EUKARYOTIC CELL, July 2011, p. 964–976 Vol. 10, No. 7
1535-9778/11/$12.00 doi:10.1128/EC.05048-11

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

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Received 25 March 2011/Accepted 11 April 2011

The unicellular eukaryote *Trypanosoma brucei* is unusual in having very little transcriptional control. The bulk 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* evades 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 been shown that the active ES in bloodstream-form *T. brucei* is depleted of nucleosomes (13, 51), indicating that chromatin remodeling plays a key role in ES activation. This is supported by the discovery that a number of proteins 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 (TSS), are enriched in the histone variants H2AZ and H2BV, as well as the histones H3K4me3 and H4K10ac. Convergent SSRs containing putative transcription termination

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‡ Supplemental material for this article may be found at http://ec.asm.org/.

▽ Published ahead of print on 13 May 2011.

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 inserted into various genomic loci, we demonstrate that TbISWI is involved in repressing an ES promoter in regions containing silent *VSG*s, 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-SA1 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 pHNES221Pur1.6 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 MC^{177} 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-VBESp, and BF-Pro are described in reference 33a as MCEP, VBEP, and EP1eGFP, respectively. *TbISWI* RNAi was induced by incubating the cells with 750 ng of tetracycline ml^{-1}. All procyclic-form reporter lines were made in a similar fashion, except that the parental cell line was *T. brucei* 29-13 (62). Reporter constructs containing the enhanced green fluorescent protein (eGFP) gene were first transfected into 29-13 cell lines, followed by the *TbISWI* RNAi construct (21).

TbISWI was epitope tagged at the C terminus with a triple myc epitope using the pMoTAG43M vector (35). Epitope tagging was performed in bloodstream-form *T. brucei* 221GPI(VO2+) cells (49) and in wild-type procyclic *T. brucei*. In order to ensure functionality of the tagged TbISWI, cell lines were also made where the second allele of TbISWI was knocked out using the pSpot5KOPhleo vector.

Constructs. The sequences of all primers used for cloning are shown in Table S1 in the supplemental material. The fragments used to target the reporter constructs to different loci included (i) a minichromosomal 177-bp repeat sequence from $p2T7^{Ti}$-177 (59), (ii) a *VSG118* basic copy array fragment amplified by PCR from *T. brucei* genomic DNA (gDNA) using the primers BCVSG118-863s and BCVSG118-2025as, (iii) an rDNA spacer targeting fragment isolated from $p2T7^{Ti}$A/GFP (27), and (iv) a procyclin targeting fragment amplified by PCR from *T. brucei* gDNA using the primers EP1_UP_2480s and EP1_UP_3991as. Constructs targeting the first two loci were made with (+ESp) or without (−ESp) an ES core promoter isolated from rDES1 (43). The reporter gene cassette consists of an *eGFP* ORF, with a tubulin splice signal at its 5′ end and a tubulin polyadenylation signal at its 3′ end. The drug resistance cassette consists of a drug resistance gene flanked by a 5′-tubulin splice signal and a 3′-actin polyadenylation signal and is driven by an rDNA promoter (48). The reporter constructs all contained the blasticidin resistance (BlastR) gene as the drug marker; however, the rDNA spacer targeting constructs were also made with the phleomycin resistance (PhleoR) gene. The tubulin array targeting reporter construct was derived from pGad8-tubulin (60) by replacing the hygromycin resistance gene (HygroR) by BlastR. All sequences are available upon request. Verification of the reporter construct integration into the different genomic loci is described in the supplemental material. Integration of reporter constructs into minichromosomes was verified by pulsed-field gel analysis and Southern blot mapping (see Fig. S1C in the supplemental material). Reporter construct integration into the *VSG118* basic copy array, the procyclin locus, and the tubulin array was verified by PCR (see Fig. S3C, S5B, and S8B in the supplemental material) (primers are listed in Table S2 in the supplemental material). Integration into the rDNA spacer was confirmed by Southern blot analysis (see Fig. S7B and Table S3 in the supplemental material).

