND METHODS

Trypanosomomes. bloodstream forms of T. brucei, MCTat 1.2 clone 221a, were maintained, transfected, and differentiated as previously described (3). at least 5 h after transfection, transformants were exposed to puromycin (2 μg ml−1), phosphonoacetic acid [PAA]), blasticidin (10 μg ml−1), hygromycin (2.5 μg ml−1), or phleomycin (2 μg ml−1) selection, as appropriate. ELP3b disruption was confirmed by PCR and Southern blotting, using standard protocols (5). for conditional strains, pHDI1313 (tet-R1) (4), was integrated at the β-tubulin locus and pRP6PPELP3b was integrated at rDNA locus in an ELP3b heterozygous strain prior to disruption of the second native allele of ELP3b. transformants were screened by immunoblotting and immunofluorescence to confirm robust regulated expression, and two strains were transformed with the final disruption construct in the presence of tetracycline (Tet; 1 μg ml−1) to generate four conditional strains. For growth assays, cultures were seeded at ~105 ml−1 and diluted back every 24 h. cell counts were made using a hemocytometer and carried out over at least 72 h. Cumulative counts were used to calculate population doubling times, assuming exponential growth. For half-maximal effective concentration (EC50) determinations, cells were seeded at 2 × 104 ml−1 in 96-well plates in a 2-fold dilution series of drug. After ~3 days of growth, 20 μl of abam blue (AbD Serotec) was added to each well, and the plates were incubated for a further 7 h. Fluorescence was determined using a fluorescence plate reader (Molecular Devices) at an excitation wavelength of 530 nm, an emission wavelength of 585 nm, and a filter cutoff of 570 nm (42). all assays were carried out in the absence of any additional antibiotics.

Plasmid construction. enhanced green fluorescent protein (eGFP) and cMYC fusions were generated using the pRP (ELPSa, ELPSb, RPB6, and RPB6) and pNAT (SNAP42 and RPC160) vectors (2). Protein-coding sequences were amplified from T. brucei genomic DNA using Phusion polymerase (New England BioLabs). Primers were designed using the publicly available T. brucei genome (www.genedb.org/genedb/tryp). For the generation of ELP3b disruption constructs, targeting fragments were amplified from genomic DNA and cloned into pBlA (Blasticidin S deaminase) and pPAC (puromycin N-acetyltransferase). ELPSa targeting fragments were cloned into pPAC; the PAC gene was subsequently replaced with BLE and HYG to generate additional disruption constructs. all transcription run-on probes were cloned in pBluescript (Strata- gene) or Pgem-T easy (Promega) with the exception of R3, which proved refractory to cloning. all primer sequences are available upon request.

Protein analysis. Immunoblotting was carried out following SDS-PAGE of whole-cell lysates and electroblotting using standard protocols (5) and an enhanced chemiluminescence kit (Amersham), according to the manufacturer’s instructions. Immunofluorescence analysis was carried out on fixed cells settled on slides pretreated with 3-aminopropyl triethoxysilane (stdsil) and processed as previously described (4), eGFP and cMYC fusions were detected with rabbit polyclonal anti-GFP (Molecular Probes) or mouse monoclonal anti-GFP (AbCam) and mouse anti-cMYC (9E10; Santa Cruz Biotechnology), respectively. NOG1 and NUP1 were detected with rabbit anti-NOG1 (38) and anti-GFP (AbCam) and mouse anti-cMyc (9E10; Santa Cruz Biotechnology), respectively. Images were captured using a Nikon Eclipse E600 epifluorescence microscope in conjunction with a Coolscan FX (Photometrics) charge-coupled device (CCD) camera and processed in Metamorph 5.0 (Photometrics) and Adobe Photoshop elements 2.0 (Adobe).

