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Probabilistic order in antigenic variation of *Trypanosoma brucei*

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Abstract

Antigenic variation in African trypanosomes displays a degree of order that is usually described as 'semi-predictable' but which has not been analysed in statistical detail. It has been proposed that, during switching, the variable antigen type (VAT) being inactivated can influence which VAT is subsequently activated. Antigenic variation proceeds by the differential activation of members of the large archive of distinct variable surface glycoprotein (*VSG*) genes. The most popular model for ordered expression of VATs invokes differential activation probabilities for individual *VSG* genes, dictated in part by which of the four types of genetic locus they occupy. We have shown, in pilot experiments in cattle, correlation between the timing of appearance of *VSG*-specific mRNA and of lytic antibodies corresponding to seven VSGs encoded by single-copy genes. We have then determined the times of appearance of VAT-specific antibodies, as a measure of appearance of the VATs, in a statistically significant number of mouse infections ($n=22$). There is a surprisingly high degree of order in temporal appearance of the VATs, indicating that antigenic variation proceeds through order in the probability of activation of each VAT. In addition, for the few examples of each available, the locus type inhabited by the silent 'donor' *VSG* plays a significant role in determination of order. We have analysed in detail previously published data on VATs appearing in first relapse peaks, and find that the variant being switched off does not influence which one is being switched on. This differs from what has been reported for *Plasmodium falciparum var* antigenic variation. All these features of trypanosome antigenic variation can be explained by a one-step model in which, following an initial deactivation event, the switch process and the imposition of order early in infection arise from the inherent activation probabilities of the specific *VSG* being switched on.

Keywords: Antigenic variation; *VSG*; VAT; Hierarchy

1. Introduction

African trypanosomes survive the antibody responses of their vertebrate hosts through antigenic variation, whereby there is a continual, clonal activation of novel variable antigen types (VATs) that are unrecognised by antibodies against other VATs. The molecular basis of antigenic variation is the parasite's variant surface glycoprotein (VSG) coat, which shields invariant antigens from antibodies and is thought to protect also against innate immune mechanisms (Ferrante and Allison, 1983). As the VSG has no known function other than forming an immuno-protective coat, its sequence can vary extensively within structural constraints, and this has given rise to the evolution of a large archive of distinct *VSG* genes in the genome. VAT switching involves the differential expression of these genes and occurs, at the population level within the host, at a rate of about 10^{-2} switches per cell per generation (reviewed by Barry and McCulloch, 2001). In common with similar systems in other pathogens, the switching process is stochastic and spontaneous, and acts independently of antibody presence. It is also divergent and not simply progressional, as seen when a clonal first peak is followed by a first relapse peak that contains at least several VATs. In addition, the composition of these first relapse peaks differs between individual infections, although certain VATs tend to

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be common to most (Miller and Turner, 1981). The peaks of growth in trypanosome infections are terminated by two main factors, anti-VSG antibody responses and the density-dependent phenomenon of differentiation to the amitotic short stumpy stage (Tyler et al., 2001). The two factors interact, but the differentiation effect has the potential to act on all trypanosomes in a peak of growth, whereas the VSG-specific antibody responses exert their effect only on the specific VAT subpopulations.

One of the mysteries of antigenic variation in *Trypanosoma brucei* is how, and to what extent, the appearance of VATs is ordered during infection. There is similarity in order of appearance, usually described as 'semi-predictable', between independent infections, but exactly how predictable has not been established. It is generally thought (reviewed by Barry and McCulloch, 2001) that there is a selective advantage in ordered (hierarchical) expression, serving to spread the infection into chronicity rather than having a disordered, and thus possibly overwhelming, initial wave of parasitaemia. Given the high switch rates and the large number of parasites that can be present early in infection, with a random activation system the entire archive of hundreds of VSGs could be expressed rapidly, which could lead to exhaustion of potential antigens or early death of the host, but this does not happen. Gray (1965) described a degree of order in independent infections, and subsequent studies involving clonal analyses showed that the same subsets of VATs tend to appear in independent first relapse peaks (McNeillage et al., 1969; Van Meirvenne et al., 1975; Miller and Turner, 1981; Liu et al., 1985). Most switching studies have examined only the first relapse peak, but two studies followed infections for longer periods, one involving *Trypanosoma equiperdum* infections of rabbits for 1 month (Capbern et al., 1977), and the other examining *Trypanosoma vivax* infections in several host species for up to several months (Barry, 1986). They confirmed that there is a general order, spread throughout infection but more apparent in the early weeks. Detailed study of one particular *VSG* gene in the first relapse phase of infection showed that it was activated independently in the same few days in separate infections (Lee and Van der Ploeg, 1987; Timmers et al., 1987). However, it remains unclear exactly how predictable or otherwise the trypanosome antigenic variation system is, which is important if we are to gain a broad understanding of how it evolved and functions within the context of chronic infections, its role in persistence and transmission in the field in the presence of any herd immunity, and in turn if a strategy for control might ever be developed.

There are several theories on how order occurs. Seed (1978) tested the hypothesis that it is due to competitive growth between VATs but found that, although clones with different growth rates arise during infection, there is not a strong correlation with the VSG expressed. Further, modelling studies based on those data suggested that the observed growth rate differences could not account for ordered emergence of VATs (Kosinski, 1980). Another proposition is that particular switching intermediates are selected against in vivo, thereby restricting the range of switch products (Agur et al., 1989). This has not received support from experiments with trypanosomes induced to artificially express two VSGs (Muñoz-Jordan et al., 1996), but is difficult to test directly. For some pathogens, immune responses against cross-reacting, or invariant, antigens can exert a limiting effect on growth as infection proceeds (Oxford et al., 2003), a mechanism that has been proposed to occur for the malaria parasite *Plasmodium falciparum* (Recker et al., 2004). For the trypanosome, however, no such antigens have been identified, and it is difficult to see how constitutively expressed invariant antigens could cause VAT-specific order, as opposed to general growth limitation. Antigenic cross-reactivity between distinct VSGs theoretically could place restriction on the succession of VATs but, at least early in infection, immunity is VAT-specific (Van Meirvenne et al., 1975; Robinson et al., 1999).

