A Host–Pathogen Interaction Reduced to First Principles: Antigenic Variation in *T. brucei*

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Abstract Antigenic variation is a common microbial survival strategy, powered by diversity in expressed surface antigens across the pathogen population over the course of infection. Even so, among pathogens, African trypanosomes have the most comprehensive system of antigenic variation described. African trypanosomes (*Trypanosoma brucei* spp.) are unicellular parasites native to sub-Saharan Africa, and the causative agents of sleeping sickness in humans and of n'agana in livestock. They cycle between two habitats: a specific species of fly (*Glossina* spp. or, colloquially, the *tsetse*) and the bloodstream of their mammalian hosts, by assuming a succession of proliferative and quiescent developmental forms, which vary widely in cell architecture and function. Key to each of the developmental forms that arise during these transitions is the composition of the surface coat that covers the plasma membrane.

The trypanosome surface coat is extremely dense, covered by millions of repeats of developmentally specified proteins: procyclin gene products cover the organism while it resides in the *tsetse* and metacyclic gene products cover it while in the fly salivary glands, ready to make the transition to the mammalian bloodstream. But by far the most interesting coat is the Variant Surface Glycoprotein (VSG) coat that covers the organism in its infectious form (during which it must survive free living in the mammalian bloodstream). This coat is highly antigenic and elicits robust VSG-specific antibodies that mediate efficient opsonization and complement mediated lysis of the parasites carrying the coat against which the response was made. Meanwhile, a small proportion of the parasite population switches coats, which stimulates a new antibody response to the prevalent (new) VSG species and this process repeats until immune system failure. The disease is fatal unless treated, and treatment at the later stages is extremely toxic.

Because the organism is free living in the blood, the VSG:antibody surface represents the interface between pathogen and host, and defines the interaction of

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the parasite with the immune response. This interaction (cycles of VSG switching, antibody generation, and parasite deletion) results in stereotypical peaks and troughs of parasitemia that were first recognized more than 100 years ago. Essentially, the mechanism of antigenic variation in *T. brucei* results from a need, at the population level, to maintain an extensive repertoire, to evade the antibody response. In this chapter, we will examine what is currently known about the *VSG* repertoire, its depth, and the mechanisms that diversify it both at the molecular (DNA) and at the phenotypic (surface displayed) level, as well as how it could interact with antibodies raised specifically against it in the host.

List of Abbreviations

VSG	Variant surface glycoprotein
BF	Bloodstream form
PF	Procyclic form
BES	Bloodstream expression site
GC	Gene conversion
TE	Telomeric exchange
ESB	Expression site body
PolI	RNA polymerase I
PolII	RNA polymerase II
DSB	Double-stranded break

1 The VSG Coat and Its Roles in Immune Evasion

In its bloodstream form (BF), *Trypanosoma brucei* lives freely in the blood, lymph, and interstitial fluids of its host, fully exposed to both innate and adaptive immune responses. In that milieu, the VSG coat shields invariant components of the cell surface from the immune system, and elicits an antibody response against only the components of the coat that are altered by antigenic variation.

VSG molecules, which comprise the most abundant protein in BF *T. brucei,* accounting for about 10 % of the total protein content of the cell (Overath & Engstler 2004), are bound to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor and displayed on the cell surface as an array of 5×10^6 identical homodimers. This dense (12–15 nm thick) surface layer does not elicit production of opsonins or allow assembly of the membrane attack complex unless mediated by bound antibodies, thus evading the action of many innate immune components in the absence of an adaptive humoral immune response (Ferrante & Allison 1983; Black et al. 2010) and effectively functioning as a physical shield.

VSGs are highly immunogenic and elicit robust antibody responses, but their structure supports tight packing on the cell surface and shielding of all but a few epitopes. All VSGs consist of two domains: a long, highly variable N-terminal

domain, and a smaller, more conserved C-terminal domain to which the GPI anchor is attached (Blum et al. 1993). Early studies characterizing the VSG coat indicated that only a small surface region of the large VSG protein is exposed to antibodies and that only a small portion of antibodies generated against soluble VSGs are able to bind VSGs expressed on living trypanosomes (Barry 1979; Masterson et al. 1988). These observations, along with measurements of coat density and thickness, and later determinations of VSG crystal structures (Blum et al. 1993; Freymann et al. 1990; Chattopadhyay et al. 2005), have informed our current model of the larger structure of the VSG coat. In this model, individual VSG homodimers extend vertically from the cell surface, and close packing between homodimers prohibits antibody access to all surfaces except for the highly variable "top" of the molecule. Thus, the more conserved C-terminal domain and many epitopes of the N-terminal domain are hidden by steric hindrance. It is generally accepted that the density of VSGs on the cell surface is also sufficient to hide other invariant surface antigens from B-cell recognition. This theory has not been rigorously tested, and it is possible that other determinants are recognized to some degree, but the bulk of the humoral response is directed against the VSG (Field et al. 2009).

Individual VSGs do not remain externally exposed for extended periods at a time; they are constantly shuttled on and off the cell surface. All endo- and exocytosis in *T. brucei* occurs at the flagellar pocket, a specialized structure that makes up 2 % of the total surface membrane, and is situated at the base of the flagellum at the posterior end of the cell. VSGs at the flagellar pocket are internalized by endocytosis in clathrin-coated vesicles (Allen et al. 2003) and delivered to RAB5-positive early endosomes. The vast majority are then sorted to RAB11-positive recycling endosomes (directly or via RAB7-positive late endosomes) where they are delivered back to the flagellar pocket and reemerge onto the cell surface (Manna et al. 2014; Pal et al. 2003; Grünfelder et al. 2003; Engstler et al. 2004). Roughly 9 % of the total cellular VSG content is in this internal recycling pathway at steady state (Engstler et al. 2004). Despite the high abundance of surface VSGs, this process is rapid and efficient. The entire surface coat goes through one round of internalization and redistribution to the cell surface in approximately 12.5 min (Engstler et al. 2004).