Transcript analysis. Total RNA was isolated using an RNeasy kit (Qiagen), fractionated on 5% polyacrylamide-7 M urea or 1.5% agarose-formaldehyde gels, and analyzed by Northern blotting according to standard protocols (5). Transcript run-on analysis was carried out using an adapted lysolecithin permeabilization protocol (51). briefly, ~2 × 106 cells were washed in transcription buffer (TB; 20 mM l-glutamic acid monopotassium salt, 3 mM MgCl2, 150 mM sucrose, 1 mM dithiothreitol [DTT], 10 μg ml l-1-3-5-leucinpeptide [Sinigal] and incubated for 1 h at 0.4 ml TB containing 2 mg lysolecithin (lα-lysophosphatidylcholine) in ice. Permeabilized cells were washed in TB and then placed in labeling mix (20 mM l-glutamic acid [monopotassium salt], 3 mM MgCl2, 1 mM DTT, 10 μg ml l-1-3-5-leucinpeptide, 25 mM creatine phosphate, 0.6 μg ml−1 carbonate phosphokinase [type I, rabbit muscle; Sigma], 2 mM ATP, 1 mM CTP, 1 mM GTP [Fermentas], 100 μCi [35S]UTP [Perkin-Elmer]) in a final volume of 200 μl for 15 min at 37°C. Total RNA was isolated using an RNeasy kit (Qiagen). Slot blots were generated in sets of three and included seven rDNA probes: R1 (rDNA promoter; −244 to +255), R2 (including the rDNA promoter and the first 400 bp of small-subunit [SSU] rDNA; RH), R3 (+1 to 3310, including SSU rDNA), R4 (+3339 to 6389, including 5.8S and large-subunit α [LSUα] rDNA), R5 (+6444 to 9455, including LSUb rDNA), R6 (nontranscribed rDNA spacer), and SS rDNA; four mRNA-associated probes, 5S2G, β-tubulin, Procyolin, and spliced-leader RNA (SL-RNA); and a pBluescript negative control. three micrograms of each plasmid (or 1.5 μg of purified R3 PCR product) was denatured in 0.4 M NaOH, transferred to nylon membrane under vacuum, and UV cross-linked. Total labeled RNA was hybridized to the slot blots overnight at 65°C and subsequently washed and processed according to standard protocols (5). Hybridization signals were detected using a Typhoon phosphor- imager (Amersham), quantified using ImageQuant (Amersham), and analyzed in MS Excel.

RESULTS

Trypanosomatid genomes encode two Elp3-related proteins. Elp3 orthologues are conserved from archaea to humans. pairs of Elp3 orthologues were identified in T. brucei, Trypanosoma cruzi, and Leishmania major (23), but we found no organism beyond the trypanosomatids with more than one Elp3 orthologue. as expected from the position in the excavata, the trypanosomatid Elp3 orthologues, designated ELPSa and ELPSb, are diverged relative to Elp3 orthologues from the Opisthokonta, i.e., metazoa, plants, fungi, and alveo- lates (1). in addition, the ELPSa and ELPSb paralogue groups are monophyletic, suggesting a single gene duplication that predates divergence of the trypanosomatids. despite the divergence, the residues required for substrate binding within both the radical SAM and acetyltrifluoracetyltransferase domains are conserved in both T. brucei ELPSa and ELPSb (Fig. 1B). Our phylogenetic and sequence analysis indicated that ELPSa and ELPSb have similar features regardless of the trypanosomatid under consideration; we have focused on the T. brucei proteins.

elp3b mutants are resistant to transcription elongation in- hibition and hypersensitive to DNA-damaging agents. to explore the function of the trypanosomatid Elp3-related proteins, we generated elp3a (Fig. 2A) and elp3b (Fig. 2B) null strains. double-null elp3a/elp3b strains were also generated (Fig. 2A and B). these strains were indistinguishable from wild type in relation to cell cycle phase distribution and differenti- ation to the insect stage (data not shown), but the elp3b strains displayed a growth defect relative to elp3a and wild-type try- panosomosomes (Fig. 2C). Interestingly, repeating the analysis after further growth suggested that the cells were adapting to the defect, with population doubling time approaching that of wild-type cells (Fig. 2C). double-null elp3a/elp3b strains also displayed a growth defect and partial reversal of this phenotype after prolonged growth. transcription elongation defects increase sensitivity to depleted nucleoside triphosphate (NTP) substrate pools. Indeed, S. cerevisiae elp3 null strains were hypersensitive to 6-azauracil (6Au) (57), which inhibits IMP dehydrogenase (IMPDH) and depletes pools of GTP and UTP (46). Surprisingly, elp3b cells displayed specific and significant resistance to 6Au relative to that of elp3a and wild-type try- panosomosomes (Fig. 2D), suggesting that ELPSb negatively controls transcription elongation. this phenotype, like the growth phenotype, was diminished after further growth (Fig. 2E). double-null elp3a/elp3b strains also displayed 6Au resistance and partial reversal of this phenotype after prolonged growth. Thus, elp3b null cells display an adaptation phenotype characterized by partial reversal of the growth and 6Au resistance phenotypes, and ELPSa is not required for this adaptation.
The data above are consistent with the idea that ELP3b negatively controls transcription, and preliminary analysis of nascent RNA did indeed reveal increased rDNA transcription in \textit{elp3b} cells (data not shown). Consistent with a link between the growth and 6AU resistance phenotypes described above, the rDNA transcription derepression phenotype was also unstable (data not shown but see below).