The simplest, and most frequently proposed, model is that hierarchical order is imposed through differences in the activation probabilities of different *VSG* genes, which is related partly to the genomic locus types they occupy. The simplicity of this model is that it is single-step, with the switch and the order being created by the same activation event. There are probably about 1700 distinct *VSG*s in the diploid genome, and they occupy four locus types, all subtelomeric (reviewed by Barry and McCulloch, 2001). There is a silent archive of ~1500 in long tandem gene arrays and up to ~200 on minichromosomes. There are also ~20 in the Bloodstream Expression Sites (BESs) and ~20 *MVSG*s that become activated in the metacyclic stage in the tsetse fly. In mammalian infections, *VSG*s from the silent archive are transcribed only by being copied into BESs, of which one is transcriptionally active at any time. A number of studies have shown that the type of locus occupied by a *VSG* gene can influence the timing of its activation (Young et al., 1983; Laurent et al., 1984; Myler et al., 1984; Liu et al., 1985; Van der Werf et al., 1990; Robinson et al., 1999). In particular, minichromosomal genes are activated early. Activation can involve duplication from telomeres, which happens particularly in trypanosomes switching at the natural, high rate, or by transcriptional switching between BESs, which is the main event in laboratory trypanosome lines. Array genes activate relatively rarely, and do so through duplication of the entire gene into a BES or partial copying, contributing segmentally to formation of an expressed gene.

One further aspect of switching is whether or not the switch-off VAT influences which VAT is switched on. Analyses of the *var* gene switching patterns of *P. falciparum* clones growing and switching in vitro (Horrocks et al., 2004), and of VSG switching in *T. brucei* infections of rats (Miller and Turner, 1981), have led to the suggestion that the switch-off antigen type has a determining effect on the type that appears subsequently. Neither study, however,

included statistical analysis. Interaction of this nature would be important for the single-step model, because imposition of a high degree of influence by the gene being switched off would add a further layer of complexity to the underlying molecular mechanisms.

Here, we examine the degree of predictability in ordered VAT expression, by undertaking detailed statistical analysis of the de novo appearance of seven individual VATs, each expressing a VSG encoded by a single-copy gene, in multiple infections. We also test whether *VSG* locus type has a significant effect on its place in the order of expression and, through statistical analysis of a substantial number of first relapse peaks reported in the literature, whether the expressed VAT dictates the particular VATs to which it switches.

2. Materials and methods

2.1. Trypanosome infections

BALB/c female mice (Harlan, UK), approximately 25 g bodyweight, were infected with various doses, as stated below. *Trypanosoma brucei* EATRO 795, a pleomorphic and meroclonal (clonal, but degenerated to expression of >1 VAT) line that switches VSG at approximately 1×10^{-2} switch.cell^{-1}.generation^{-1} (Turner, 1997). Trypanosomes were grown from stabilate in a cyclophosphamide-immunosuppressed mouse (25 mg.kg bodyweight^{-1} via the i.p. route, 24 h prior to infection). Trypanosomes (at least 80% long slender form) from the initial parasitaemic peak were diluted into samples of 1×10^{7} cells in guinea pig serum (GPS; Harlan UK), and were incubated for 1 h at 37 °C in specific antisera against each of the seven VSGs under study, at a final antibody concentration of 10%. This neutralisation step ensured that these VSGs arose in experimental infections only from de novo switching events. The effectiveness of the neutralisation step was tested by incubating each antiserum with clonal trypanosomes expressing the corresponding VAT. In each case, no surviving parasites were observed in several hundred examined. Experimental infections were initiated with 1×10^{6} motile cells inoculated i.p. (trial infections revealed that 1×10^{6} parasites produced patent parasitaemia from day 5 or 6 post inoculation, and a chronic infection over the 35 days of the experiment). Parasitaemia was estimated every 2 days by haemocytometer counts and plasma was collected every fourth day.

Infections in cattle were initiated with the *T. brucei* ILTat 1.2 clonal trypanosome line, which is a line of EATRO 795 that antigenically varies at approximately 1×10^{-5} switch.cell^{-1}.generation^{-1} (Robinson et al., 1999). Two Friesian steers approximately 18 months old were obtained from Rift Valley Province, Kenya, an area free from trypanosomiasis. The experiment was carried out at the International Livestock Research Institute (ILRI), Nairobi, Kenya, also free from trypanosomiasis and vectors. The animals were confirmed free of antibodies against somatic trypanosome antigen by standard ILRI serodiagnosis. Trypanosomes were grown from stabilate in a Harlan MF1 mouse (ILRI), immunosuppressed 24 h previously by sub-lethal irradiation. Trypanosomes were prepared as for the mouse infections, except that fresh serum from ILRI guinea pigs was used for antibody neutralisations. One$\times10^{6}$ motile organisms were inoculated intravenously and the infections were monitored for 70 days. Of 5 ml blood collected in an EDTA vacutainer (Beckton Dickinson) every 24 h, 0.2 ml was injected i.p. into an irradiated Harlan MF1 mouse to amplify any parasite population present, both as a method of detecting low levels of trypanosome and also in order to make representative stabilates of the daily populations. Euthanasia and exsanguinations were performed when the parasitaemia reached at least $1\times10^{8.1}$ parasites.ml^{-1} (Herbert and Lumsden, 1976). With the remainder of the cattle daily blood sample, parasitaemia was measured by haemocytometer counts, and by examining the buffy coat, and plasma was also collected from 2 ml of the blood.

