TbISWI Regulates Multiple Polymerase I (Pol I)-Transcribed Loci and Is Present at Pol II Transcription Boundaries in *Trypanosoma brucei*\(^\ddagger\)

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The unicellular eukaryote *Trypanosoma brucei*, the causative agent of African sleeping sickness, has some unorthodox features. Most of the *T. brucei* genome is constitutively transcribed by RNA polymerase II (Pol II) as extensive polycistronic transcription units. Exceptions to this rule include several RNA Pol I transcription units such as the VSG expression sites (ESs), which are mono-allelically expressed. TbISWI, a member of the SWI2/SNF2 related chromatin remodeling ATPases, plays a role in repression of Pol I-transcribed ESs in both bloodstream- and procyclic-form *T. brucei*. We show that TbISWI binds both active and silent ESs but is depleted from the ES promoters themselves. TbISWI knockdown results in an increase in VSG transcripts from the silent VSG ESs. In addition to its role in the repression of the silent ESs, TbISWI also contributes to the downregulation of the Pol I-transcribed procyclin loci, as well as nontranscribed VSG basic copy arrays and minichromosomes. We also show that TbISWI is enriched at a number of strand switch regions which form the boundaries between Pol II transcription units. These strand switch regions are the presumed sites of Pol II transcription initiation and termination and are enriched in modified histones and histone variants. Our results indicate that TbISWI is a versatile chromatin remodeler that regulates transcription at multiple Pol I loci and is particularly abundant at many Pol II transcription boundaries in *T. brucei*.

As an early branching eukaryote, *Trypanosoma brucei*, the causative agent of African sleeping sickness, has some unorthodox features. Most of the *T. brucei* genome is constitutively transcribed by RNA polymerase II (Pol II) as extensive polycistronic arrays, and there is very little transcriptional control (11). Another unusual feature is that RNA Pol I transcribes not only the multicopy ribosomal DNA (rDNA) but also the genes encoding the trypanosome major surface proteins variant surface glycoprotein (VSG) and procyclin, which are controlled in a life cycle-specific fashion (17, 25, 42). Bloodstream-form *T. brucei* evade immune attack by the mammalian host through antigenic variation of its VSG surface coat by switching between different VSG variants. It is therefore critical for bloodstream-form trypanosomes to tightly control expression of their VSG repertoire. Only one of about 1,500 different VSG genes and pseudogenes is expressed in a mono-allelic fashion from one of about 15 telomeric VSG expression site (ES) transcription units (8, 10, 36). Upon differentiation to the procyclic-form insect midgut stage, the VSG coat is replaced by an invariant coat of procyclin (41).

Very few regions of the trypanosome genome are not transcribed. These include the silent VSG genes, which are located in subtelomeric VSG basic copy arrays, as well as at the telomeres of large chromosomes, intermediate chromosomes, and mini-chromosomes (4, 31, 61). Minichromosomes are primarily composed of arrays of 177-bp repeats and are transcriptionally silent (61). In addition, the rDNA spacer is an 8-kb nontranscribed region in between tandem arrays of rDNA transcription units (58). Finally, the transcription units containing the *T. brucei* coat protein genes are turned off in the life cycle stage where they are not needed. All VSG ESs are downregulated in procyclic-form *T. brucei* where VSG is not expressed, and the procyclin transcription units are silenced in bloodstream-form *T. brucei*, where it would be deleterious for an invariant surface protein to be expressed on the cell surface (56).

It is unclear how *T. brucei* keeps these transcriptionally inactive regions of the genome silent, as well as the extent to which epigenetic modifications such as chromatin remodeling are involved in this process. In the case of the ESs, it has recently become clear that epigenetic marks are also involved in epigenetic control play a role in ES regulation, including the *T. brucei* imitation switch homologue TbISWI (21), the telomere-binding protein RAP1 (64), and the histone methyltransferase DOT1B (14).

It has recently become clear that epigenetic marks are also present at the strand switch regions (SSRs) located between the polycistronic *T. brucei* Pol II transcription units (50, 63). The divergent SSRs, which contain putative transcription start sites (TSSs), are enriched in the histone variants H2AZ and H2BV, as well as the histones H3K4me3 and H4K10ac. Convergent SSRs containing putative transcription termination
sites (TTS) are enriched in the histone variants H3v and H4v (50). The proposed histone labeling of transcription stop sites is another unique feature of the trypanosome genome.

In the present study, we investigated the role of TbISWI in regulating gene expression in different genomic regions of T. brucei. We show that TbISWI binds in the T. brucei genome within silent DNA repeats, in silent and active transcription units transcribed by Pol I, and at Pol II-transcribed loci. We also found that TbISWI is particularly enriched at a number of SSRs between Pol II transcription units containing the presumed Pol II transcription initiation and termination sites. Using eGFP reporter constructs integrated into various genomic loci, we demonstrate that TbISWI is involved in repressing an ES promoter in regions containing silent VSGs, including the VSG basic copy arrays and the minichromosomes, as well as genes transcribed by Pol I, including procyclin. Knockdown of TbISWI resulted in an increase in VSG transcripts from the silent telomeric VSG ESs, indicating that it plays a role in keeping the entire ES silent. Our results indicate that the chromatin remodeler TbISWI is involved in transcription by both Pol I and Pol II in T. brucei.

