Nuclear repositioning of the *VSG* promoter during developmental silencing in *Trypanosoma brucei*

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Interphase nuclear repositioning of chromosomes has been implicated in the epigenetic regulation of RNA polymerase (pol) II transcription. However, little is known about the nuclear position–dependent regulation of RNA pol I–transcribed loci. *Trypanosoma brucei* is an excellent model system to address this question because its two main surface protein genes, *procyclin* and *variant surface glycoprotein* (*VSG*), are transcribed by pol I and undergo distinct transcriptional activation or downregulation events during developmental differentiation. Although the monoallelically expressed *VSG* locus is exclusively localized to an extranucleolar body in the bloodstream form, in this study, we report that nonmutually exclusive *procyclin* genes are located at the nucleolar periphery. Interestingly, ribosomal DNA loci and pol I transcription activity are restricted to similar perinucleolar positions. Upon developmental transcriptional downregulation, however, the active *VSG* promoter selectively undergoes a rapid and dramatic repositioning to the nuclear envelope. Subsequently, the *VSG* promoter region was subjected to chromatin condensation. We propose a model whereby the *VSG* expression site pol I promoter is selectively targeted by temporal nuclear repositioning during developmental silencing.

Introduction

Trypanosoma brucei is an extracellular protozoan parasite responsible for a reemerging tropical disease known as sleeping sickness in humans. There are two main proliferative forms of the parasite: the bloodstream form in the mammalian host and the midgut insect stage or procyclic form in the tsetse vector. Changes in the variant surface glycoprotein (VSG) type on the surface allow the bloodstream form of the parasite to elude the host immune antibody response, ensuring a persistent infection (Cross et al., 1998; Barry and McCulloch, 2001; Pays et al., 2004). The monoallelically expressed *VSG* gene is always located at the end of a telomeric *VSG* expression site (ES). Previous estimations suggest the presence of 20 different telomeric *VSG* ESs that share highly homologous promoter sequences. The *VSG* ES promoter, which is located 40–60 kb upstream of the telomere, drives the polycistronic transcription of developmentally regulated genes named ES-associated genes (for review see Pays et al., 2004). In the bloodstream form, only one *VSG* ES is fully transcribed at a given time so that each cell displays a single VSG type on the surface. Transcriptional switching among ESs results in antigenic variation. In the procyclic

form, VSG is not expressed, but an invariant family of glycoproteins called procyclins are constitutively expressed and replace VSG on the parasite surface (Roditi et al., 1989). Previous data suggest two distinct mechanisms for ES regulation: a developmental silencing of the ES in the procyclic form and a coupled mechanism for ES activation/inactivation in the bloodstream form (Navarro et al., 1999).

In eukaryotic cells, RNA polymerase I (pol I) transcribes ribosomal loci (ribosomal DNA [rDNA]) and is highly compartmentalized in the nucleolus (for review see Scheer and Hock, 1999). Interestingly, in *T. brucei*, pol I also transcribes *procyclin* and *VSG*. Previously, we have proposed a model whereby the recruitment of a single *VSG* ES to a discrete pol I–containing extranucleolar body (ES body [ESB]) defines the mechanism responsible for *VSG* monoallelic expression (Navarro and Gull, 2001; for review see Borst, 2002).

In this study, we investigate the nuclear localization of pol I–transcribed chromosomal sites in the context of pol I machinery and transcription activity. Our results show that the nonmutually exclusive *procyclin* gene family is transcribed at the nucleolus periphery in contrast to the monoallelically expressed *VSG* ES, which is associated with the extranucleolar ESB. Furthermore, we address the possible repositioning of bloodstream pol I–transcribed loci during differentiation to the insect procyclic form. We found that upon developmental silencing,

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Abbreviations used in this paper: BC, basic copy; ES, expression site; ESB, ES body; IF, immunofluorescence; pol, polymerase; rDNA, ribosomal DNA; SM, single marker; VSG, variant surface glycoprotein.

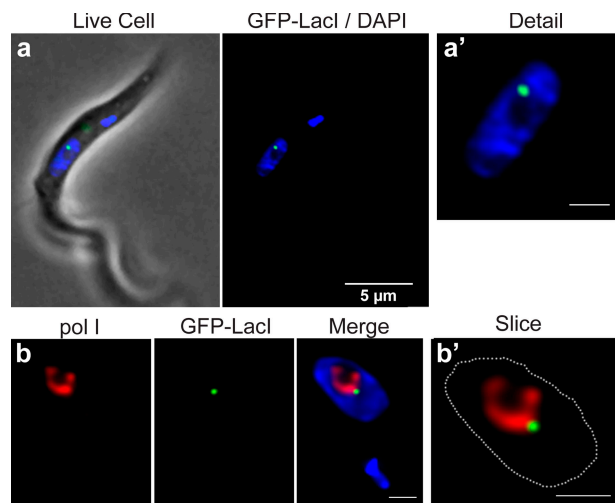The online version of this article contains supplemental material.