Parasitaemia measured by haemocytometer counting, with a detection threshold of 1×10^{5} trypanosomes.ml^{-1}, revealed only an initial peak in both cattle, on days 12–14 in cow A, and on days 13 and 14 in cow B. No parasites were detected by this method during the remaining 70 days of infection in cow A, and only on days 19, 20, 24, 31, 65 and 66 in cow B, each incidence at the detection threshold. The buffy coat technique, which is less accurate but has a greater sensitivity of approximately 1×10^{2} parasites.ml^{-1}, revealed trypanosomes more frequently. The most sensitive means of observing parasitaemia was the indirect approach of subinoculation into mice. Scoring the time (days) taken for achievement of a mouse parasitaemia of $1\times10^{8.1}$ parasites.ml^{-1} provided a reflection of the corresponding cow parasitaemia (Fig. 1). It was assumed that, if no parasites could be detected in the mice by 10 days post-inoculation, there had been no viable parasites in the inoculum.

2.2. RNA isolation, cDNA generation and PCR

In the cattle experiments, RNA was extracted from each 0.2 ml blood sample using the RNeasy® kit (Qiagen). cDNAs were generated by reverse transcription (RT) using the Superscript II® first-strand synthesis system for RT-PCR kit (Invitrogen). The following sets of primers were designed for PCR. For trypanosome tubulin: Tubfor2 and Tubrev2 (AGCCAGGTACAATGGACTCC, CGTTCATG TTGGACTCTGCC); for bovine actin: bovactfor and bovactrev (GCGGCATTCACGAAACTACC, TGGGAA GGCAAAGGACTTCC); and for the respective ILTat *VSG*s (primer names refer to the respective *VSG*): 1.2for3 and 1.2rev3 (AATCGCAAATACCGGCCTGG, AAGG TTTGCTGTTGCATGCC); 1.21for and 1.21rev (CAAAGGCAGGCGGTTCGATC, TGTTTGCTTTGCC

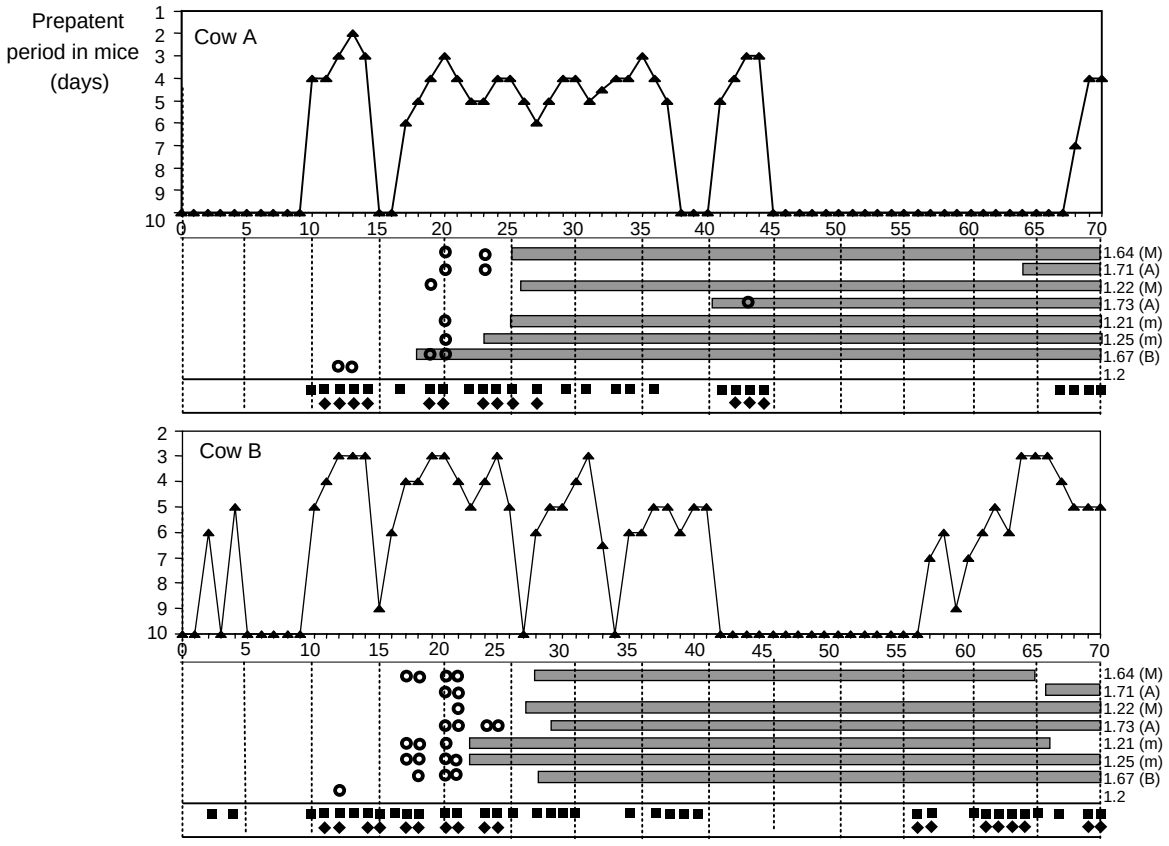

Fig. 1. Summary of 70 day *Trypanosoma brucei* ILTat 1.2 infections in cattle A and B. Upper half of each diagram: the *x*-axis follows the timeline of infection, from days 0–70. Parasitaemic profiles of cattle A and B are plotted as measured by inversely plotting the prepatent period in days (*y*-axis), from inoculating 0.2 ml cattle blood into an immunosuppressed mouse to achievement of a parasitaemia of $1\times10^{8.1}$ trypanosomes.ml^{-1}, a measure that is approximately proportional to the parasite density in the cow. Lower half of each diagram: the onset and duration of variant surface glycoprotein (VSG)-specific immune responses, as measured by the in vitro complement lysis assay, are represented by solid bars. Open circles indicate days on which *VSG*-specific transcripts were detected by reverse transcriptase (RT)-PCR. Solid squares indicate days on which *Bos taurus* actin transcripts were amplified from samples. Solid diamonds represent detection of *T. brucei* β-tubulin RT-PCR products. (B = bloodstream expression site, m = minichromosomal, M = metacyclic expression site, A = megachromosomal subtelomeric array).