MATERIALS AND METHODS

Trypanosomes. Bloodstream- and procyclic-form T. brucei 427 cell lines were cultured as described previously (49), except that the bloodstream form was grown in the presence of 20% fetal calf serum without the addition of SerumPlus. The bloodstream-form T. brucei 121-S1A cell has an active VSG121 ES, eGFP in the silent VSG221 ES, and a TbISWI RNA interference (RNAi) construct (described in reference 21). Additional bloodstream-form T. brucei lines were made containing a reporter eGFP gene inserted into various loci, including the VSG basic copy array, the 177-bp repeats of minichromosomes, the procyclin locus, the rDNA spacer, and the tubulin array. All bloodstream-form reporter lines were derived from T. brucei S16.221, a derivative of the T. brucei S16 “single-marker” cell line (62). S16.221 was obtained by transfecting S16 with the pHINES221Parl.6 construct (unpublished) that inserts a puromycin resistance gene downstream of the active VSG221 ES promoter. Next, the relevant eGFP containing reporter construct was transfected into S16.221, followed by the MC177 TbISWI-A RNAi construct (21). The lineage of the different cell lines used for the reporter assays is shown in Fig. 2. Reporter cell lines BF-MCESp, BF-Pro, and BF-VBESp, and BF-Pro are described in reference 33 as MCEP, VBEP, and PVBEP, respectively. BF-MCESp, BF-Pro, and BF-VBESp were derived from bloodstream-form T. brucei. The bloodstream-form T. brucei 106 to 20 × 10^6 cells (equal numbers of cells per lane in a gel) was separated by SDS-PAGE. Proteins were transferred to nitrocellulose and probed with either a monoclonal anti-myc antibody (clone 4A6, catalog no. 05-724; Upstate) to detect C-terminally tagged TbISWI or polyclonal anti-BIP (kindly provided by Jay Bangs, University of Wisconsin) as a loading control. Wild-type TbISWI was detected by using polyclonal anti-TbISWI antibody (21), and an anti-SMC3 antibody (6) was used as a loading control. Secondary antibodies conjugated to horseradish peroxidase were used to detect bound antibodies, and signals were visualized by using a Western Lightning Plus ECL system (Perkin-Elmer Life Sciences).

Flow cytometry. Flow cytometry was performed on T. brucei cell lines containing an eGFP reporter gene in different genomic locations after induction of RNAi against TbISWI for the times indicated. A Becton Dickinson FACSCalibur (BD Biosciences) was used to monitor the fluorescence of the cells in the FL-1 channel. The mean of 100,000 events was calculated using CellQuest software (BD Biosciences). The fold derepression was determined by dividing the mean FL-1 values of RNAi-induced cultures at each time point by the mean FL-1 values of uninduced cultures. For each cell line, three independent experiments were performed, and the standard deviations (SD) are indicated with error bars.

ChIP assays and quantitative PCR. ChIP was performed in T. brucei cells expressing wild-type or myc-tagged TbISWI essentially as described previously (51). In brief, cultures were fixed in 1% formaldehyde for 1 h at room temperature, and chromatin from 7 × 10^7 cell equivalents was incubated for 16 h with an anti-histone H3 antibody (ab1791; AbCam) as a positive control, no antibody as a negative control, and a monoclonal anti-myc antibody (clone 9E10, M5546; Sigma) to detect TbISWI. The protein-DNA complexes were incubated with protein A-beads (CL4B beads; GE Healthcare) and eluted in 1% SDS−0.1 M NaHCO3. DNA was purified using QIAquick PCR purification kit (Qiagen). Quantitative PCR (qPCR) was used to amplify regions of interest using the LightCycler 480 Real-Time PCR System (Roche) (primer details can be found in Tables S1 and S5 in the supplemental material). For the quantitation of TbISWI ChIP experiments, signals are expressed as a percentage of input immunoprecipitated using an anti-myc antibody after subtraction of signal from the non-antibody control for both the wild-type TbISWI and the myc-tagged TbISWI cell lines. Quantitation of histone H3 shows the percentage of input immunoprecipitated after subtraction of signal from the no antibody control. The data shown is from bloodstream-form T. brucei and is the average of three to five independent ChIP experiments, with the SD indicated by error bars. ChIP-qPCR was also carried out in procyclic-form T. brucei and produced results very similar to those found for bloodstream-form T. brucei in all regions analyzed.
ChIP material was also analyzed on slot blots using radiolabeled probes as described previously (51). Hybond-XL membranes (GE Healthcare) were hybridized with probes specific for the 177-bp repeats (61), the 50-bp repeat regions flanking VSG ESs (65), or the telomere repeats (7). Blots were quantitated by using a PhosphorImager (Bio-Rad), and the percentage of DNA immunoprecipitated was determined by using QuantityOne software (Bio-Rad). Signals are expressed as a percentage of input as described above.

RNA isolation and quantitative reverse transcriptase PCR. The bloodstream-form T. brucei 121-SA1 cell line (21) was used for quantitative reverse transcriptase PCR (qRT-PCR). RNAi against TbISWI was induced, and total RNA was isolated at various time points using a RNeasy kit (Qiagen). RNA was DNase treated by using a Turbo DNA-free kit (Ambion), and cDNA was synthesized by using the Omniscript RT kit (Qiagen) with random hexamer primers (Promega). qPCR was performed on a LightCycler 480 Real-Time PCR System using a LightCycler 480 SYBR green I Master Mix (Roche) in triplicate. The reaction product of the correct size. The sequences of the primers used are detailed in Table S4 in the supplemental material. Control reactions without reverse transcriptase (RT) were made using DNase-treated RNA from each time point. The levels of transcript were plotted as the fold change relative to the 0-h time point after subtraction of the no-RT controls. Three independent experiments were performed, and the SD is indicated with error bars.

RESULTS

TbISWI binds silent DNA repeats as well as the VSG basic copy arrays. TbISWI was originally identified as binding the 177-bp repeats in DNA affinity purification experiments and was subsequently shown to be involved in silencing VSG ESs in both bloodstream- and procyclic-form T. brucei (21). Since the 177-bp repeats are primarily located in the transcriptionally silent minichromosomes (61), we investigated whether TbISWI binds to and represses other silent areas of the T. brucei genome. We performed ChIP analysis using an epitope-tagged copy of TbISWI. Bloodstream and procyclic-form T. brucei lines expressing a TbISWI-myc fusion protein from one endogenous TbISWI allele were generated. Expression of the myc-tagged TbISWI was verified using Western blot analysis (Fig. 1A). The functionality of the myc-tagged TbISWI was confirmed by knocking out the other TbISWI allele. The TbISWI-myc/TbISWI-KO strains grew normally compared to cells expressing wild-type TbISWI in both life cycle stages (data not shown). The myc tag therefore did not detectably interfere with TbISWI function. This cell line was used for all successive ChIP experiments.