Supplemental Material can be found at:
http://www.jcb.org/cgi/content/full/jcb.200607174/DC1

Live Cell	GFP-LacI / DAPI	Detail
a		a'

pol I	GFP-LacI	Merge	Slice
b			b'

Figure 1. **GFP-LacI tagging of the procyclin locus in the nucleus of the T. brucei procyclic form.** (a) In vivo fluorescence detection of the GFP-LacI bound to lac operator sequences (green) inserted into the procyclin locus. DNA was stained with DAPI (blue), and the cell was visualized by phase contrast (gray). A single optical section from a deconvolved two-channel 3D dataset is shown using a 0.5-μm z step (slice animation available as Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200607174/DC1). (a') Higher magnification of the nucleus showing GFP-LacI and DAPI fluorescence signals. (b) 3D IF analysis of a procyclic-form interphase nucleus revealing localization of the GFP-LacI–tagged procyclin chromosomal locus using an anti-GFP monoclonal antibody (green) and localization of pol I using a polyclonal antibody against pol I (TbRPA1; red). DAPI staining (blue) reveals the nucleus as well as the mitochondrial DNA and indirectly indicates the position of the nucleolus because of its lack of DAPI staining. Pol I is exclusively localized in the nucleolus and displays a characteristic U-shape structure. The procyclin locus is detected in the nucleolar periphery in living and fixed cells. Maximum intensity projection of a three-channel 3D stack (b) or maximum anti-GFP intensity slice (b') are shown. Dotted line indicates the nuclear outline. Bars, 1 μm.

the active *VSG* ES promoter is subjected to nuclear envelope repositioning concomitant with ESB disassembling and is followed by chromatin condensation.

Results and discussion

Nuclear positioning dynamics of developmentally regulated chromatin domains is involved in coordinating transcriptional activation and repression. For a precise positional analysis of a particular sequence in nuclei, we have adapted the in vivo GFP tagging of chromosomes (Robinett et al., 1996) to bloodstream and procyclic trypanosomes. By expressing GFP-*Lac*I in a tetracycline-inducible system (Wirtz et al., 1999), we are able to localize a particular DNA sequence in the nucleus, as visualized by GFP-*Lac*I binding to *lac* operator sequences inserted in a chromosome site in vivo and in fixed cells, thereby exploiting the advantages of this tool (for review see Gasser, 2002). *T. brucei* immunofluorescence (IF) analysis has been considerably improved by adapting 3D deconvolution wide-field fluorescence microscopy (Engstler and Boshart, 2004) to the study of nuclear architecture in this paper.

Researchers have reported that heterologous genes transcribed from the *procyclin* locus generate mRNAs that are localized either to the nucleolus (Rudenko et al., 1991; Chung et al., 1992) or to the nucleoplasm (Chaves et al., 1998), as

Table I. **Nuclear positional analysis by 3D IF in procyclic-form GFP-tagged cell lines**

GFP-tagged loci	Detectable GFP dot	Perinucleolar	Nuclear periphery
	%	%	%
Procyclin promoter (n = 102)	88.2	78.9 (18.9)	2.2
rDNA promoter (n = 100)	88[a]	81.8 (17.1)[a]	1.1[a]
ES promoter (n = 103)	63.1[b]	30.8 (27.7)[b]	41.5[b]

Different GFP-tagged cell lines were analyzed by 3D double IF with DAPI staining, anti-GFP, and anti–pol I antibodies. Interphase GFP-*Lac*I–expressing cells were scored as GFP dot positive or negative (second column). Perinucleolar position as defined by pol I labeling and DAPI staining is shown in the third column. Nuclear periphery position as determined by DAPI staining is shown in the fourth column. Cells matching both categories (nucleolar periphery signal in the nuclear periphery) are shown in parentheses in the third column (see Materials and methods for further details). Normalized percentages against GFP dot–positive cells are presented in the third and fourth columns. Statistical significance was calculated with a chi-square test against the procyclin promoter GFP-tagged cell line. *Procyclin* and *rDNA* promoters are located at the nucleolar periphery, whereas the ES promoter is significantly located at the nuclear periphery (P < 0.001). The ES promoter–tagged cell line showed a reduced percentage of GFP dot–positive cells.
[a]No significant difference.
[b]Significant difference (P < 0.001).