For chromatin immunoprecipitation (ChIP) studies, one of the endogenous TbISWI alleles was tagged at the C-terminal end with a triple myc epitope as follows. The C-terminal end of *TbISWI*, excluding the stop codon, was amplified from *T. brucei* 427 genomic DNA using Spot5CTtag_s and Spot5tag4934_as to yield a 701-bp fragment (positions 2780 to 3480 of the TbISWI open reading frame). For the downstream region, an 827-bp fragment was produced using the primers Spot5DRtag_s and Spot5DRtag_as. Both of these fragments were cloned into the pMoTAG43M vector, containing HygR for selection (35).

The untagged allele of TbISWI was knocked-out through replacement with PhleoR to ensure functionality of myc-tagged TbISWI. A 499-bp fragment immediately upstream of the *TbISWI* open reading frame was amplified from *T. brucei* 427 genomic DNA by using Spot5-957s-UPF and Spot5-1455as-US. For the downstream region, a 586-bp fragment was amplified by using the primers Spot5-4935s-DSF and Spot5-5520as-DS. These fragments were cloned on either side of PhleoR in the pBlueScript vector to produce pSpot5KOPhleo. The MC^{177} TbISWI-A RNAi construct used for all reporter lines is described in reference 21, and the MC^{177} Tb11.01.2500 RNAi construct is described in the supplemental material.

Western blotting. Protein lysates were made from bloodstream- and procyclic-form *T. brucei* essentially as described previously (21), and total lysate from 8×10^6 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 $NaHCO_3$. 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 S4 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 no-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 an 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 conditions for each primer pair were individually optimized to obtain a single 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 arrays located 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.

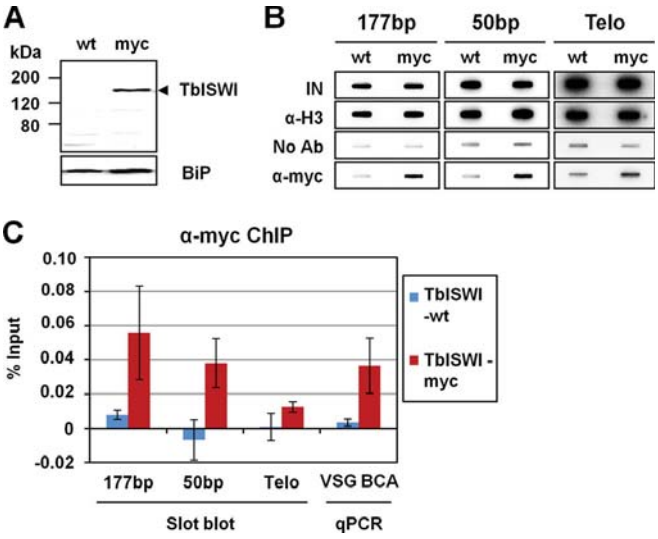

FIG. 1. TbISWI binds transcriptionally silent DNA repeats as well as the *VSG* basic copy arrays. (A) Bloodstream-form (BF) *T. brucei* strains expressing wild-type (wt) or myc-tagged TbISWI (myc) were analyzed with Western blots using an anti-myc antibody (top panel) or an anti-BiP antibody (bottom panel) as a loading control. The position of TbISWI-myc is indicated with an arrowhead on the right, and size markers in kilodaltons are shown on the left. (B) ChIP was performed on bloodstream-form (BF) *T. brucei* expressing wild-type TbISWI (wt) or myc-tagged TbISWI (myc). The chromatin in the input material (IN) was immunoprecipitated with an anti-histone H3 antibody (α-H3) as a positive control, no antibody (No Ab) as a negative control, and an anti-myc antibody (α-myc) to immunoprecipitate TbISWI-myc. The immunoprecipitated DNA was analyzed by slot blots hybridized with radiolabeled probes specific for the 177-bp, 50-bp, and telomere repeats (Telo). A representative slot blot is shown for each probe. Although 100% of the DNA immunoprecipitated using an anti-myc antibody or no antibody was analyzed on the slot blots, only 0.1% of the input DNA and 1% of the anti-H3 immunoprecipitated DNA was analyzed to avoid saturation and enable accurate quantitation. (C) TbISWI distribution in BF *T. brucei* was analyzed by quantitation of slot blots or by qPCR for *VSG118* (located in the VSG basic copy array). Signals are expressed as the percentage of input immunoprecipitated by anti-myc antibody using cell lines expressing TbISWI (wt) or TbISWI-myc. The data shown are the averages of three to five independent ChIP experiments, with the SD indicated using error bars. Significant amounts of TbISWI were found binding these *T. brucei* genomic regions ($P < 0.05$; see Table S6 in the supplemental material).

