

Regulation of *vsg* expression site transcription and switching in *Trypanosoma brucei*

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Abstract

Current understanding of expression-site transcription in *Trypanosoma brucei*, has been refined by recent results of promoter manipulations at *vsg* expression sites (ES) and examination of the behavior of ES promoters in ectopic locations both within the ES and at other loci. In summary, ES promoter sequences inserted into non-transcribed rRNA spacers are generally inactive, or have low activity, in bloodstream and procyclic forms. Some mechanism apparently operates to ensure full activation of a single ES in bloodstream-form trypanosomes and the inactivity of all ES promoters in procyclic forms. As previously shown, a rRNA promoter can replace an ES promoter. In bloodstream forms, the replacement rRNA promoter was down-regulated in a 'silent' ES but it was active in procyclic forms [1,2]. In addition to manipulations of endogenous promoters, we have recently shown that, when an ES promoter is replaced by a T7 promoter, the T7 promoter is unregulated but transcription is attenuated before the *vsg*, and another ES switches on to maintain cell viability. However, T7 transcription is repressed in the context of core ES-promoter sequences in both stages, particularly in procyclic forms. These observations strongly argue that sequences in the vicinity of the ES core promoter play a role in ES control by nucleating critical events in silencing as well as in activation. Deletions of sequences surrounding the ES core promoter, in situ, did not affect its activity or regulation. In bloodstream forms, rRNA or ES promoters inserted adjacent to silent telomeres or to a non-telomeric 'basic-copy' *vsg* were > 98% repressed [3,4]. After transformation to procyclic forms, the sub-telomeric rRNA promoter regained about 10% of its maximal activity but the 'basic-copy' rRNA promoter was fully active. Similarly-positioned ES promoters remained silent in procyclic forms. These results suggest that telomere-proximal or *vsg*-proximal sequences might mediate suppression of transcription via position-effects that could be sufficient to suppress the expression of chromosome-internal *vsgs* or telomeric metacyclic *vsgs*, in bloodstream-form trypanosomes. Recent experiments with T7 promoters indicate that sequences within the ES core promoter might be responsible for silencing ES promoters in procyclic forms. Precedents for regulatory mechanisms that modulate transcription over large chromatin domains are reviewed and possible models for ES regulation are presented. © 1998 Francqui Foundation. Published by Elsevier Science B.V. All rights reserved.

Keywords: *Trypanosoma brucei*; Antigenic variation; Variant surface glycoprotein; Gene regulation; Transcription; Chromatin structure; T7 polymerase

Abbreviations: bp, base pairs; CTR, co-transposed region; ES, expression site; *esag*, ES-associated gene; TPE, telomere position effect; Vsg, variant surface glycoprotein; *vsg*, Vsg gene.

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1. Introduction

The genome of *Trypanosoma brucei* encodes hundreds of variant surface glycoprotein (Vsg) genes (*vsgs*) or pseudogenes. The actual number is probably quite variable and largely irrelevant. The generally quoted figure of 1000 *vsgs* is an estimate derived from the proportion of clones in a cosmid DNA library that reacted with a probe for the canonical '70-bp' repeats that have been linked to all *vsgs*, except for some that are expressed in metacyclic trypanosomes [5]. It is (or should be) a great source of embarrassment that we (collectively, the laboratories studying this phenomenon) have little idea of the variation in the total number of *vsgs*, or the full range of their diversity, or their organization in the genome, beyond the observation that they are clustered in cosmid clones [5]. The first clear step towards improving this situation, the mapping of chromosome I of *T. brucei* strain 927 by Sara Melville and her colleagues, has produced evidence for an arrangement of *vsgs* that is striking and, if true for other chromosomes, has major implications for how we think about the regulation of antigenic variation. The available data (S. Melville, personal communication), which suggests that *vsgs* are clustered close to the ends of chromosome I, is based on mapping *vsg*-specific probes onto a library of P1 clones that form three 'contigs' covering most of chromosome I. These clusters of *vsgs* and separate clusters of *ingi* retrotransposon-like elements [6–8] could account for much of the enormous size variation between sister chromatids, indicated by gel electrophoresis of chromosomal DNA. The sizes of sister chromatid DNAs of chromosome I vary from 1.1 to 1.2 Mbp in strain 927 and from 1.6 to about 4 Mbp in strain 427 (S. Melville, personal communication). This chromosomal arrangement could also account for the 'haploid' state of most silent *vsgs* that have been studied.

The expressed *vsg* is always located adjacent to a telomere, only 1–3 kbp upstream of the TTAGGG hexameric telomeric repeats, although at least one additional gene unit can be inserted between the *vsg* and the telomere without apparent phenotype [4]. All telomeres so far identified contain *vsg* 'expression sites' (ES). There are two

major ES subsets: those that are transcribed in metacyclic forms, in the tsetse salivary gland, and those that are transcribed in the mammalian stage of the life cycle, the so-called bloodstream forms. Bloodstream-form ESs are polycistronic transcription units containing about eight ES-associated genes (*esags*), transcribed from a promoter located 30–50 kbp upstream of the telomere, but metacyclic ESs are transcribed from a promoter immediately upstream of the *vsg* [9,10] and appear not to contain functional *esags* or the large arrays of 70-bp repeats that are found between the *vsg* and the upstream *esag1* in bloodstream-form ESs [11,12]. There are approximately 20 of each type of ES. The precise number is probably variable and unimportant, but their different structures probably reduces recombination between them and preserves them as two distinct subclasses. This distinct organization may be particularly important for maintaining the *vsg* diversity expressed by metacyclic forms. Most of our impression of bloodstream-form ES organization is based on the detailed characterization of one ES [13].