assessed by RNA-FISH. However, the nuclear position of the *procyclin* chromosomal loci has not been investigated. In this study, we address the nuclear position of the *procyclin* chromosomal locus, which is transcribed by pol I (Rudenko et al., 1990) and is developmentally regulated (Roditi et al., 1989). For this purpose, the *lac* operator repeats were chromosomally inserted upstream of a *procyclin* promoter within the *procyclin* (GPEET-PAG3) locus. First, to avoid possible fixing artifacts, the position of the procyclin locus was determined in vivo. After DAPI staining of DNA in the nucleus of live cells, the position of the nucleolus was indirectly determined by the absence of DAPI staining, and localization of the GFP-*Lac*I bound to the *procyclin* locus was visualized upon GFP-*Lac*I induction. A fluorescent GFP dot was clearly visible upon induction, and its localization was determined to be at the periphery of the nucleolus (Fig. 1 a). Live cell 3D microscopy confirmed that the *procyclin* locus was confined to the border of the nucleolus (Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200607174/DC1). Second, to more precisely determine the position of this sequence in the nucleus with respect to the nucleolus, we performed IF analysis in PFA-fixed cells. The localization of GFP-*Lac*I was detected using an anti-GFP monoclonal antibody, and pol I was stained using affinity-purified anti–pol I large subunit (anti-TbRPA1) antiserum (Navarro and Gull, 2001). Analysis of deconvolved 3D datasets indicated that the GFP-*Lac*I–tagged *procyclin* locus is associated with the nucleolus (Fig. 1 b).

Interestingly, this position was observed in 97.8% of GFP dot–positive cells (Table I), suggesting a highly constrained chromosomal position, which was confirmed by time-lapse fluorescence imaging in living cells (Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200607174/DC1). Because the *procyclin* gene family is transcribed at a similar level for all allelic variants (Acosta-Serrano et al., 1999), there seems to be no need to associate them with a single extranucleolar body, which is
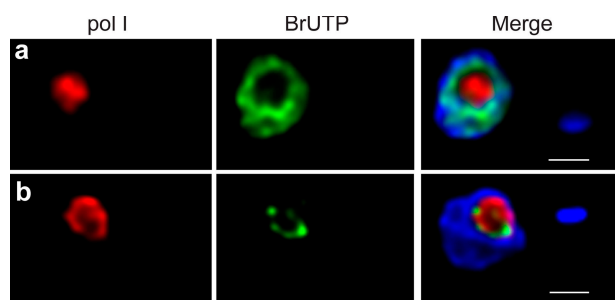
pol I	BrUTP	Merge
a		
b		

Figure 2. **BrUTP labeling of nascent RNA in permeabilized procyclic-form nuclei.** Double IF using anti–pol I antiserum (red) and a monoclonal antibody against BrdUTP (green) together with DNA staining using DAPI (blue) in PFA-fixed cells. (a) Total nuclear transcription in situ as revealed by the incorporation of BrUTP into nascent RNA in the absence of α-amanitin. (b) BrUTP-labeled nascent RNA transcribed by pol I in the presence of 100 μg/ml α-amanitin. Pol I–mediated transcription is confined to several perinucleolar foci (green), indicating that pol I transcription occurs largely along the periphery of the nucleolus. Single deconvolved slices are shown. Bars, 1 μm.

in contrast to the model of the monoallelic expression of *VSG* ES in the bloodstream form (Navarro and Gull, 2001). Although in vitro differentiation from the bloodstream to the procyclic form is efficient in *T. brucei*, the converse is not feasible. Thus, we are unable to address possible nuclear localization changes for the *procyclin* locus in the procyclic form (Fig. 1) upon differentiation to the bloodstream stage. The same lac operator construct that was used to tag the *procyclin* locus in procyclics was repeatedly used in bloodstream parasites with no success, suggesting that the procyclin promoter is developmentally down-regulated in the bloodstream form by a chromatin-mediated mechanism, as suggested previously (Hotz et al., 1998).

