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 this study, we report that nonmutually exclusive *procyclin* genes are located at the nucleolar periphery. Previously, we proposed a model whereby the recruitment of a single VSG ES to a discrete pol I–containing extranucleolar 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,
the active VSG ES promoter is subjected to nuclear envelope repositioning concomitant with ESB disassembly 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-LacI tagging of chromosomes (Robinett et al., 1996) to bloodstream a particular sequence in nuclei, we have adapted the in vivo GFP activation and repression. For a precise positional analysis of nuclear architecture in this paper.

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

<table>
<thead>
<tr>
<th>GFP-tagged loci</th>
<th>Detectable GFP dot</th>
<th>Perinucleolar</th>
<th>Nuclear periphery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Procyclin promoter</td>
<td>88.2%</td>
<td>78.9% (18.9%)</td>
<td>2.2%</td>
</tr>
<tr>
<td>(n = 102)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rDNA promoter</td>
<td>88%</td>
<td>81.8% (17.1%)</td>
<td>1.1%</td>
</tr>
<tr>
<td>(n = 100)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ES promoter</td>
<td>63.1%</td>
<td>30.8% (27.7%)</td>
<td>41.5%</td>
</tr>
<tr>
<td>(n = 103)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Different GFP-tagged cell lines were analyzed by 3D double IF with DAPI staining, anti-GFP, and anti-pol I antibodies. Interphase GFP-LacI-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 nuclear 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.

*No significant difference.

Significant difference (P < 0.001).

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 procyclin-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.
Interestingly, pol I was found to be subcompartamentalized 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-LacI–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-LacI–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-LacI 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.
in vitro differentiation, the GFP-tagged active procyclin. Finally, 24 h uponing the full surface expression of ES nuclear reposition silencing is preced-
VSG was higher than the number of procyclin-positive cells (22%),
active ES promoter to the nuclear periphery in 70% of the cells
riphery 5 h upon differentiation (Fig. 4 c). The relocation of the
I dot at the nuclear pe-
70% of the nuclei display the GFP-
Lac exclusively localizes to the nucleolus in the established procyclic
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-LacI–tagged VSG121 BC and inactive VSG ES promoter chromosome sites showed no significant nuclear envelope repositioning upon early differ-
entiation (Fig. 4 c).

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 enve-
lopop late 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-LacI dot at the nuclear pe-
riphery 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 prece-
ding the full surface expression of procyclin. Finally, 24 h upon in vitro differentiation, the GFP-tagged active VSG ES promoter

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 antisem (red), and DAPI staining (blue). 5 h after the induction of differentiation, the extranucleolar ESB is not detect-
able, and the GFP-tagged active VSG ES is now located at the nuclear enve-
lope (as determined by the edge of DAPI staining). (b) In contrast, the GFP-LacI–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 num-
ber of GFP dot–positive cells where the GFP-LacI dot is in contact with the nuclear periphery in cells tagged in the rDNA, 121VSG 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-LacI dot is visible in cells tagged in the rDNA, 121VSG BC, inactive ES, or active ES promoter regions. The profound lack of detection of the GFP-LacI bound to chromatin in the active ES promoter suggests a reduced chromosomal ac-
cessibility 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 VSG121 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-LacI 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-LacI dot (Fig. 4 d). In contrast, detection of the GFP-LacI 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 VSG121 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-LacI dot for the rDNA locus that was easily detectable. In contrast, 24 h upon differentiation, when the active ES was tagged, the GFP-LacI dot was almost undetectable even though the cells displayed diffuse GFP expression in their nuclei (Fig. 4 d).

These differential results suggest that GFP-LacI 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-LacI. In this context, changes in chromatin seem to dramatically affect the accessibility of GFP-LacI 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 VSG121 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 [M141 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 8 [X81; Olympus], 63×/1.4× 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-LacI 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-LacI was expressed in a tetracycline-inducible manner (Wirtz et al., 1999). We localized a particular DNA sequence in the nucleus by
Lac I was induced with (Sigma-Aldrich) for bloodstream and procyclics, for 16 h. Simultaneously induced in early exponential cultures with 1–0.1 g/ml doxycycline that is not present in the endogenous locus. GFP-I expression was in the construct, we included an ES promoter to drive the selectable marker repeats of similar promoters. However, in the case of the 121 BC target-A A T T C G A G C T C A T A T A G T T G G -3 (5′-GGAATTCCTCTAGATGAATG-3′) and 5′-GCGGAT- GGGAGGAAGGAGGATCCGATG-3′. To tag the DNA spacer located between two rDNA repeats, the targeting sequence was a PCR fragment (5′-CGGAGGCACGATGAGCTGCTGTCGACGAGAACGAC-3′) using pT7ε2 as a template (Wirtz et al., 1999). To drive a bleomycin selectable marker, we used a ribosomal promoter obtained by PCR using oligonucleotides (5′-GGGAGGCGATGAGCTGCTGTCGACGAGAACGAC-3′ and 5′-GGGAGGCGATGAGCTGCTGTCGACGAGAACGAC-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. Lac-I expression was induced in early exponential cultures with 0.01–0.1 μg/ml doxycycline (Sigma-Aldrich) for bloodstream and procyclins, for 16 h. Simultaneously with differentiation induction, the expression of Lac-I was induced with 0.1 μg/ml doxycycline.

Statistical position analysis

GFP-Lac expression in SM-75 and 1313-75 cell lines displayed a proportion of nuclei that did not express the GFP-Lac fusion after induction even without the lac operator repeats. Thus, this variable expression was not caused by a toxic effect but rather by varied activity of the procyclin promoter driving the expression of GFP-Lac. Thus, all statistical analyses in both developmental stages described in this paper are based on Lac-I-positive nuclei cells recognized by the unbound GFP-Lac that was detected in a dispersed manner in the nucleoplasm. Statistical analysis of 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. Video 2 shows in vivo visualization of the highly transcribed procyclin chromosomal site tagged with GFP in a procyclin-PCR-based transposase. Video 3 shows 3D deconvolved slice animation through the whole fixed nuclei. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200607174/D1C1.

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large-scale chromatin organization using lac operator/repressor recognition.