ATCCGCG); 1.22for2 and 1.22rev2 (TCGAAAA CGAAGTTTCCGCG, ATCGTCTTGACATGGATCCC); 1.25for and 1.25rev (CACCAGAAGCATGCAAGGCG, TGCTCCATTTGCAACCATCG); 1.64for and 1.64rev (AAGCGGTAGCAGCAGCTGCG, GCGTCGTCTAGGG TACCGCG); 1.67for and 1.67rev (GAAGCGGAATA TATGCAATC, GCCGTCCTGCTATGCCTGCG); 1.71for and 1.71rev (GGCAGTGTTTGCCTTAGCCG, TTGCCGGGTTGTTTCCGCCG); 1.73for and 1.73rev (AACAAGCAGCAGCAAGCGGC, CTTGTTGAGGC TTTTCCGCG); and for the control *VSG* from another trypanosome stock: 221 5′ and 221 3′ (CCGAATTCCG CATGCCTTCCAATCAGGAGGC, CGCGGATCCGC TGTATCGGCGACAACTGCAG). PCRs had an initial 5 min denaturation cycle at 95 °C, followed by 30 cycles of 1 min at 95 °C, 1 min at 60 °C and 2 min at 72 °C, and ended with 10 min at 72 °C. The products were resolved on a 0.7% agarose gel. The detection limit of this PCR approach was determined initially through addition to blood of trypanosomes growing in vitro and stably expressing the 221 *VSG*. By titration from 1×10^{1} to 1×10^{5} trypanosomes.ml^{-1}, the minimum, reproducible detection limit was found to be 1×10^{3} trypanosomes.ml^{-1}.

Cloning of PCR products was carried out using the TOPO® TA cloning kit (Invitrogen). Automated sequencing was performed on the plasmids by the Molecular Biology Support Unit (University of Glasgow).

2.3. Serology

2.3.1. Antisera

VAT-specific antiserum for use in the in vitro complement lysis assay was prepared from ICR mice infected with clonal ILTat stabilates. At the initial parasitaemic peak, the mice were treated with cymelarsen (5 mg.kg^{-1})(Rhône Mérieux). Blood was collected 72 h later, centrifuged at 8000*g* for 10 min in a microcentrifuge, and the plasma supernatant was collected and was stored at −20 °C. VSG-specific polyclonal antisera were prepared in lop-eared rabbits (Diagnostics Scotland, Lanarkshire, UK), by serial

immunisation with purified soluble VSG (4 inoculations of 0.5 mg, 1 month apart, serum being collected 1 week post-injection). Antisera from different boosts, rich in either IgM or IgG, were used in ELISA tests.

2.3.2. Complement lysis assay

Clonal ILTat trypanosomes grown from stabilate in a cyclophosphamide-immunosuppressed ICR mouse (Harlan UK) were suspended in GPS (Harlan) to 1×10^7 cells.ml^{-1} (except clonal lines ILTats 1.25 and 1.73, which were suspended to 5×10^6 cells.ml^{-1}). Five-microlitres samples of mouse plasma, diluted 1:10 and 1:50 in GPS, were mixed with 5 μl trypanosome suspension in a well of a Terasaki plate (Greiner Labortechnik), which was then incubated at room temperature for 1 h in a humid chamber. The extent of lysis was then determined, by counting 100 trypanosomes under phase contrast microscopy. Controls included GPS alone as a negative control; VSG-specific antiserum specific to each clonal line diluted 1:10 in GPS as a positive control; and antiserum specific to a different VSG diluted 1:10 in GPS as a further negative control. The assay was performed within 1 h of blood collection and the trypanosomes were stored on ice while the dilutions were prepared.

2.4. Statistical methods

Two methods were used to test for order in appearance of VATs. In the first approach, times of onset of VAT-specific immune responses were normalised by transformation as log 10 (time+), where the occasions where no immune response was observed were scored as 'time+', i.e. 36 days, the last day of experiment +1. General Linear Models were used to analyse the data, with log 10 (time+) as the response variable, and VAT code and mouse batch as factors. Parametric assumptions were checked by analysing normal distribution and by testing for equal variances. Any effect of mouse batch was also examined.

The second method involved ranking the onset of VAT-specific immune responses according to their order of appearance within individual mouse infections. Thus, the first VAT immune response was assigned a value of 1, the second 2, and so on. If immune responses to different VATs appeared at the same time, they were assigned the same value. If no immune response was observed, the VAT was assigned a ranking one greater than the highest within that infection. 'Rank' data were normalised by transformation as reciprocal of rank. General Linear Models were used to test the hypothesis, with reciprocal of rank as the response variable, and VAT code and mouse batch as factors. Parametric assumptions were checked by analysing normal distribution and testing for equal variances.

To test correlation between locus type and timing of immune response onset, data were analysed by a directional heterogeneity test (Rice and Gaines, 1994). To test whether there was variation in the rate at which particular VATs were switched to, and whether this was affected by the switch-off VAT, we analysed the rat infection data of Miller and Turner (1981), scoring whether each VAT appeared in the single-relapse peak. These data were analysed with the Proc GENMOD routine in the SAS statistical package, using binomial errors and with the number of rats in which a particular VAT appeared divided by the total tested as the response variable, and VAT as the predictor variable.

2.5. Animal ethics approval

All animal experimentation was undertaken under appropriate legislature; standard operating procedures designated by ILRI's Institute Animal Care and Use Committee, or the UK Animals (Scientific Procedures) Act, 1986. Experimental cattle were maintained on ad lib. diets, and were monitored for changes in appetite, weight, body temperature and white blood cell concentrations. A total of 5 ml blood was sampled each day, by venupuncture of the left jugular vein. The cattle were drug cured at the end of the monitored infection period (3.5 mg.kg^{-1} Diminazene aceturate, s.c.).