IF analysis showed that pol I was exclusively localized to the nucleolus, and no substantial extranucleolar signal was detected (Fig. 1 b), ruling out the possibility of a specific pol I–containing body responsible for *procyclin* expression (Pays et al., 2004). Interestingly, pol I was found to be subcompart-

mentalized in the nucleolus, with distinct foci peripherally distributed in a U-shaped pattern that was easily detectable by 3D microscopy (Fig. 1 b). To investigate this unexpected pol I distribution, we performed BrUTP labeling of nascent RNA in situ (Navarro and Gull, 2001) in PFA-fixed procyclic cells to determine the sites of pol I transcription. To exclusively detect pol I transcriptional activity in the nucleus of permeabilized cells, experiments were performed in the presence of high concentrations of α-amanitin (100 μg/ml), which is known to inhibit pol II and III transcription. Indeed, although many transcriptional foci were distributed along the nucleus in the absence of the drug (Fig. 2 a), in the presence of α-amanitin, nascent RNA was solely detected in the nucleolus (Fig. 2 b). Furthermore, within the nucleolus, BrUTP-labeled RNA was confined to distinct foci located predominantly in a peripheral position similar to that of the GFP-*Lac*I–tagged *procyclin* locus (Fig. 1 a).

To further investigate pol I–dependent transcriptional activity, we determined the position of the rDNA in the procyclic form. Several independent clones were analyzed, and all revealed a perinucleolar position for the GFP-*Lac*I–tagged rDNA chromosomal site (Fig. 3 a and Video 3, available at http://www.jcb.org/cgi/content/full/jcb.200607174/DC1). Again, the position of the GFP-*Lac*I bound to the rDNA locus associated with the position of pol I and showed a stable perinucleolar position (98.9% of GFP dot–positive cells) when examined by 3D microscopy (Table I). The peripheral nucleolus location of *procyclin* and rDNA loci, together with pol I transcription foci along the nucleolus periphery (Fig. 2) instead of an inner central position, may explain the lack of colocalization of these two loci that were described previously using RNA-FISH (Chaves et al., 1998).

To determine whether the peripheral distribution of pol I–transcribed loci in the nucleolus is a unique feature of the insect form of the parasite or is also present in the bloodstream form, we addressed the position of the rDNA locus. We performed 3D IF of bloodstream-form cells upon PFA fixation
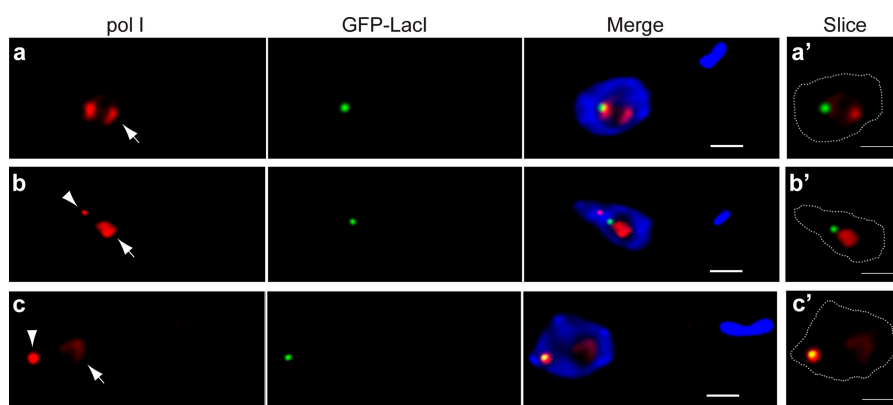
pol I	GFP-LacI	Merge	Slice
a			a'
b			b'
c			c'

Figure 3. **Localization of a GFP-*Lac*I–tagged rDNA chromosomal site in the procyclic and bloodstream forms of *T. brucei*.** (a) Localization of the rDNA locus tagged with *lac* operators in the procyclic form visualized by an anti-GFP monoclonal antibody (green) and anti–pol I antiserum (red) in the nucleolus (arrow). (b) As in panel a, but using bloodstream-form parasites. Anti-pol antibody (red) stained the nucleolus (arrow) and the extranucleolar pol I–containing body, the ESB (arrowhead). In both developmental stages, the rDNA chromosomal sites are located in the nucleolar periphery. (c) The ESB (arrow) is associated with the GFP-tagged active *VSG* ES promoter, which was detected by double labeling using anti–pol I (red) and anti-GFP (green) antibodies in the PFA-fixed bloodstream form. Note the U-shape distribution of pol I in the nucleolus (arrowhead). Maximum intensity projections of 3D stacks (a–c) or maximum anti-GFP intensity slices (a'–c') are shown. Dotted lines indicate the nuclear outline. Bars, 1 μm.

in suspension (Engstler and Boshart, 2004), which preserves nuclear structure better than the previously used fixation conditions (Navarro and Gull, 2001). The position of rDNA locus in the bloodstream form localized to the nucleolus and, similar to the procyclic form, was peripheral with an equivalent constrained position (98% of GFP dot–positive cells; Fig. 3 b).