2. Defining the problem

Vsg switching occurs as a result of two fundamental processes: concerted change in the transcriptional status of a pair of ESs, involving silencing of one with activation of another, or recombination events, which allow silent *vsgs* to move to an active ES. Recombination events can either be tidy, whereby 'chromosome-internal' 'basic-copy' *vsg* cassettes are shuttled into the active site, by a process of duplicative transposition akin to gene conversion, or they can be less specific, as when there is duplicative transposition of large silent telomeric regions into the active ES, or reciprocal recombinations, at random regions of homology, between active and silent telomeres.

A major focus of current investigations is the regulation of ES transcription. The central problem is how to account for the apparent exclusiveness of ES transcription, which appears to be the key to the ordered expression of *vsg* genes. There is a second related question with implications for

the first: what is the mechanism responsible for the developmental regulation of ES transcription? Finally, we must consider what mechanisms silence the majority of *vsgs* that are not in ESs, but whose chromosomal organization remains unclear. Some of these silent *vsgs* may be associated with putative promoters [14]; others may need to be insulated from readthrough transcription by RNA polymerases initiating at remote promoters.

Before we consider potential mechanisms that could regulate the exclusiveness of ES transcription, we should reconsider the evidence for the tenet that ES transcription is exclusive. There is only one documented example of the simultaneous expression of two *vsgs*, from different bloodstream-form ESs, and the double-expressing cells were quite stable in culture but unstable in animals, reverting to the expression of one of the two doubly expressed *Vsgs* [15]. This observation implied that two ESs can be transcribed simultaneously but, on reconsideration, this cannot be concluded from the available data. Given that *Vsg* has a half-life of 30–40 h and *vsg* mRNA has a half-life of 4.5 h [16–19]), cells that express two *Vsgs* could easily be switching from expression of one to the other less than once per cell division. On the other hand, we cannot ignore the possibility that expression of multiple *vsgs*, from multiple ESs, is a common occurrence. The large *vsg* repertoire, and the relatively few reagents available to explore multiple expression, have limited our ability to pursue this question. A recent report of multiple *vsg* cDNAs, cloned from an apparently antigenically homogeneous population of *T. brucei* [20], needs to be accommodated by any hypothesis that purports to explain *Vsg* exclusiveness.

In the immunocompetent host, failure to cease transcription of the previous *vsg* would be fatal: double-expressing trypanosomes would not benefit from a switch, but, for several generations, would presumably remain susceptible to antibodies against the original *Vsg*. Thus, there must be strong selection in vivo against trypanosomes that do not switch decisively. As we will describe later, this situation does not exist in culture, and failure to stably switch is observed in some clones [21].

3. Precedents for regulating transcription of multiple alleles

There are several situations in which eukaryotic cells repress, or silence, the expression of multiple alleles. The parallel that has been most frequently drawn, for trypanosomes, is mating-type switching in yeast. Another phenomenon that has been well described in yeast, which shares several components with HML/HMR silencing, is the suppression of promoters close to telomeres. This so-called telomere position effect (TPE) is metastable and depends on promoter strength and distance from the telomere [22,23]. Almost the same set of components is involved in TPE and HML/HMR silencing, except that *SIR1* (silent information regulator gene 1) is not required for telomeric silencing. Both HML/HMR silencing and TPE appear to depend upon alterations in chromatin structure, either heterochromatinization or some telomere-specific chromatin structure, but neither is completely understood, although most of the key components and their interactions have probably been defined genetically [24–26]. Unfortunately, we're a long way behind in understanding the genetics of chromatin structure in trypanosomes. Some significant observations have been made. There are differences in chromatin structure between bloodstream-form and procyclic-form trypanosomes [27], in nuclease effects on active and silent ESs [28,29], and in the rate of elongation of telomeres at active and silent ESs [30]. There is also a novel base modification, β -D-glucosyl-hydroxymethyluracil, which exists in bloodstream forms but not in procyclic forms [31]. Although the existence of this modification was discovered because of differences in restriction endonuclease sensitivity of *vsgs* in active versus silent ESs [32,33], most of the modification is found in the telomeric repeats [34] and it is not clear if its abundance differs when ESs are silent or transcribed. One thing is clear, however. If the modification is related to ES silencing in bloodstream forms, it cannot be involved in ES silencing in procyclic forms.

In diploid mammalian cells, genomic imprinting and allelic exclusion are widespread phenomena that can be attributed to differences in DNA

methylation that are heritable [35–37]. CpG methylation also specifies a chromatin structure that is resistant to site-specific immunoglobulin V(D)J recombination [38]. It has been suggested that the differential methylation in imprinted genes is achieved by a dynamic mechanism that senses gene dosage and adjusts methylation accordingly [39].