Saccharomyces cerevisiae strains lacking Elp3 (28) or with increased rDNA transcription (22) are hypersensitive to DNA-damaging agents, and notably, \textit{elp3b} null cells were also hypersensitive to phleomycin (Fig. 2F), an agent that damages DNA via a mechanism involving chelation of metal ions and the generation of free radicals.

To facilitate studies of ELP3b function in a controlled environment, we generated strains with a conditional (Tet-on) copy of \textit{GFPELP3b} in an \textit{elp3b} null background. Cells in which \textit{GFPELP3b} expression was inactivated (Fig. 3A) displayed decreased growth rate (Fig. 3B), reduced 6AU sensitivity (Fig. 3C), and increased sensitivity to methyl methanesulfonate (MMS) (Fig. 3D), an alkylating agent that damages DNA and is thought to stall replication forks. These results recapitulate the \textit{elp3b} null phenotypes and demonstrate that \textit{GFPELP3b} functionally complements the \textit{elp3b} defect.

ELP3b negatively controls ribosomal DNA transcription elongation. We next measured nascent transcripts emanating from different loci in cells expressing \textit{GFPELP3b} or in cells
where GFP-ELP3b expression had been inactivated for 3 or 7 days (Fig. 4). In this case, we used a series of five probes (R1 to R5) covering the length of the rDNA transcription unit (Fig. 4A). Slot blots were also loaded with probes for VSG2, the active VSG in all clones analyzed (RNAP-I), β-tubulin genes (RNAP-II), spliced-leader (SL-RNA) genes (RNAP-II), 5S rDNA genes (RNAP-III), and several negative controls: nontranscribed rDNA intergenic spacer, an insect-stage-
specific transcript that is not expressed in the bloodstream-form cells used for this analysis (procyclin), and plasmid vector DNA. The transcription run-on analysis indicated significantly increased transcription through the rDNA unit following GFPELP3b inactivation (Fig. 4B). In the 7-day samples, transcription was significantly increased (by $60\%$) across a region encompassed by probes in the middle and at the distal end of the rDNA unit (Fig. 4B). The equivalent 3-day samples also displayed increased transcription across this region but without achieving statistical significance. Probes encompassing the proximal end of the rDNA unit failed to show an increase in transcription, suggesting little attenuation in this region in wild-type cells. Thus, consistent with increased 6AU resistance, cells depleted for ELP3b displayed a relative increase in nascent rDNA promoter-distal transcripts; the large increase is remarkable given the major contribution that rDNA genes are thought to make to total transcription in unper- turbed cells. Interestingly, VSG2 transcription was significantly reduced (by $20\%$) in these cells (Fig. 4B), which could reflect depletion of the extranucleolar pool of RNAP-I. No negative-control transcript, including nontranscribed rDNA intergenic spacer, was significantly above background (data not shown), and neither the RNAP-II (SL-RNA) or RNAP-III (5S rRNA) transcripts displayed significant change (Fig. 4B).

FIG. 3. ELP3 downregulation phenocopies ELP3b knockout. (A) Western blotting with anti-GFP confirmed Tet-on (1 $\mu$g ml$^{-1}$) regulation of GFPELP3b in an elpb background. The lower panel shows an equivalent Coomassie blue-stained gel as a loading control. Data from one representative cell line are shown. (B and C) Population doubling times (B) and 6AU (50 $\mu$g ml$^{-1}$) sensitivities (C) in wild-type (WT) and GFPELP3b strains. (D) Methyl methanesulfonate (MMS; 0.0004%) sensitivity in GFPELP3b strains (EC$\text{~50}: 0.00036\%$ Tet, 0.000015%; 0.00024\% Tet, 0.000016%). The $-\text{Tet}$, GFPELP3b-depleted population was assessed 4 to 7 days after Tet removal. Data in panels B to D were derived from four independent clones. Standard deviations are indicated, and $P$ values were derived using a paired Student $t$ test. ***, $P < 0.001$; **, $P < 0.01$.