To investigate a possible nuclear position–dependent regulation of pol I–transcribed chromosomal sites in the bloodstream developmental form, we first analyzed the position of the active *VSG* ES promoter. Double IF using an anti–pol I antibody and an anti-GFP antibody showed that the active *VSG* ES tagged with GFP-*Lac*I localizes to the ESB as previously described (Navarro and Gull, 2001), whereas pol I was present in the ESB as well as in the nucleolus (Fig. 3 c). We also addressed the nuclear position of the internal chromosomal *VSG* basic copy (BC) tandem genes. These copies of different *VSG* genes serve as substrates for recombination events into the active ES telomere, resulting in an antigenic switch. The GFP-*Lac*I–tagged *VSG*121 BC locus showed no association with the ESB, which is similar to an inactive 121 ES promoter region (Navarro and Gull, 2001). Importantly, statistical IF position analysis of both the BC and inactive ES promoter sequences revealed no considerable association to the nuclear envelope (2% of GFP dot–positive cells). We show that in bloodstream form, the telomeric silencing of *VSG* ES proposed previously (Horn and Cross, 1995) is not associated to either nuclear periphery repositioning or chromatin condensation.

We next determined whether the active *VSG* ES undergoes nuclear repositioning upon developmental differentiation from the bloodstream to the procyclic form, where no VSGs are expressed. For this purpose, the differentiation of bloodstream- to procyclic-form parasites was induced in vitro, and nuclear localization changes were analyzed early (5 h) or late (24 h) during differentiation. To assess the differentiation process, we monitored the developmental expression of the surface glycoprotein procyclin by double IF using anti–EP procyclin and anti-VSG221 antibodies. 22% of the cells displayed procyclin on the surface 5 h upon in vitro differentiation. This value increased 24 h upon differentiation, with 83% of cells exclusively displaying procyclin on the cell surface and 5% displaying a mixed coat of procyclin and VSG. The remaining 10% of cells that solely displayed VSG on the surface can be interpreted as differentiation retarded or defective in the asynchronous differentiation process that occurs in this monomorphic cell line. 3D IF analysis showed that the active *VSG* ES promoter relocated to the nuclear envelope early during differentiation (5 h; Fig. 4 a). Importantly, at the same time, extranucleolar pol I (ESB) was no longer detected, which is consistent with our observation that pol I exclusively localizes to the nucleolus in the established procyclic form (Fig. 1). Statistical analysis of the position indicated that 70% of the nuclei display the GFP-*Lac*I dot at the nuclear periphery 5 h upon differentiation (Fig. 4 c). The relocation of the active ES promoter to the nuclear periphery in 70% of the cells was higher than the number of procyclin-positive cells (22%), suggesting that *VSG* ES nuclear reposition silencing is preceding the full surface expression of *procyclin*. Finally, 24 h upon in vitro differentiation, the GFP-tagged active *VSG* ES promoter

was located to the nuclear periphery in 88% of the cells (Fig. 4 c) displaying *procyclin* on their surface.

To determine whether such rapid developmental repositioning was a unique feature of the active ES promoter, we determined the localization of various other chromosomal sequences. For example, the rDNA locus showed no change in nuclear localization either 5 (Fig. 4 b) or 24 h upon differentiation and was always detected in a perinucleolar location (100% of GFP dot–positive cells; Fig. 4 c). Similarly, statistical analysis on the location of the GFP-*Lac*I–tagged *VSG*121 BC and inactive *VSG* ES promoter chromosome sites showed no significant nuclear envelope repositioning upon early differentiation (Fig. 4 c).
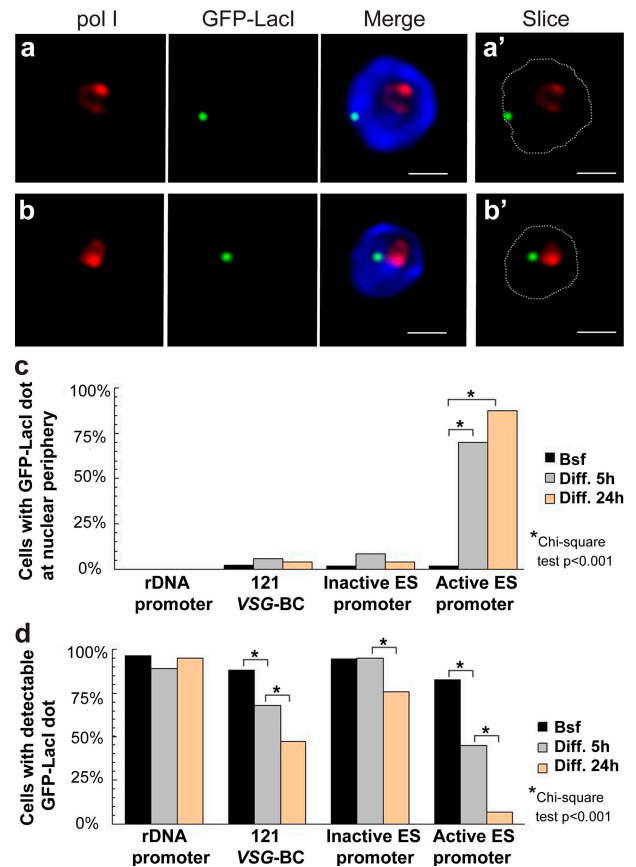

