Nuclear architecture underlying gene expression in *Trypanosoma brucei*

Miguel Navarros, Xenia Peñate and David Landeira

Instituto de Parasitología y Biomedicina López-Neyra, Consejo Superior de Investigaciones Científicas (Spanish National Research Council), Avda. del Conocimiento s/n, 18100 Granada, Spain

The influence of nuclear architecture on the regulation of developmental gene expression has recently become evident in many organisms ranging from yeast to humans. During interphase, chromosomes and nuclear structures are in constant motion; therefore, correct temporal association is needed to meet the requirements of gene expression. *Trypanosoma brucei* is an excellent model system in which to analyze nuclear spatial implications in the regulation of gene expression because the two main surface-protein genes (procyclin and VSG) are transcribed by the highly compartmentalized RNA polymerase I and undergo distinct transcriptional activation or downregulation during developmental differentiation. Furthermore, the infective bloodstream form of the parasite undergoes antigenic variation, displaying sequentially different types of VSG by allelic exclusion. Here, we discuss recent advances in understanding the role of chromosomal nuclear positioning in the regulation of gene expression in *T. brucei*.

**Trypanosomes and regulation of gene expression**

African trypanosomes display intriguing characteristics at the transcriptional level that set them apart from most eukaryotes with respect to developmentally regulated genes in addition to housekeeping genes. On the one hand, developmental-stage-specific surface protein genes are transcribed by RNA polymerase I (pol I). On the other hand, housekeeping genes are clustered into long polycistronic transcriptional units and transcribed by RNA polymerase II (pol II) (reviewed in Ref. [1]). The *T. brucei* genome consists of 11 diploid megabase chromosomes (1–6 Mbp), 1–5 intermediate chromosomes (200–900 kbp) and ~100 minichromosomes (50–150 kbp). The fact that RNA pol II promoters for housekeeping genes have proved elusive so far, together with differential regulation of polycistronically transcribed mRNA, led to the conclusion that constitutive gene expression is predominant in trypanosomes [2,3]. Recently, a pol II promoter has been described for the splice leader RNA [4], albeit showing rather weak activity. This weak level of activity could explain the difficulty in identifying other pol II promoters. Most of the more well-characterized promoters are associated with pol I and drive the developmentally regulated robust transcription of variant surface glycoprotein (VSG) genes and procyclins, in addition to rDNA (Figure 1). In contrast to diploid housekeeping genes, VSG genes are subject to monoallelic expression from haploid chromosomal telomeres (reviewed in Refs [5–7]). There are ~1000 VSG genes spread throughout different chromosomes and the copy number and location can vary largely between strains [8,9].

In this review, we discuss recent results concerning trypanosome nuclear architecture in the context of gene expression, and search for insights into the mechanisms involved in gene regulation. Rather than review the large amount of information available on antigenic variation or the trypanosome life cycle, we discuss developmentally regulated gene expression in the context of chromosomal position and associations with nuclear structures in an effort to provide new insights on epigenetic regulation.

**Developmentally regulated gene expression of surface proteins**

The parasite *Trypanosoma brucei* alternates between its mammalian host and tsetse fly vector during a complex life cycle involving several developmental stages (reviewed in Refs [10,11]). The infective, extracellular bloodstream form is covered by a dense coat that consists mainly of one type of glycosylphosphatidylinositol (GPI)-anchored VSG. The sequential expression of VSG genes causes a persistent infection by enabling bloodstream parasites to elude the immune response of the host in a process known as antigenic variation. Upon differentiation to the procyclic form (or tsetse mid-gut stage), the parasite expresses no VSG and, conversely, an invariant glycoprotein named procyclin covers its surface (reviewed in Refs [6,7,10]).