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

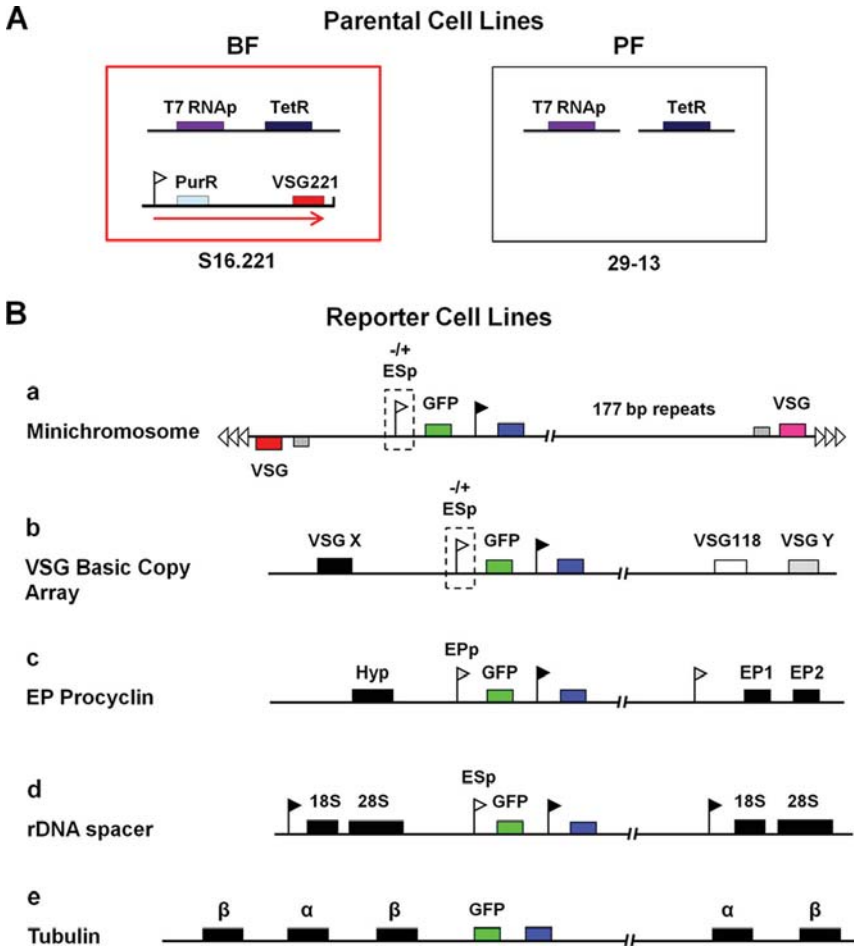

FIG. 2. Schematic of the *T. brucei* reporter cell lines constructed for the present study. (A) Parental cell lines for the *T. brucei* reporter lines used. The bloodstream-form (BF) *T. brucei* S16.221 cell line has a puromycin resistance gene (PurR) inserted in the active *VSG221* expression site (transcription 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 *VSG*s 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 *VSG*s 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.