It has long been observed that live trypanosomes rapidly redistribute surface-bound antibody to the flagellar pocket (Barry 1979). VSG and bound antibody are then internalized together, but upon internalization, the antibody is separated from the VSG, transported to the lysosome, and degraded (Pal et al. 2003; O'Beirne et al. 1998). It is not entirely clear how VSGs are specifically sorted from other proteins in the endosome, but the current model suggests that this selection is based on default sorting of GPI-anchored proteins to the recycling pathway (Manna et al. 2014; Grünfelder et al. 2003). VSG recycling thus provides a mechanism for rapid clearance of host antibodies from the trypanosome surface. Trypanosomes have been demonstrated to fully remove a single layer of surface-bound IgG in 120 s at 37 °C (Engstler et al. 2007), far faster than the rate of internalization of the VSG coat. This increased antibody clearance rate has been accounted for by the influence of hydrodynamic forces created by parasite motility. Beating of the

trypanosome flagellum produces forward motion, causing bound antibodies to act as "molecular sails," which preferentially drives VSG–antibody complexes to the posteriorly located flagellar pocket (Engstler et al. 2007). Overall, the high rate of endocytosis and surface coat recycling appears to be another adaptation for immune evasion in the mammalian host.

2 Antigenic Variation of VSG Coats

2.1 The VSG Genomic Repertoire

In 1909, Ross and Thomson applied a new method for counting parasites to the blood samples of a patient infected with *T. brucei gambiense*. Their methodical counting revealed, for the first time, the periodic peaks and valleys in parasitemia characteristic of *T. brucei* infections (Ross and Thomson 1910). These waves, we now understand, are the result of periodic "switching" of the parasite's variant surface glycoprotein (VSG) coat.

Recent work sequencing the "VSGnome" of *T. brucei* (Lister427 strain) has provided great insight into the genomic *VSG* repertoire (Cross et al. 2014). In contrast with previous predictions (Van der Ploeg et al. 1982), this analysis revealed more than 2000 different *VSG*-encoding genes. Interestingly, the great majority of these are incomplete *VSGs* or *VSG* pseudogenes. This might at first suggest a very limited repertoire of *VSG* genes from which the parasite can draw during an infection. What purpose could a repertoire of partial *VSG* genes serve? It appears that this repertoire of partial *VSGs* and *VSG* pseudogenes may be extremely important to *T. brucei* infection. When expressed *VSGs* were cloned from late stages of *T. equiperdum* infections in rats, it was discovered that of three clones, isolated based on recognition by a single polyclonal antibody, all were distinct and derived from three or four *VSG* pseudogenes (Roth et al. 1989). This type of chimeric *VSG* sequence is referred to as a "mosaic *VSG*," and the appearance of such mosaic *VSGs* late in infection suggests a role for the large pool of *VSG* pseudogenes in the *T. brucei* genome.

Mosaic *VSGs* have also been described in *T. brucei* infections (Kamper & Barbet 1992). A recent study looked in depth at the formation of mosaic *VSGs* in vivo by cloning and sequencing hundreds of expressed *VSGs* over the course of mouse infections (Hall et al. 2013). This work again confirmed the late emergence of mosaic *VSGs* during infection. In 4–5 week-long infections, mosaic *VSGs* only began to emerge at week 3 of infection. Moreover, these experiments demonstrated that mosaics with similar sequence were nonetheless antigenically distinct, as antibodies raised against one variant would not cross-react with a related variant from the same infection. Modeling of the protein structure of these VSGs showed that many of the distinct residues on related mosaics occurred in regions likely to be exposed on the trypanosome surface and thus most immunogenic. Altogether,

reports of mosaic *VSGs* suggest that the repertoire of complete *VSG* genes at a given time is perhaps not sufficient for escape from the host immune system in a chronic infection. Switching may occur at a rate that exhausts the intact *VSG* repertoire relatively early during infection. As a result, the much larger repertoire of *VSG* pseudogenes and partial *VSGs* must be accessed to produce mosaic *VSGs*, in which segments of partial *VSGs* are combined by gene conversion to build a new, intact, and (ideally) antigenically distinct VSG coat. Some studies have suggested an even greater extension of the *VSG* repertoire through the introduction of point mutations during gene conversion events (Lu et al. 1993, 1994), although others have suggested this is not a significant source of VSG diversity (Graham & Barry 1996).

It is important to note that while mosaic *VSGs* are expressed later in *T. brucei* infection, it is not known when or how these variants are actually formed within the genome. Hall et al. described mosaic *VSGs* whose boundaries of segmental gene conversion between donors showed absolutely no homology (Hall et al. 2013). Donors typically showed homology with one another, but it did not appear that homology at the site of gene conversion was required for the formation of mosaic *VSG* genes. Moreover, it is not clear where in the genome mosaic *VSGs* form or how frequently they arise.

2.2 Immune Response to VSG Coats

Given the immense repertoire of *VSGs T. brucei* may use during infection, and the parasite's extracellular lifestyle, it is no surprise that the immune response to *T. brucei* is primarily B-cell mediated. The immune response to the parasite has been thought to be mediated by IgM B cells. This is based on a study tracking VSG-specific antibody titers during *T. brucei* infections (Dempsey & Mansfield 1983), which reported that IgM appeared earliest in infection and persisted longest. VSG-specific IgG did not appear until the first peak of parasitemia was cleared, leading the authors to conclude that the IgM response was responsible for clearance of each variant. Other studies, however, paint a more complex picture of the arms race between host and parasite. One study reported polyclonal B-cell activation (nonspecific) in response to *T. brucei* infection (Diffley 1983), while VSG-specific antibodies could also be detected during infection (Dempsey & Mansfield 1983; Musoke et al. 1981). In addition, certain B-cell populations have been reported to be lost during *T. brucei* infection, specifically IgM marginal zone B cells in the spleen. Similarly, coinfection with *T. brucei* resulted in a demonstrated loss in vaccine-induced protection against *B. pertussis* infection in mice (Radwanska et al. 2008). Based on these observations, it is interesting to speculate why a parasite that undergoes antigenic variation might simultaneously destroy immune memory.