The procyclin gene family (EP and GPEET) is arranged in tandem copies in internal chromosomal positions. However, the expressed VSG is always located adjacent to a telomere at the end of one out of ~20 polycistronic loci known as expression sites (ESs) (Figure 1a). Only one ES is fully transcribed at a time, yielding a single VSG on the surface of the infective trypanosome. A set of ES-associated genes [12] are polycistronically transcribed together with the VSG from a promoter located 40–60 kb upstream, depending on the ES. The majority of antigenic variation is achieved by recombination events involving the replacement of VSG genes into the active ES [13] (reviewed in Ref. [5]); however, antigenic variation can also occur by the transcriptional activation and inactivation of ESs in a process named *in situ* switching.

Many models to explain the expression of a single subtelomeric VSG-ES have been suggested thus far. Three of the more important models are: (i) telomeric position, (ii)
differential elongation–RNA maturation, and (iii) a unique nuclear transcription site. Here, we discuss these models and consider them within the framework of nuclear architecture. Furthermore, these models are probably not mutually exclusive; instead, the mechanisms involved might work together or at different times during the regulation of VSG expression.

The VSG-ES telomeric position

The telomeric position of VSG genes in VSG-ESs and minichromosomes has recently been the focus of much attention (reviewed in Refs [5,14]). These studies have led to the conclusion that the telomeric position of VSG genes facilitates the major mechanism of antigenic switching: recombination involving duplicative gene conversion and reciprocal telomere translocation. A search for the machinery involved in these events has proven difficult [15]; however, RAD51 T. brucei mutants did show a reduced ability to undergo VSG switching [16]. Other proteins such as Ku and TERT have been shown to be involved in telomere maintenance but do not significantly alter switching rates [17–20].

Furthermore, antigenic switching can also occur by VSG-ES in situ transcriptional switching by an epigenetic mechanism. No sequence alterations, DNA rearrangements or chromatin changes that correlate with an in situ transcriptional switch have been detected in the promoter region, although chromatin differences have been described in the active telomeric VSG (reviewed in Ref. [7]). The position of VSG at chromosome ends has led researchers to hypothesize that a mechanism similar to telomeric silencing might operate on inactive ESs in bloodstream form trypanosomes. However, if a silencing mechanism were involved, it is probably unrelated to yeast telomeric silencing for the following reasons: (i) in a null mutant of the trypanosome ortholog of yeast silent information regulator SIR2 (TbSIR2P), no effect on antigenic switching rates was observed [21]; (ii) two ESs cannot be fully active at the same time [22]; and (iii) no association with the nuclear envelope has been detected for an inactive VSG-ES promoter [23]. Taken together, these results suggest that a classical telomeric silencing mechanism does not operate on either inactive telomeric VSG-ESs or in situ transcriptional switching (reviewed in Refs [7,24]). In contrast to trypanosomes, Plasmodium antigenic variation of var genes seems to be dependent on PiSIR2 for the silencing of inactive var genes [25,26]. Importantly, inactive var genes tend to be associated with the nuclear periphery [27].

The role of RNA polymerase I subnuclear compartments in the regulation of VSG expression

The recent recognition of subnuclear compartments as modulators of transcription in eukaryotes has placed emphasis on research involving transcriptional regulation at the level of nuclear architecture (reviewed in Refs [28,29]). In contrast to pol II, pol I localizes exclusively in the nucleolus of eukaryotic cells where it transcribes rDNA loci (reviewed in Ref. [30]). Because such compartmentalization occurs for pol I, much attention has been focused on investigation of the nuclear spatial organization of rDNA, procyclin and VSG loci transcribed by this machinery in T. brucei.

Early studies focused on the nuclear position of telomeric and minichromosome sequences using fluorescence in situ hybridization (FISH) analysis [31]. The authors concluded that the majority of these sequences are found together in approximately ten clusters, with a tendency to be localized

