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Antigenic variation in the African trypanosome: molecular mechanisms and phenotypic complexity

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Summary

Antigenic variation is an immune evasion strategy that has evolved in viral, bacterial and protistan pathogens. In the African trypanosome this involves stochastic switches in the composition of a variant surface glycoprotein (VSG) coat, using a massive archive of silent VSG genes to change the identity of the single VSG expressed at a time. VSG switching is driven primarily by recombination reactions that move silent VSGs into specialized expression sites, though transcription-based switching can also occur. Here we discuss what is being revealed about the machinery that underlies these switching mechanisms, including what parallels can be drawn with other pathogens. In addition, we discuss how such switching reactions act in a hierarchy and contribute to pathogen survival in the face of immune attack, including the establishment and maintenance of chronic infections, leading to host–host transmission.

Antigenic variation in African trypanosomes

African trypanosomes, such as Trypanosoma brucei, Trypanosoma congolense and Trypanosoma vivax, are protistan parasites that infect a range of mammals and are transmitted between successive hosts by the tsetse fly. Subspecies of T. brucei cause disease in humans (termed sleeping sickness) but are relatively asymptomatic in cattle and goats, which act as reservoirs for human infection. T. congolense and T. vivax are not human infective but are major cattle parasites, causing disease (termed Nagana) similar to that in humans. In all mammals, the parasites replicate extracellularly in the tissue fluids and bloodstream. Despite continuous exposure to immune attack trypanosome infections can be very prolonged, causing a range of pathological manifestations (Barrett et al., 2003). Trypanosome persistence in the mammal is due to antigenic variation, which involves changes in the identity of the variant surface glycoprotein (VSG) that forms a dense cell surface coat to shield invariant surface antigens from immune recognition. Antibodies against the VSG kill the trypanosome, but the population survives due to stochastic switching to a coat composed of an antigenically distinct VSG. The rate of VSG switching (up to $10^{-3}$ switches/cell/division) (Turner and Barry, 1989) is typical of what Moxon and colleagues termed ‘enhanced phenotypic variation’ (Moxon et al., 1994) in subset of genes in viral, bacterial and eukaryotic pathogens (Deitsch et al., 2009).

Trypanosomes have made a huge investment in antigenic variation. The genome of T. brucei contains > 1600 VSG genes (Marcello and Barry, 2007), hugely in excess of antigenic variation gene families elsewhere (Table 1). Most (80–90%) of this VSG archive is in 11 diploid, megabase-sized chromosomes; the subtelomeres harbour silent VSGs at their ends, suggesting they evolved to expand the telomeric pool of VSGs (Barry et al., 2003). Intermediate chromosomes are related to minichromosomes (Wickstead et al., 2004) and contain ES (Fig. 2), are found directly proximal to the telomeres (Hertz-Fowler et al., 2008). Only a minority (~5–10%) of VSG array genes are functional, with the large majority being pseudogenes (Marcello and Barry, 2007), which (in common with other pathogens) provide a critical resource during antigenic variation. T. brucei has also evolved novel, aneuploid chromosomes to facilitate antigenic variation. Minichromosomes (Wickstead et al., 2004) are present in ~100 copies per cell and harbour VSGs at their ends, suggesting they evolved to expand the telomeric pool of VSGs (Barry et al., 2003). Intermediate chromosomes are related to minichromosomes (Wickstead et al., 2004) and contain ES. These chromosomes may also be a product of the evolutionary drive to expand the VSG archive, or may indicate selection for increased flexibility in ES-associated gene (ESAG) expression (see below).

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Two modes of VSG switching occur: recombination reactions that move silent VSGs into the ES, or transcriptional switches in which the single actively transcribed ES is silenced and a silent ES becomes actively transcribed. Given that the overwhelming number of archival VSGs are outwith the ES, recombinational switching is the main player in *T. brucei* antigenic variation.

Recombination mechanisms in *T. brucei* antigenic variation

Three recombination pathways contribute to VSG switching. The extent to which the underlying recombination machinery is shared by these pathways, and whether or not each can occur by different recombination reactions, is being dissected. Additionally, VSG switching by recombination operates in a hierarchy (Capbern et al., 1977; Morrison et al., 2005), the basis for which is not fully understood. In the hierarchy, telomeric VSGs are activated earliest in infections, followed by intact subtelomeric array VSGs, and finally pseudogenes, which are recombined to form functional VSG ‘mosaics’ (Thon et al., 1990; Morrison et al., 2005; Marcello and Barry, 2007).