FIG. 4. ELP3b negatively regulates transcription elongation at rDNA loci. (A) Schematic of a T. brucei rDNA transcription unit and the location of the R1 to R5 probes (horizontal bars) for nascent transcript analysis. Each rDNA unit is approximately 10 kbp in length (55). The promoter (flag) and the rDNA subunit coding regions are indicated. (B) Transcription run-on analysis during depletion of GFPELP3b. Phosphorimager signals were corrected against $\text{β-tubulin}$ transcript abundance and expressed relative to the $+\text{Tet}$ value (set to 1). The inset shows a sample slot blot. V2, VSG2; $\beta T$, $\beta$-tubulin. VSG2 is a single-copy gene ~60 kbp from its promoter that is transcribed by RNAP-I. The spliced-leader RNA (SL-RNA) is derived from a tandem gene array and contributes a fragment that is trans-spliced to the 5’ end of every mRNA in trypanosomes. This RNA and $\beta$-tubulin, used as a loading control and also derived from a tandem gene array, are transcribed by RNAP-II. 5S rRNA is also derived from a tandem gene array and is transcribed by RNAP-III. Data were derived from four independent GFPELP3b strains. Error bars represent one standard deviation, and $P$ values were derived using a paired Student $t$ test. *, $P < 0.05$. 

ELP3b suppresses transcription of a reporter integrated within ribosomal DNA. We next sought an independent approach to confirm negative control of transcription by ELP3b. Trypanosomes are unusual in that all mature mRNAs are fused to an RNAP-II-transcribed, trans-spliced leader sequence (19). This allows RNAP-I to transcribe the protein-coding segment of mRNA (45), as is the case for VSG genes. We took advan-
tage of this feature and used a selectable marker as a reporter of transcription through rDNA (Fig. 5A). A neomycin phosphotransferase (NPT) gene was inserted between the 5.8S and LSU/H9251 genes in cells engineered for conditional expression of GFPELP3b in an elp3b null background, and three independent clones were analyzed (Fig. 5B); correct integration was confirmed using PCR assays (data not shown).

NPT expression was increased 3 and 7 days after inactivation of GFPELP3b expression, achieving statistical significance in the 3-day samples (Fig. 5B). The results confirm negative control of transcription through rDNA by ELP3b and suggest suppression of transcription through individual rDNA units rather than complete transcription blockade in a subset.

Having established that ELP3b negatively controls transcription at rDNA loci, we now revisited the adaptation phenotype in the elp3b strains described above. We predicted that adaptation would involve reduced rDNA transcription, either through change in rDNA gene copy number or through the action of a second negative regulator. There are nine complete rDNA units annotated in the haploid T. brucei genome sequence, with one unit each on chromosomes 1 and 7, three on chromosome 2, and four on chromosome 3 (6). Using elp3b null cells grown in culture for several weeks (elp3b*), we saw no evidence for altered rDNA gene dosage (data not shown), but as predicted, transcription run-on analysis revealed reduced rDNA transcription (Fig. 6B). In elp3b* cells, transcription was reduced by >50% compared to the wild type in a region encompassed by three probes at the proximal end of the rDNA unit. This does not reflect more transcription in the distal region but is readily explained by a two-stage process involving increased elongation after ELP3b loss which is compensated for by less initiation (compare Fig. 6B and Fig. 4B).

Consistent with adaptation in the absence of ELP3a (in elp3a/elp3b double-null strains [Fig. 2C and E]), nascent transcript analysis in elp3a strains did not reveal changes in rDNA transcription that were statistically significant (Fig. 6C), implicating another, unknown factor in reduced initiation and adaptation in elp3b null cells.