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). Both of these reporter lines were transfected with a TbISWI RNAi construct (21), and two independent RNAi clones per line were analyzed (BF-MCe1, BF-MCe2, BF-MCESp1, and BF-MCESp2). Western blot analysis confirmed TbISWI protein

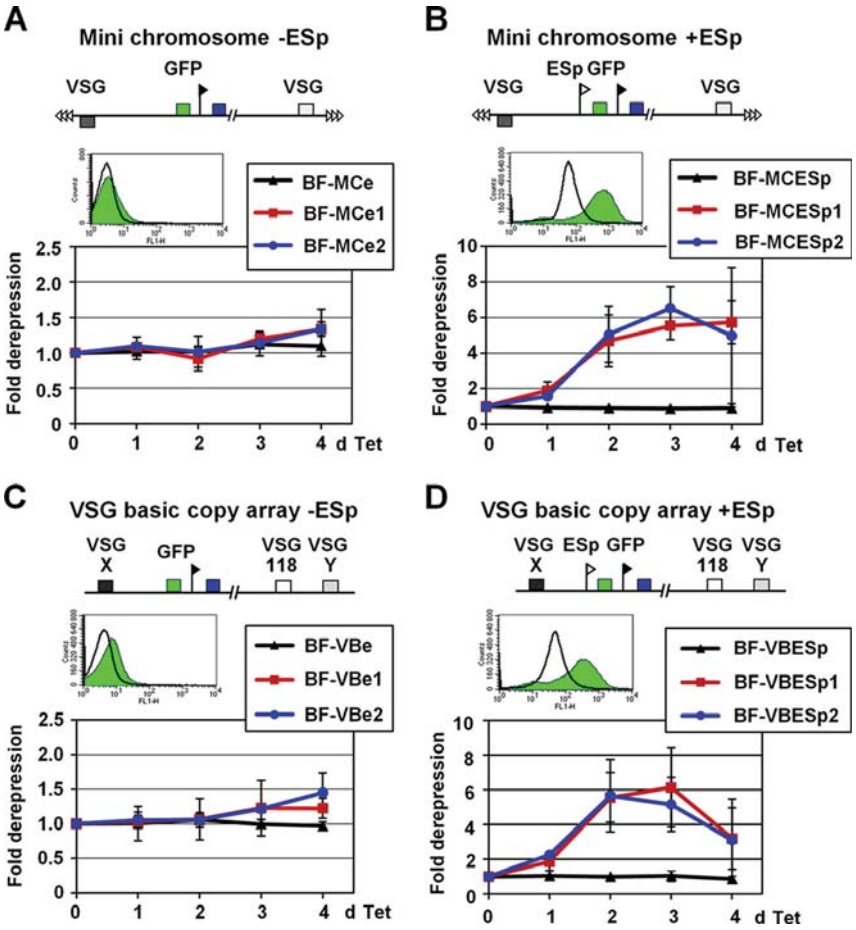

FIG. 3. TbISWI downregulates an exogenous expression site promoter (ESp) located within the silent minichromosomes or *VSG* basic copy array by 6-fold in bloodstream-form *T. brucei*. (A) The schematic shows an *eGFP* reporter construct integrated into the 177-bp repeat arrays in the minichromosomes of BF *T. brucei*. Downstream of the *eGFP* gene is an rDNA promoter (black flag) and a blasticidin resistance gene (blue box). *VSG* genes are indicated with boxes, and telomere repeats are indicated with triangles. A *TbISWI* RNAi construct was transfected into this cell line and two independent clones were isolated. The cell lines analyzed are the parental reporter cell line (BF-MCe) and two independent *TbISWI* RNAi clones (BF-MCe1 and BF-MCe2). Flow cytometry was used to monitor eGFP expression after inducing *TbISWI* RNAi with tetracycline (Tet) for the time indicated in days (d). A representative flow cytometry trace is shown where the uninduced sample (indicated by a thick black line) is overlaid with a shaded trace obtained after 4 days induction of *TbISWI* RNAi. The graph shows quantitation of the fold derepression of *eGFP* after induction of *TbISWI* RNAi. Fold derepression was calculated as the ratio of mean fluorescence of induced cells to that of the uninduced cells ($n = 3 \pm$ the SD). (B) Same as in in panel A, except the *eGFP* reporter construct integrated in the minichromosomal 177-bp repeats contains an ES promoter (ESp) upstream of *eGFP*. The parental reporter cell line (BF-MCESp), as well as two independent RNAi clones (BF-MCESp1 and BF-MCESp2), was analyzed after the induction of *TbISWI* RNAi. (C) Same as in panel A, except that the *eGFP* reporter construct is integrated into the *VSG* basic copy array. The average fold derepression of *eGFP* was quantitated for the parental reporter cell line (BF-VBe), as well as two independent RNAi clones (BF-VBe1 and BF-VBe2), after the induction of *TbISWI* RNAi. (D) Same as in panel A, except that the reporter construct contains an ES promoter upstream of *eGFP* and is integrated into the *VSG* basic copy array. Quantitation of the average fold derepression of the *eGFP* gene after tetracycline induction of *TbISWI* RNAi is plotted for the parental reporter cell line (BF-VBESp), as well as two independent *TbISWI* RNAi clones (BF-VBESp1 and BF-VBESp2).

