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Microreview

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 *VSG*s 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 *VSG* arrays (Berriman *et al.*, 2005), while sites for *VSG* transcription, termed the *VSG* expression sites (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 *VSG*s at their ends, suggesting they evolved to expand the telomeric pool of *VSG*s (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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Table 1. Antigenic variation systems in eukaryotic and bacterial pathogens.

Pathogen	Infection strategy	Surface antigen[a]	Gene no. in archive	% of archive pseudogene	Expression site no.	Switch mechanism	RecA/Rad51 dependency
T. brucei	Extracellular	VSG	~1600	85	14–23	Recombination and transcription	Rad51-dependent and -independent
P. carinii	Intracellular	MSG	~80	0	1	Recombination	?
G. lamblia	Extracellular	VSP	~150	0	~150	Transcription	N/A
B. bovis	Intracellular	VES1	~150	~50	24–42	Recombination and transcription	?
P. falciparum	Intracellular	PfEMP1	~60	0	~60	Transcription	N/A
B. hermsii	Extracellular	VLP/VSP	~60	0	1	Recombination	?
B. burgdorferi	Extracellular	VLS	~15	100	1	Recombination	RecA-independent
N. gonorrhoeae	Extracellular	PIL	~20	100	1–2	Recombination	RecA-dependent
A. marginale	Intracellular	MSP2	< 10	100	1	Recombination	?

a. 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; VLS, Vmp-like sequence; PIL encodes the type IV pilus; MSP2, major surface protein 2.
N/A, not applicable.

Two modes of VSG switching occur: recombination reactions that move silent *VSG*s 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 *VSG*s 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 *VSG*s are activated earliest in infections, followed by intact subtelomeric array *VSG*s, 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 *VSG*s (~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 *VSG*s 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 *VSG*s are copied into the ES (Fig. 1C). However, given that a substantial minority of *VSG*s 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