**ELP3b localizes to the nucleolus but not to the VSG expression site body.** We proceeded to explore the subcellular location of GFPELP3a and GFPELP3b in bloodstream-form trypanosomes engineered for tetracycline (Tet)-inducible expression (3). Microscopic analysis of these strains did not reveal any substantial GFP signal in uninduced cultures, while fluorescence or immunofluorescence analysis of induced cells indicated specific accumulation in distinct subnuclear compartments (data not shown). To directly compare these compartments, we established trypanosomes constitutively expressing both GFPELP3a and MYCELP3b (Fig. 7A). Immunofluorescence analysis revealed little overlap in the location of the two proteins; the subnuclear compartment occupied by GFPELP3a is punctate and typically at the nuclear periphery, while MYCELP3b occupies a more central compartment (Fig. 7B). We obtained similar results using insect-stage, procyclic trypanosomes (data not shown). Interestingly, the accumulation of MYCELP3b was specifically disrupted after transcription inhibition (Fig. 7B), which could indicate engagement with active transcription factors.

Diploid T. brucei nuclei contain a single nucleolus, the site of rDNA transcription driven by RNAP-I, and apparent ELP3b accumulation at this site is consistent with the phenotypes described above. However, bloodstream-form T. brucei trypanosomes are unusual in that they also use RNAP-I to transcribe VSG mRNA at an extranucleolar site known as the expression site body (ESB) (32). To examine ELP3b localization with respect to both RNAP-I compartments, we estab-
lished trypanosomes expressing MYC-ELP3b and GFP-RPB6z (Fig. 7A), the latter being a well-characterized RNAP-I subunit found in both RNAP-I compartments (12, 33); N-terminally tagged RPB6z was previously shown to be fully functional and does not interfere with RNAP-I activity in vitro (33). Immunofluorescence staining of GFP-RPB6z revealed the nucleolus and the ESB as expected (Fig. 7C). Dual detection of GFP-RPB6z and MYC-ELP3b revealed a strong MYC-ELP3b signal at the nucleolus, but no detectable MYC-ELP3b signal that coincided with the smaller ESB, the compartment involved in VSG transcription (Fig. 7C).

The data above indicated nucleolar sequestration of ELP3b and little or no association with the ESB. However, we detected some nuclei with a second compartment of MYC-ELP3b staining, and we speculated that these represented nascent nucleoli. This was confirmed using cells expressing MYC-ELP3b (Fig. 3A). Dual immunofluorescence detection of GFP-ELP3b and NOG1, a nucleolar G protein (38) that is not detected in the ESB, indicated that the second focus of GFP-ELP3b staining always colocalized with NOG1 (Fig. 7D). In addition, nuclear and mitochondrial (kinetoplast) DNAs, stained with DAPI (4',6-diamidino-2-phenylindole), provide excellent cytological markers that define the position in the cell cycle (58), and as expected, two nucleoli and two GFP-ELP3b foci in a single nucleus were observed in trypanosomes only in the G2/M phases. Thus, ELP3b was sequestered in the nucleolus and showed little or no association with the ESB regardless of whether the protein was fused to GFP or a MYC epitope. The results show that nucleolus-enriched GFP-ELP3b complemented the growth, 6AU resistance, and transcription phenotypes seen in elp3b cells (see above). We also demonstrated that elp3b cells were indistinguishable from the wild type in relation to ELP3a, NOG1, and RPB6z localization (data not shown).

We used a similar approach to further examine the compartment(s) occupied by the other Elp3-related protein, ELP3a. GFP-ELP3a was coexpressed with other tagged transcription factors, and expression of proteins of the predicted size was confirmed by Western blotting (Fig. 8A). ELP3a appeared to occupy a compartment that was distinct from all three major RNA polymerases (Fig. 8B). A peripheral nuclear localization appeared more pronounced during mitosis (Fig. 8C), and partial colocalization with NUP-1, a putative nuclear lamina component (44), supported an association with the nuclear envelope (Fig. 8D). We also examined spindle microtubule acetylation, telomere position-effect repression (16), and VSG expression site silencing (54) in elp3a cells, as well as cytokine methylation (31) in elp3a and elp3b cells (data not shown), but detected no significant differences from the wild type.