ChIP analysis was performed on bloodstream-form cell lines containing either wild-type or myc-tagged TbISWI. An α-myc antibody was used to immunoprecipitate TbISWI, an α-histone H3 antibody was used as a positive control, and no antibody was used as a negative control. Input chromatin and immunoprecipitated material were analyzed using either slot blots probed for repetitive regions or qPCR for individual genes. As expected, we found that TbISWI binds the 177-bp repeats (Fig. 1B) with 0.05% of the input immunoprecipitated (Fig. 1C). TbISWI was also found to bind other silent regions, including the 50-bp repeat regions upstream of VSG ESs (0.04% immunoprecipitated) (48, 65), the telomere repeats (0.01% immunoprecipitated), and the subtelomeric VSG118 basic copy array (0.04% immunoprecipitated). Significant amounts of TbISWI were therefore found binding all of these nontranscribed regions (P < 0.05; see Table S6 in the supplemental material for statistical analyses). Similar results were obtained using procyclic-form cells (data not shown). The minichromosomes and the VSG basic copy arrays house the silent VSG repertoire used during antigenic variation. Since TbISWI was found binding both of these regions at similar levels, we next investigated the role of TbISWI in silencing these areas of the genome.

TbISWI is involved in downregulating transcription in T. brucei minichromosomes and VSG basic copy arrays. In order to investigate the role of TbISWI in silencing, we made T. brucei reporter cell lines where the parental bloodstream-form T. brucei S16.221 line or the procyclic-form T. brucei 29-13 line had an eGFP containing construct inserted into the minichromosomes, the VSG118 basic copy array, the EP procyclin locus, the rDNA spacer, or the tubulin transcription unit (Fig. 2). The advantage of using eGFP to monitor for transcription is that gene expression at the population level can be analyzed using flow cytometry. This allows one to uncover cell-cycle-specific
effects, which is not straightforward with a reporter such as luciferase.

Constructs integrated into the minichromosomes or the VSG118 basic copy array, either did (+) or did not (−) contain an ES promoter (ESp). Unlike other T. brucei Pol I promoters, the ESpS are unusually small and lack an upstream control element (39), rendering transcription highly sensitive to the chromatin state of their genomic context (20, 53). We therefore used an ESp as a “probe” for repressed chromatin. These T. brucei reporter cell lines were subsequently transfected with a TbISWI RNAi construct allowing tetracycline-inducible knockdown of TbISWI. See Materials and Methods for details on the various constructs and cell lines.

To determine whether TbISWI has a role in silencing the T. brucei minichromosomes, we integrated an eGFP reporter gene into the 177-bp repeat arrays in bloodstream-form T. brucei (Fig. 2Ba, BF-MCe in Fig. 3A; see also Fig. S1A in the supplemental material) and made a similar reporter line with an ESp upstream of eGFP (BF-MCESp in Fig. 3B; see Fig. S1B in the supplemental material). Correct integration of eGFP was confirmed by pulsed-field gel analysis and Southern blot mapping (see Fig. S1C in the supplemental material). An exogenous ES promoter (ESp) is indicated by a white flag. The eGFP reporter construct is integrated within the polycistronic tubulin transcription unit. Flanking α- and β-tubulin genes are indicated by black boxes.

FIG. 2. Schematic of the T. brucei reporter cell lines constructed for the present study. (A) Parental cell lines for the T. brucei reporter lines used. The bloodstream-form (BF) T. brucei S16.221 cell line has a puromycin resistance gene (PurR) inserted in the active VSG221 expression site (transcription is indicated with an arrow). Both T. brucei S16.221 and procyclic-form (PF) T. brucei 29-13 lines contain genes encoding the T7 RNA polymerase (T7 RNAp) and the tetracycline repressor (TetR), allowing tetracycline-inducible transcription. (B) T. brucei reporter lines generated by transfecting different eGFP containing reporter constructs into the parental T. brucei S16.221 or 29-13 lines. Experimental details concerning the construction and verification of these cell lines are given in the supplemental material. A TbISWI RNAi expressing construct was subsequently introduced into each of these lines to allow monitoring for derepression of eGFP after the induction of TbISWI RNAi. (a) Reporter cell line with a construct containing eGFP (green box) either with (+) or without (−) an expression site promoter (ESp, white flag) inserted in a T. brucei minichromosome. Telomeric VSGs are indicated with red boxes, and the telomere repeats are indicated with horizontal arrows. Transcription of the selectable marker (blue box) is directed by an rDNA promoter (black flag). (b) As described for panel a, except the reporter constructs are integrated into the VSG118 basic copy array. Flanking VSGs are indicated as VSGX and VSGY. (c) The eGFP containing reporter construct is integrated behind an endogenous EP1 promoter (EPp, gray flag) in the EP procyclin locus. The EP procyclin genes and an unknown hypothetical protein (Hyp) are indicated. (d) Reporter construct integrated between two ribosomal DNA (rDNA) transcription units (the 18S and 28S rRNA genes are indicated). An exogenous ES promoter (ESp) is indicated by a white flag. (e) The eGFP reporter construct is integrated within the polycistronic tubulin transcription unit. Flanking α- and β-tubulin genes are indicated by black boxes.
knockdown after the induction of *TbISWI* RNAi (see Fig. S2 in the supplemental material), and flow cytometry was used to monitor eGFP expression. At 4 days after the induction of *TbISWI* RNAi there was no significant increase of eGFP expression observed in the lines not containing an ES promoter (BF-MCe1 and BF-MCe2 in Fig. 3A) compared to the negative control (BF-MCe). This indicates that blocking *TbISWI* synthesis does not lead to a significant increase in fortuitous initiation of transcription in the minichromosomes. In cell lines where an ES promoter was present upstream of eGFP, knockdown of *TbISWI* leads to 6-fold derepression of eGFP (BF-MCESp1 and BF-MCESp2 in Fig. 3B) compared to the negative control (BF-MCESp). This indicates that *TbISWI* plays a role in maintaining a repressed chromatin structure around the ES promoter in *T. brucei* minichromosomes.