The T-cell response to *T. brucei* has also been characterized (Schleifer et al. 1993), and it appears to be very important in eliciting an IFN-γ response during infection, which is a major determinant in resistance and susceptibility to infection (Hertz et al. 1998). Interestingly, although T cells could theoretically recognize peptide fragments from the entire VSG protein, including the more

conserved C-terminal domain, an analysis of T cells activated during *T. brucei* infection indicated that these cells only recognized epitopes originating from the more variable N-terminal domain (Dagenais et al. 2009). Somehow, the parasite manages to avoid immune recognition at invariant regions of the VSG. Thus, both arms of the adaptive immune system primarily recognize the variable, and exposed, N-terminal domain of the VSG.

2.3 Characteristics of Antigenic Variation (VSG Coat Switching)

It is obvious that the parasite interferes with the host immune response in a number of ways. It is unclear, however, what the mechanism of this interference is. There are many factors that would contribute to *VSG* expression dynamics in vivo. These can be grouped into two general categories: (1) the rate of switching (is the parasite exhausting humoral immunity simply by virtue of very frequent switching?) and (2) the characteristics of each new variant (including growth rate differences and immune cross-reactivity between VSGs).

Despite *T. brucei's* large repertoire of *VSGs*, it is thought that the rate of switching must be tightly controlled. If switching is too slow, the immune system will recognize *T. brucei* and clear the infection, preventing transmission to the tsetse fly. If switching is too fast, it is possible that the *VSG* repertoire will be exhausted prematurely. Many measurements of *VSG* switching rates have been made, both in vivo and in vitro. In laboratory-adapted strains, the rate of switching ranges between 10^{-5} and 10^{-7} switch events per parasite per population doubling both in vivo (Lamont et al. 1986; Doyle et al. 1980; Turner 1997) and in vitro (Lamont et al. 1986; Doyle et al. 1980; Aitcheson et al. 2005). Many interpret the in vitro data to suggest that the process of switching is purely stochastic. However, some studies have shown the rate of switching in naturally occurring (fly-transmitted) infections to be much higher, perhaps as high as 1 in 100 (Turner 1997; Turner & Barry 1989). These findings suggest that there may be some environmental cue initiating a *VSG* switch, or at least an environmental influence on the frequency of *VSG* switching.

Determining the true rate of *VSG* switching presents experimental challenges. Trypanosomes expressing different *VSGs* may grow at different rates, or trypanosomes may switch to a *VSG* for which the immune system has already mounted a full or partial response. Moreover, trypanosomes can sequester in extravascular tissues (Seed et al. 1984), and most work examines only populations in the bloodstream. As a result, even if a switch occurs, that switched variant may never establish in the measured population. It is also difficult, if not impossible, to examine the response to a changing antigen without an understanding of how that antigen changes. Unfortunately, while our understanding of the *VSG* repertoire has improved greatly in recent years due to next-generation sequencing approaches, our understanding of how that repertoire is accessed in vivo has not yet benefitted from more modern techniques (Fig. 1).

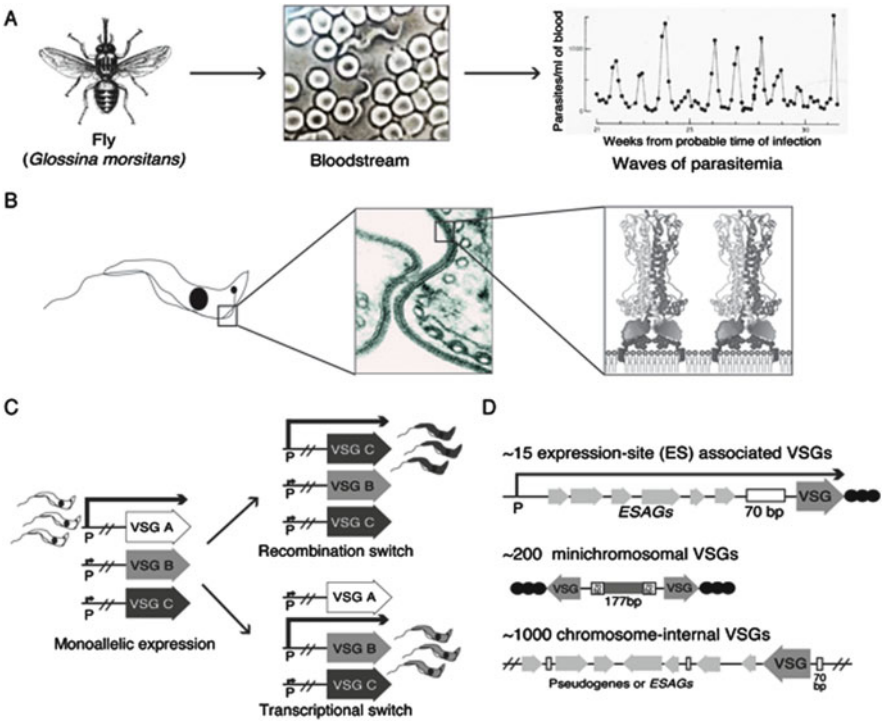

Fig. 1 (**a**) *Trypanosoma brucei* is transmitted from the salivary gland of the fly, *Glossina morsitans*, to its mammalian host. There it lives extracellularly in the bloodstream. Patients infected with *T. brucei* display waves of parasitemia that reflect the interaction between the parasite and the host immune system. Host antibodies are able to mostly clear parasites from the bloodstream, but a small number of parasites are able to escape the response, leading to a resurgence in parasite number (image adapted from Ross and Thomson 1910). Escape from the immune system is largely mediated by the Variant Surface Glycoprotein (VSG) that densely coats the parasite surface, as shown by the electron micrograph (http://tryps.rockefeller.edu/trypsru2_avariation_intro.html) displayed in (**b**). Ten million VSG molecules homodimerize on the surface of the parasite cell, shielding all remaining surface epitopes from exposure to the host immune system (image courtesy of Markus Engstler). (**c**) VSG genes are expressed from ~15 telomeric expression sites, only one of which is transcriptionally active at any given time. The parasite can switch expression to an antigenically distinct VSG by (1) silencing the initial expression site and transcriptionally activating a new one, thus activating a new VSG (*bottom*, transcription switch) or (2) copying a VSG from a separate expression site or from elsewhere in the genome into the active transcription site (*top*, Recombination switch). Using a repertoire of ~2000 distinct VSG genes, the parasite is able to effectively disguise itself from existing host antibodies by remodeling its surface coat. (**d**) The extensive repertoire of VSG genes is located in multiple places throughout the genome. Around 15 VSGs are associated with expression sites, where they are transcribed with a cohort of Expression Site Associated Genes (ESAGs). Expression sites also contain repetitive 70 bp repeat elements. Around 200 VSGs are located on minichromosomes, which contain 177 bp repetitive elements. Finally, a large number of VSGs are located in chromosome-internal regions, where they are often found in combination with ESAGs and repetitive elements