The most striking example of allelic control in mammals is X-chromosome inactivation, which silences most or all genes on one of the two X chromosomes in female cells. X-inactivation appears to be mediated in *cis* by a mechanism that is not fully understood. The product of the *Xist* gene is a non-coding RNA that interacts with the X-chromosome from which it is transcribed [40,41]. The interaction starts at the X-inactivating center, *Xic*, which is within the *Xist* locus, then spreads throughout the chromosome. X-chromosome regulation is considered to be a counting problem. How does the cell count identical elements and inactivate all except one? The parallel to ES regulation is intriguing. In *Drosophila*, males compensate for having only one X chromosome by up-regulating its transcription. This requires *trans*-acting proteins, encoded by four *msl* (male-specific lethal) genes, that associate along the length of the X chromosome, together with non-coding RNAs that can act in *cis* or in *trans* [42,43].

Thus, there is an abundance of precedents that may apply to ES regulation but, as in other situations, trypanosomes may have devised an altogether novel mechanism, whose elucidation will continue to challenge us.

4. Models for positive or negative ES regulation

Although common observation argues against the existence of a single mobile ES-activating element, we cannot discount the possibility that, along with the many gene rearrangements occurring in this system, there could lurk the occasional duplication of a single mobile promoter, or other mobile activating element. However, this hypothesis can probably be discounted. A related hypothesis would be that some form of chromosomal

rearrangement, either gross or subtle, occurs during ES activation and inactivation events. Although sporadic chromosomal rearrangements have been reported after ES switches ([44–46]), this issue has not been systematically studied. Characterization of panels of switches, to be discussed in more detail below, show conclusively that ES switching can occur without detectable DNA rearrangements.

As new tools yield better insights into the complexity of nuclear organization in other systems, the long-standing idea that there is a single nuclear transcription site or other physical site-specific effect, at which only one ES transcription complex can be assembled or stabilized, merits renewed investigation. A variation on the single nuclear site model is that ES activation, which likely involves a major reorganization of chromatin structure, requires a critical concentration of a key activator or a series of factors that are present in limiting amounts in the nucleus. The activation of one ES would be a stochastic event, but could be influenced by subtle differences in DNA sequence at the activation/nucleation site, which could cause some ESs to predominate. Because none of the putative participating factors have been identified, this is a somewhat vague concept, but it does make predictions that will be testable in the foreseeable future.

The idea that the default ES state is off and activation requires positive intervention is implicit in the foregoing models. The silent state is not necessarily a passive state, but probably involves specific silencing factors, as in other systems, from which any silent ES can occasionally escape. As already noted, ES silencing and activation appear to be closely coordinated events, suggesting direct crosstalk between ESs. The activated ES could secure its situation by expressing a transcription or translation product that acts in *trans* to tighten the silencing of other ESs. We are currently exploring testable predictions of this hypothesis. If it were true, all manner of situations that interfered with efficient transcription of the currently active ES might cause an ES switch, including the insertion of transcription attenuators or other gratuitous DNA rearrangements, or any cellular event or extracellular intervention that allowed a nega-

tive regulator to decay. Variations in ES promoter strength, leading to differences in the efficiency of the presumed specific initiation of ES transcription, would be expected to influence ES dominance and switching rates.

Most available evidence is circumstantial and is compatible with any of these models. Once activated, ES transcription is pretty stable. Certain disruptions of the ES reduce stability or cause a rapid switch (see later and [47]). Metastability of old and new ESs can exist after a switch in vitro [21]. Differential ES stability, or preference for activation of one ES over another, could involve subtle mutations that could affect activation, repression, nuclear localization, or changes in ES products that nucleate chromatin silencing.

Vsg itself is one ES product that, it has been suggested, could select against multiple ES transcription. Although this could select, it is difficult to see how it could regulate. The dimeric Vsg three-dimensional structures are thought to be highly conserved [48]. The question of whether simultaneous expression of two VsGs is disadvantageous for replication or infection of trypanosomes, or whether constraints intrinsic to Vsg synthesis or coat assembly might enforce the exclusiveness of *vsg* expression, has been the subject of continuing speculation [49–54]. We explored this question experimentally, by inserting a second *vsg* into an active ES, and concluded that parasites expressing two VsGs have no intrinsic growth disadvantage in vivo or in vitro [55].

5. DNA sequence requirements for ES promoter function

There is strong but indirect evidence that the *parp* and ES loci are transcribed by RNA Polymerase I (Pol I) ([56]). However, neither the core polymerase subunits nor any accessory transcription factors have been identified, so it is not known whether the three promoters are recognized by the same DNA-binding factors, despite the lack of conservation of their DNA sequences. A 315-bp region containing the core ES promoter can be replaced by a 518-bp fragment containing the rRNA promoter in situ. The remodeled ES

remains capable of activation and inactivation in bloodstream forms, but is not fully silenced in procyclic forms [2]. Although the sequences of the core rRNA, bloodstream-form ES, metacyclic-form ES and *parp* promoters are quite distinct [10,57,58], hybrid rRNA-*parp* [59] or rRNA-ES promoters [60] can drive reporter-gene expression. In procyclic trypanosomes, a silent chromosomal ES promoter could also be activated by fusing it to a sequence that had no intrinsic promoter activity, but was derived from upstream of the *parp* promoter [61]. These observations suggest an interplay between positive and negative regulatory elements but provide no direct evidence to illuminate the real requirements for regulation of transcription from the ES or *parp* promoters.