The *VSG* basic copy arrays are another transcriptionally silent genomic location in *T. brucei*. Therefore, we also performed similar experiments using cell lines where eGFP reporter constructs (with or without an ES promoter) were integrated into the *VSG118* basic copy array (Fig. 2Bb, 3C, and 3D; see also Fig. S3 in the supplemental material). Similar to the cell lines where the reporter construct was integrated in minichromosomes, there was no significant increase in eGFP expression seen in the absence of an ES promoter (BF-VBe1 and BF-VBe2) in the absence of an ES promoter (BF-VBe1 and BF-VBe2).
Vbe2 in Fig. 3C). However, if an ESp was present (BF-VBESp1 and BF-VBESp2 in Fig. 3D) ~6-fold derepression of eGFP was observed after the induction of a block in TbISWI synthesis.

Comparable experiments were also performed in procyclic-form T. brucei, and similar results were obtained (see Fig. S4 in the supplemental material). These data indicate that TbISWI plays a role in maintaining a repressed chromatin structure in both the T. brucei minichromosomes and VSG basic copy arrays in both bloodstream- and procyclic-form T. brucei. We cannot exclude the possibility that the presence of an rDNA promoter directing transcription of the plastidic resistance gene downstream of the reporter cassette could affect the local chromatin state. However, since the results are plotted as the fold derepression (i.e., the ratio of the mean fluorescence of induced cells to that of uninduced cells), the observed derepression can be inferred to be a consequence of TbISWI depletion.

TbISWI is present in both silent and active VSG ESs but is depleted from ES promoters. The active VSG is transcribed by Pol I from 1 of ~15 highly similar polycistronic VSG ES transcription units (depicted schematically in Fig. 4A) (19). We determined the distribution of TbISWI along the length of these VSG ESs by using ChIP on wild-type or myc-tagged TbISWI cell lines, followed by qPCR as described above. Using six different primer pairs (labeled “a” to “f” in Fig. 4A and B), which detect most, if not all VSG ESs (i.e., the 14 silent ESs in addition to the one active ES), we determined that TbISWI is present at significant levels (P < 0.05) along the length of the ES, with the exception of the “core” ES promoter (22), where TbISWI is relatively depleted (primer pair a in Fig. 4A and B) (P < 0.05; see Table S7 in the supplemental material). Primer pair a is located immediately upstream of the putative site of ES transcription initiation. The relative depletion of TbISWI at ES promoters is particularly striking, considering that most if not all ESs can be detected with these particular primers. It is thought that the VSG ES “core” promoters of the 14 “silent” ESs contain “paused” Pol I complexes, which lead to a low amount of transcription (Fig. 4B) (2, 43, 55). It is possible that these “paused” Pol I transcription complexes result in exclusion of TbISWI. This pattern of TbISWI binding is similar to that observed for histone H3 at this locus (13, 51) and is consistent with TbISWI playing a role in nucleosome sliding and remodeling (30, 51).

Having determined the relative distribution of TbISWI along the VSG ESs (Fig. 4A and B), we investigated whether there is a correlation between histone occupancy and TbISWI abundance in silent versus active VSG ESs. We used the bloodstream-form T. brucei 221GPI(VO2+) line shown in Fig. 4C, which has a neomycin resistance gene inserted behind the

FIG. 4. TbISWI is distributed along active and silent VSG ESs but is depleted from ES promoters. (A) Schematic of a typical VSG ES (modified from AnTat1.3A) (5, 29), with the ES promoter (flag), the expression site-associated genes (ESAGs) (numbered boxes), and characteristic ES repeat arrays (striped boxes) indicated. The region surrounding the ES promoter is expanded below. The positions of the primer pairs used for qPCR analysis are indicated with lettered brackets. These primers can be expected to recognize most, if not all ESs. (B) Distribution of TbISWI over the length of VSG ESs. ChIP was performed on TbISWI (wt) and TbISWI-myc expressing BF T. brucei cell lines using an anti-myc antibody (α-myc). qPCR was used to amplify the regions indicated in panel A. Signals are expressed as the percentage of input immunoprecipitated by the α-myc antibody in the two cell lines (n = 3 to 5 ± the SD). Significant amounts of TbISWI were found binding ESs (P < 0.05; see Table S7 in the supplemental material) with the exception of the ES promoter region (primer a). (C) Schematic of the BF T. brucei 221GPI(VO2+) cell line used for ChIP experiments. Two VSG ESs are shown, with the ES promoters indicated by white flags and transcription of the active VSG ES by an arrow. A neomycin resistance gene (Neo) is located downstream of the ES promoter in the active VSGVO2 ES. The silent VSG221 ES contains a puromycin resistance gene (Puro) downstream of the ES promoter, as well as a single copy VSG pseudogene (ψ). (D) Distribution of TbISWI over silent and active VSG ESs. ChIP analysis was carried out on BF T. brucei cell lines expressing TbISWI (wt) and TbISWI-myc using α-myc, and chromatin was immunoprecipitated as described in panel B. qPCR was used to quantitate amounts of genes shown in panel C (n = 3 to 5 ± the SD). These genes are all single copy with the exception of VSGVO2, which has an additional copy present in a VSG basic copy array (BCA). Significant amounts of TbISWI were found binding both active and silent ESs (P < 0.05; see Table S8 in the supplemental material).
The promoter of the active VSGVO2 ES (49). The silent VSG221 ES contains a puromycin resistance gene downstream of the ES promoter, as well as a VSG pseudogene (\(\psi\)). These are all single-copy genes with the exception of VSGVO2, which has an additional copy in a VSG basic copy array. To determine the distribution of TbISWI over these silent and active ESs, ChIP and qPCR analyses were performed. Interestingly, significant amounts of TbISWI (\(P < 0.05\); see Table S8 in the supplemental material) were found to bind both active and silent ESs at approximately comparable levels (Fig. 4D).