To date, the resolution of most studies measuring switching rate in *T. brucei* has been quite low. In fact, nearly all of the studies on *VSG* switching rate and *VSG* switching hierarchy have measured less than ten *VSGs*, for which either the sequence of the *VSG* gene was known or antibodies against the *VSG* existed (Turner 1997; Turner & Barry 1989; Miller & Turner 1981; Liu et al. 1985; Morrison et al. 2005). Even more recent work cloning hundreds of *VSGs* over the course of infection (Hall et al. 2013) could not detect rare variants at any given point, due to limited sampling. Thus, if some variants arise but never establish within the population (due to cross-reactivity, physical location, or intrinsic fitness), they cannot be measured using existing methods. This makes it difficult to estimate the effects of competition between variants or immune cross-reactivity.

Although limited in resolution, studies looking at a handful of *VSGs* have shed some light on the characteristics of new variants that arise during infection. It has been observed in many in vivo studies that certain VSGs are more likely to arise early in infection than others (Miller & Turner 1981; Liu et al. 1985; Morrison et al. 2005; Myler et al. 1985). While some work suggests that this can be explained in part by differences in growth rate (i.e., a variant that grows the fastest will appear first) (Myler et al. 1985; Lythgoe et al. 2007), this view has been challenged (Kosinski 1980), and it is clear that the genomic position of a *VSG* has an influence on when that *VSG* appears during infection. Telomeric *VSGs* are much more likely to arise early in infection (Liu et al. 1985), while more complicated forms of recombinant *VSGs* tend to appear later (Hall et al. 2013). The mechanism for this preference is not well understood, although it makes logical sense that *VSGs* that comprise segmental gene conversion events may be more likely to arise later in infection, when the repertoire of complete *VSGs* has been exhausted. Finally, other studies report that the immune system is the major player in determining *VSG* order, primarily through cross-reactive epitopes (Antia et al. 1996; Recker et al. 2004), thus highlighting the role of the immune repertoire in this host:pathogen arms race.

Recently, we developed a high-throughput method for identifying and measuring expressed *VSGs* during infection (Mugnier et al. 2015). Our study of expressed *VSGs* over the course of chronic mouse infections confirmed many of the findings from previous low-resolution studies. Indeed, *VSG* populations appear to be biased toward certain *VSGs* at different times during infection, mosaic *VSGs* tend to appear later infection, and immune cross-reactivity appears to play a key role in determining variant success. On the other hand, this work brought to light aspects of infection that could not be accessed using other approaches. During infection, many *VSGs* are expressed at a single time (28 variants, on average), and most of these variants never establish to levels at which they could be detected using low-resolution techniques. Thus, the complete functional *VSG* repertoire (~400 *VSGs* in Lister 427) is almost certainly insufficient to support the long *T. brucei* infections frequently observed in the field. *VSG* diversification through the formation of mosaic *VSGs*, or perhaps other yet to be identified mechanisms, may play a more significant role in sustaining infection than previously thought.

A better understanding of how the genomic *VSG* repertoire is accessed during infection would certainly shed light on the mechanisms of switching in *T. brucei*.

The recently catalogued VSGnome reference (Cross et al. 2014) can now be used to study how the *VSG* repertoire is used, in high resolution. Simultaneously, the immune response of the parasite can be measured. Now that the system in vivo can be described accurately, it may be possible to disentangle some of these many variables and reveal the fundamental mechanistic parameters that define this extremely dynamic host–pathogen interface.

3 Mechanisms of *VSG* Switching

Antigenic variation is required for *T. brucei* to effectively evade the host immune response during the bloodstream stage of the life cycle. Switching from the expression of one *VSG*-encoding gene to the next occurs through specific genetic processes that allow access to an extensive genetic repertoire (discussed in Sect. 2.1). At the same time, it is essential that only one VSG be displayed on the cell surface at a given time to ensure that the parasite has time to replicate before the immune system marshals its forces. This dual requirement for switching *VSG* expression and for monoallelic expression of each coat (discussed in detail in Sect. 4) controls antigenic variation at the molecular level.

Both requirements are facilitated by the special structure of the locus that harbors the expressed *VSG*. Transcription of the active *VSG* gene occurs from one of approximately 15 specialized subtelomeric Bloodstream Expression Sites (BES) that have a similar organization. Each BES can be likened to an operon with several unique features: (1) transcription initiates from an RNA Polymerase I promoter (PolI), which in all other organisms studied is exclusively used for ribosomal RNA production; (2) the promoter is followed by a series of expression site associated genes (ESAGs); (3) then a long region of repetitive DNA; and (4) a single *VSG* gene in close proximity to the telomeric repeats of the chromosome (Shea et al. 1986; Hertz-Fowler et al. 2008). The active BES produces a single polycistronic transcript (30–60 kb in length depending on the specific site) that is subsequently spliced and targeted for nuclear export (Alarcon et al. 1994). BESs are highly similar at the nucleotide sequence level with minor variations in the number and order of ESAG genes (Hertz-Fowler et al. 2008). *VSG*-encoding genes are very dissimilar at the level of DNA sequence, bearing only a short conserved tract at the 3′-end. Thus, the most unique BES region is the *VSG* gene.