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**Figure 1.** Genomic organization of RNA polymerase I transcribed loci and expression pattern in two developmental forms of *Trypanosoma brucei*. Genes are represented as black boxes, except for genes encoding surface proteins (orange and green, VSG; blue, procyclin family). Flags indicate promoter position; full transcriptional activity is outlined by red dashed arrows and abortive transcription is indicated by small brown dashed arrows. Telomere repeats are represented by horizontal triangles and other repetitive sequences are indicated with vertically hatched boxes. Figures are not to scale. (a) The bloodstream form trypanosome eludes the mammalian immune response by frequently changing its VSG coat, which is transcribed polycistronically from a subtelomeric region called the expression site (ES). *T. brucei* has ~20 ESs but only one is fully active at any given time. Abortive transcription occurs in the promoter-proximal region of at least some of the ~19 inactive ESs [24]. The invariant surface protein or procyclin genes are not transcribed. rDNA tandem arrays are constitutively transcribed. (b) Upon ingestion by the tsetse fly vector or when triggered in vitro, bloodstream forms differentiate to the procyclic form. In the procyclic stage, no VSG transcription occurs and the surface coat is covered by the procyclins. This gene family is expressed from two polycistronic loci located on chromosomes VI and X. rDNA tandem arrays retain constitutive expression.
in the periphery of the nucleus in the procyclic form and in a more central position in bloodstream form nuclei. Later analyses confirmed these results and investigated the implications in the dynamics of chromosome segregation [32,33].

It was not until years later that a series of elegant studies by the Borst group re-established an interest in the investigation of nuclear position-dependent expression regulation. Chaves et al. [34] demonstrated for the first time that active VSG-ES transcription does not occur in the nucleolus. FISH analysis using the 50 bp repeat sequences upstream of every VSG-ES promoter showed that inactive ESs are widespread in the nucleoplasm and do not cluster towards the nuclear envelope in the bloodstream form [34]. Furthermore, double selection of two different ES promoters tagged with selectable markers showed that the distance between the two nascent RNAs is less than half the distance of pol II control loci [22], hinting at the existence of a unique nuclear sub-compartment that allows full ES transcription. The unstable nature of dual VSG expressers and, therefore, a possible intermediate switching state [22], together with the inability to obtain triple-marked ES expressers [35], suggest that this unique site only allows expression of a single ES.

Another interesting result is that deletion of the active ES promoter leads to the stable activation of a different telomeric ES, resulting in an in situ switching event that normally occurs with low frequency [36]. This study indicated that activation and inactivation are coupled processes, and a model was hypothesized whereby the active ES is located in a unique transcriptional site, a disruption of which leads to occupancy by another ES [36]. Working with this model, the search for a putative nuclear site led to the identification of a coherent and transcriptionally active extra-nucleolar body, named the expression site body (ESB), which associates with the active VSG-ES promoter (Figure 2a) [23]. Thus, a model was proposed to explain VSG monoallelic expression whereby ESB-dependent VSG-ES recruitment leads to the activation of a single VSG-ES, while inactive ESs are excluded [23]. It seems that the architecture of the ESB might define the singularity of the active ES, thereby enabling a high level of both transcriptional elongation and RNA processing by recruiting transcriptional and RNA processing machinery [24].

![Image of Figure 2](image_url)
Differential transcriptional elongation and RNA processing

The existence of a unique nuclear site is in agreement with the efficient processing of transcripts from the active ES [37] and includes the model for ES regulation based on the differential control of transcriptional elongation and RNA processing proposed by Pays and coworkers [6,24]. This model points to monoallelic expression of a single VSG-ES through differential transcriptional elongation and processing of the RNA in the active ES [6,24], based on the detection of transcription in inactive ES proximal regions by RT-PCR [37–39]. Furthermore, a significant transcription level has been detected from inactive ES promoters through the insertion of selectable marker genes downstream of inactive promoters [35,40–42] (reviewed in Ref. [7]). Thus, an important question emerges as to exactly where a partially active ES might localize in the nucleus, challenging the model of allelic exclusion based on ESB-dependent recruitment of a single ES.