Sampling of experimental mice was undertaken every second day over the 35 days infection period. Blood was collected following tail prick with a lancet, and not more than 35 μl was collected over a 7 days period. Mice were monitored for clinical signs of trypanosomiasis, which include inactivity, a hunched appearance, and the development of a bristled coat. Mice were euthanased if clinical signs were observed, or at the end of the infection period, by placing in >70% carbon dioxide.

3. Results

3.1. Chronic infections in cattle

From daily cattle blood samples, *VSG* transcripts were detected by RT-PCR analysis specific to the N-terminus coding region of each of our selected *VSG*s. Control reactions included primer pairs for a constitutively expressed trypanosome sequence, β-tubulin, and for bovine actin. Bovine actin gene products were obtained in the majority of cattle samples analysed; 78.8% from cow A (26 samples of 33 analysed), and 80.4% from cow B (37/46) , a relatively low level of detection indicating limitations in the RNA extraction or the reverse transcription methods. Trypanosome β-tubulin transcripts were obtained from about half of these: 39.4% (13/33) of samples from cow A and 41.3% (19/46) from cow B, reflecting the fluctuating nature of the parasitaemia. The samples that were positive for trypanosome β-tubulin were then examined for the presence of specific *VSG* transcripts and products for all seven *VSG*s were identified in both cattle. *VSG* transcripts were identified in 53.8% (7/13) of the samples from cow A and 36.8% (7/19) from cow B. The period within each infection during which *VSG*s were detectable is similar

(Fig. 1). In cow A, *VSG* transcripts were detectable on days 12 and 13, corresponding to the initial parasitaemia peak, on days 19, 20, and 23, which relate to the first relapse peak(s), and on day 43, which matches with a small independent peak after the first relapse. For cow B, *VSG* mRNAs were first detected on day 12 of the infection, again matching the initial parasitaemia peak, and thereafter on days 17, 18, 20, 21, 23 and 24, during the first relapse peak(s). Several of these products were sequenced and confirmed as the expected *VSG* product.

RNA for only the ILTat 1.2 *VSG*, which was expressed by the trypanosomes initiating the infections, was detectable in the first parasitaemia peak. In both cattle, all except one of the *VSG*s were detected during the first relapse peak (Fig. 1). In cow A, ILTats 1.22 and 1.67 were detected first, on day 19, and there were transcripts of ILTats 1.21, 1.25, 1.64, 1.67 and 1.71 on day 20. No trypanosome RT-PCR products were obtained in the 17, 18, 21 and 22 day samples. By day 23, only ILTats 1.64 and 1.71 were detectable, and for the remainder of the first relapse peak no specific transcripts were detected. The one exception was ILTat 1.73, which was detected on day 43, during a peak of short duration (4 days) that occurred 4 days following the end of the first relapse peak. Thereafter there were no PCR products detectable for the remainder of the infection. For cow B infection, ILTats 1.21, 1.25 and 1.64 were detected on day 17, and ILTat 1.67 arose on day 18. Day 19 did not have any detectable bovine or trypanosome material. On day 20, it was possible to amplify products for all seven *VSG*s, except 1.22. On day 21, there was no longer a 1.21 product, but 1.22 appeared for the first time, along with 1.25, 164, 1.67, 1.71 and 1.73. By days 23 and 24, only 1.73 was present. No PCR products were detectable for the remainder of the infection. In this infection, the appearance of the seven candidate *VSG*s did span the course of one distinct parasitaemia peak, indicating that this peak comprises several sub-populations. In the case of cow A, the VATs other than ILTat 1.73 arose over a period similar to that in cow B, but seemed to form a subpeak at the beginning of a large period of continuous parasitaemia.

Immune responses to each of the VATs were measured by the in vitro complement lysis assay. As a positive control for lysis, reference clonal populations homogeneously expressing each VAT were tested against their specific antisera. A specificity control involved each of these reference populations incubated with antiserum against another VAT and a negative control for lysis omitted the test antiserum or plasma. All controls behaved as expected. Responses against ILTat 1.21, 1.25 and 1.67 appeared between days 18 and 27, and those against 1.22, 1.64, 1.71 and 1.73 appeared later, between days 25 and 66 (Fig. 1). Almost without exception, the responses remained detectable by this method until the end of the 70-day monitoring period. The exceptions were those against 1.21 and 1.64 in cow B, the former becoming undetectable on day 66 and the latter on day 65.

3.2. Chronic infections in mice

Having established a correlation between appearance of VAT and specific antibody, the analysis was extended to mice, with which a statistically significant number of infections can be analysed and in which trypanosomes switching at a very high rate can be examined. As the frequency and volume of samples were limited, for humane reasons and in the interests of maintaining physiological conditions in the host, the only feasible approach was to assess VAT appearance by antibody responses. Fifty mice were infected with EATRO 795 trypanosomes neutralised with antisera specific to each of the VSGs under study. Parasitaemias followed a general pattern, the initial peak having almost uniform timing, on days 6–7, but with variation in its height (Fig. 2(A)). Thereafter, there was typically a distinct first relapse peak, occurring at around 14 days, followed by a varying number of relapse peaks, which increased in height and width but displayed variance in these parameters between individual mice. Grouping parasitaemias with similar height of first peak did not show any less variance in height and width of relapse peaks (Fig. 2(B)). With the exception of one mouse that became ill by day 21, all displayed a gradual increase in parasitaemia, generally leading to humane termination of infection at up to day 35.