Several studies have demonstrated that the minimal DNA sequence required for ES promoter function is short [45,62–64]. An upstream element, which enhances transcription from the rRNA and *parp* promoters [65,66], is apparently unnecessary for ES promoter activity. Dissection of the essential sequence elements for ES promoter activity suggests that the same elements are necessary for transcription-initiation in plasmid and chromosomal contexts [60,64]. Both bloodstream and procyclic forms are able to initiate transcription from an episomal ES promoter, but promoter efficiency, relative to the endogenous locus, is unknown. Early attempts to identify ES promoter-specific double-stranded DNA-binding proteins were unsuccessful [60], although this situation has recently changed [67].

None of these studies were performed on the ES promoter in situ in bloodstream-form trypanosomes. The ES region flanking the core promoter is highly conserved. We decided, therefore, to make deletions of these flanking regions and look for effects on ES transcription regulation in both bloodstream and procyclic forms. No *cis*-acting regulatory sequences that affected ES promoter activity in situ were found in the regions upstream (–64 to –729) or downstream (+8 to +962) from the transcription start site, in either active or silent ESs in bloodstream-forms, or in procyclic forms (M. Navarro and G.A.M. Cross, submitted for publication). These deletions also had no effect on ES switching rates.

6. Gene transfection in bloodstream-form *T. brucei*

The early generations of ES studies, which occupied the 1980s, were highly informative about *vsg* and ES organization but were largely limited to observation rather than experiment, and were performed on several strains of *T. brucei*. The essentially complete sequence of one ES was determined [13], in an effort that was largely responsible for identifying a series of *esags* whose structures tantalize us but whose functions remain largely unknown, with the spectacular exception of the heterodimeric transferrin receptor encoded by *esags* 6 and 7 [68–70]. However, we do not know how widely conserved this prototypical ES structure is.

Arguments about switching rates, the relative merits of laboratory and recently transmitted strains, and the question of whether some ESs are favored over others and, if so, what determines which shall predominate, have persisted, in the absence of rational experiments or common understanding of the issues involved. In the past few years, however, we have developed the ability to genetically manipulate the ES. Insertion of reporter genes into active or silent ESs allows us to capture panels of specific switching events, to measure ES switching frequencies, to explore intrinsic and extrinsic parameters that might affect switching, to more readily map switch-related chromosome rearrangements, and to more easily study cellular and genetic aspects of the developmental regulation of *Vsg* expression. Hopefully, we will be able to extend these techniques to identify genes that regulate these phenomena.

Despite early resistance to attempting transfection in bloodstream forms *in vitro*, there really was no alternative for trying to understand antigenic variation. We were therefore pleasantly surprised to discover, when we tackled this problem, that bloodstream forms have several advantages over procyclic forms. The doubling time of bloodstream-form *T. brucei* strain 427 is 6–8 h, compared to 12–16 h for procyclic forms, and bloodstream forms can be easily grown on agar [71–73]. They can grow from

single cells with no special nurturing. They have no minimum density requirements and no need for conditioned medium. They have a 10- to 20-fold higher drug sensitivity and sensitive cells die quickly. They retain full animal infectivity and can easily be grown in large quantities. Some selective drugs can be used *in vivo* [74,75], allowing large populations to be screened for switches or mutations. They can be easily transformed to procyclic forms, whereas the reverse is not possible without using tsetse. The only disadvantages we have encountered are that electroporation kills a higher proportion of target cells, therefore the overall transfection efficiency is lower, and stable episomal vectors are not available. These last two problems combine, currently, to rule out the possibility of genetic complementation by transfection in bloodstream forms, although this can be achieved in procyclic forms [76].

7. ES mapping during multiple independent and consecutive switches

As a starting point for a new generation of genetic studies, we decided to revisit the question of whether chromosome rearrangements could be consistently detected during ES switching. We used two approaches to insert selectable markers into silent ESs. We could place a reporter cassette at any position in a silent ES if we included a promoter and a downstream selectable marker, to enable the recombinant cells to be recovered [3]. We had some concern that the necessary inclusion of a promoter in this cassette could influence the parameters, like switching rate, that we sought to study. We found no evidence of this. The other approach was to use very low drug concentrations to insert a promoterless drug-resistance gene downstream of the endogenous ES promoter, relying on the low level of transcription initiation at a 'silent' ES promoter [1,75]. Again, this approach could be criticized for selecting cells with incompletely silenced promoters, but this criticism could be dispelled by inserting the cassette into an active ES, then turning the ES off and sim-

ply measuring the reporter transcript without selection. We found no significant difference in the 'off' activity regardless of whether the initial insertion was made into a silent or an active ES (M. Navarro and G.A.M. Cross, unpublished observations).