We had shown earlier that TbISWI is important for silencing genes directly downstream of a silent ES promoter, although in those experiments we had not found clear evidence for an increase in transcripts from telomeric VSGs present in a number of silent ESs (21). Considering that TbISWI binds along the length of VSG ESs (Fig. 4), we wondered whether it could play a role in keeping the entire ES silent, including the telomeric VSG. We therefore decided to reinvestigate this question using newly available ES sequence data (19).

Depleting TblISWI leads to an increase in previously silent telomeric VSG transcripts. The repertoire of 15 VSG ESs from T. brucei 427 has recently been sequenced (19). These new data enabled us to reinvestigate whether downregulation of TbISWI results in derepression of telomeric VSGs located in ESs, in addition to upregulation of the ES promoter-proximal regions. The T. brucei 121-SAl TblISWI RNAi cell line has an active VSG121 ES and contains eGFP downstream of the promoter of the silent VSG221 ES (21). Induction of TblISWI RNAi led to a decrease in TblISWI transcript and a concurrent 60-fold increase of eGFP transcript (Fig. 5A). These results agree with previously published data (21). Transcript levels for \(/H9253\)-tubulin remain relatively unchanged after TblISWI knockdown, which was also observed using a reporter construct integrated into the \(/H9251\)-\(/H9252\) tubulin array (Fig. 6E). Transcripts from the active VSG121 decrease by about 20%.

We next monitored transcripts from 11 silent ES-located VSGs, as well as VSG118, which is located in a VSG basic copy array (Fig. 5B). Transcripts from all except one of the telomeric ES-located VSGs increased significantly (\(P < 0.05\)) after depletion of TblISWI for up to 72 h. The observed increase in VSG transcripts varied between the different VSGs from 5-fold (for VSG16) to 70-fold (for VSG1.8) (see Table S9 in the supplemental material). Variability in the degree of derepression of different ES-located silent VSGs was also observed after the knockdown of RAP1 and presumably reflects a hierarchy of different VSG activation frequencies (64). Interestingly, no statistically significant increase in VSG14 transcript was observed after TblISWI knockdown. VSG14 is located in a drastically truncated ES (BES8), which contains only ESAG7 (19). We have never found evidence for activation of BES8 in vitro (19), and it is likely that this tiny ES is dysfunctional.

Transcript levels from the VSG118 basic copy also did not increase significantly after TblISWI knockdown, presumably since there are no promoters (which could be derepressed) in...
FIG. 6. TbISWI binds within both Pol I and Pol II transcription units; however, TbISWI knockdown leads to increased transcription from Pol I-transcribed genes only. (A) Distribution of TbISWI in the Pol I-transcribed procyclin transcription unit in BF and PF T. brucei. At the top is a schematic of the EP procyclin locus (PARP B1) (44). The procyclin promoter (gray flag), procyclin genes (black boxes), and regions analyzed by qPCR (lettered brackets) are indicated. The graph shows quantitation of ChIP analysis carried out on TbISWI (wt) and TbISWI-myc expressing BF and PF T. brucei cell lines. Signals are expressed as a percentage of the input immunoprecipitated using anti-myc antibody (α-myc) in the TbISWI (wt)- and TbISWI-myc-expressing cell lines (n = 3 to 5 ± the SD). Significant amounts of TbISWI were found to bind the procyclin coding region (primer c) (P < 0.05; see Table S10 in the supplemental material) in both BF and PF T. brucei and the procyclin promoter (primer a) in BF T. brucei. (B) Effect of depleting TbISWI on expression of eGFP inserted into the procyclin locus in BF T. brucei. A schematic of the cell line containing eGFP downstream of the endogenous procyclin promoter is shown. The procyclin genes EP1 and EP2, as well as a gene encoding a hypothetical protein (Hyp), are indicated (black boxes). The eGFP (green box) containing construct is integrated behind an endogenous procyclin promoter (gray flag), with the single-crossover-mediated insertion resulting in duplication of the promoter. A downstream rDNA promoter (black flag) and blasticidin resistance gene (blue box) are also indicated. This cell line (BF-Pro) was transfected with a TbISWI RNAi construct, and two independent clones were isolated (BF-Pro1 and BF-Pro2). Flow cytometry was used to monitor eGFP expression after the induction of TbISWI RNAi with tetracycline (Tet) for the time indicated in days (d). A representative flow cytometry trace is shown with an outline of the trace from the uninduced cell line (black line) overlaid with the trace showing fluorescence after 4 days of induction of TbISWI RNAi (green-shaded trace). The graph shows quantitation of the fold derepression of eGFP calculated as the ratio of mean fluorescence of induced cells to that of uninduced cells (n = 3 ± the SD). (C) Distribution of TbISWI in the Pol I-transcribed rDNA transcription unit compared to Pol II-transcribed genes in bloodstream-form T. brucei. A schematic of the region around a ribosomal DNA (rDNA) transcription unit is shown (58), with the rDNA promoter (black flag), the rRNA genes (black boxes), and the regions analyzed by qPCR indicated by lettered brackets. The graph shows quantitation of ChIP-ed DNA using qPCR to amplify regions indicated above, as well as the Pol II-transcribed genes encoding either the large subunit of RNA polymerase I (Pol I) or α-tubulin (α-Tub), which are located within Pol II polycistronic transcription units. Signals are expressed as a percentage of input immunoprecipitated as in panel A (n = 3 to 5 ± the SD). Significant amounts of TbISWI were found to bind the rDNA region (primers a to d) and the Pol I and tubulin transcription units in BF T. brucei (P < 0.05; see Table S11 in the supplemental material). (D) Same as in panel B, except the β-tubulin reporter construct contains an ES promoter (ESp) (white flag) upstream of eGFP. The construct is integrated into the nontranscribed spacer between two rDNA transcription units (region of primer pair a in panel C). The parental reporter line is BF-tDESp1 and the two TbISWI RNAi clones are BF-tDESp1 and BF-tDESp2 (n = 3 ± the SD). (E) Same as in panel B, except the eGFP reporter construct is integrated into the tubulin array of bloodstream-form T. brucei and lacks an rDNA promoter upstream of the blasticidin resistance gene (blue box). The schematic shows the eGFP construct integrated between alternating α-tubulin and β-tubulin genes (black boxes). Representative flow cytometry traces are shown for a bloodstream-form TbISWI RNAi clone. The parental reporter line is BF-tubeG, and the RNAi clones are BF-tubeG1 and BF-tubeG2 (n = 3 ± the SD).
this area of the genome. In addition, there does not appear to be an increase in levels of fortuitous initiation of transcription in the VSG basic copy arrays. A comparable increase in telomeric ES located VSG transcripts was also observed in the T. brucei T3-SAI cell line, where the VSGT3 ES is active (data not shown). Although we detected VSG transcripts derived from the silent VSG ESs, we did not detect VSG protein (result not shown), presumably because Western blot analysis is much less sensitive than qPCR. These data indicate that TbISWI could play a role in keeping the entire ES silenced. Since Pol I transcribes VSG ESs, we next sought to determine whether TbISWI is involved in silencing other Pol I-transcribed loci.