Nuclear position analysis of the GFP-tagged (Box 1) inactive 121ES selected for partial activation shows that it is not associated with the ESB but rather with the nucleolus (Figure 2b; D. Landeira and M. Navarro, unpublished), and can still be distinguished from the fully active ES because the parasites still display 221VSG on the membrane. This result suggests that the partially active ES promoter in the nucleolus allows the ES to be accessible to the pol I machinery. However, the nucleolus probably does not contain the proper transcriptional elongation and processing machinery for VSG mRNA production, which is located exclusively in the ESB. Thus, partial transcription of inactive ES sequences might be mediated by ES promoter positioning in the nucleolus instead of the ESB.

Previous results show that active VSG-ES promoter replacement with an rDNA promoter did not affect ES regulation [43]. This suggests that the machinery recruited by both the rDNA promoter (in the nucleolus) and the VSG-ES promoter (in the extra-nuclear ESB) is similar. However, later nuclear position analysis showed that an ES driven by an rDNA promoter was extra-nuclear [34] and presumably located in the ESB, suggesting that other cis- or trans-acting sequences different from the promoter are responsible for ES recruitment to the ESB.

Positional analysis by FISH using probes common to all ESs showed that inactive ESs are randomly located in the nucleus [34,44]. Differential nuclear positioning of VSG-ESs could help to explain the different degrees of transcriptional initiation detected from inactive ES promoters [37], both in the nucleolus (predominant RT-PCR product, or partially active ES) and in the nucleoplasma (minor RT-PCR product). As discussed previously [7], partially active ESs do not result in a higher frequency of in situ activation, suggesting that they are not involved in switching. However, transcription of partially active ESs might provide advantageous expression of different transferring receptor types located downstream of all ES promoters.

Nuclear architecture and subnuclear compartmentalization

Vickerman’s early work on trypanosome nuclear ultrastructure using electron microscopy (EM) describes many nuclear components including nuclear pores and dense chromatin along the internal side of a mitotic-persistent nuclear envelope (NE) [45]. Chromatin structure in T. brucei is different from that of other eukaryotes because a 30 nm fiber is not formed and mitotic chromosome condensation is also absent [45]. This could be explained by the divergence of core histones in this parasite [46]. Transcriptional regulation by the ‘histone code’ in T. brucei might have a crucial role, as suggested by recent data [47–50]. In T. brucei, histone methylation and acetylation have been described [50], different histone modifying enzymes have been identified [46,49,51], and there are at least two interesting histone variants [47,48]. One of these, H2AZ, contrary to yeast, does not co-localize with pol II transcriptional foci in T. brucei but rather associates with repetitive DNA [47], suggesting a different role in this parasite.

A combination of FISH and EM provides a more detailed picture of T. brucei mitotic and interphase nuclei [32]. The authors show electron-dense chromatin aggregates that form clusters close to the NE and disperse in the nucleoplasm, and a prominent nucleolus that does not disintegrate during mitosis. The trypanosome nucleolus as viewed by EM is not as clearly structured into different concentric components as in higher eukaryotes [52], although areas of granular and fibrillar appearance have been described [32]. Additional structural analyses by EM suggest differences between isolated nuclei in the two developmental stages [53]. Procylic form nuclei appear to be rounded with a clear NE, a large nucleolus and heterochromatin arranged into small regions, whereas the nucleus in the bloodstream form is irregular in shape and smaller, with a less distinct NE, a smaller nucleolus and more contiguous heterochromatin [53]. Structural components of the main subnuclear compartments in T. brucei have been purified and analyzed [53]; however, protein function has been

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**Box 1. Tetracycline-induced GFP–lacI tagging of chromosomes in Trypanosoma brucei**

To obtain a precise positional analysis of a particular locus in the nucleus, in vivo GFP tagging of chromosomes [66,67] has been applied to the two main proliferative trypanosome developmental stages [23,54]. Classical FISH can alter nuclear architecture [68], whereas GFP tagging is better at maintaining nuclear structure.