The onset of the immune responses was measured by the in vitro complement lysis assay, with the same controls as in the cow experiments. A total of 30 chronic infections were analysed, in four batches, with sampling days differing between batches. An initial batch of eight mice was a control in which the VATs under study had not been removed from the inoculum by incubation with antibody. In these mice, responses occurred 2–10 days earlier than in those initiated with the antibody-neutralised trypanosomes, except for the 1.71 response. It can be concluded that ILTats 1.21, 1.22, 1.25, 1.64 and 1.67 were present in varying, probably very small, numbers in the initial inoculum, whereas ILTat 1.71 may have been absent. The immune response analysis of all other mice indicates that all seven VATs were expressed routinely in the 35–40 day period of the infections (Fig. 2(C)). There were only four instances in which no immune response was detected: once for 1.22 and 1.64, and twice for 1.71. In the majority of cases, the VAT-specific immune response remained detectable to the end of the infections, although in some the response became undetectable. This happened in one mouse each for 1.25, 1.71 and 1.73, in three mice (13.6%) for 1.22 and in seven mice (35%) for 1.64.

The relative order of appearance of VATs in mice, as measured by the onset of specific antibody responses, was analysed statistically in two ways. The first approach determined if there was a significant difference between the times of onset of responses against the different VATs. The second ranked the timing of onsets within individual infections, and compared the rankings between infections,

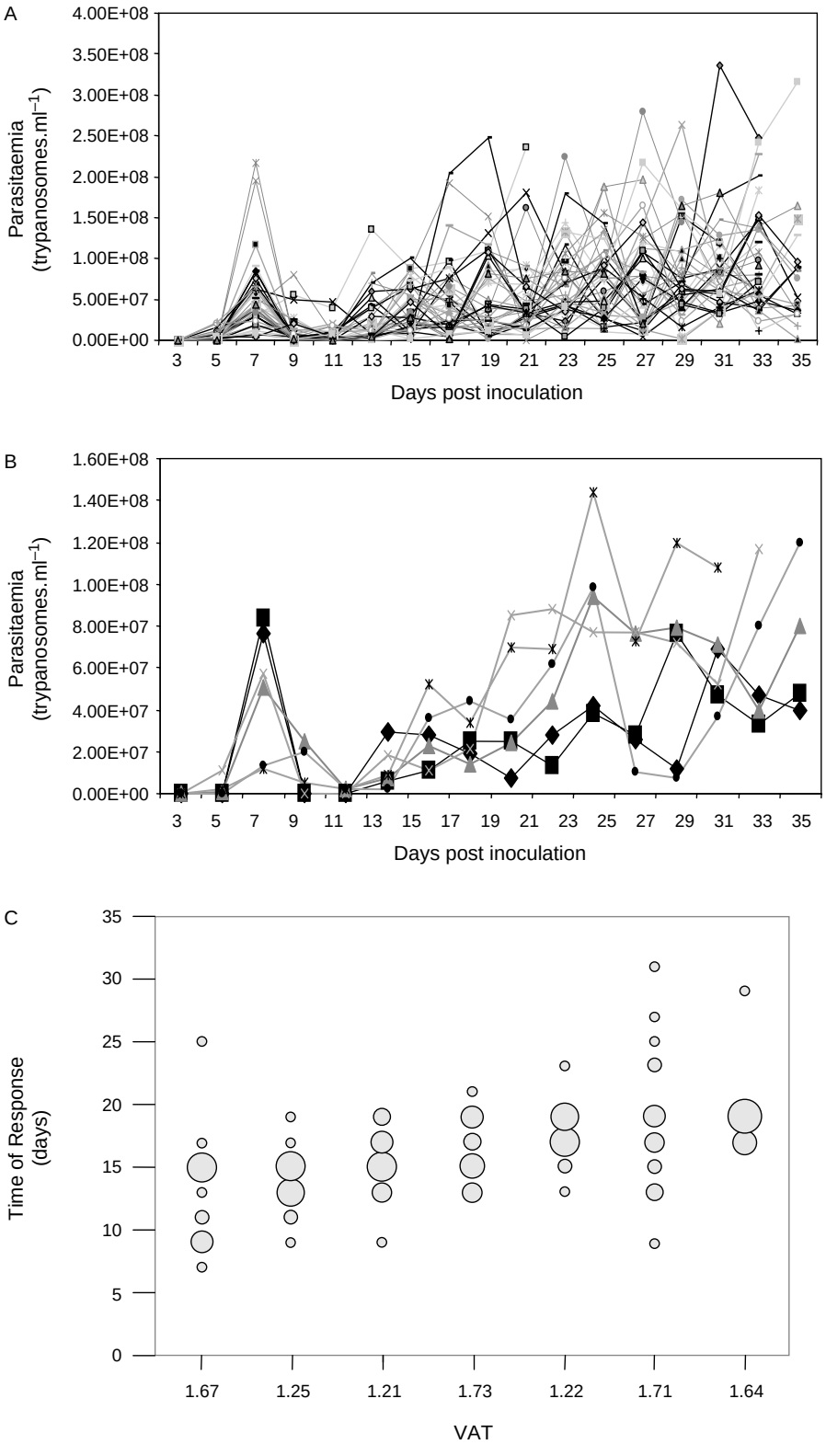

Fig. 2. Mouse infections. (A) *Trypanosoma brucei* EATRO 795 parasitaemia profiles of infections in 50 BALB/c mice, demonstrating the reproducibility of the initial and, to a lesser extent, first relapse peaks, followed by a more divergent and unpredictable pattern. (B) A sample of parasitaemia profiles from graph A, with pairs displaying high, medium and low initial parasitaemic peaks, illustrating that the height of the initial peak does not correlate with subsequent parasitaemia profile. (C) Onset of variable antigen type (VAT)-specific antibodies. The spread of time of first detection of VAT-specific immune responses (days, *y*-axis) for each VAT (*x* axis), in three experimental groups of mice (total $n=22$). The area of each circle is proportional to the number of mice first responding on that day.