**TbISWI binds within Pol I and Pol II transcription units but is only involved in silencing Pol I-transcribed loci.** Pol I not only transcribes VSG genes but also the EP and GPEET procyclin genes encoding the invariant surface proteins of insect-form T. brucei (17, 25, 41, 42), as well as the rDNA. In bloodstream-form T. brucei the procyclin loci are downregulated, whereas in procyclic-form T. brucei they are actively transcribed (56). Since TbISWI was found to bind along both silent and active VSG ESs, we investigated TbISWI occupancy in one of the procyclin transcription units (depicted schematically in Fig. 6A) in both life cycle stages. Using ChIP-qPCR, we found that significant amounts of TbISWI (P < 0.05; see Table S10 in the supplemental material) bind the EP procyclin coding region in both bloodstream- and procyclic-form T. brucei. TbISWI is relatively depleted from the EP promoter (Fig. 6A) in bloodstream-form T. brucei (P < 0.05), as was also observed at the promoters of the many “silent” ESs which contain paused Pol I complexes (Fig. 4B) (2, 43, 55). It has been proposed that “paused” Pol I transcription complexes are also present on “silent” procyclin promoters in bloodstream-form T. brucei (54), possibly resulting in the displacement of TbISWI from this region.

We next investigated whether TbISWI is involved in silencing the procyclin locus in bloodstream-form T. brucei. To address this, we created a reporter cell line with eGFP targeted downstream of an endogenous procyclin promoter (Fig. 6B; see also Fig. S5 in the supplemental material). This cell line was transfected with a TbISWI RNAi construct, and two independent clones were isolated. TbISWI protein knockdown was confirmed by Western blot analysis (see Fig. S2 in the supplemental material). Flow cytometry was used to monitor levels of eGFP after TbISWI knockdown. After TbISWI was depleted for 4 days, there was a 6-fold increase in eGFP expression (Fig. 6B). These results are similar to the levels of derepression observed in the minichromosomes and VSG basic copy array (Fig. 3B and D). A similar experiment was carried out in procyclic-form T. brucei, where depleting TbISWI led to a modest 2.5-fold derepression (see Fig. S6A in the supplemental material). Therefore, TbISWI appears to play a role in modulating transcription of the EP procyclin locus in both life cycle stages of T. brucei.

In mammals, the ISWI containing nucleolar remodeling complex (NoRC) mediates silencing of the inactive rRNA genes (46). We were therefore interested in whether TbISWI binds in and around T. brucei rDNA transcription units (Fig. 6C). ChIP was performed in bloodstream-form T. brucei, and qPCR was used to detect binding to four regions within the rDNA locus. In addition, two Pol II-transcribed genes were analyzed: the genes encoding the large subunit of Pol I and α-tubulin. Significant levels of TbISWI were found binding all of these regions (P < 0.05; see Table S11 in the supplemental material). TbISWI was particularly enriched in the transcriptionally inactive rDNA spacer (primer pair a in Fig. 6C) compared to the 18S rRNA coding region (primer pair d, Fig. 6C) (P < 0.05). Similar results were found in procyclic-form T. brucei (result not shown). TbISWI distribution in the rDNA closely resembled that of histone H3 (51), a finding consistent with ISWI-containing complexes playing a role in nucleosome remodeling.

To determine whether TbISWI plays a role in silencing the rDNA locus, we created a bloodstream-form reporter line containing a construct with eGFP downstream of an ES promoter located in an rDNA intergenic region between two rDNA transcription units (Fig. 6D and see Fig. S7 in the supplemental material). After 2 days of induction of TbISWI RNAi, there was a 4-fold increase in the levels of eGFP (Fig. 6D). Comparable derepression was observed in procyclic-form T. brucei (see Fig. S6B in the supplemental material). These results indicate that TbISWI has a role in silencing all Pol I transcription units analyzed: the VSG ESs, procyclin, and the rDNA spacer region in both life cycle stages.

We saw that in most cases in these different bloodstream-form T. brucei reporter lines, the T. brucei TbISWI protein is greatly depleted 24 h after the induction of TbISWI RNAi (see Fig. S2 in the supplemental material). An observable growth arrest is typically seen about 48 h after the induction of TbISWI RNAi (see Fig. S10 in the supplemental material). The timing of this growth inhibition coincides with the time point of maximal derepression of eGFP in most of these reporter cell lines, with the exception of the minichromosome reporter line, where maximal derepression of eGFP was seen a bit later (after 3 days). This delay between the reduction of TbISWI protein to very low levels, and the appearance of a growth or derepression phenotype could indicate that even small amounts of TbISWI protein are sufficient to maintain normal levels of silencing (21).