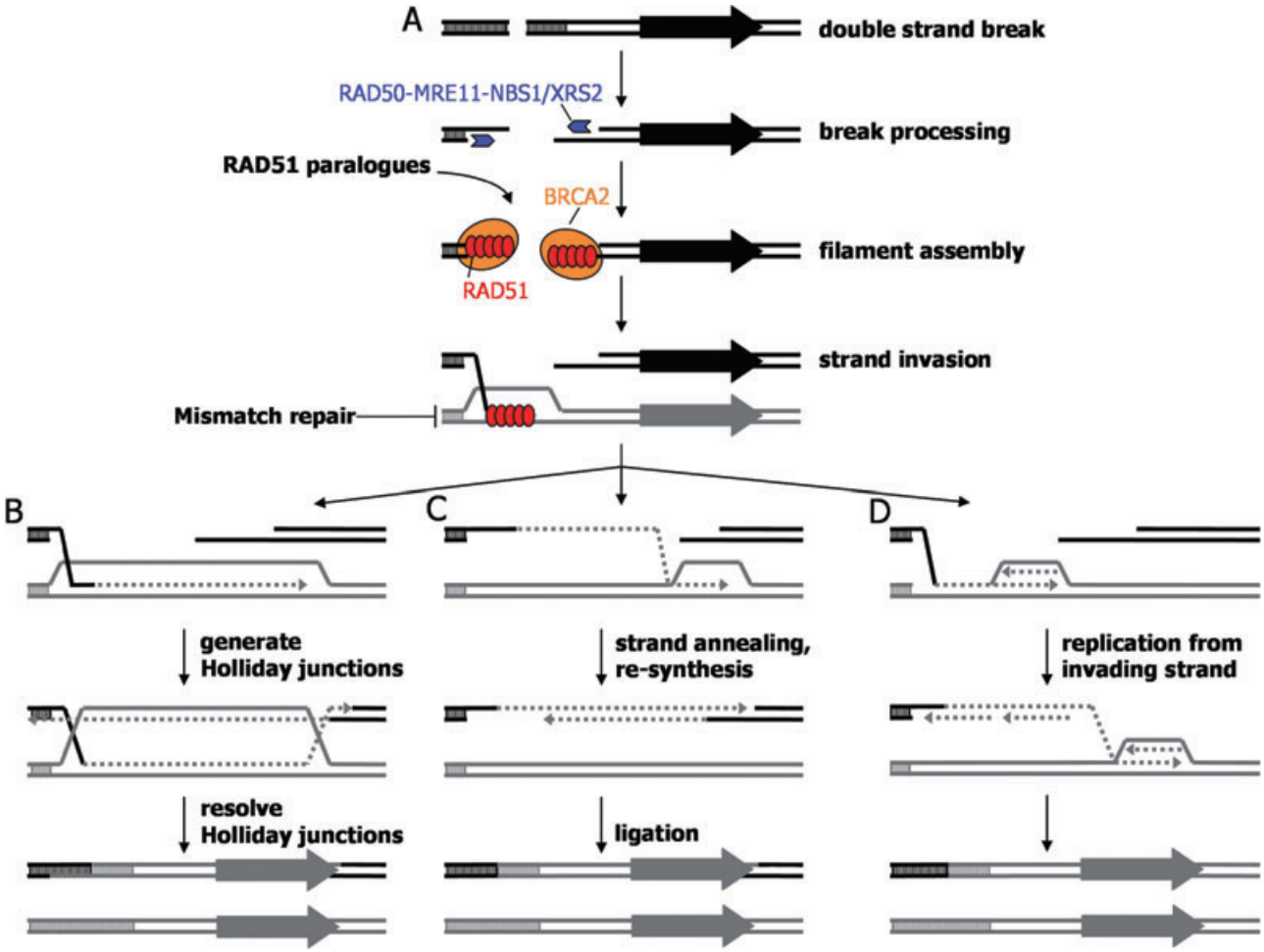

Fig. 1. Recombination models for VSG switching by gene conversion.
A. Recombination is shown initiated by a DNA double strand break (DSB) in the 70 bp repeats (hatched) upstream of the *VSG* (black arrow) in the active expression site. DSB processing reveals 3′ single-stranded ends, a reaction in which MRE11-RAD50-XRS2/NBS1 acts, providing a substrate for RAD51 to form a nucleoprotein filament. RAD51 function is aided by a number of factors, including BRCA2 and multiple RAD51 paralogues (not shown). RAD51 catalyses homology-dependent invasion of the single-stranded end into intact DNA duplex (grey lines) containing a silent *VSG* (grey arrow); mismatch repair (MMR) limits recombination to sufficiently homologous sequences. Three pathways for DSB repair can be used. B shows double strand break repair, where newly synthesized DNA is copied from the intact DNA duplex and remains base-pared, leading to Holliday junction recombination intermediates, whose enzymatic resolution can lead to gene conversion with (not shown) or without (shown) cross-over of flanking sequences. C shows synthesis-dependent strand annealing, where newly replicated DNA is displaced from the intact duplex, re-anneals with homologous sequence at the DSB, allowing synthesis of the other strand. In D, break-induced replication, an origin-independent replication fork, with leading and lagging strand synthesis, forms on the strand invasion intermediate allowing replication to the chromosome end.

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 *VSG*s, 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 *ESAG*s 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 *VSG*s 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 *VSG*s, 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 *VSG*s 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 *VSG*s, 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 pili antigenic variation (Hill and Davies, 2009), which occurs by gene conversion of silent, non-functional *pilS* 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 *VSG*s, 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 *vlsE* 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 recombinatorial 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 VSG*s 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 *VSG*s 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 *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 *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 *T. brucei* cells expressing VSG from an *ES* engineered to lack 70 bp repeats. Neither study could detect differences in switch frequency compared with *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 *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 *VSG*s as donors (> 80% residing in silent ES). This raises the question of whether DSBs also elicit switching of intact array *VSG*s 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 *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 *pilE* expression locus that is critical for *pilS* gene conversion and forms a guanine-quartet structure. Intriguingly, to be effective the G-rich sequence must be located adjacent to the expressed *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 *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 PolI-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 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 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).