The most commonly described recombination pathway is gene conversion, where a silent, functional VSG is copied and transferred to the ES, replacing the resident VSG (Fig. 1). The sequence copied in this reaction normally extends beyond the VSG open reading frame (ORF). Upstream, the boundary is often degenerate 70 bp repeats (Liu et al., 1983), which flank virtually all VSGs (~90%) (Marcello and Barry, 2007), whereas gene conversions in which a silent ES acts as VSG donor can extend much further upstream (Pays et al., 1983), even including the VSG promoter ~50 kb distant (Hertz-Fowler et al., 2008). Downstream, gene conversion of array VSGs can extend to the 3′ coding or non-coding parts of the gene (Bernards et al., 1981), or to the chromosome end (de Lange et al., 1983). A number of recombination models can account for VSG gene conversion. Synthesis-dependent strand annealing (SDSA) does not generate cross-overs and has been invoked (Borst et al., 1996; Barry, 1997) as it avoids potentially lethal translocations when array VSGs are copied into the ES (Fig. 1C). However, given that a substantial minority of VSGs are telomeric, break-induced replication (BIR) has also been suggested (Barry and McCulloch, 2001; Dreesen et al., 2007). BIR (Fig. 1D) is enzymatically and kinetically distinct from SDSA (Lydeard et al., 2007), primarily in that it involves repair by a processive DNA replication fork established from one end of a DNA double strand break (DSB). In yeast, BIR can stabilize telomeric repeats using subtelomeric sequences (Lydeard et al., 2007), and this may be promoted in *T. brucei* VSG switching by the preponderance of conserved subtelomeric sequences, such

Table 1. Antigenic variation systems in eukaryotic and bacterial pathogens.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Surface antigen</th>
<th>Infection strategy</th>
<th>Gene no. in archive</th>
<th>% of archive pseudogene</th>
<th>Expression site no.</th>
<th>% of archive</th>
<th>Switch mechanism</th>
<th>RecA/Rad51 dependency</th>
<th>RecA-independent and -dependent</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. brucei</em></td>
<td>VSG</td>
<td>Extracellular</td>
<td>~1600</td>
<td>85</td>
<td>14–23</td>
<td>Reconversion and transcription</td>
<td>Recombination and transcription</td>
<td>Recombination and transcription</td>
<td>Recombination and transcription</td>
</tr>
<tr>
<td><em>P. carinii</em></td>
<td>MSG</td>
<td>Intracellular</td>
<td>~80</td>
<td>0</td>
<td>0</td>
<td>Transcription</td>
<td>Recombination</td>
<td>Recombination</td>
<td>Recombination</td>
</tr>
<tr>
<td><em>G. lamblia</em></td>
<td>VSP</td>
<td>Extracellular</td>
<td>~150</td>
<td>0</td>
<td>0–150</td>
<td>Transcription</td>
<td>Recombination</td>
<td>Recombination</td>
<td>Recombination</td>
</tr>
<tr>
<td><em>B. bovis</em></td>
<td>VES1</td>
<td>Intracellular</td>
<td>~150</td>
<td>0</td>
<td>0–150</td>
<td>Transcription</td>
<td>Recombination</td>
<td>Recombination</td>
<td>Recombination</td>
</tr>
<tr>
<td><em>P. falciparum</em></td>
<td>PfEMP1, variant specific surface protein</td>
<td>Intracellular</td>
<td>~60</td>
<td>0</td>
<td>0–150</td>
<td>Transcription</td>
<td>Recombination</td>
<td>Recombination</td>
<td>Recombination</td>
</tr>
<tr>
<td><em>B. hermsii</em></td>
<td>VLP/VSP</td>
<td>Extracellular</td>
<td>~60</td>
<td>0</td>
<td>0–150</td>
<td>Transcription</td>
<td>Recombination</td>
<td>Recombination</td>
<td>Recombination</td>
</tr>
<tr>
<td><em>B. burgdorferi</em></td>
<td>VLS</td>
<td>Extracellular</td>
<td>~15</td>
<td>100</td>
<td>1</td>
<td>Recombination</td>
<td>Recombination</td>
<td>Recombination</td>
<td>Recombination</td>
</tr>
<tr>
<td><em>N. gonorrhoeae</em></td>
<td>PIL</td>
<td>Extracellular</td>
<td>~20</td>
<td>100</td>
<td>1–2</td>
<td>Recombination</td>
<td>Recombination</td>
<td>Recombination</td>
<td>Recombination</td>
</tr>
<tr>
<td><em>A. marginale</em></td>
<td>MSP2</td>
<td>Intracellular</td>
<td>&lt;10</td>
<td>100</td>
<td>1–2</td>
<td>Recombination</td>
<td>Recombination</td>
<td>Recombination</td>
<td>Recombination</td>
</tr>
</tbody>
</table>