By using a tetracycline-inducible system [69] and GFP fused to the lacI repressor, it is possible to localize a particular DNA sequence in the nucleus at any given time, as visualized by GFP–lacI binding to the lac operator sequences inserted into the chromosome. Stable transformants in Trypanosoma brucei occur by homologous recombination, which enables the insertion of a tagging cassette containing 256 lac operators through a single crossover event. For the purpose of localization of a particular locus, lac operator repeats can be inserted a few Kb upstream of the promoter of interest. The use of tetracycline-induced expression of GFP–lacI also enables consideration of whether a chromatin region is accessible to the binding of the lacI repressor or not at any given time.

Analysis of the position of a GFP-tagged promoter can be carried out by in vivo microscopy in the procyclic form. 3D deconvolution microscopy can be applied to both developmental forms upon double indirect immunofluorescence using anti-GFP antibodies to provide high-resolution nuclear position analysis. Overall, this system is a powerful new tool in the study of chromosomal positioning in T. brucei.
inferred by subnuclear localization only. For instance, NUP-1 localizes to the nuclear envelope [32] and might serve as a component of the trypanosome nuclear lamina [53]. Lamina proteins are believed to have a major role in reformation of the NE during late telophase in eukaryotes. In yeast, for example, nuclei do not disassemble their NEs during mitosis and lack lamina proteins (reviewed in Ref. [29]). Because trypanosomes maintain their nuclear envelope during mitosis, the presence of the putative nuclear lamina protein NUP-1 suggests a function other than postmitotic NE reformation (reviewed in Ref. [33]). Lamin A might influence RNA polymerase II activity: as a negative regulator of transcription that localizes to the NE, it might favor the incorporation of target genes into a repressive environment [29]. Thus, a possible role for trypanosome NUP-1 might be involvement in the organization of heterochromatin associated with the nuclear envelope in trypanosomes.

Labeling of nascent RNA with Br-UTP in the nuclei of permeabilized cells has facilitated the localization of transcriptionally competent areas in the nucleus of trypanosomes. Many transcriptional foci were detected throughout the nucleoplasm, however, these foci were absent towards the nuclear envelope in both developmental stages [23,39,54]. This suggests that the spatial organization of chromosomes in the trypanosome nucleus might serve to facilitate or to impede transcription, and thus nuclear repositioning could regulate gene expression.

**Procyclin chromosome locus positioning suggests a functionally compartmentalized nucleolus**

The nuclear localization of the highly transcribed procyclin chromosome loci in the procyclic form of trypanosomes has been the subject of considerable study. Heterologous genes transcribed from the procyclin locus generate mRNAs that are localized either to the nucleolus [55,56] or to the nucleoplasm [34], as assessed by RNA-FISH. However, no information regarding the nuclear position of the chromosome loci was available until recently [54]. Analysis by 3D microscopy indicates that the GFP-lacI tagged procyclin locus localizes to the nucleolar periphery (Figure 2c), similar to the position of rDNA loci [54] (Figure 2d).

Furthermore, pol I is subcompartmentalized in the nucleolar periphery in a horseshoe-shaped manner [54]. Pol I transcriptional activity is detected in the nucleolar periphery in the procyclic form, consistent with the localization of the procyclin promoter and pol I [54]. This perinucleolar distribution of transcriptional activity is related to the subnuclear location of the exosome complex, which is involved in rRNA processing and mRNA degradation in trypanosomes [57]. The exosome localizes to speckles in the nucleoplasm but it is enriched at the periphery of the nucleolus, revealing a perinucleolar ring-like pattern [58].

The confinement of pol I-transcribed DNAs (rDNA and procyclin) to the nucleolar periphery, similar to the distribution of pol I and transcriptional activity, suggests that the structure of the nucleolus in T. brucei is different from that of other eukaryotes. Mammalian nucleoli show a conserved and concentric arrangement of three elements detectable by electron microscopy: the fibrillar center, the dense fibrillar component and the granular component. Each element corresponds to a different step in ribosome biogenesis [52]. The peripheral organization of the trypanosome nucleolus suggests a model whereby transcription and splicing of procyclin mRNA take place towards the nucleoplasm, where coding RNA maturation factors can be found.