**ELP3b-depleted cells are hypersensitive to translation inhibition.** We considered the possible benefits of limiting transcription through rDNA. This could facilitate DNA replication and DNA damage tolerance (Fig. 2F and 3D) (22) or could contribute to modulating rRNA synthesis to satisfy cellular demands for translation capacity. Indeed, rDNA transcription elongation is the rate-limiting step for rRNA synthesis in human cells (48). To explore a role for ELP3b in regulating rRNA synthesis, we examined the downstream consequences of increased rDNA transcription in ELP3b-deficient cells (Fig. 9). The trypanosomatid rDNA transcription unit is unusual in that it encodes several small rRNAs (55), but no major change in the relative steady-state abundance of any of these transcripts was seen in elp3a, elp3b*, or elp3a/elp3b null cells (Fig. 9A). Furthermore, Northern blot analysis revealed no major change in relative SSU or LSU transcript abundance in ELP3b-depleted cells (Fig. 9B). To ask whether increased rDNA transcription is reflected at the level of sensitivity to translation inhibition, we assessed growth in G418. Surprisingly, ELP3b-depleted cells were hypersensitive to G418 (Fig. 9C). This was also the case in elp3b* cells, while elp3a cells were indistinguishable from the wild type (data not shown). This result could reflect disruption of the ribosome assembly process, causing limiting factors to be channeled into a non-productive pathway, or ELP3b could play an additional role in tRNA modification (21). Taken together, the results are consistent with a role for rDNA transcription control by ELP3b in maintaining genome stability rather than in modulating translation capacity.
DISCUSSION

rDNA genes encode the core components of the ribosomes, the molecular machines that drive mRNA translation into protein. Sixty to 80 percent of transcription in rapidly growing yeast cells is at rDNA loci mediated by RNAP-I. The elongation rate has been estimated to be ~60 nucleotides/s with a reinitiation rate of ~1 s (14). Despite the important link to genome stability (22), the balance between silencing and activating complexes and their contributions to the formation of alternative chromatin states at these loci remain poorly understood (30). A number of factors that typically control multiple classes of RNA polymerase have been shown to exert positive (7, 59) and negative (30) control on RNAP-I elongation at rDNA genes. In addition, structural analysis reveals that S. cerevisiae RNAP-I contains a built-in elongation factor related to the RNAP-II-associated factor TFIIF (27), and a mutated phosphorylation site on S. cerevisiae RNAP-I increases resistance to 6AU, consistent with a role in negative control of elongation (15). We have demonstrated negative control by ELP3b that is specific to nucleolar rDNA genes in trypanosomes, and our reporter analysis suggests suppression of individual rDNA transcription units rather than complete blockade of a subset.

Nucleosomes are depleted or disordered at actively transcribed rDNA loci (30). Thus, ELP3b may generate a more stable or “closed” chromatin state that promotes premature termination. How might this be achieved? rDNA gene regulation involves histone modification and DNA methylation (50), and ELP3b, like other Elp3 orthologues, has two major domains, an acetyltransferase domain and a radical SAM domain. Histone acetylation is important for transcription initiation and elongation, and a specific enrichment of histone H4K10 acetylation, H2AZ and H2BV histone variants, and the BDF3 bromodomain factor is seen at probable RNAP-II transcription start sites in trypanosomes (47). These same variants are depleted within the nontranscribed rDNA spacer, and

FIG. 7. ELP3b localizes to the nucleolus but is not detected in the ESB in bloodstream-form T. brucei. (A) Western analysis of strains used for ELP3 localization studies. Blots were incubated with anti-MYC or anti-GFP. A Coomassie blue-stained gel is included to show loading. Predicted molecular masses of the fusions: GFP-ELP3a, 104 kDa; MYC-ELP3b, 77 kDa; GFP-RPB6z, 42 kDa. The lower anti-GFP panel is a shorter exposure. (B) Dual localization of GFP-ELP3a and MYC-ELP3b. The effect of actinomycin D treatment is also shown. The regions outlined in the phase images indicate the regions shown in the immunofluorescence panels. The nucleus (n) and kinetoplast (k) were stained with the DNA intercalating dye 4′,6-diamidino-2-phenylindole (DAPI), and these images were merged with the immunofluorescence images. Bar, 5 μm. (C) MYC-ELP3b localizes specifically to the nucleolus but not to the smaller ESB (arrowhead), as revealed by GFP-RPB6z. (D) Colocalization of GFP-ELP3b and the nucleolar protein, NOG1, through the cell cycle. G1/S cells have a single nucleolus, while a second nucleolus can be seen during G2/M (identified by two kinetoplasts). Postmitotic (Post-M) cells have a single nucleolus in each nucleus. Other details are as in panel B above.
BDF3 is depleted in the transcribed rDNA region, revealing a distinct chromatin architecture at these loci (see the region encompassing Tb927.3.3421 to Tb927.3.3455 at http://tritrypdb.org/). Importantly, histone acetylation has also been associated with negative control of transcription (9, 24, 29, 53). Thus, acetylation by ELP3b may negatively control rDNA transcription elongation. The radical SAM domain could equally be responsible for negative control, possibly via DNA demethylation (36).