VSG, variant surface glycoprotein; MSG, major surface glycoprotein; VSP, variant specific surface protein; VES1, variant erythrocyte surface antigen, of both α and β types; PfEMP1, *P. falciparum* erythrocyte membrane protein 1, encoded by *var* genes; VLP/VSP, variable large protein/variable small protein; PIL encodes the type IV pilus; MSP2, major surface protein 2. N/A, not applicable.
as the 70 bp repeats. In addition, long-range VSG gene conversions in the ES during antigenic variation (Hertz-Fowler et al., 2008) may be better explained by BIR than SDSA. Nevertheless, no genetic or mechanistic dissection has shown that either SDSA or BIR act in VSG switching. Moreover, if both reactions do act, it is highly likely that BIR is important only for activation of telomeric VSGs, while SDSA can activate any gene in the archive.

A second recombination pathway has been termed reciprocal, telomeric VSG exchange (Pays et al., 1985). Here, chromosome ends are exchanged by cross-over, moving a silent telomeric VSG into the active ES and retaining the previously active VSG on the other chromosome, most likely by DSB repair (Fig. 1B). It seems this is a minor pathway of VSG switching. Indeed, recent work has suggested that the ES undergo extensive recombination within the ESAGs upstream of the VSG (Hertz-Fowler et al., 2008), and it is possible that VSG reciprocal exchange might be a by-product of this activity.

A final recombination pathway (Fig. 2) is used to generate novel, functional VSGs by combining portions of the ORFs from at least two pseudogenes to yield VSG mosaics (Thon et al., 1990). A number of observations suggest this is a distinct pathway to intact VSG gene
conversion. First, this appears to be a relatively inefficient reaction, with VSG mosaics observed late in infections (Marcello and Barry, 2007). Nevertheless, the huge number of VSG pseudogenes suggests this is very important (Barbet and Kamper, 1993), expanding the VSG coat repertoire beyond that encoded directly from the genome. Second, in contrast to the predominant use of flanking homology during gene conversion of intact VSGs, mosaic gene conversion involves extensive recombination within the VSG ORFs, often manifest as short conversion tracts. Third, mosaic VSG formation involves multiple donor genes, rather than the conversion of a single, intact VSG. Finally, activation of intact VSGs almost certainly targets the ES (Boothroyd et al., 2009), but it is unclear if this is also the case for mosaic VSG formation. Sequencing the bloodstream stage ES repertoire in one T. brucei strain revealed that each site possesses a functional VSG adjacent to the telomere (Hertz-Fowler et al., 2008) and it is unclear if the VSG pseudogenes sometimes observed upstream are assembly intermediates (see below).