The procyclin family of surface protein genes is constitutively transcribed at a similar level for all allelic variants [59] and such genes localize to the nucleolar periphery [54]. By contrast, the monoallelically expressed VSG-ES promoter was found to be segregated to the ESB, which serves as a unique recruitment transcription site [23]. A model based on the recruitment of a single VSG-ES to the ESB is in agreement with the model described for odorant receptor monoallelic expression, where a single trans-acting DNA element allows the activation of only one odorant receptor allele in a neuron at a time [60]. In trypanosomes, the unique transcription site (the ESB) was first described because extra-nucleolar pol I is easily detectable. Whether this unique ESB is the result of a single enhancer trans-acting DNA element that is responsible for its nucleation remains to be shown, although this is a testable hypothesis.

**VSG-ES promoter chromatin repositioning to the nuclear envelope during developmental silencing**

During B lymphocyte development, selective activation for transcription and rearrangement of immunoglobulin loci correlate with localization away from the nuclear periphery [61]. In Saccharomyces cerevisiae, transcriptional silencing of genes adjacent to telomeres is associated with nuclear envelope positioning (reviewed in Ref. [29]). When bloodstream forms are ingested by the tsetse fly, the parasite replaces its VSG coat with procyclin and no VSGs are expressed (Figure 1b). To investigate possible repositioning of a chromosomal site during differentiation from bloodstream to procyclic form, GFP tagging of chromosomes (Box 1) has been successfully employed in trypanosomes [54]. To perform these complex transgenic experiments, a culture-adapted strain was used. Although this strain does not reach the short stumpy bloodstream stage, parasites had efficiently undergone differentiation to the procyclic stage after 24 h, as assessed by the large percentage of cells expressing procyclin on the surface (88%). The active VSG-ES promoter selectively relocates to the nuclear envelope 5 h after differentiation [54]. At the same time, the extra-nucleolar pol I ESB is no longer detected (Figure 3). An eventual repositioning of all inactive ES promoters to the nuclear envelope also occurs, as shown by FISH experiments [44] and by GFP tagging of a single ES promoter in established procyclic trypanosomes [54]. Such nuclear repositioning upon early differentiation to the procyclic form precedes full VSG downregulation because VSG mRNA is still detected in this strain, as described elsewhere [62]. During the transformation of dividing slender forms into non-replicative short stumpy bloodstream forms, an analysis of VSG-ES transcription showed transcripts from many inactive ESs, suggesting the loss of monoallelic control of the active ES [39]. However, nascent nuclear RNA labeling suggested that short...
stumpy transformation results in an overall reduction of transcriptional activity. The authors conclude from run-on data that pol I is still associated with the promoter-proximal region of the active ES, thus establishing a decreasing transcriptional gradient. Selective repositioning of the active ES promoter to the nuclear envelope (in contrast to inactive ESs at early differentiation) could explain the loss of monoallelic control (Figure 3).

Using GFP tagging of chromosomes, it was also observed that after differentiation only 8% of GFP-positive nuclei showed a detectable GFP–lacI dot, whereas detection in control loci was unaffected. This suggests that GFP–lacI binding might reflect changes in chromatin condensation. This supports previous findings regarding the accessibility of ES chromatin in the bloodstream form and its subsequent inaccessibility upon differentiation. Recently, a chromatin decondensation event upon gene activation was detected in mammalian cells using the GFP–lacI system.