Most of the T. brucei genome is constitutively transcribed by Pol II as extensive polycistronic units. Since TbISWI was found to bind to two Pol II transcribed genes (Fig. 6C), we investigated whether TbISWI could play a role in regulating Pol II transcription. We inserted an eGFP reporter construct into the tubulin array, depleted the cells of TbISWI, and monitored the levels of eGFP (Fig. 6E and see Fig. S8 in the supplemental material). We found no significant change in the levels of eGFP after blocking TbISWI synthesis in both life cycle stages of T. brucei (Fig. 6E and see Fig. S6C in the supplemental material).

**TbISWI is enriched in Pol II strand switch regions.** Recent data have identified enrichment of various modified histones and histone variants in regions between polycistronic Pol II transcription units in T. brucei (50, 63). Since ISWI complexes are involved in Pol II transcription initiation and termination in other organisms (30), this led us to hypothesize that TbISWI may play a role in establishing the epigenetic marks found at strand switch regions (SSR). We analyzed three “divergent” and three “convergent” SSRs on T. brucei chromosome 10 using ChIP-qPCR analysis (Fig. 7A). Regions chosen included those within the SSRs (primers c to e), in the genes directly
flanking the SSRs (primers b and f), as well as in genes at the approximate midpoint of the flanking polycistronic units (primers a and g). Using anti-histone H3 antibody, histone H3 was found to be relatively uniformly distributed over divergent and convergent regions (see Fig. S9 in the supplemental material).

The SSRs between two diverging Pol II transcription units are likely to contain Pol II promoters. Significant levels of TbISWI ($P < 0.05$) were found binding three “divergent” SSRs (D1 to D3) and flanking transcription units (Fig. 7B; see Table S12 in the supplemental material). TbISWI was found to be significantly enriched in the “divergent” SSRs (primers c to e) compared to genes within the neighboring polycistronic transcription units (primers a and g) in divergent regions D1 and D2, although this was less clear for divergent region D3.

The SSRs between two converging Pol II transcription units are likely to contain Pol II terminators. Lower but significant amounts of TbISWI ($P < 0.05$; see Table S13 in the supplemental material) were found binding three “convergent” SSRs (C1 to C3) and flanking transcription units (Fig. 7C). There was evidence for enrichment of TbISWI on the SSRs of “convergent” regions C2 and C3, although this was not found for region C1. TbISWI therefore appears to be enriched at many (particularly divergent) SSRs, specifically colocalizing with nucleosomes enriched in modified histones and histone variants known to have roles in regulating transcription by RNA Pol II in other organisms (3, 47, 50). TbISWI therefore appears to be associated with both Pol I and Pol II transcription units in T. brucei.

DISCUSSION

Members of the ISWI family of chromatin remodelers are involved in a wide variety of processes including transcription
activation, repression, and termination, as well as the maintenance of higher-order chromatin structure and DNA replication (12). The specialized function of individual ISWI complexes depends on the protein partners associated with ISWI. Here, we found that TbISWI is involved in downregulating regions containing silent VSGs, as well as other Pol I transcription units. TbISWI was found to be particularly enriched in many Pol II strand switch regions, which are the proposed sites of Pol II transcription initiation and termination and are known to contain histone variants and specific histone modifications (50, 63).

**TbISWI is involved in preventing transcription of the silent VSG repertoire.** For antigenic variation to work, it is critical for bloodstream-form *T. brucei* to tightly control expression of its extensive VSG repertoire. VSGs are located at most, if not all, telomeres, as well as in subtelomeric VSG basic copy arrays (4, 31, 61). We found that TbISWI binds minichromosomes, the VSG118 basic copy array, as well as VSG ESs. Using eGFP reporter constructs inserted into the 177-bp repeats of minichromosomes and the VSG118 basic copy array, we found that TbISWI depletion does not lead to a significant increase in transcription from these nontranscribed genomic regions. It is possible that even if TbISWI depletion results in a more “open” chromatin configuration, the absence of promoters in these regions prevents a more significant increase in transcription.

In order to study the role of TbISWI in maintaining a repressed chromatin state at these regions, constructs containing a small and genome context-sensitive ES promoter were integrated into these loci. Depletion of TbISWI resulted in 4- to 6-fold derepression of the exogenous ES promoter in both BF and PF *T. brucei*. Although it can be argued that this effect of TbISWI depletion is related to the ES promoter itself, the observation that levels of derepression from an ES promoter vary from ~6-fold in BF-MCESp1 or BF-VBESp1 to ~60-fold in a silent ES (21) suggests that TbISWI-mediated repression of the ES promoter is dependent on genomic context. ES promoter derepression is not simply a consequence of a lethal phenotype, since RNAi-mediated knockdown of another unrelated SNF2 domain containing protein also led to a significant growth arrest but no observable ES derepression (see Fig. S10 in the supplemental material). It is likely that TbISWI is involved in preventing inappropriate transcription of the various pools of silent VSG genes, presumably by maintaining a repressive chromatin structure which plays a role in inhibiting fortuitous initiation of transcription. Our results are in agreement with studies from other eukaryotes, where ISWI chromatin remodeling complexes have been shown to be involved in transcription repression (12, 16, 30, 45).

Previously, we reported that TbISWI is important for silencing the promoters of the silent VSG ESs, although it was unclear whether TbISWI depletion leads to processive transcription down to the telomeric VSG (21). Using new sequence data for *T. brucei* 427 VSG ESs (19), we reexamined whether TbISWI plays a role in silencing telomeric ES located VSGs. After blocking TbISWI synthesis, we observed significant and reproducible increases in telomeric VSG transcripts. This implies that, contrary to the previous model, TbISWI has a role in keeping the entire silent ES inactive. The level of derepressed transcription from the silent ESs observed after TbISWI knockdown was much lower than levels of transcription from an active ES (discussed in reference 21). The active ES is located in an expression site body (ESB) (34), which is a subnuclear structure thought to contain a high concentration of Pol I, as well as transcription and RNA processing factors essential for high levels of expression. Since the ESB is thought to be only able to accommodate a single ES, this restriction could limit the amount of ES derepression observed after TbISWI depletion.