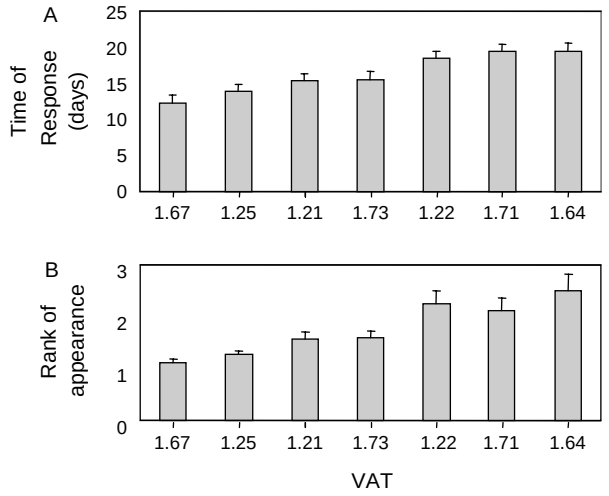

Fig. 3. Ordered appearance of variable antigen type (VAT)-specific antibody responses. A. Least square mean of time of onset of immune response against VATs. B. Least square mean of rank of order of appearance of immune response against VATs. Error bars represent ±SEM, $n=22$.

an approach that reduced the possibility of extremes (statistical outliers) impacting on the mean outcome. Time of onset of immune response differed significantly between the VATs ($F_{6106}=7.49$, $P<0.0001$) (Fig. 3(A)). There was also a significant difference between the two batches ($F_{1106}=5.82$, $P=0.018$), as was expected due to one of the batches being sampled asynchronously from the others. Importantly, however, there was no interaction between mouse batch and VAT ($F_{6106}=1.39$, $P=0.23$), and therefore the relative time of appearance of the VATs was not different between batches.

Immune responses against the different VATs showed significantly different rankings ($F_{6112}=8.03$, $P<0.0001$) (Fig. 3(B)). There was no significant mouse batch effect ($F_{1112}=1.68$, $P=0.20$), and no interaction between batch and VAT ($F_{6106}=0.83$, $P=0.55$). Again, the lack of a batch by VAT interaction demonstrates experimental consistency.

3.3. Gene locus types

To examine, on a limited scale, whether any difference in timing of onset of immune responses corresponded with different silent *VSG* locus types, the data for different genes of the same locus type were combined and then tested in the order of prediction. Data for genes belonging to one of these groups were pooled; ILTats 1.21 and 1.25 VSGs are both encoded by minichromosomal genes, both 1.22 and 1.64 VSGs are encoded by *MVSG* genes, and 1.71 and 1.73 VSGs are products of array genes. This gave three groups of data, and the fourth group, BES genes, comprised just ILTat 1.67. Predictions were based also on our previous study of six of these genes in rabbit infections (Robinson et al., 1999). Thus, the VAT encoded by a *VSG* gene already in a BES was expected earliest, followed by VATs encoded by minichromosomal genes, then those encoded by *MVSG*s,

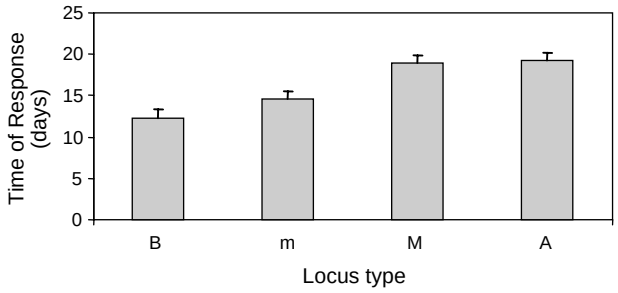

Fig. 4. Ordered appearance of responses against variable surface glycoprotein (*VSG*) locus types. Least square mean of time of onset of immune response against category of *VSG* locus; B=bloodstream expression site, m=minichromosomal, M=metacyclic expression site, A=megabase chromosome subtelomeric array. Error bars represent ±SEM, $n=22$.

and finally those encoded in the silent gene arrays. The directional heterogeneity test revealed that the order of appearance between these four groups in the 22 mouse infections was as predicted ($P<0.0001$) (Fig. 4). This ordered appearance was also apparent in Cow A and, to some extent, in Cow B.

3.4. Is there a deterministic relationship between the switch-off VAT and the switch-on VAT?

This question was addressed by statistical analysis of the data of Miller and Turner (1981), who used the trypanosome line that we studied in cattle. In their study, 47 single relapse rat infections, each initiated by one of six individual VATs, were analysed at single time points for which of the other five VATs, as well as another early VAT, had been activated. The VATs examined included three in the present study: ILTat 1.21, 1.22, 1.25. The probability that a particular VAT would be switched on varied, with some antigens more likely to appear than others, irrespective of the initial VAT ($F_{6,28}=4.99$, $P=0.0014$) (Fig. 5). As an example, VAT 1.21 appeared in 78% of rats, whereas VAT 1.23 appeared in only 6%. In contrast, there was no evidence that the initiating VATs varied in their propensity to switch to new VATs ($F_{5,30}=0.40$, $P=0.84$). Furthermore, there was no evidence, for any switched-on VAT, that the probability of its appearance depended on the type of initiating VAT (goodness of fit tests, null hypothesis of equal probabilities $P>0.15$ in all cases).