Figure 4. **Changes in nuclear localization and chromatin accessibility of the active ES promoter upon in vitro differentiation.** (a) The GFP-tagged active ES promoter (green) localized to the nuclear periphery upon differentiation, as indicated by double labeling with an anti-GFP monoclonal antibody (green), anti–pol I antiserum (red), and DAPI staining (blue). 5 h after the induction of differentiation, the extranucleolar ESB is not detectable, and the GFP-tagged active *VSG* ES is now located at the nuclear envelope (as determined by the edge of DAPI staining). (b) In contrast, the GFP-*Lac*I–tagged rDNA chromosomal site does not show nuclear repositioning. Maximum intensity projections of three-channel 3D datasets (a and b) or maximum anti-GFP intensity slices (a' and b') are shown. Dotted lines indicate the nuclear outline. Bars, 1 μm. (c) Statistical analysis on the number of GFP dot–positive cells where the GFP-*Lac*I dot is in contact with the nuclear periphery in cells tagged in the rDNA, 121*VSG* BC, inactive ES, or active ES promoter loci. BSF, bloodstream form. (d) Statistical analysis of the number of GFP-expressing cells in which a clear GFP-*Lac*I dot is visible in cells tagged in the rDNA, 121*VSG* BC, inactive ES, or active ES promoter regions. The profound lack of detection of the GFP-*Lac*I bound to chromatin in the active ES promoter suggests a reduced chromosomal accessibility of this locus upon differentiation.

Together, our data indicate that the active *VSG* ES promoter sequences reposition to the nuclear periphery concomitantly with the ES transcription silencing during differentiation to the insect form (Navarro et al., 1999). Importantly, rapid nuclear repositioning of the *VSG* promoter detected at 5 h after differentiation induction precedes the full down-regulation of *VSG* transcription given that *VSG* mRNA is still clearly detectable at 12 h after differentiation (Janzen et al., 2006). This is the case despite that *VSG* mRNAs are down-regulated by the 3′-untranslated region in the procyclic form (Berberof et al., 1995). This mechanism seems to be specific for the active ES promoter, as such rapid repositioning was not observed for the inactive 121 ES promoter or *VSG*121 BC loci at early differentiation stages (Fig. 4 c).

Interestingly, although 83% of GFP-positive nuclei tagged at the active *VSG* ES promoter showed a clear GFP-*Lac*I dot in an exponentially growing bloodstream culture, upon 24 h of differentiation and nuclear repositioning, only 8% of the GFP-positive nuclei showed a detectable GFP-*Lac*I dot (Fig. 4 d). In contrast, detection of the GFP-*Lac*I bound to rDNA was evident in 98% of the GFP-positive nuclei even 24 h upon differentiation. Cell lines tagged either at the inactive 121 ES promoter region or in the *VSG*121 BC region showed an intermediate situation, with 53–76% of the cells displaying a visible GFP dot 24 h upon differentiation (Fig. 4 d). Similar data were also obtained by in vivo GFP fluorescence direct visualization. In late differentiation (24 h), cells showed a GFP-*Lac*I dot for the rDNA locus that was easily detectable. In contrast, 24 h upon differentiation, when the active ES was tagged, the GFP-*Lac*I dot was almost undetectable even though the cells displayed diffuse GFP expression in their nuclei (Fig. 4 d).