This location also facilitates the two major classes of *VSG* switching, namely the transcriptional switch (or "in situ") between BESs (i.e., inactivation of one and activation of another) or recombination-based switching, which results in a new *VSG* gene in the actively transcribed BES [reviewed extensively Barry & McCulloch (2001), Borst & Ulbert (2001), Pays et al. (1994), Borst et al. (1998)]. The 15–20 *VSGs* that reside in BESs are a small portion of the total *VSG* archive (~2000) encoded in the *T. brucei* genome (Cross et al. 2014). The majority of *VSG* genes are encoded in gene arrays on megabase chromosomes and the remainder is subtelomerically located on intermediate chromosomes (~10) or minichromosomes

(~100), which have the potential for bearing a *VSG* at each telomere (Cross et al. 2014; Berriman et al. 2005; Wickstead et al. 2004). Thus, 1–5 % of the *VSG* archive is encoded in sites that can be expressed (BESs), while the remainder resides in physically distinct, transcriptionally inactive (comparatively), and actively silenced (see Sect. 4) genomic sites. The following sections will describe the discoveries and remaining questions regarding BES enforced monoallelic expression as well as review current knowledge regarding the two broad classes of *VSG* switching and their contribution to the repertoire.

3.1 Transcriptional Switching

VSG expression is not only required for infection, it is essential for life in African trypanosome species. Safeguards must be in place within the cell to ensure that expression of the active *VSG* is not compromised during switching from one *VSG* gene to the next. Transcription of the active BES by PolI occurs rapidly and processively through many tens of kilobases and, ultimately, the *VSG*-encoding gene (Zomerdijk et al. 1990, 1991). Modest amounts of transcription occur from silent PolI promoters, but only one VSG coat is expressed on the surface of the cell. Transcriptional *VSG* switching occurs by the simultaneous activation of a new BES and inactivation of the previously expressed site (Johnson & Borst 1986; Horn & Cross 1997). This type of switch can only promote access to the small fraction of the *VSG* repertoire that is encoded on BESs. It is nonetheless worth considering the dynamics of transcriptional switching, why it occurs, and whether or not transcription is a layer of switching regulation.

The actively expressed BES resides in a subnuclear compartment termed the expression site body (ESB), from which the inactive BESs are believed to be excluded [reviewed in Navarro et al. (2007)]. Similarly, the active BES has a much lower level of nucleosome occupancy than its silenced counterparts (both of these phenomena are covered in detail in the monoallelic exclusion section of this chapter) (Figueiredo & Cross 2010; Stanne & Rudenko 2010). Thus, a transcriptional switch of *VSG* expression would be accompanied by both a considerable change in the chromatin state of the newly active site and the active repositioning of this site into the ESB coupled with the predicted ESB exclusion of the newly silenced site. The natural conditions under which transcriptional switching occurs are unknown; however, transcriptional switching has been monitored in a number of potentially informative laboratory experiments.

Genetic analysis of trypanosomes that have switched during in vivo infections under laboratory conditions suggests that transcriptional switching accounts for approximately 20–60 % of the events observed (Myler et al. 1984a, b). As with most early in vivo analysis, these measurements are based on very few switched isolates. More recent in vitro approaches have enabled enrichment of switchers from a population for more quantitative genetic determination. These in vitro studies suggest that transcriptional switching in wild-type strains occurs in as few

as 2 % and as much as 30 % of the total switchers, depending on the isolation method (Kim & Cross 2010; Hovel-Miner et al. 2012). Thus, while the exact amount that transcriptional switching contributes to switching in vivo, in vitro, or during natural infections is unclear, it is a mode of switching that must be considered.

Due to its low diversity contribution (<10 % of the total repertoire) and potentially infrequent occurrence, some have wondered: why have more than one BES and why retain a system of transcriptional switching? About 20 years ago, a theory emerged that the system provided a safeguard against irreparable BES damage. Keeping in mind that *VSG* expression is essential, it was proposed that transcriptional switching only occurs when the active BES can no longer produce sufficient *VSG* transcript. Subsequent experiments have shown that when the active BES promoter is artificially shut off, or if the end of the active chromosome is sufficiently damaged, transcriptional switching is favored (Glover et al. 2007). These data support the hypothesis that either a damaged active site or loss of *VSG* expression can trigger the transcription switch. However, exactly how BES damage is sensed and whether this is the only source of transcriptional switching are unknown.

One of the most unusual and fascinating aspects of *T. brucei* genetics is that transcription is largely unregulated. Transcription of most of the genome occurs in a largely constitutive manner by RNA Polymerase II (PolII) without the benefit of transcription factors and expression is primarily regulated at the level of translation (De Gaudenzi et al. 2011; Vasquez et al. 2014). In contrast, BESs have PolI promoters that are not only identical to one another at the DNA sequence level but also indistinguishable from most rRNA promoters, which can be swapped with BES promoters to retain active BES function (Rudenko et al. 1995). Until recently these observations seemed to preclude any traditional model of transcriptional regulation of switching, in which signaling may occur through transcription factors to control genetic outcomes. Recent studies have identified and characterized a multicomponent novel Class I Transcription Factor A (CITFA) that binds to PolI promoters and is required for BES transcription (Brandenburg et al. 2007; Nguyen et al. 2012). In addition, CITFA subunits predominantly occupy the active BES promoter and are localized with the ESB (Nguyen et al. 2014). Whether CITFA directs activation and ESB localization of the active BES or reinforces the existing state (active or silent) of the BES is unclear. It is intriguing to speculate, and worth investigating, if this newly identified level of transcriptional regulation could be a component in a pathway that results in transcriptional switching.