Molecular players in antigenic variation by recombination

Variant surface glycoprotein switching, at least of intact VSGs, occurs by exploiting homologous recombination (HR), a universally conserved DNA repair mechanism (San Filippo et al., 2008). Mutation of three key factors of HR has been shown to impair (not abrogate) VSG switching: RAD51, the central enzyme of HR (McCulloch and Barry, 1999), and BRCA2 (Hartley and McCulloch, 2008) and RAD51-3 (Proudfoot and McCulloch, 2005), distinct proteins that mediate RAD51 activity (San Filippo et al., 2008). These data show striking parallels with Neisseria gonorrhoeae type IV pil genes to a pilE expression locus. This is dependent on RecA, the bacterial homologue of eukaryotic RAD51. Some evidence suggests that this exploits each available subpathway of RecA-dependent HR, initiated by a RecF-like route (Mehr and Seifert, 1998) or by recBCD (Hill et al., 2007), while other evidence appears to rule out recBCD-mediated initiation (Helm and Seifert, 2009). T. brucei VSG gene conversion clearly exploits different recombination subpathways, but the machinery is less clearly defined. Residual VSG gene conversion in RAD51 mutants suggests that RAD51-independent recombination occurs. Further evidence for flexibility in the recombination pathways is suggested by findings that four RAD51 paralogues appear to make non-equivalent contributions to the process (R. Dobson, C. Stockdale and R. McCulloch, unpublished) (Proudfoot and McCulloch, 2005). Intriguingly, the contribution of mismatch repair (MMR) to antigen gene conversion in the two pathogens also shows parallels. MMR recognizes and repairs base mismatches, which can occur during recombination between sequence-diverged DNA molecules, in which context MMR suppresses recombination. Surprisingly, mutation of neither mutS in N. gonorrhoeae nor the eukaryotic orthologue, MSH2, in T. brucei alters antigenic variation frequency, despite elevating rates of mutation and HR (Bell and McCulloch, 2003; Hill and Davies, 2009). This could suggest the gene conversion reactions are not subject to normal MMR surveillance, which for T. brucei could implicate an MMR-independent HR reaction (Barnes and McCulloch, 2007). Alternatively, in addition to regulating antigen recombination MMR may function during the initiation of the process (see below), nullifying the phenotype of any mutation.

The use of both antigen pseudogenes and intact genes for gene conversion in a single pathogen is rare (Table 1). To date, approaches in T. brucei have only assayed for recombination of intact VSGs, by investigating the factors that contribute to the reaction, or modelling the process experimentally (Boothroyd et al., 2009). Nevertheless, comparison with other pathogens may provide clues regarding mosaic VSG formation. Borrelia burgdorferi antigenic variation involves recombining silent vls segments into a vls ES (Norris, 2006) and looks superficially similar to that of N. gonorrhoeae, but is RecA-independent (Liveris et al., 2008). The explanation for this difference may reside in the fact that VlsE antigenic variation involves apparently random segmental conversions from multiple vls donors, without any clear homology sequences guiding the reaction (Zhang and Norris, 1998). This contrasts with the ‘mini-cassette’ process of N. gonorrhoeae pilS gene conversion (Hill and Davies, 2009), which is normally guided by upstream and downstream flanking sequence homology, similar to the recombination of intact vlp/vsp genes in Borrelia hermsii (Dai et al., 2006). In principle therefore B. burgdorferi antigenic variation appears more similar to Anaplasma marginale msp2 gene conversion, which employs only a very small donor archive and uses segmental gene conversion to generate huge numbers of variants (Futse et al., 2005). This reaction appears to be ‘anchored’ by sequence homology at one flank, but is resolved in regions of limited homology, allowing huge recombinosaurl flexibility (Futse et al., 2005). Two speculations seem warranted. First, it is likely that B. hermsii antigenic variation compares with gene conversion of intact T. brucei VSGs and N. gonorrhoeae pilS genes in being driven by RecA/Rad51-dependent recombination. Second, the segmental gene conversion reactions that underlie T. brucei VSG and A. marginale msp2 mosaic gene formation, like B. burgdorferi VlsE switching, are (at least partly) RecA/Rad51-independent. We still have much to learn about segmental gene conversion. Functional mosaic VSGs identified thus far
have been generated from multiple donor genes, and it has been inferred that this construction is stepwise, involving successive gene conversions (Marcello and Barry, 2007). However, VSG intermediates that would be generated and then overwritten in such a scenario have not been described, so we cannot yet exclude that a functional mosaic may result from a single, complex construction. Do the silent ES, as has been suggested (Barry and McCulloch, 2001), provide sites where VSG mosaics can be assembled over a number of cell divisions, or might the reaction have kinetics that allow a functional VSG mosaic to form within the active ES without leading to the expression of non-functional VSG protein? More broadly, segmental gene conversion pathways may have evolved to elaborate antigenic variation, allowing highly protracted infections marked by huge antigen variability and allowing superinfection in partly immune hosts. What features are common to those pathogens that employ only intact genes are less clear; this is not limited to intracellular pathogens (Table 1), but may be used where a relatively precise surface antigen activation hierarchy is seen, such as in \textit{B. hermsii} (Barbour et al., 2006).