Accurate measurements of ES activation rates could be made with positive selectable markers (encoding drug-resistance genes) inserted into ESs. The initial measurements [21,75] held no surprises: 'neutral' ES insertions showed activation frequencies in the range of 10^{-6} , as previously noted for 'off' rates in the same clones [77]. As will be discussed below, other ES manipulations could dramatically increase the switching rate.

In one study, restriction mapping within and beyond the ES failed to detect any DNA rearrangements in multiple independent identical switches [21]. In another study [75], we mapped independent consecutive on-off-on switches between two specific ESs. Direct analysis of ES promoters, showed that their numbers (average 27 ± 3) varied. The first activation of the 121 ES was always associated with deletion of the upstream tandem promoter in this ES, but no further rearrangements were detected in consecutive off/on switches of this ES. It is interesting to note that an identical deletion of a duplicated promoter in the same ES had previously been characterized in great detail during inactivation of this ES [45,46]. However, in another set of experiments, we detected no rearrangement in the same duplicated promoter region following inactivation of this ES in nine independent clones [21]. These and related observations suggest that recombination might occur at a significant frequency between the highly conserved sequences around ES promoters and we suggest that, at least in some cases, ES switching may occur after a period of chromosomal interactivity that may or may not leave tangible evidence in the form of detectable sequence changes.

The overall conclusion from these new studies is that chromosomal rearrangements are not essential for ES switching, which confirms established prejudice based on more limited data.

8. Telomere stability at active and silent ESs

Changes in telomere length have been reported to be associated with ES switching [78]. We measured the telomere restriction fragment length, at several silent and activated ES, in sets of clones that had or had not undergone ES switching [21]. The data showed that silent telomeres were relatively stable but an actively transcribed ES telomere was unstable, as was also previously shown for a rRNA promoter in a mini-chromosome [79], and the length becomes heterogeneous within the cell population during clonal growth. Cloning, therefore, involves selection of cells with different telomere lengths rather than inducing shortening, as previously suggested [30,80].

In some organisms, telomeres appear to interact with each other and with the nuclear envelope [81]. One model for ES switching would have the ESs interact physically during the switch process, leading to end exchange, which could be appealing if telomeric chromatin structure were responsible for silencing and this state could be transferred by end exchange. This does not appear to be the case, however, as we saw no increases in telomere length, in any of the switched clones, beyond what would be expected during normal growth [30,80]. Other observations suggest that there is no relation between telomere length and ES switching, except in one case where a telomere became extremely short and could neither grow nor be activated (D. Horn and G.A.M. Cross, unpublished observations).

9. Is there a role for telomere-induced silencing in antigenic variation?

We found strong suppression of ES, *parp* and rRNA promoters placed in the immediate vicinity of a silent telomeric *vsg* in bloodstream-form trypanosomes [3,4]. Repression was reduced about 10-fold when the rRNA promoter cassette was moved 14 kbp further upstream, but it was not abolished at this distance, as it would have been in yeast. It seems entirely possible that this 'telomeric' silencing would be sufficient to minimize transcription from metacyclic promoters in blood-

stream forms, but repression nucleated by changes in telomeric chromatin structure would be unlikely to be responsible for silencing of far-upstream ES promoters.

How are non-telomeric *vsgs* maintained in a silent state? It seems likely that most *vsgs* are organized in large clusters [5], sometimes associated with *ingi* retrotransposon-like elements [6–8]. Their location and organization could simply leave them promoterless and insulated from readthrough by the trypanosome Pol II, which seems to transcribe polycistronically over long distances. On the other hand, these *vsgs* may lie in regions of chromatin that are actively silenced, perhaps in the immensely variable subtelomeric regions of the chromosome (S. Melville, personal communication). rRNA or ES promoters inserted adjacent to a non-telomeric ‘basic copy’ *vsg118* had < 5% or undetectable activity, respectively, in bloodstream forms [4]. This also suggests that a *vsg*-associated or a promoter-associated sequence may be important for silencing. Sub-telomeric repeats, 29-bp motifs, or telomeric TTAGGG repeats, are not found at this locus [82], but conserved octamer and hexadecamer sequences are found 3′ of all *vsgs* [83].

In the same study, after transformation to procyclic forms, the activity of a telomere-proximal rRNA promoter increased to about 10% of its maximum level, but the ES promoter remained inactive. At the ‘basic-copy’ *vsg118*, the ES promoter regained about 15% activity but the rRNA promoter was completely de-repressed. Previous studies, in procyclic forms, had shown no evidence of telomere-dependent silencing of the *parp* promoter on small linear artificial chromosomes [84] or on an endogenous mini-chromosomal rRNA promoter lying about 7 kb upstream of telomeric repeats [79]. However, although these cells were resistant to high levels of G418, the steady-state level of *neo* mRNA was significantly lower than when the same cassette was integrated into a 2-Mbp chromosome, so some telomere-associated suppression may have occurred on the artificial chromosomes.