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 ESp (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 BFMCESp2 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 ESp 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 ESp (BF-VBe1 and BF-

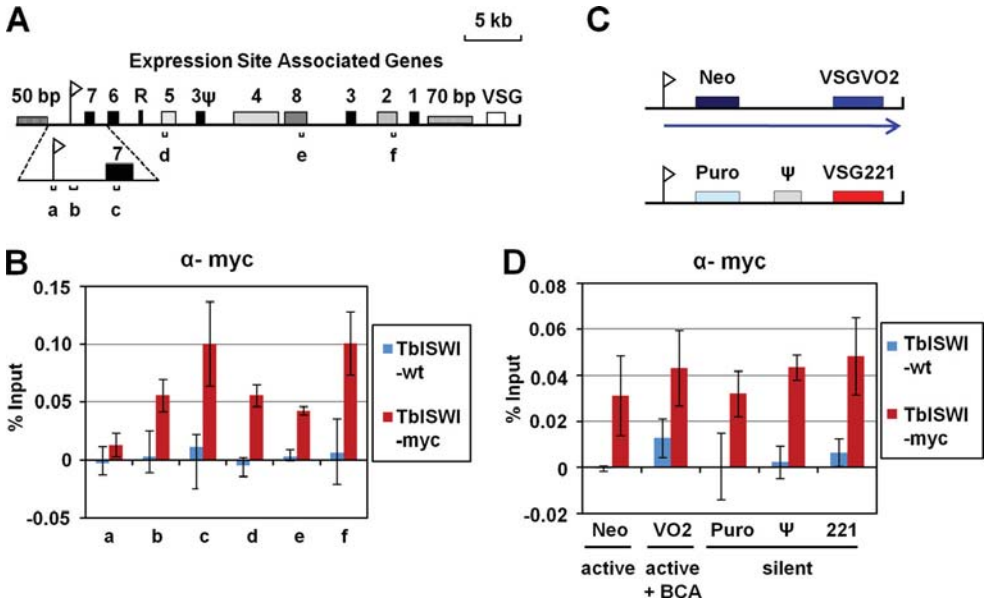

FIG. 4. TbISWI is distributed along active and silent *VSG* ESs but is depleted from ES promoters. (A) Schematic of a typical *VSG* ES (modified from AnTat1.3A) (5, 29), with the ES promoter (flag), the expression site-associated genes (ESAGs) (numbered boxes), and characteristic ES repeat arrays (striped 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).

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 blasticidin 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