3.2 *Recombinatorial Switching*

Chronic infection with *T. brucei* is dependent upon its ability to activate the expression of *VSGs* encoded throughout the genome. Since most *VSG* genes are located in transcriptionally silent sites, recombination-based mechanisms exist to

copy a silent *VSG* gene into an actively expressed BES. The two major types of recombinatorial switching are reciprocal telomeric exchange and duplicative gene conversion. Telomeric exchange (TE) is the result of a homologous recombination event between telomeric ends that results in the reciprocal translocation of a *VSG* from a silent site into the active BES and the previously active *VSG* into the corresponding silent site. By definition, TE facilitates selection of a limited repertoire because it can only occur between telomeric ends that contain functional bloodstream *VSGs* (~200). TE is also not considered to be a major contributor to the overall rate of switching. In contrast, duplicative gene conversion (GC) is an unbalanced chromosomal translocation in which a donor *VSG* encoded in a silent site is duplicated into the active BES resulting in deletion of the previously active *VSG*. Thus, TE and GC can be distinguished experimentally by their genetic outcomes. There are multiple subtypes of GC associated with their specific genetic outcomes; for instance, expression site gene conversion (ESGC) is a GC event in which the entire BES sequence is replaced with another BES. Unlike transcriptional switching and TE, GC can (theoretically) access the entire functional *VSG* repertoire regardless of genomic site (BES, internal array, or MC) as well as being the putative source of mosaic *VSG* formation (discussed in Sect. 2). GC is observed to be a major contributor to switching in vitro and considered by many to be the predominant mechanism in natural infections (Robinson et al. 1999). While transcriptional switching and TE are naturally restricted to telomeric *VSGs*, subtelomeres are also preferred sites of *VSG* donor selection during GC. In fact, there is a natural hierarchy in *VSG* donor selection that first favors BES-encoded *VSGs*, followed by non-BES subtelomeres (MC and others), and finally chromosome internal arrays (Morrison et al. 2005; Lythgoe et al. 2007). The observed semi-predictable order and inherent considerations of switching frequency and timing during natural infections have resulted in many speculations regarding how recombinatorial switching is monitored and controlled. The following sections will provide an overview of what is known and what remains to be discovered about recombinatorial switching with an emphasis on gene conversion.

Gene conversion events have been observed in most chromosomally organized genomes. In mammalian genomes, they are usually spontaneous, often detrimental, and can result in genetic disorders and specific cancers (Chen et al. 2007; Kobayashi 1992). The essential steps of GC, which include DNA break formation, ssDNA strand migration, and DNA duplication, are likely conserved among eukaryotic genomes (Kobayashi 1992), but how they are accomplished in *T. brucei* antigenic variation is an active research area. BES-encoded *VSGs* are located near the end of the chromosome downstream from a long region (4–16 kb) of "70-bp" DNA repeats and upstream from the *VSG* and telomeric repeats (Hertz-Fowler et al. 2008). Naturally occurring DNA lesions that may precipitate GC have been observed throughout BESs (Glover et al. 2013a) with possible preferable formation within the 70-bp repeats of the active BES (Boothroyd et al. 2009). Induction of site-specific DNA double-stranded break (DSB) in the active BES results almost exclusively in switching by GC, but the exact position of the DNA break formed can affect the amount of switching and the specific genetic outcomes

(Glover et al. 2008, 2013a; Boothroyd et al. 2009). A proposed source of naturally occurring DNA breaks is subtelomeric fragility of the actively expressed site, which may acquire DNA lesions from collisions between transcription and DNA replication machinery [reviewed in Dreesen et al. (2007)]. In support of this hypothesis, it was shown that artificial shortening of the active site telomere results in an increase in switching and GC (Hovel-Miner et al. 2012). It is also intriguing to note that natural *T. brucei* isolates have shorter telomeres than laboratory-adapted strains (Dreesen & Cross 2008), which may, in part, account for the fact that laboratory-adapted strains have lower rates of switching.

VSG switching by GC occurs by homologous recombination events in which homologs of specific recombination proteins have been implicated. A deficiency in *T. brucei* RAD51, which forms nucleoprotein filaments on ssDNA at the site of damage and catalyzes the transfer to a homologous sequence, results in reduced switching (McCulloch & Barry 1999). Mutations of *T. brucei* BRCA2 homolog, which is a key mediator of RAD51 in other systems, impair *VSG* switching and display genome instability (Hartley & McCulloch 2008). In contrast, mutation of MRE11, a sensor of DSBs in other organisms, resulted in DNA damage and chromosomal rearrangements but did not affect *VSG* switching (Robinson et al. 2002). Another interesting question is why GC occurs in response to DNA break formation rather than the crossover events that result in TE. Mutation of *T. brucei* homologs of either TOPO3a or RIM1, which function in the same genome maintenance pathway in other eukaryotes, tips the balance between these events toward TE (Kim & Cross 2010, 2011). While many of the events and factors associated with recombinatorial switching can be determined based on conservation with other eukaryotes, *T. brucei's* genomic divergence results in unexpected differences and the potential for novel protein functions.

The fact that conserved recombination proteins are required for GC and that *VSG* expression occurs from unstable genomic sites supports a stochastic model for *VSG* switching and its genetic outcomes. In this model, DNA breaks form randomly as a result of genomic positioning, the resulting ssDNA uses 5′ 70-bp homology and 3' *VSG* homology to pair with the new *VSG* donor gene, and conserved DNA duplication and recombination proteins complete translocation into the active site [reviewed recently Horn (2014)]. The implication of this model is that no switching specific factors exist that either control the amount of switching or direct donor selection toward specific *VSG*-encoding sites. Yet, the frequency and source of naturally occurring DNA breaks in BESs is still speculative and it is not known if the breakage observed in vitro is sufficient to support antigenic variation in a natural infection (Glover et al. 2013a; Boothroyd et al. 2009). Selection of the *VSG* donor during gene conversion is semi-ordered (as described above) and predicted to be driven by homology of 70-bp upstream and *VSG* 3′ conserved sequences in the donor sites. However, conservation of the 3′ end is consistent among all *VSG* genes (Cross et al. 2014) and recently data suggest that the repetitive element that composes the 70-bp repeat has a highly conserved sequence throughout the genome (BES, internal array, and MC—Hovel-Miner et al. unpublished data). Thus, variations in the conserved DNA elements flanking *VSG* genes may not be a sufficient

predictor of *VSG* donor selection. Similarly, BESs have both long regions of 70-bp repeats and extensive sequence identity in common with one another (Hertz-Fowler et al. 2008) and yet sites other than BESs are selected frequently even early in switching (Boothroyd et al. 2009). Alternative explanations to homology-driven donor selection include physical proximity of *VSG* containing sites in the nucleus or hypothetical factors that guide ssDNA in homologous pairing with donor sites. Much has been, and will continue to be, learned using functional homologs from other eukaryotic genomes. It is important to keep in mind that the findings from those studies do not preclude the involvement of functionally novel or highly divergent factors in *T. brucei* antigenic variation that have not yet been identified.