At present, we cannot reconcile our observations of ectopic ES promoters with those reported elsewhere [85], which showed that an ES promoter

inserted into a rRNA untranscribed spacer could be active in both bloodstream-form and procyclic-form trypanosomes. The ES promoter sequences used in the two studies are subtly different, which may account for the differing results. Also, it’s not entirely clear how active rRNA promoters are in ectopic locations, in general. Recent experiments (M. Navarro and L.E. Wirtz, unpublished observations) suggest that rRNA promoters are significantly repressed in bloodstream forms and are up-regulated in procyclic forms.

Our current conclusions are that ES promoters and rRNA promoters inserted into bloodstream-form ESs are restrained by position effects related to their proximity to telomeres, *vsgs*, or other features of the ES, or by virtue of some counting mechanism relying on Pol I core promoter elements themselves. The manifestation of repression could be due to restraints on transcription initiation and/or elongation. In procyclic forms, inserted rRNA promoters appeared to be fully de-repressed, except at a telomere, but ES promoters remained silent in any context, which we attribute to the absence of developmentally regulated ES promoter-specific factors or to promoter-specific active silencing, rather than a position effect.

10. ES alterations that affect switching rates

Disruption of the co-transposed region (CTR), immediately upstream of the expressed *vsg*, can cause a dramatic increase in the rate at which the ES is switched off and a new ES is switched on [47]. Deletion of most of the CTR in two ESs caused a greater than 100-fold increase in the rate of ES switching. Other insertions into an active ES also increased the switch frequency. A more dramatic effect was observed when the entire CTR and the 5′ coding region of the expressed *vsg221* were deleted. In this case a new ES was activated within a few cell divisions. The low transfection efficiency, and the continuous growth in the presence of drug selection, ensured that the selection of a sub-population from a contaminant in the starting cell line would be highly improbable. All of the observed switches occurred without

additional detectable DNA rearrangements in the switched ES. Deletion of the 70-bp repeats or a *vsg* pseudogene upstream of the CTR did not affect ES stability. Several speculative interpretations of these observations are possible, the most intriguing of which is that the CTR plays some role in modulating chromatin conformation at an ES. An alternative and perhaps more likely explanation is that the disruption caused a rather non-specific effect on transcription through the ES, which allowed (and required) another ES to be activated.

The other situation in which we have observed an essentially immediate ES switch is upon replacement of the active ES promoter with the T7 promoter, or insertion of a T7 promoter upstream of the active ES promoter. Both of these manipulations, for reasons that we can speculate about, appear to inactivate the ES, although the T7 promoter itself is fully active in this context (M. Navarro and L.E. Wirtz, manuscript in preparation). Once again, it was striking that the cells did not die but switched. All of these observations lead irresistibly to the conclusion that disabling an ES, by various interventions, can permit the cells to switch to an alternative ES. The switches can only be detected when the drug-resistance gene, associated with the targeting cassette, is transcribed to the necessary level at its insertion site. With the associated T7 promoter, which is fully functional, this is no problem. One possible explanation of these observations would be that some product of ES transcription, either RNA or protein, either stabilizes, in *cis*, the active ES or stabilizes, in *trans*, the repression of inactive sites. Another explanation is that deletion of these elements from the active ES could cause it to vacate a subnuclear transcription site and allow another ES to occupy it.

11. Stability of the switched state

In yeast, silencing of HML or HMR loci can be split into three distinct phases, establishment, maintenance and inheritance [25], and this provides a good paradigm for the ES. Establishment is the genetic switch; separate, additional or over-

lapping components may be necessary to maintain the silent state; inheritance of the silenced state in the replicated chromosome may involve considerations additional to those involved in establishment and maintenance [25]. We have evidence that not all progeny clones may have the same stability for some time after a switch. After drug selection, which ensured that the switched populations were initially pure, some clones became heterogeneous after further growth in the absence of drug [21]. Examination of individual cells, with antibodies to VsGs, showed the presence of cells expressing both parental and progeny Vsg, and cells that had reverted to expressing only the parental Vsg. Such a situation would not be seen after switches in immunocompetent animals because unstable switches and revertants would be strongly selected against.

12. Using the T7 promoter as a probe for epigenetic control at the *vsg* ES

The recent recognition of chromatin remodeling as a major modulator of transcription, in higher eukaryotes, has emphatically refocused the problem of transcription regulation at the level of template accessibility [86–90]. The interaction of the single-subunit bacteriophage T7 polymerase with its promoter has been used in other systems as a reporter for *cis* elements mediating chromatin accessibility [91,92] (Fig. 1). We have used chromosomally integrated T7 promoters, with linked luciferase reporters, to investigate sequence requirements of epigenetic control mechanisms at ESs. T7 transcription provides a means of overcoming the difficulty inherent in distinguishing silenced from mutationally inactivated promoters, by separating the obvious role of ES core promoter elements in transcription initiation from any role they might have in silencing. Two targeting strategies were used to replace an active ES promoter by a T7 promoter. In the first, the entire ES promoter was replaced with the concise 20-bp T7 promoter. In the second, the T7 promoter was flanked upstream by an ES core promoter, intact except for a start site mutation to block elongation by the endogenous polymerase. The inte-