Further work will be necessary to elucidate whether T. brucei mechanisms of ES nuclear repositioning are a cause or a consequence of developmental chromatin silencing. Recent work by the Gasser laboratory has shown that SIR-mediated repression can occur independently from nuclear envelope repositioning in a yKu mutant. However, it cannot be ruled out that temporal nuclear repositioning is required to establish silencing. In this context and similar to yeast, TbKu80-deficient trypanosomes are unable to halt VSG-ES developmental transcriptional silencing. However, no information on possible nuclear repositioning is available for this mutant. TbSIR2RP1 localizes to the nuclear periphery together with telomeric sequences. However, transcription of a reporter gene located downstream from the ES promoter was unaffected by TbSIR2 disruption upon differentiation to the procyclic form, indicating that TbSIR2 is unlikely to have a direct role in the developmental control of the VSG-ES (M. Hoek, PhD thesis, Rockefeller University, 2001). These data suggest that trypanosome pol I-mediated transcription of the VSG-ES is silenced by a TbSIR2-independent mechanism. In addition, ES promoters in large episomes that lack telomeres are effectively downregulated, indicating that silencing of ES promoters in the insect stage of T. brucei, although influenced by the genomic context, is not dependent upon telomere sequences. The important new conclusion from these studies is that the rapid and dramatic repositioning rate of the active ES promoter to the NE can be detected early in the differentiation process, suggesting that temporal nuclear periphery repositioning contributes to the establishment of VSG-ES silencing (Figure 3).

**Concluding remarks and future perspectives**

Nuclear repositioning of chromosomes has an important role in accomplishing proper control of gene expression in eukaryotes. Correct timing in the positioning of chromosome sites and association with particular nuclear structures is necessary to coordinate and achieve gene...
regulation. Recent findings in trypanosome nuclear chromosome dynamics suggest that these phenomena are extremely conserved because *T. brucei* is in a distal position in the eukaryotic cell line. It is clear that NE relocation affects RNA pol II transcription and, furthermore, this relocation also influences pol I transcription in *T. brucei* [54]. This suggests that nuclear repositioning has a global role in the regulation of transcription.

It now seems clear that procyclin gene transcription of all allele variants is unnecessary for association with a single extra-nuclear body [54], in contrast to the monoallelic expression of the VSG-ES segregated in a single ES body [23]. Upon differentiation from the bloodstream to the procyclic stage, the active VSG-ES promoter undergoes a rapid repositioning to the nuclear periphery with concomitant loss of the ES body. After repositioning, the active ES promoter undergoes chromatin condensation (Figure 3). We favor a model based on current data that suggests the existence of two mechanisms for VSG-ES regulation influenced by nuclear position. Firstly, a single ES is activated by the ES body in a recruitment-dependent manner, which enables monoallelic expression and transcriptional switching by ES replacement with a transcriptionally competent inactive ES. Secondly, during differentiation, the ES promoter repositions to the NE and this repositioning is followed by chromatin condensation.

We have discussed several models for VSG-ES regulation, however, these do not represent mutually exclusive mechanisms; rather, they might exist concomitantly. Notwithstanding recent findings, many questions remain to be answered, some of which are listed in Box 2. The combination of GFP chromosome tagging with high-resolution fluorescence microscopy and functional analysis is likely to provide new insights concerning the mechanisms that underlie transcriptional regulation and antigenic variation in *T. brucei*.

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**Box 2. Unanswered questions**

- Are there any *cis*- or *trans*-active sequences required for monoallelic expression?
  - Certain sequences enable a single VSG-ES to be expressed either in *cis* (linked to the active VSG-ES), or in *trans* (a unique enhancing sequence located in a different chromosome). The latter is similar to the situation described for olfactory receptors [60].

- Where are the individual telomeres containing VSG-ESs located in the nucleus?
  - To answer this question, GFP tagging of chromosomal telomeric sites will probably be required.

- Is there any difference between the RNA pol I machinery that is involved in rDNA and the one involved in VSG-ES and/or procyclin transcription?
  - It would be necessary to identify specific transcription factors that promote VSG and/or procyclin transcription, in addition to those that regulate the RNA pol I core complex involved in rRNA production.

- Are there any VSG-ES-specific factors?
  - These would include ESB-specific transcription factors (for initiation, elongation or RNA maturation) and ESB structural components (if any) that enable the body to be structurally coherent and singular.

- How does VSG-ES developmental silencing occur in *Trypanosoma brucei*?
  - Do nuclear envelope repositioning and chromatin condensation involve novel proteins that are different to those described in yeast?
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