**TbISWI is involved in downregulating Pol I transcription.** Pol I transcribes the multicopy rDNA transcription units as well as the VSG and procyclin genes. Protein coding genes transcribed by Pol I are tightly regulated, unlike the constitutively transcribed Pol II transcription units (11). Silent ESs in both bloodstream- and procyclic-form *T. brucei* have “poised RNA polymerases” at their promoters and low levels of nonprocessive transcription (2, 43, 55). A similar situation is also seen in the procyclin loci that are transcribed at a low level in bloodstream-form *T. brucei* (54). Using ChIP-qPCR, we show that TbISWI is present on silent VSG ESs and on the silent EP1 procyclin locus in bloodstream-form *T. brucei* but is relatively depleted from the ES (both silent and active) promoters, as well as procyclin promoters. One possibility is that poised RNA polymerase complexes on these different promoter regions displace TbISWI from binding. Alternatively, since TbISWI is a nucleosome remodeler, this relative absence could be a consequence of the relative depletion of nucleosomes observed on ES and procyclin promoters (51).

Both of these Pol I-transcribed loci are derepressed when TbISWI synthesis is blocked. Although silent ESs are derepressed 30- to 60-fold in bloodstream-form *T. brucei* and 10- to 17-fold in procyclic-form *T. brucei* (21), a silent procyclin locus in bloodstream-form cells is derepressed ~6-fold. Surprisingly, expression from even the active procyclin locus is upregulated by 2.5-fold in procyclic *T. brucei* after TbISWI depletion. This suggests that EP1 procyclin is not transcribed at its maximal capacity in procyclic *T. brucei* due to a TbISWI-mediated repressive mechanism. These observations all suggest that TbISWI is involved in the regulation of transcription elongation in Pol I transcription units. This function of TbISWI is different from that seen in the regions containing silent VSG in either the VSG basic copy arrays or minichromosomes.

Here, TbISWI presumably modulates chromatin structure, possibly preventing fortuitous initiation of transcription from cryptic promoters.

Our ChIP-qPCR data also show that TbISWI binds the active ES in bloodstream-form *T. brucei*, as well as the active EP1 procyclin locus in procyclic-form *T. brucei*. However, the fact that TbISWI is found to bind both active and silent transcription units does not necessarily argue that it is doing the same thing in these two very different places, since ISWI complexes can have different functions depending on exactly which proteins ISWI is partnered up with. In *Saccharomyces cerevisiae* an ISWI complex is present on the active rDNA transcription units which facilitates transcription (24). In fact, the ISWI protein Isw1p is a member of two different ISWI chromatin remodeling complexes: the Isw1a complex (consisting of Isw1p partnered with Ioc5p) downregulates transcription initiation (32), while the Isw1b complex (consisting of Isw1p in association with the Ioc4p and Ioc2p proteins) can facilitate both
transcription elongation or termination (33). In addition, in other organisms, different ISWI complexes have been shown to perform distinct functions in Pol I transcriptional control. The ISWI containing B-WICH complex is involved in activating Pol I transcription by promoting elongation (37, 38), whereas the ISWI containing NoRC complex is involved in repression of Pol I transcription and keeping the silent rRNA genes inactive (52). It is therefore possible that one TbISWI complex downregulates transcription elongation from Pol I transcription units, while another maintains a chromatin structure at the active loci which facilitates transcription.

TbISWI is implicated in chromatin remodeling around Pol II transcription units. Recently, in *T. brucei* and the related *T. cruzi*, acetylated and methylated histones were shown to be enriched in regions where polycistronic transcription units diverge and which are proposed to be the sites of transcription initiation (40, 50, 63). The histone variants H2AZ and H2BV are also enriched in these regions. Convergent Pol II SSRs in *T. brucei* containing putative transcription termination sites are marked with histone variants H3v and H4v (50).

It has been suggested that chromatin remodeling is required to allow for the recognition of the histone N-terminal tails by histone-modifying enzymes (26). Evidence is mounting that ISWI-containing complexes are necessary for histone methyltransferases and acetyltransferases to gain access to their histone-modifying enzymes (26). ISWI remodeling complexes have also been found to be involved in histone variant H2A.Z replacement (18, 57) and have greater activity on H2A.Z-containing nucleosomes (15). Siegel et al. have presented a model for the sequence of events leading to Pol II transcription initiation in *T. brucei* which requires a chromatin remodeling complex to replace canonical histones with the variants H2AZ and H2BV (50). We found that TbISWI is enriched at divergent SSR, which are the proposed sites of Pol II transcription initiation. Possibly, TbISWI has a role in allowing histone chaperones and histone modification enzymes to gain access to their target substrates in the *T. brucei* SSRs.

In summary, our results indicate that TbISWI has at least four different roles in *T. brucei* transcriptional regulation. First, TbISWI appears to be involved in the maintenance of a repressive chromatin structure in various silent regions of the *T. brucei* genome. Second, TbISWI regulates transcription elongation at the Pol I transcription units encoding the silent VSG and procyclin coat protein genes in both bloodstream- and procyclin-form *T. brucei*. Third, TbISWI binds to multiple regions containing putative Pol II transcription initiation sites, possibly influencing chromatin remodeling or epigenetic marks in these regions. Fourth, based on the observed ISWI enrichment at different pol II convergent SSRs, TbISWI may also play a role in termination of Pol II transcription, as has been found for other chromatin remodeling complexes (1, 33). It will now be crucial to determine the partners of TbISWI. Elucidating the composition and role of the various TbISWI-containing chromatin remodeling complexes will allow us to dissect the different functions of TbISWI and unravel the mechanisms that *T. brucei* uses to inherit specific chromatin states from one generation to the next.

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