**Initiation of VSG switching**

A question long unanswered in any pathogen, is how are recombination-based switching events initiated? DSBs within the antigen ES have been suggested as the likeliest culprit (Borst et al., 1996; Barry, 1997; Hill and Davies, 2009) and recently Boothroyd et al. (2009) provided evidence that this may be so for \textit{T. brucei} VSG switching. Controlled generation of a DSB adjacent to the 70 bp repeats in the active ES increased the rate of VSG switching and initiated VSG gene conversion. In addition, using ligation-mediated PCR, DNA breaks could be detected within the 70 bp repeats upstream of the VSG in the active ES, but much less readily (if at all) in an inactive ES. The 70 bp repeats have been predicted as the site of initiation previously (Barry, 1997; Liu et al., 1983), but further work is needed to determine how they act (Barry and McCulloch, 2009). This may not be straightforward, as several observations appear at odds with the suggestion that the 70 bp repeat breaks always initiate VSG switching. First, both previous work by McCulloch et al. (1997) and the Boothroyd et al. (2009) study examined VSG switching in \textit{T. brucei} cells expressing VSG from an ES engineered to lack 70 bp repeats. Neither study could detect differences in switch frequency compared with \textit{T. brucei} expressing VSG from the same ES when a large array of 70 bp repeats was retained. Second, in all eukaryotes DNA breaks elicit a DNA damage response, allowing the cell to co-ordinate repair with cell cycle progression. For DSBs, a key factor is the Mre11-Rad50-Nbs1/Xrs2 complex (Harper and Elledge, 2007), but mutation of MRE11 in \textit{T. brucei} has no discernible effect on VSG switching (Robinson et al., 2002), even though the protein is clearly important for genome stability (Robinson et al., 2002; Tan et al., 2002). Finally, generation of a DSB appeared not to recapitulate the full diversity of VSG switching events (Boothroyd et al., 2009), in that all switching reactions used telomeric VSGs as donors (>80% residing in silent ES). This raises the question of whether DSBs also elicit switching of intact array VSGs or VSG pseudogenes. Indeed, we do not yet know if DSBs in the ES recruit RAD51 for HR, as seen elsewhere in the genome (Glover et al., 2008).

In \textit{N. gonorrhoeae}, the route for initiation of antigenic variation has also recently been uncovered (Cahoon and Seifert, 2009). Here, a small G-rich sequence has been identified upstream of the \textit{pilE} expression locus that is critical for \textit{pilS} gene conversion and forms a guanine-quartet structure. Intriguingly, to be effective the G-rich sequence must be located adjacent to the expressed \textit{pilE} locus in an orientation and position-specific manner, and the available evidence suggests that it is DNA nicks, not DSBs, which form within the motif that initiate \textit{N. gonorrhoeae} antigenic variation.

**What is the role of VSG transcriptional switching, and how is it catalysed?**

Antigenic variation relies on the expression of a single ES out of many, a process termed allelic exclusion (Borst and Genest, 2006). An important context to understand this process is the observation (Navarro and Gull, 2001) that the active ES, which is transcribed by RNA polymerase (Pol) I, does not localize to the nucleolus (as expected for Pol-transcribed genes such as rRNA) but to a putative subnuclear site termed the expression site body (ESB). A model for the functioning of the ESB (whose composition remains unknown) is that it accommodates only a single ES, explaining why attempts to select for trypanosomes expressing >1 ES yield only unstable, rapidly switching intermediates (Chaves et al., 1999). Recently, a number of factors have been described whose mutation or RNAi-mediated knockdown results in derepression of transcription from the silent ES. Most factors that have been analysed influence chromatin structure, and in each case derepression affected differing extents of the ES transcription units (Fig. 2). RNAi of \textit{TbISWII}, a SWI2/SNF-2-related chromatin remodelling factor, leads to a ~30-fold increase in transcripts of genes proximal to the ES promoter (Hughes et al., 2007). Conversely, RNAi of \textit{TbRAP1}, which in yeast binds telomeres and contributes to heterochromatin formation, causes a 2–50 fold increase in VSG transcripts from the silent ES, but lesser derepression proximal to the promoter (Yang et al., 2009).
A similar telomere-mediated effect is seen in histone deacetylase TbSir2 mutants (Alsford et al., 2007), but limited to the region downstream of the VSGs. Finally, RNAi of DOT1B, one of two T. brucei histone H3 methyltransferases, results in ~10-fold derepression of the whole silent ES transcription unit (Figueiredo et al., 2008). In addition, DOT1B RNAi caused accumulation of cells expressing two VSGs on the cell surface, indicating a perturbation in VSG coat transition during switching. These studies reveal that a number of epigenetic processes contribute to singular ES expression, but it is striking that the extent of silent ES derepression never leads to transcription at the levels seen in the active site, and the frequency of VSG switching appears not to change (where tested). It seems likely therefore that these are secondary processes that follow from the establishment of singular expression. Indeed, the singularity of the ESB may not be perturbed in these studies, as transcription of the silent ES may not emanate from the ESB, but from the nucleolus, which would explain why transcriptional switching does not increase. In contrast, a very recent study by Landeira et al. (2009) used partial RNAi against one component of the cohesin complex, which is responsible for ensuring newly replicated sister chromatids remain associated until mitotic segregation, and caused an increase in transcriptional switching. Partial cohesin RNAi appears to cause premature dissociation of the newly replicated ES from the ESB, and thereby presumably undermines the inheritance of the signals that dictate ES activity. This suggests that cell division (and perhaps DNA replication) is a crucial cell cycle stage at which transcriptional switching might occur. Nevertheless, the physiological triggers that might promote transcriptional switching remain to be determined for trypanosomes, as for pathogens exclusively reliant on this process, such as Plasmodium falciparum (Scherf et al., 2008).