The nucleus is highly heterogeneous, containing euchromatic and heterochromatic compartments thought to be permissive and repressive for transcription, respectively. RNAP-I transcribes rDNA genes in the nucleolar compartment, and, in T. brucei, RNAP-I also synthesizes a subset of abundant pre-mRNAs. In fact, T. brucei is the only known eukaryote with a multifunctional RNAP-I and presents a unique opportunity to study RNAP-I regulators. The trypanosomatid RNAP-I complex (34, 52) contains three “specialized” subunits, RPB5z, RPB6z, and RPB10z (12), as well as RPA31 (33) and the RPB7 subunit, typically associated with RNAP-II (39). Trypanosome RNAP-I transcription also depends upon a novel complex known as class I transcription factor A (8). ELP3b has not been identified in fractions containing T. brucei RNAP-I (8, 33, 34, 52), possibly due to weak or little direct interaction or because the majority of RNAP-I is engaged in processive transcription. Remarkably though, ELP3b specifically impacts relatively short RNAP-I transcription units (rDNA, 10 kbp) and appears to be excluded from a much longer RNAP-I transcription unit at the ESB (VSG, 60 kbp). Several additional nucleolar factors are undetectable in the ESB, but ELP3b is the first of these factors shown to distinguish between rDNA and VSG mRNA synthesis. The distinct promoters that operate at these loci could determine this differential association.

FIG. 8. ELP3a occupies nuclear territories distinct from those occupied by the transcription machinery. (A) Western analysis of strains used for ELP3 localization studies. Predicted molecular masses of the fusions: 6MYC-RPB6z, 24 kDa; 6MYC-RPB6, 25 kDa; GFP-RPB6, 43 kDa; SNAP4212MYC, 60 kDa; RPC16012MYC, 188 kDa. (B) GFP-ELP3a and the tagged polymerase subunits occupy distinct nuclear territories. 6MYC-RPB6z, RNAP-I; 6MYC-RPB6 and SNAP4212MYC, RNAP-II (colocalization of these factors is associated with sites of SL-RNA transcription [30, 52]); RPC16012MYC, RNAP-III. (C) Localization of GFP-ELP3a and SNAP4212MYC through the cell cycle. (D) GFP-ELP3a shows partial colocalization with NUP1. Other details are as in Fig. 7B.
Microarray analysis of steady-state transcripts in Elongator-defective *S. cerevisiae* revealed 52 genes that were downregulated and 44 genes that were upregulated (26). Under the conditions examined here, ELP3b negatively controls rDNA transcription and can be considered an “anti-Elongator.” We suggest that this may also be the case at the upregulated loci in *S. cerevisiae*. Indeed, Elp3 was recently shown to have a role in maintaining silencing at telomeres and at mating-type loci in *S. cerevisiae* (28). Our results indicate a negative role for ELP3b in processivity, while, at this stage, the role of ELP3a remains unknown.

The purpose of limited transcription through rDNA appears to be to improve genome stability (22). Our results are consistent with the idea that unregulated transcription of rDNA genes is indeed toxic and compromises the capacity for DNA repair at these loci. Our findings could reflect a conserved role for Elp3 proteins in this process. Indeed, Elp3 is concentrated in nucleoli in HeLa cells (20). We favor a model whereby ELP3b, like Elp3 in *S. cerevisiae*, associates with elongating RNAP and modifies chromatin structure, but we cannot rule out other possible scenarios to explain negative control of RNAP-I processivity at this stage. Targeted meganuclease cleavage (17) could now be used to further explore the impact of ELP3b transcription control on double-strand break repair within rDNA transcription units. It will also be important to determine what contributions the ELP3b acetyltransferase and radical SAM domains make to the novel anti-Elongator function described here.

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