4 *VSG* Monoallelic Expression

As already mentioned, trypanosomes actively transcribe a single *VSG* gene whose product is displayed on the surface of the cell while transcriptionally repressing the rest. The parasite is thus faced with the problem of expressing one of the variants at a high enough level to completely coat the surface of the cell, while keeping nearly a quarter of the genes in its genome transcriptionally silenced. This is referred to as monoallelic expression and is essential for a number of developmental processes in mammalian cells. These include olfactory gene receptor usage (Rodriguez 2013), differential rhodopsin gene usage in the retina (Rister et al. 2013), as well as immunoglobulin allelic exclusion in the development of B and T cells of the immune system (Vettermann & Schlissel 2010). In these diverse systems, regulation of chromatin structure has emerged as a common mechanism by which monoallelic expression is maintained (Peng & Chen 2007; Magklara et al. 2011; Stanhope-Baker et al. 1996). Recent evidence from the malaria field has also implicated chromatin structure in the maintenance of monoallelic expression of the *var* genes (Volz et al. 2012). It is indeed striking that evolutionarily distinct organisms use such similar mechanisms to maintain monoallelic expression, and trypanosomes prove to be no exception to this rule. Elucidating the mechanisms by which chromatin structure is used to maintain monoallelic expression in *T. brucei* is sure to provide insight into how these systems evolved across evolutionary time.

4.1 Chromatin State Facilitates Expression

Monoallelic expression is regulated, at least in part, through chromatin state. Early evidence for chromatin regulation of monoallelic *VSG* expression came from the finding that a transcriptionally active T7 promoter placed at the BES in bloodstream forms became inactivated upon differentiation to procyclic forms, implicating a chromatin remodeling mechanism that rendered the locus “inaccessible” in procyclic forms (Navarro et al. 1999). While the histones in *T. brucei* are extremely

diverged from those found in yeast and metazoans, there is ample evidence for the existence of both acetylation and methylation marks on the highly diverged N-terminal tails [reviewed in Figueiredo et al. (2009)]. The existence of histone variants has also been verified in *T. brucei*, and their location at sites of transcription initiation and termination indicates that they may play a role in the regulation of PolII transcription (Siegel et al. 2009). The fact that the active BES is depleted of nucleosomes when compared to its silent counterparts indicates that chromatin structure may also play a role in PolI transcriptional regulation and the maintenance of monoallelic expression (Figueiredo & Cross 2010). More definitive evidence comes from the fact that depletion of both H3 and the linker histone H1 results in higher transcription at inactive BES promoters (Povelones et al. 2012; Alsford & Horn 2012). The factors responsible for histone deposition have also been shown to be important for maintaining transcriptional repression at inactive BESs, as depletion of FACT, NLP (Narayanan et al. 2011), Asf1A, or Caf-1b (Alsford & Horn 2012) results in derepression at these sites. Finally, nucleosome remodelers have been implicated in this regulation, as inhibition of ISWI causes depression at the BES promoters as well as the *VSGs*. Thus, the presence or absence of the histones themselves appears to be important in maintaining monoallelic expression at BESs. With respect to maintaining high levels of transcription at the active BES, the high mobility group protein TDP1 is enriched at PolI transcription sites and its depletion results in lower levels of transcription at the active BES as well as the rDNA loci, which are also transcribed by PolI (Narayanan & Rudenko 2013). The transcription factor CITFA binds to PolI promoters and is also essential for both PolI-driven rDNA transcription and BES transcription (Nguyen et al. 2012).

In addition to their physical location on the DNA, the "code" provided by the covalent modifications on the histone tails provides an additional layer of regulation for transcriptional activity. It is thus not surprising that perturbing proteins that "write" or "erase' these covalent modifications in *T. brucei* result in disruption of monoallelic *VSG* expression. This appears to be the case for the histone deacetylase DAC3, as inhibition results in derepression of BES promoters (Wang et al. 2010). Additionally, inhibiting the methyltransferase Dot1B, which is responsible for modifying H3K79, results in a striking derepression phenotype, whereby two distinct VSG proteins can be detected on a single cell (Figueiredo et al. 2008). While histone acetyltransferase HAT1 and DAC1 maintain silencing of a telomeric reporter, disruption of these factors does not appear to lead to derepression of the *VSGs* themselves (Wang et al. 2010; Kawahara et al. 2008). The role for histone "writers" and "erasers" in regulating monoallelic expression in *T. brucei* has been well established, but not as much work has been done to investigate how histone "readers," proteins that recognize specific modifications on the histone tails and coordinate downstream transcriptional effects, affect monoallelic expression. However, an intriguing early report for a role for Bdf5 indicates that these proteins may be worthy of future study (Alsford & Horn 2012).

4.2 A Role for Telomeric Silencing

In yeast, telomeric silencing is controlled by the sirtuin family of proteins (Pillus & Rine 1989). As *VSGs* are located near telomeres, a similar silencing mechanism could be in play in *T. brucei*. Surprisingly, disrupting the sirtuin SIR2rp1 leads to derepression of a telomeric reporter while not affecting transcription of *VSGs* in inactive BESs, a phenotype similar to the one seen upon HAT1 and DAC1 inhibition (Alsford et al. 2007). However, the factor RAP1, which is recruited to telomeres through its interaction with telomere-bound TRF2, has been shown to be essential for silencing telomeric *VSGs*, but not *VSGs* located elsewhere in the genome (Yang et al. 2009). Surprisingly, when a telomere is removed through a programmed DNA break, telomere-mediated silencing is disrupted, but the *VSG* proximal to the removed telomere remains transcriptionally repressed, suggesting that there may be both telomere-dependent and independent modes of *VSG* silencing (Glover et al. 2007).