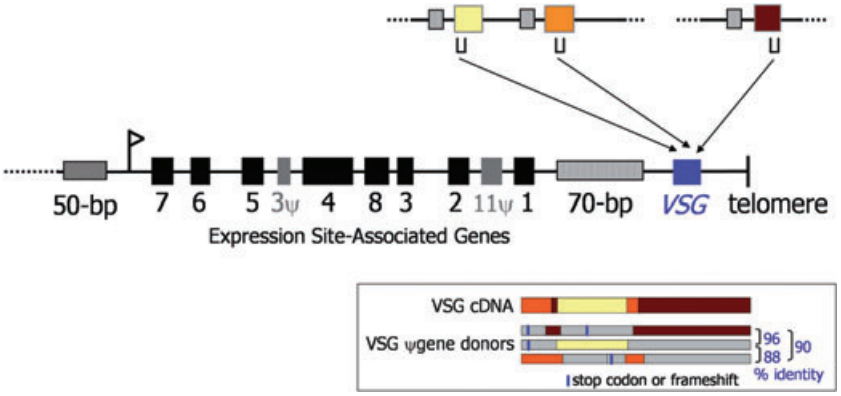

Fig. 2. VSG mosaic formation by segmental gene conversion. A *VSG* gene (blue box) is shown within an expression site (ES), which is represented as an archetype of such a transcription unit: expression site associated genes are shown as functional (black box) or pseudogenes (grey box) and are identified by number; repeat sequences within (70 bp repeats) or upstream (50 bp repeats) of the ES are indicated by hatched boxes; the promoter is indicated by an arrow; and the telomere by a vertical line. Segmental gene conversion of three silent *VSG* pseudogenes from the subtelomeric array archive is diagrammed, indicating segments from each ORF being combined (arrows) to replace the transcribed *VSG*. The inset shows details of an experimentally verified *VSG* mosaic (adapted from Marcello and Barry, 2007). Three silent *VSG* pseudogene donor ORFs are shown below the mosaic sequence of the functional *VSG* (identified as an expressed cDNA): colours indicate regions of sequence homology between the donors and the cDNA, and the potential junctions in the gene conversion(s); overall sequence identity is shown.

A similar telomere-mediated effect is seen in histone deacetylase TbSir2 mutants (Alsford *et al*., 2007), but limited to the region downstream of the *VSG*s. 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 *VSG*s 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 *ESAG*s, not the *VSG*s (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 *ESAG*s 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?