A broader question about transcriptional switching in trypanosomes is the purpose of this reaction. Given that the overwhelming numbers of silent VSGs that must be activated by recombination, does the presence of ~20 ES add much to antigenic variation, or does it contribute another function? It has been suggested that the reason for possessing multiple ES may lie in the ESAGs, not the VSGs (Bitter et al., 1998; Young et al., 2008). ESAG6 and ESAG7 form a heterodimeric receptor for host transferrin, supplying the parasite with iron. A host-range hypothesis has stemmed from this, suggesting that transcriptional switching reveals different transferrin receptors adapted to the different mammalian hosts trypanosomes infect (Bitter et al., 1998). This has been extended to other ESAGs and, though questioned by others (Steverding, 2006), would suggest that transcriptional switching provides a means for infection establishment. However, we cannot yet exclude that transcriptional switching provides antigenic variation functions throughout a chronic infection: the silent ES may provide sites for the generation of VSG mosaics (Barry and McCulloch, 2001), or might provide a failsafe switch mechanism when VSG pseudogenes are switched into the active ES. Understanding the evolution of the VSG system for antigenic variation would shed light on this (Barry and McCulloch, 2001). For instance, do transcriptional and recombinational switching reactions share a common trigger?
Infection dynamics and antigenic variation

The essence of the antigenic variation system in trypanosomes is to ensure survival within the mammal to allow transmission to the next host, via the tsetse vector. The system is clearly efficient, because infections in experimental cattle can last for hundreds of days (Luckins and Mehlitz, 1976), while human infections can have a pre-patent period of years (during which it is assumed antigenic variation occurs) before clinical signs emerge (Barrett et al., 2003). This picture of a chronic disease is not mirrored by the majority of the trypanosome antigenic variation literature, which, largely due to experimental restrictions, has concentrated on the first peaks of parasitaemia, essentially the initiation and establishment of infection. Despite these shortcomings, studies that have ventured into the chronic phase of infection (Capbern et al., 1977; Thon et al., 1990; Kamper and Barbet, 1992; Morrison et al., 2005; Marcello and Barry, 2007) have revealed some intriguing insights into the dynamics and functioning of antigenic variation.