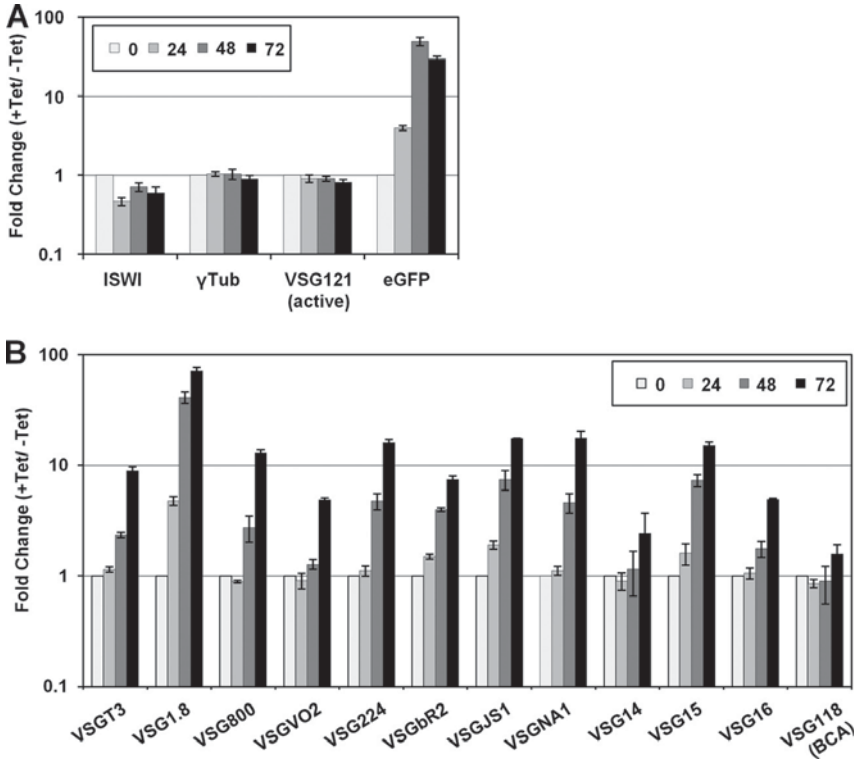

FIG. 5. Depleting TbISWI leads to increased transcription of silent telomeric ES-located *VSG*s. RNA was isolated from the bloodstream-form *T. brucei* 121-SA1 cell line (21) 0, 24, 48, and 72 h after the induction of *TbISWI* RNAi. cDNA was synthesized, and qPCR was used to quantitate the levels of transcript. (A) The change in transcript levels after the induction of *TbISWI* RNAi relative to the 0-h time point are plotted for *TbISWI*, γ-tubulin (γTub), the active VSG (*VSG121*), and *eGFP* located downstream of a silent ES promoter ($n = 3 \pm$ the SD). (B) Changes in transcript levels from silent *VSG*s after the induction of *TbISWI* RNAi. *VSG* transcripts from 11 telomeric ES-located *VSG*s, as well as a *VSG* located in the *VSG* basic copy array (*VSG118*) were analyzed by qPCR ($n = 3 \pm$ the SD).

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 (ψ). 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 *VSG* 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 *VSG*s 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 TbISWI 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 *VSG*s located in ESs, in addition to upregulation of the ES promoter-proximal regions. The *T. brucei* 121-SA1 *TbISWI* RNAi cell line has an active *VSG121* ES and contains *eGFP* downstream of the promoter of the silent *VSG221* ES (21). Induction of *TbISWI* RNAi led to a decrease in TbISWI transcript and a concurrent 60-fold increase of *eGFP* transcript (Fig. 5A). These results agree with previously published data (21). Transcript levels for γ-tubulin remain relatively unchanged after TbISWI knockdown, which was also observed using a reporter construct integrated into the α-β tubulin array (Fig. 6E). Transcripts from the active *VSG121* decrease by about 20%.

We next monitored transcripts from 11 silent ES-located *VSG*s, 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 *VSG*s increased significantly ($P < 0.05$) after depletion of TbISWI for up to 72 h. The observed increase in *VSG* transcripts varied between the different *VSG*s 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 *VSG*s 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 TbISWI 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 TbISWI knockdown, presumably since there are no promoters (which could be derepressed) in