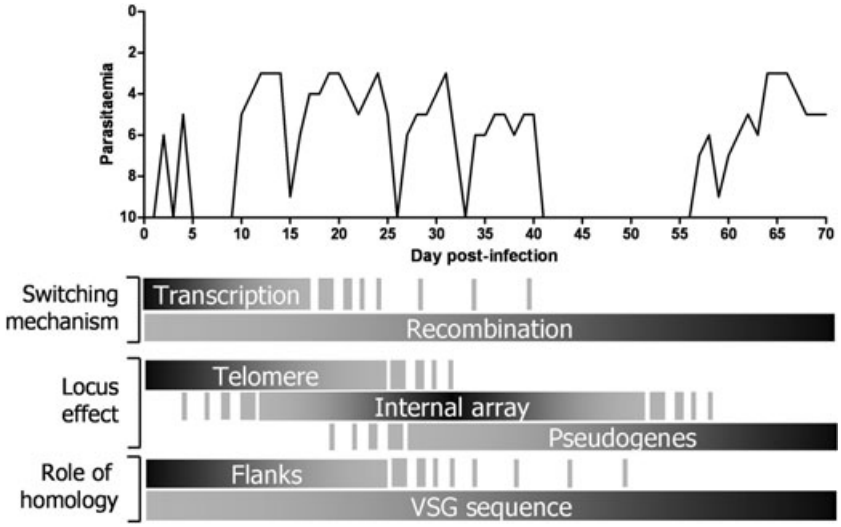

Fig. 3. VSG switching hierarchy in *T. brucei*. The graph shows the numbers of *T. brucei* cells (parasitaemia) measured in a cow up to 70 days post infection (plotted as the pre-patent period, in days, that a 0.2 ml inoculum of cattle blood achieves a parasitaemia of $1 \times 10^{8.1}$ trypanosomes ml^{-1}units in a mouse; adapted from Morrison *et al.*, 2005). Below is a depiction of the timing of *VSG* switch mechanism usage and gene activation. Transcriptional switching is proposed to occur at the outset of an infection, whereas recombination switching is used throughout; note, the former proposal is not experimentally verified. Silent telomeric *VSG*s are activated more frequently than intact, subtelomeric array *VSG*s, which are activated more frequently than *VSG* pseudogenes. During recombinational switching, VSG flanking sequence (e.g. 70 bp repeats) is proposed to mediate the *VSG* switching reactions of intact genes early in infections, whereas the formation of *VSG* mosaics relies on recombination using the genes' ORF sequences later in infections. The intensity of shading in each bar represents the effective switching rate, i.e. the proportion of switches that give rise to novel VSGs.

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 prepatent 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 *VSG*s) 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*., 2002; Glover *et al*., 2008) suggests a pathway, but this remains to be tested. There may be further mechanistic differences in VSG switching at the species level. The *VSG* archive of *T. brucei* is now fairly well characterized, but those of *T. congolense* and *T. vivax* have not been similarly analysed. Genome projects are present for both (http://www.genedb.org/), and preliminary analysis suggests that *T. congolense* does not have the 70 bp repeat system of *T. brucei* (Marcello, 2006), hinting that phenotypic similarities in infection profiles may hide mechanistic differences in VSG switching.

Recombination-based determinants of switch hierarchy have been described during antigenic variation in other pathogens. In *B. hermsii* the activation probability of silent (functionally intact) *vlp/vsp* genes appears to be dependent on the sequence homology and positioning, respectively, of upstream and downstream homology elements (Barbour *et al*., 2006). *A. marginale* also displays a hierarchy, but using only pseudogenes: simple *msp2* mosaics appear preferentially early in infection and more complex mosaic genes predominate later, in the presence of a patent-specific immune response to the earlier, simple mosaics (Palmer *et al*., 2007). It has been suggested that the simple mosaic confer a fitness advantage over the complex mosaics, but this fitness advantage is balanced by the simple mosaics being more immunogenic (Zhuang *et al*., 2007). A potential explanation for antigens expressed early in infection being more immunogenic is that if the switching probability matrix is maintained throughout infection, they will be continuously switched to, giving rise to a persistent stimulation of the immune response to those epitopes. Once the antibody response against these epitopes is patent, further switches to these commonly switched to antigens will be redundant, but the maintenance of this spectrum of switching probability throughout infection will result in the characteristic profile (in trypanosomes and *Anaplasma*) of large, early parasitaemic peaks followed by intermittent and smaller peaks (Lythgoe *et al*., 2007) (i.e. a reduction in the effective switching rate). Variant fitness requires addressing in trypanosome chronic infections, as this may be due to cross-reactive epitopes, as suggested in *Anaplasma*, but also potentially to the creation of non- (or less-) functional VSGs as the more complex (and less probable) mosaic formation occurs, and consequently reduced fitness of the trypanosome expressing that VSG. In addition, homology-driven switching is likely to generate VSGs with epitopes that are cross-reactive with patent immune responses, and such cross-reactivity has been suggested to play a vital role in determining *Plasmodium* infection dynamics (Recker *et al*., 2004). Cross-reaction between immune responses to VSGs has not been observed in trypanosome infections (Van Meirvenne *et al*., 1975; Capbern *et al*., 1977; Robinson *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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