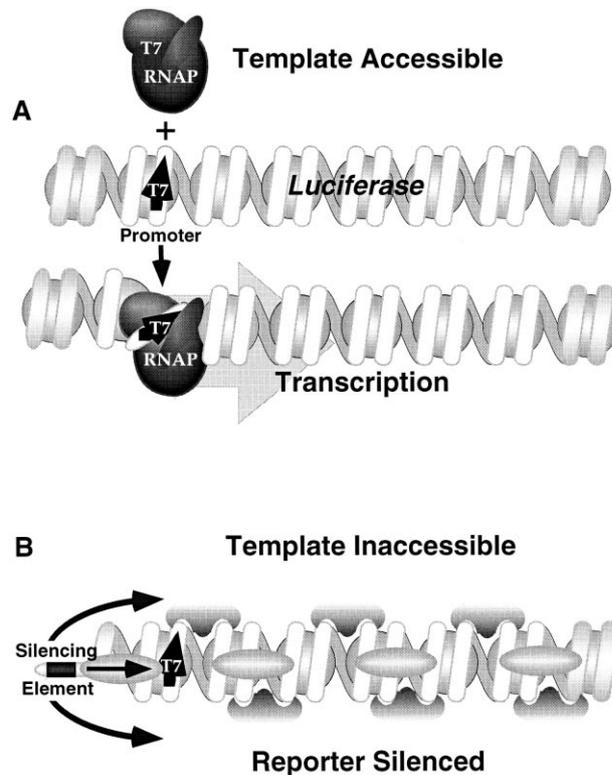


Fig. 1. T7 transcription as a reporter for *cis* elements mediating silencing of chromatin domains. (A) T7 promoters integrated into trypanosome chromatin can direct efficient reporter gene expression when the phage polymerase is expressed endogenously (L.E. Wirtz and G.A.M. Cross, manuscript in preparation). (B) Regulatory strategies relying on silencing or heterochromatinization that impact the accessibility of the polymerase to its promoter and template are reflected at the level of T7-mediated reporter activity, providing an experimental approach for the definition of *cis* elements influencing chromatin structure.

grated T7 promoter was down-regulated (strongly so in procyclic forms) only in the context of the ES core promoter elements, revealing a role for the core promoter in ES repression.

The overlap of the ES core promoter region with elements involved in silencing is suggestive of competitive or antagonistic interaction between activation and silencing mechanisms, consistent with chromatin structure analyses showing conservation of DNase hypersensitive sites between silent and active ES promoters (see below).

13. Support for a 'repressed and ready-to-go' model for ES transcription

There are previous findings suggesting at least a low level of transcription initiation occurs at more than one, and maybe many, ES promoters in bloodstream forms and, at a much lower level, from many ES promoters in procyclic forms [1]. The ratio of transcripts originating from the active versus silent ESs was about 33:2 in bloodstream forms. Secondly, there is the implication, from other results, that extensive tran-

scription of multiple ESs can occur in certain situations. In particular, multiple *vsg* transcripts were detected in a line of *T. rhodesiense* that was expressing the predominant *vsg* from a metacyclic ES [20]. However, this line may not represent a typical situation: it could represent a mutation leading to specific de-repression of telomeric *vsgs* driven by proximal promoters rather than far-upstream ES promoters.

Insertion of selectable markers into silent ESs has allowed us to detect ES promoter activity in other than the active ES (M. Navarro and G.A.M. Cross, Molecular Parasitology Meeting, Woods Hole, 1995). In some experiments, we tried to activate a *neo*-tagged silent 121 ES in cells where the 221 ES was active. We obtained G418-resistant clones that did not switch, but continued to express *vsg* 221. Thus, in these clones, two ES promoters were active while only one ES was fully transcribed, suggesting that productive transcription (defined as transcription that generates viable amounts of Vsg) requires more than initiation at the ES promoter.

The idea that facilitating or lifting restraints on elongation might be the regulating step in ES transcription has also been considered previously [1,2]. Perhaps the strongest support for the existence of such a mechanism comes from studies of *parp* (procyclin) transcription. *parp* transcription, like ES transcription, is thought to be a consequence of Pol I, but expression of *parp* and *vsg* are restricted to the opposite life-cycle stage. However, in bloodstream forms, the *parp* locus is transcribed to a significant extent, but elongation appears to be attenuated within 1 kbp of the promoter [93,94].

Early studies showed that chromatin adjacent to the transcribed *vsg* was hypersensitive to endonuclease [28]. Other studies, however, showed no evidence for different nucleosome structures between active and silent ESs [29]. In contrast, we find that nucleosome structure is conserved around active and silent ES promoters, but is disordered downstream. We also looked for chromatin alterations around the ES promoter that could be associated with *cis*-acting regulatory sequences. A marker gene, inserted 1 kbp downstream of an ES promoter, was used to map the position of nuclease hypersensitive sites, using an indirect end-labeling technique. Treatment with either DNase I or Micro-

coccal nuclease indicated the presence of a prominent hypersensitive site within the core ES promoter. This hypersensitive site was present in both active and silent ES promoters, suggesting that a protein complex is bound to the core promoter, irrespective of the transcriptional state. In addition, less intense nuclease-hypersensitive sites were detected upstream and downstream of the core promoter, in both active and silent ESs (M. Navarro and G.A.M. Cross, submitted for publication).