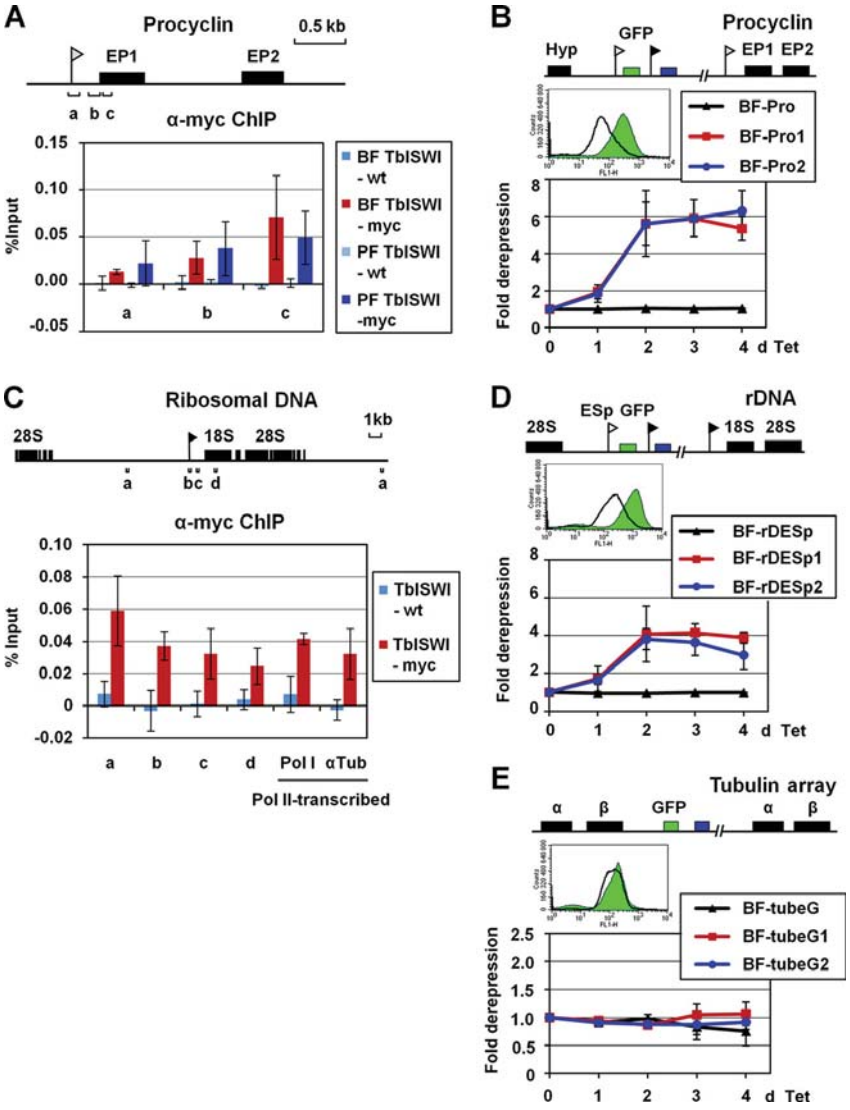

FIG. 6. TbISWI binds within both Pol I and Pol II transcription units; however, TbISWI knockdown leads to increased transcription from Pol I-transcribed genes only. (A) Distribution of TbISWI in the Pol I-transcribed procyclin transcription unit in BF and PF *T. brucei*. At the top is a schematic of 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 BF 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-rDESp1 and the two *TbISWI* RNAi clones are BF-rDESp1 and BF-rDESp2 ($n = 3$ ± 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-SA1 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