These differential results suggest that GFP-*Lac*I binding to the lac operator sequences inserted into distinct chromosomal positions reflect differences in chromatin accessibility and, thus, allow us to detect changes in chromatin condensation. These data are supported by the previously described *VSG* ES chromatin remodeling of the bloodstream *VSG* ES after differentiation to the procyclic form to yield a structure that is no longer permissive for T7RNAP transcription in vivo (Navarro et al., 1999; Janzen et al., 2004). Recently, Dietzel et al. (2004) detected an opposing chromatin decondensation event upon gene activation utilizing the accessibility of GFP-*Lac*I. In this context, changes in chromatin seem to dramatically affect the accessibility of GFP-*Lac*I to the lac operators inserted in the active *VSG* ES promoter region, as indicated by the drastic decrease in the number of nuclei with a detectable GFP dot (Fig. 4 d). Although chromatin in the rDNA locus is not affected at all upon differentiation, a moderate degree of chromatin condensation was also found for the *VSG*121 BC and inactive 121 ES promoter regions even though these loci are not transcribed in the bloodstream form. Moreover, an eventual repositioning of inactive ES promoter to the nuclear envelope does occur, as tagging the inactive ES promoter regions in established procyclic form revealed that these chromosomal loci localized to the nuclear envelope in 41.5% of nuclei (Table I). The active *VSG* ES promoter repositioning in 88% of cells at early stages of the differentiation process is in contrast with the 41.5% of nuclei detected for the promoter locus in established procyclics (Fig. 4 c and Table I). Thus, our results show that nuclear repositioning targets more efficiently at early stages during the differentiation process and suggest that the establishment of silencing requires a transient perinuclear localization.

Despite many correlations between nuclear localization and gene activity, it remains unclear whether nuclear repositioning is the cause or the result of such activity. Like yeast (Gartenberg et al., 2004), TbKU80-deficient trypanosomes are unable to halt *VSG* ES developmental silencing (Janzen et al., 2004) or the silencing of all *VSG* ESs but one in the bloodstream form (Conway et al., 2002), but no information on possible nuclear repositioning is available for this mutant. Although we cannot conclude that nuclear repositioning causes silencing, importantly, our data provide new insights into this problem. First, the active *VSG* ES promoter, located 60 kb upstream of the telomere, is the sole target for nuclear envelope relocation during differentiation, which is in contrast to inactive *VSG* ES promoters. Second, this rapid repositioning precedes chromatin condensation during differentiation (Fig. 4, c and d).

Nuclear envelope repositioning and chromatin condensation events have been suggested to affect pol II promoter activities in yeast and mammalian cells (Spector, 2003). Our data represent the first example of a pol I–transcribed chromatin domain targeted by a nuclear position–dependent silencing mechanism, indicating that such regulation is not restricted to pol II and that nuclear architecture plays a universal role in the epigenetic regulation of transcription.

Materials and methods

Trypanosomes and 3D IF
T. brucei bloodstream-form (Molteno Institute Trypanozoon antigenic type 1.2 [MITat 1.2]; clone 221a) and 427 procyclic-form DNA transfections and selection procedures were described previously (Wirtz et al., 1999). For these studies, the bloodstream cell lines were differentiated in vitro to procyclics using standard conditions but with SDM-79 medium (Overath et al., 1986). IF was performed on cells in suspension (Engstler and Boshart, 2004) except that fixation was performed for 2.5 h on ice with 4% PFA and permeabilized with 1% NP-40 for 1 h at room temperature. IF was performed in 1% blocking reagent (Roche) in PBS (Sigma-Aldrich) using the monoclonal anti-GFP (Invitrogen) and affinity-purified anti–pol I (TbRPA1) rabbit antiserum (1:600; Navarro and Gull, 2001). Alexa-Fluor488- or -594–conjugated goat species–specific antibodies (Invitrogen) were used as secondary antibodies, and cells were DAPI stained and mounted as described previously (Engstler and Boshart, 2004). Stacks (0.1-μm z step) acquisition was performed with a microscope system (Cell R IX81; Olympus), 63×/100× objectives, illumination system (MT20; Olympus), and camera (Orca CCD; Hamamatsu). Deconvolution of 3D images was performed using Huygens Essential software (version 2.9; Scientific Volume Imaging) using an experimentally calculated point-spread function with 0.2-μm TetraSpeck microspheres (Invitrogen). All images displayed in the figures are maximum intensity projections from digitally deconvolved multichannel 3D image datasets. Pseudocoloring and maximum intensity projections were performed using ImageJ software (version 1.37; National Institutes of Health). Nascent RNA labeling in permeabilized procyclic form was essentially performed as described previously for the bloodstream form (Navarro and Gull, 2001) except that cells were fixed with 2% PFA for 20 min. The single-slice deconvolution shown in Fig. 2 was performed using Huygens software with 0.3 μm as a z sample size.

GFP-*Lac* repressor tagging of chromosome sites
We have adapted the in vivo GFP tagging of chromosomes (Robinett et al., 1996; Straight et al., 1996) to bloodstream and procyclic trypanosomes. GFP-*Lac*I was expressed in a tetracycline-inducible manner (Wirtz et al., 1999). We localized a particular DNA sequence in the nucleus by

detection of the GFP-*Lac*I bound to the *lac* operator sequences inserted in a chromosome. Stable transformants in *T. brucei* occur by homologous recombination, allowing us to insert a *lac* operator tagging cassette by a single crossover.