Antigenic variation occurs independently of host immune responses as a stochastic process. However, it is antibody selection that results in the observed hierarchy of VSG expression (Fig. 3). Experimental infections and mathematical modelling suggest the first peak of parasitaemia is controlled by a combination of density dependent growth arrest and immunity (Morrison et al., 2005; Lythgoe et al., 2007). The sequential expression of VSGs that follows (Fig. 3) is a product of cumulative antibody responses that select against the more frequently activated VSGs as the infection progresses. As we have argued above, VSG switching is two-tiered, using one form of recombination (mosaic VSG formation) that is VSG-sequence homology-dependent, and another (gene conversion of intact VSGs) driven by sequence homology in the flanking regions. Mosaic VSG formation is relatively rare (Thon et al., 1990; Kamper and Barbet, 1992; Barry, 1997) and predominates later in infection (Marcello and Barry, 2007). This difference in rate of switching between VSG classes provides some basis for understanding the VSG hierarchy, but a modelling analysis (Lythgoe et al., 2007) suggests further subtleties (Fig. 3). Within the limitations of the model, only by grouping genes into subsets with distinct ranges of switching probabilities could a realistic representation of in vivo infection dynamics be generated. These groupings may correspond to (a) those genes that comprise intact and functional ORFs that are switched to by a simple single-step recombination reaction, and (b) pseudogenes requiring more than a single recombination event to create an intact ORF and functional protein. Within these probability clusters there is further hierarchy; for example, within (a), probability of switching has been experimentally demonstrated to be dependent upon the locus position of the donor sequence (Aline et al., 1985; Liu et al., 1985; Robinson et al., 1999;
Morrison et al., 2005) or (b), dependent upon the number of recombination events required to create a functionally expressed VSG, from involving few sequences (Pays et al., 1983) to multiple and complex recombinations (Kamper and Barbet, 1992; Marcello and Barry, 2007). Several aspects remain unclear, however. Most prominently, we do not yet know if the transition to mosaic VSG formation involves a change in dominance of switching mechanisms per se (i.e. specific recombination pathway differences), mainly because recombination pathway responsible has not been characterized. The demonstration of RAD51-independent recombination in T. brucei requiring as little as 7–13 bp of homology (Conway et al., 1975; Capbern et al., 1977; Robin- son et al., 1999; Morrison et al., 2005), although it is a phenomenon that has not specifically been searched for. Immunosuppression during trypanosome infections might also contribute to antigenic variation by allowing increased tolerance of cross-reactive epitopes and a wider range of VSG mosaics in the late stages of infection. Furthermore, the recently reported ‘extreme’ loss of memory B cells (Radwanska et al., 2008) may explain the reappearance of early variants late in infections observed in mice, rabbits and goats (but not cattle) (Barry, 1986). Interestingly, pathogen interference with immunological memory is not restricted to trypanosome infections, but is seen also in Anaplasma (Han et al., 2008) and Plasmodium (Wykes and Good, 2006). Immune selection at a population level and chronicity, combined with homology-driven switching, may drive VSG archive evolution down homology-mediated routes, explaining how strain divergence in VSG repertoire develops (Hutchinson et al., 2007). The continual generation of antigenic diversity at the repertoire level is essential to allow the parasite to overcome herd immunity (Futse et al., 2008).

Antigenic variation undoubtedly combines with other parasite- and host-driven mechanisms to maintain chronicity. Probably the most important parasite determinant for maintaining chronic infections, other than the antigenic variation system, is the self-regulation of growth by the density-dependent differentiation of mitotically dividing long slender bloodstream form cells to non-dividing short stumpy form cells, which are pre-adapted to initiate infection in the tsetse fly midgut (Barry and McCulloch, 2001). The interplay of differentiation and antigenic variation are key (Tyler et al., 2001; Lythgoe et al., 2007), and the recent identification of a key marker of differentiation will allow this relationship to be examined in detail (Dean et al., 2009). Further fitness advantages that allow an increased time window for a successful switch to novel variants may be at the population level and more generic, such as hydrodynamic uptake and removal of bound antibody (Engstler et al., 2007), or the advantages conferred by different trypanosome transferrin receptors (Bitter et al., 1998). These multiple mechanisms, along with the VSG switching dynamics, will contribute to the evolution
of fitness within the host. Certainly, when superinfections with a different strain of trypanosome are attempted in an animal with an established infection, the strain that is established does seem to have a fitness advantage (Morrison et al., 1982).

In summary, the availability of post-genomic tools has begun the exploration of several key aspects of antigenic variation, revealing underlying mechanisms and VSG switching dynamics in the chronic stage of infection. Understanding this stage may explain the apparently uniquely enormous antigen repertoire in trypanosomes, and identification of the molecular mechanism of mosaic VSG formation will inform how infection time and potential for transmission is maximized. Appreciating the diversity of host–parasite interactions is important. Combining markers for both parasite (VSG switching and stumpy formation) and host (immune response and antibodies) will result in better overall understanding of how trypanosomiasis manifests as a chronic disease, and potentially provide opportunities for disease intervention.

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