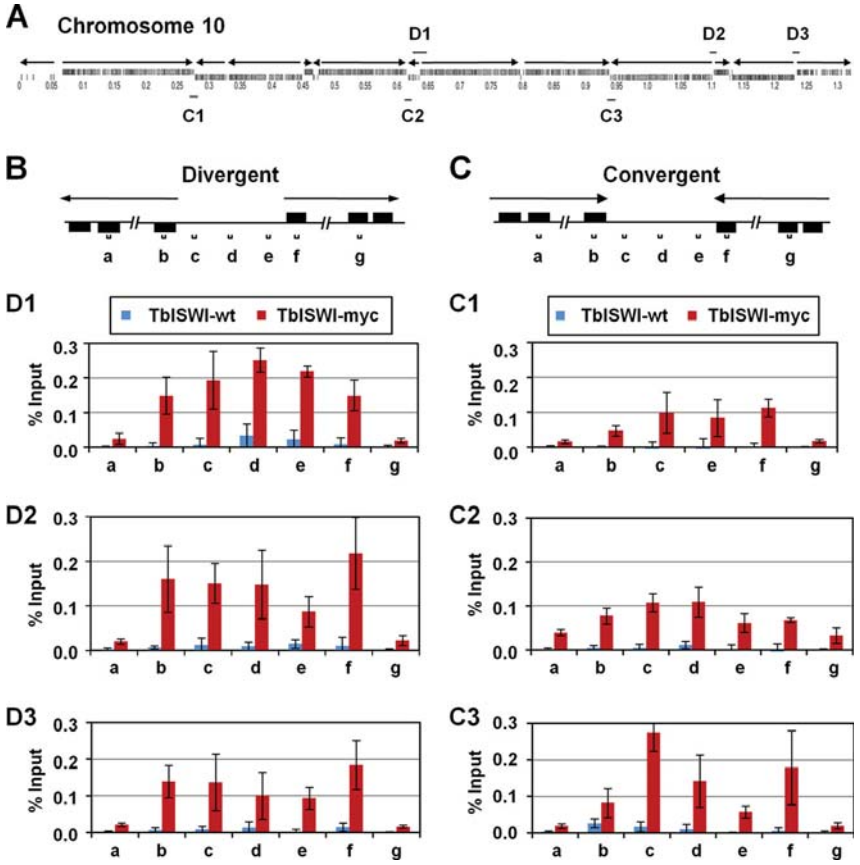

FIG. 7. TbISWI is enriched in the strand switch regions (SSR) between RNA polymerase II-transcribed polycistronic transcription units. (A) Schematic representation of a region of chromosome 10 according to Siegel et al. (50), where gray boxes represent open reading frames and arrows indicate the direction of transcription. The positions of three different divergent SSRs (D1 to D3) (containing putative transcription initiation sites) and three different convergent SSRs (C1 to C3) (containing putative transcription termination sites) used for ChIP experiments are indicated. (B) The schematic shows a divergent SSR (top panel), with the positions of the primer pairs used for qPCR analysis indicated with lettered brackets. Primer pairs a and g are located at the approximate midpoint of the flanking polycistronic transcription units. The graphs below show distribution of TbISWI over three divergent regions (D1 to D3), as indicated in panel A. ChIP analysis was carried out on TbISWI (wt) and TbISWI-myc expressing BF *T. brucei* cell lines. qPCR was used to amplify the indicated regions. Signals are expressed as percentage of input immunoprecipitated by anti-myc antibody in the TbISWI (wt) and TbISWI-myc expressing cell lines ($n = 3 \pm$ the SD). Significant amounts of TbISWI were found binding most regions of the D1-D3 divergent strand switch regions ($P < 0.05$; see Table S12 in the supplemental material). (C) Above is a schematic of a convergent SSR, with the positions of the primer pairs used for qPCR analysis indicated with lettered brackets. The graphs show the distribution of TbISWI over three convergent regions (C1 to C3) as indicated in panel A. ChIP was performed, and qPCR was used to amplify regions shown schematically with signals expressed as a percentage of the input ($n = 3 \pm$ the SD). Significant amounts of TbISWI were found to bind most regions of the C1 to C3 convergent strand switch regions ($P < 0.05$; see Table 13 in the supplemental material).

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 *VSG*s, 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. *VSG*s 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 reinvestigated whether TbISWI plays a role in silencing telomeric ES located *VSG*s. 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-form *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*s 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 Ioc3p) 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 targets (9, 26, 28). Histone variant H2A.Z-containing nucleosomes are less stable and help maintain an accessible chromatin structure at transcription start sites (23). 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 procyclic-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.

ACKNOWLEDGMENTS

We thank Jane Mellor and David Clynes (Department of Biochemistry, University of Oxford) for advice about ChIP and ISWIs and allowing us to use the BioRuptor sonicator. We are grateful to Thomas Seebeck (University of Bern) for providing epitope tagging constructs and to Marcus Gould for some constructs. We thank Jay Bangs (University of Wisconsin) for providing anti-BiP antibody and Klaus Ersfeld for providing anti-SMC3 antibody. We thank Mani Narayanan, Megan Lindsay Povelones, Simone Wiesler, Marcin Dembek, and other members of the Rudenko lab for discussions and comments on the manuscript.

This study was supported by the Wellcome Trust. G.R. is a Wellcome Senior Fellow in the Basic Biomedical Sciences. M.K. is funded by the Inlaks Foundation and the Clarendon fund.

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