From these observations, and the conservation of nuclease hypersensitive sites at silent and active ES promoters, we suggest that all ES core promoters are occupied by a specific DNA-binding protein complex (Fig. 2). The same proteins could be bound to both silent and active ES promoters, with the outcome of their presence (activation or repression) depending upon other proteins or nuclear interactions. Alternatively, different proteins could bind to identical or adjacent sites within a silent or active promoter. Low-level transcription from a 'silent' ES promoter, as described above, could be a consequence of leaky silencing, leading to intermittent exchange of silencing and activating proteins in the promoter-bound complex. We are currently exploring these alternatives.

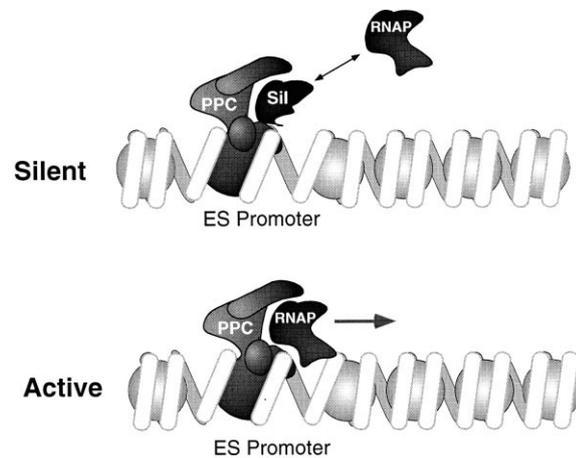


Fig. 2. Occupancy of the core promoter region by DNA-binding protein complexes at silent or active ESs: a speculative model of a transcriptional complex in bloodstream-form *T. brucei*. The promoter protein complex (PPC) bound to DNA may contain silencing factors (Sil) responsible for repression in silent ESs. Upon activation, PPC would recruit the RNA polymerase holoenzyme (RNAP) and, possibly, other activating factors.

14. Conclusions

We have considered several models for ES regulation, drawing parallels to other potentially relevant systems, but with an appreciation that these do not represent mutually exclusive mechanisms. Multiple mechanisms might work in concert. As suggested elsewhere [95], the activity of ES promoters might still be controlled by sequence changes far upstream of the regions that have been mapped. However, our recent analyses of multiple independent switches show that ES inactivation can occur without detectable rearrangements as far upstream as 200 kbp from the *vsg*, but we almost always see telomere length changes, for which a regulatory role has been suggested [78] in switched clones, but not restricted to the telomeres involved in the switch. Taken together, our experiments suggest, tentatively, that ES switching may occur after a period of subtle and infrequent chromosomal interactivity that may or may not leave tangible evidence in the form of detectable sequence changes.

There remains a strong probability that productive ES transcription is determined by a change in chromatin topology: maybe there is a unique subnuclear compartment in which the necessary components for ES transcription can assemble. Based on the possible involvement of Pol I in ES transcription, the nucleolus has been suggested as a candidate site [14]. However, many dispersed rRNA genes [96] are presumably transcribed, so it seems likely that the nucleolus can accommodate several transcription units on multiple chromosomes. Thus, the nucleolar location idea is not persuasive, although another unique subnuclear location may provide the basis for exclusiveness. Digestion with certain restriction endonucleases and single-strand-specific nucleases S1 and *Bal31* clearly distinguished active and silent ESs, even when transcription was interrupted [29]. The existence of single-stranded regions within the active ES was interpreted to indicate that the active ES was under torsional stress due to the telomere being constrained by attachment to a nuclear matrix structure. The nature of this attachment and whether it can account for the exclusiveness of ES transcription remains unknown.

The study of antigenic variation has moved from observation to experimentation. Recent experiments suggest several levels of regulation of *vsg* expression but have not defined the mechanisms that allow productive transcription from one among many ES promoters in bloodstream-form *T. brucei*. Many observations now suggest that ES switching is not accompanied by consistent or obligatory rearrangements of ES sequences and that more subtle chromosomal interactions are required to hand off transcription from one ES to another. Our ability to insert selectable markers into silent ESs allows us to measure *vsg* switching rates at the genomic level, and identify contextual factors that influence the sequence of *vsg* expression.

A great deal of further work will be necessary to elucidate the mechanisms of *vsg* regulation and chromatin silencing in *T. brucei*. Even in better studied systems, especially in yeast where genetic experiments are relatively simple, the mechanisms of silencing are not fully understood, although most of the key components have probably been identified. Whether we will be able to develop real genetic approaches to identifying key components of genetic regulation in trypanosomes remains to be discovered. Although there are hints that genetic selections could be developed, the obstacles remain daunting. Success might come sooner through knowing the complete inventory of the *T. brucei* genome, which would allow us to make more connections with other better characterized systems, via genomic analysis.

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