With respect to the broader nuclear architecture, the actively PolI transcribed BES is located in the ESB (Navarro & Gull 2001), a region distinct from the nucleolus where rDNA transcription takes place. Interestingly, upon differentiation to the procyclic form, the active BES promoter is repositioned to the nuclear envelope, an event that is followed by chromatin condensation (Landeira & Navarro 2007). While direct evidence is lacking for the nuclear location of the silent BESs, heterochromatic localization is implied by the fact that depletion of NUP-1, a large repetitive protein that in *T. brucei* appears to functionally substitute for mammalian lamins, results in derepression of inactive BESs (DuBois et al. 2012). Little is known about how the inheritance of the epigenetic state for the active and silent BESs is maintained across generations, but DNA replication processes may play a role. It appears that the separation of the sister chromatids belonging to the active BES is delayed relative to other regions of the genome and that this is at least partially mediated by cohesin. Depletion of cohesin results in a rapid transcriptional switch to a new BES, indicating that it may have a role in epigenetic VSG inheritance (Landeira et al. 2009). Studies have also implicated ORC1/CDC6 as being important to maintain the repressive state of the inactive BESs. ORC1/CD6 is functionally related to both Orc1 of the mammalian Origin Recognition Complex and CDC6, a factor that mediates interaction with the minichromosome maintenance (MCM) helicase. Knocking down ORC1/CDC6 derepresses BESs in both procyclic and bloodstream form cells, as well as metacyclic *VSGs* in procyclic form cells (Tiengwe et al. 2012). Attention was also drawn to the MCM helicase when it was found that an MCM binding protein (MCM-BP), a member of a complex including MCM4-8, is essential for maintaining repression of both BESs and the procyclin surface protein genes that cover the parasite during the procyclic life cycle stage (Kim et al. 2013). Since ORC1 has been shown in other systems to interact with cohesin, this might be the mechanism by which epigenetic inheritance is perturbed (Guillou et al. 2010).

In conclusion, *VSG* monoallelic expression is maintained by both the nuclear architecture and chromatin state of the DNA in which the *VSGs* reside, while DNA replication processes are likely involved in the placement of epigenetic marks and their inheritance. A recent comprehensive review on monoallelic *VSG* expression and other aspects of antigenic variation is available from Glover et al (2013b).

5 Concluding Remarks: Open Questions

Unlike many other pathogens, *T. brucei* exists in the bloodstream of the mammalian host, completely exposed to the host's immune system. In vivo, the parasite must switch its VSG coat in order to evade recognition by the immune system. By packing extremely densely on the parasite's surface, VSG also obscures invariant antigens that might be recognized by the host immune system. This packing poses an interesting requirement, however, on the expressed VSG repertoire: VSGs must be structurally conserved, in order to pack densely on the parasite surface, but also antigenically distinct, to evade detection by host antibody.

It turns out that the parasite balances these requirements quite well. Only two (partial) VSG crystal structures exist (Blum et al. 1993; Freymann et al. 1990), but these structures are superimposable despite having only 16 % sequence identity (Blum et al. 1993). Analysis of the sequence of *VSG* genes has identified conserved cysteine residues that likely play a role in maintaining VSG structure while allowing for significant sequence divergence (Carrington et al. 1991). Global analysis of the genomic repertoire of *VSG* genes, both full length and partial, has provided useful information regarding the depth of the genomic repertoire. However, how this repertoire is used during an infection and how that correlates with utilization of the antibody repertoire are key questions that remain open. Recently developed high-throughput approaches for examining expressed *VSGs* during infection show great promise for answering these questions (Mugnier et al. 2015).

The relative contribution of surface antibody clearance to immune evasion has not been determined. The notion that this process is, in fact, an immune evasion strategy is supported by observed differences in endocytosis rates between BF trypanosomes and those in other life cycle stages, which are not subject to similar immune pressure. BF trypanosomes induced to differentiate to the tsetse fly midgut-inhabiting procyclic form show downregulation of components of the endocytic pathway and a concurrent tenfold reduction in endocytosis rate (Natesan et al. 2007). A recent report indicated that mice infected with trypanosomes with a motility defect did not have a survival advantage over mice infected with motile trypanosomes (Kisalu et al. 2014). However, the trypanosomes used for these experiments were derived from the highly virulent Lister 427 strain, and all mice succumbed to infection within 2 weeks. Thus, the capability of trypanosomes defective in internalization of surface antibody to persist within the host has not been properly addressed in an in vivo model of chronic infection.

Furthermore, the molecular mechanisms of monoallelic expression as well as of switching remain to be fully elucidated. Switching is a semi-ordered process, and there is a loose relationship between the expressed *VSG* and the identities of the new *VSGs* that might arise as a result of switching. This relationship might be partially based on sequence homology or on nuclear proximity, but must have additional layers of dynamic complexity. For example, if *VSG-X* possesses a preference to pair with *VSG-Y*, this results not only in replacement of *VSG-X* but also in duplication of *VSG-Y*. The subsequent recombination event must now avoid the locus with highest homology, namely, the original donor. Though genetic experiments continue to be informative, we have very little information on the *trans*-acting factors that might mediate these events in the cell.

Finally, once a new *VSG* is recombined into, and expressed from the active BES, it must fully replace the old VSG coat. Because trypanosomes cannot survive without their coat, this requires the rapid turnover and replacement of 10 % of cellular protein. Yet, the half-life of VSG proteins is quite long [determined to be approximately 32.6 h in two independent studies Bülow et al. (1989) and Seyfang et al. (1990)], and this is likely a consequence of the trypanosome's need to consistently maintain a sufficient surface coat density. Unless the process of coat replacement is specifically accelerated as a consequence of switching, a switched cell could well express the original VSG on their surface for an extended period after expression of the new VSG begins, and would be at significant risk of antibody clearance. Because antigenic variation occurs in a prohibitively small fraction of trypanosome populations in vitro, this coat replacement process has not been directly observed. Thus, questions regarding the rate of VSG coat replacement in the context of switching remain, for the moment, unanswered; however, new tools can and will be applied to this and all the other problems we note, and we are looking forward to significant new knowledge in the near future. This new knowledge will directly impact treatment modalities for this neglected tropical disease, but beyond that, it is our hope that it will also (re)introduce *T. brucei* as a model system for studies well beyond VSG switching, such as telomere biology, DNA repair, and even cancer biology through the modeling of chromosomal translocations.

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