The bloodstream single marker (SM) cell line (Wirtz et al., 1999) and the procyclic cell line 1313-1333 (Alibu et al., 2005) were used for tetracycline-inducible expression. To express the GFP-*Lac*I fusion in a tetracycline-dependent manner, we used pMig75, which was described previously (Navarro and Gull, 2001), in procyclic (1313-75) and bloodstream forms (SM-75). These two cell lines were used to obtain all transformants with the *lac* operator tagging constructs (described in the next paragraph) in the absence of tetracycline induction.

To GFP-*Lac*I tag any locus of interest, we developed a series of constructs containing variable target DNA upstream of a 256–*lac* operator–containing fragment (Navarro and Gull, 2001) and downstream of the promoter of the locus under study, which will drive expression of the selectable marker. To GFP tag the *procyclin* (*GPEET/PAG3*) locus, the targeting sequence located 60 bp upstream of the endogenous *procyclin* promoter was a PCR fragment generated using oligonucleotides (5′-CGAGCTCATA-CCGCTGCCGGCCTAAATGC-3′ and 5′-CAAGCTTCATTTTGCACAAAAT-GCACTATTG-3′). To drive the expression of the GFP-*Lac*I selectable marker, we used a *procyclin* promoter obtained by PCR using oligonucleotides (5′-GTGGATCCTCCATTTTGTGGCAGTGATGG-3′ and 5′-CGCCAT-GGAAAGGGAACGAGGTGCCATTG-3′). To tag the rDNA spacer located between two rDNA repeats, the targeting sequence was a PCR fragment (5′-AATTCGAGCTCATATAGTTGG-3′ and 5′-CGCGAAGCTTCGGTGTGT-TGCCAAAGACATTC-3′) using pLew82 as a template (Wirtz et al., 1999). To drive a bleomycin selectable marker, we used a ribosomal promoter obtained by PCR using oligonucleotides (5′-CGAGGGATCCACCCAGCGC-GGGTGCATTC-3′ and 5′-GGCATATGCAGTCCTGCTCCTCGGCC-3′). The 121VSG BC target sequences that we used were the full 121VSG cDNA and the ES promoter described previously (Navarro et al., 1999). The constructs to tag the active 221 ES and the inactive 121 ES were previously described (Navarro and Gull, 2001). All constructs were inserted upstream of the promoter of the locus under study, resulting in tandem repeats of similar promoters. However, in the case of the 121*VSG* BC targeting construct, we included an ES promoter to drive the selectable marker that is not present in the endogenous locus. GFP-*Lac*I expression was induced in early exponential cultures with 1–0.1 μg/ml doxycycline (Sigma-Aldrich) for bloodstream and procyclics, for 16 h. Simultaneously with differentiation induction, the expression of GFP-*Lac*I was induced with 0.1 μg/ml doxycycline.

Statistical position analysis

GFP-*Lac*I expression in SM-75 and 1313-75 cell lines displayed a proportion of nuclei that did not express the GFP-*Lac*I fusion after induction even without the *lac* operator repeats. Thus, this variable expression was not caused by a toxic effect but rather by variegated activity of the *procyclin* promoter driving the expression of GFP-*Lac*I. Thus, all statistical analyses in both developmental stages described in this paper are based on GFP-*Lac*I–positive nuclei cells recognized by the unbound GFP-*Lac*I that was detected in a dispersed manner in the nucleoplasm. Statistical analysis of GFP-*Lac*I–expressing nuclei that were positive or negative for the GFP dot was performed in 100–120 interphase nuclei. The positive ones were grouped in different categories based on the GFP dot nuclear position within the DAPI staining (nuclear periphery, nucleolus, and nucleoplasma) and the relative position between the GFP dot and pol I signals. The scoring was performed by direct optical observation. Questionable cells were analyzed by 2D or 3D digital imaging. At least 20 representative cells were analyzed by 3D deconvolution microscopy. Tagged chromosome position and GFP dot detection probability distributions were compared between categories indicated with an asterisk in Fig. 4 using chi-square analysis. Statistical significance was determined by using a 95% confidence interval.

Online supplemental material

Video 1 shows a *T. brucei* live cell in which the procyclin chromosomal site is tagged with GFP-*Lac*I. Video 2 shows in vivo visualization of the highly transcribed procyclin chromosomal site tagged with GFP in a procyclic-form trypanosome. Video 3 shows 3D deconvolved slice animation through the whole fixed nucleus. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200607174/DC1.

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