4. Discussion

We have determined, and analysed in statistical detail, the order in appearance of seven defined VATs in cattle and mouse infections, have questioned whether gene locus type is associated with order and have applied statistical methods to a previous large-scale analysis of switching (Miller and Turner, 1981) to determine whether the expressed VAT influences what follows it. We have done so to attempt to

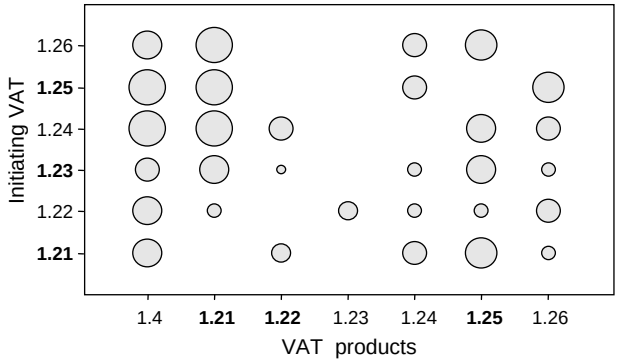

Fig. 5. Variable antigen type (VAT) products detected in first relapse peaks of multiple rat infections. Data from Miller and Turner (1981) are redrawn in this bubble chart, in which circle areas are proportional to numbers of events. For each initiating VAT (*y*-axis), rats were infected with 10 trypanosomes and switch-on VATs (*x*-axis) were detected on one occasion only in the first relapse peak; presence of the switch-on VAT is scored as an event. VATs common to the present study are highlighted in bold.

define better some of the basic features of antigenic variation. For example, ordered expression has been described only loosely, as 'semi-predictable'. Despite there having been a reasonable number of experiments, the influence of switch-off VAT on what succeeds it has not been subjected to detailed statistical analysis to test rigorously for correlation. Although we do not add more than a few loci to the question of the role of locus type, the only other study of *VSG*s known to belong to all locus types concentrated on merely four infections (Robinson et al., 1999), compared with the statistically significant number analysed here. Our findings show that the order in antigenic variation is highly structured, with each analysed VAT (with the exception of ILTat 1.71, which may be artifactual, as we discuss below) having a surprisingly predictable rank in the expression hierarchy. To put this in perspective, these are the most competitive genes, as seen by their repeated isolation from first relapse peaks in different laboratories (Miller and Turner, 1981; Robinson et al., 1999), and even in this restricted subset there is order of expression. It also emerges that the order of antigenic variation early in infection is not determined by the preceding VATs. Additionally, the limited data available (no more than two VATs per set) provide significant correlation of locus type with order of appearance. The simplest model consistent with these findings is that each *VSG* has a distinct probability for activation that gives it a distinct position in the rank order of antigenic variation; the system follows probabilistic order. Rather than being related to *VSG* coding sequence, the mechanisms providing the order are associated with the environment of each *VSG*, including where it is located in the genome and possibly elements in its flanks. This model provides a simple, one-step mechanistic basis for order in antigenic variation, in that the switch event and the determination of rank order are the same process (Fig. 6). As we discuss below, this latter point applies to

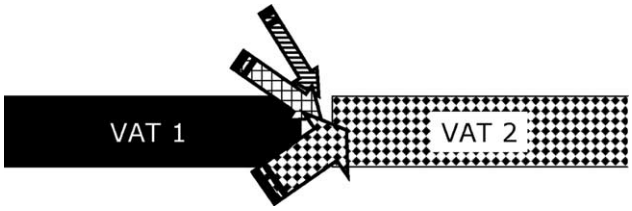

Fig. 6. One-step model of variable surface glycoprotein (VSG) switching. The first event is the *VSG* specifying variable antigen type (VAT) 1 becoming inactivated by an unknown mechanism, presumably involving a DNA break. This precipitates the switch, which is driven by the new donor *VSG*. Each donor gene has a distinct probability for activation, proportional to the width of its arrow, and that is what resolves if it will complete the switch and hence determine the order.

early VATs, but is unlikely to occur for later VATs in chronic infection, where order is likely to be dictated also by coding sequence.

Our analysis brings statistical methods and outcomes to the phenomenon of ordered expression, elements of which were first noted decades ago (very early work is described in McNeillage et al., 1969). Gray (1965) demonstrated similar order of expression in different chronic infections, Capbern and colleagues (1977), through extensive clone analysis, showed that VATs can be attributed to groups appearing at different phases of infection ('précoce', 'semi-précoce' and 'tardif' groups) and Barry (1986) showed that general order of VAT expression in *T. vivax* infections is independent of the host species, a finding not inconsistent with order being determined inherently by the parasite. Apart from these surveys of chronic infection, there have been a large number of studies of single-relapse infections in which the switch products of clonal populations have been identified. Each single-relapse switch produces mixtures of VATs (Lourie and O'Connor, 1937; McNeillage et al., 1969; Van Meirvenne et al., 1975; Le Ray et al., 1977; Miller and Turner, 1981; Liu et al., 1985; Robinson et al., 1999) and it seems clear that VATs appearing at this early stage of infection are destined to be activated with high probability. Detailed study of one *VSG* gene in first relapses (Lee and Van der Ploeg, 1987; Timmers et al., 1987) has shown that it was activated in predictable periods of several days, with each isolated clone displaying evidence for independent activation. One explanation for this is that all individuals in the population within a host have the same probability for activating a given gene and, as antibodies remove trypanosomes that have already activated higher-probability genes, the survivors include many individuals that have independently activated those next in the hierarchy. The fact that different activations of the same gene arise within such a limited period is further evidence for a high degree of structure in ordered expression, and argues against a major role for indirect factors such as differential VAT growth rates.

Key to our analysis is the large number of independent infections, in each of which the studied VATs arose de

novo. For humane and physiological reasons, adequate sampling of material for *VSG* mRNA analysis from mice is not possible, so we adopted the well-tried approach of detecting specific antibodies (Capbern et al., 1977; Barry, 1986), having first validated it by correlation between mRNA presence and antibody responses in the cattle infections. The immune lysis reaction was chosen because it is highly VAT-specific, but it is also insensitive. We attempted to use, in addition, ELISA assay against purified, soluble VSGs, but this gave an unacceptably high level of non-specific reaction, due to the cross-reacting determinant that is created artefactually when VSG is solubilised (Shak et al., 1988). In the analysis of whether the switch-off VAT influences what is switched on, the data of Miller and Turner are extensive and obviate the need for further infections, especially as the trypanosome line used was the same as in the present study. It is likely that the number of VATs being switched on in that study is underestimated, because the first relapse peaks were examined on 1 day only (Miller and Turner, 1981), whereas we know that the relapse peaks comprise a number of sequentially appearing, and overlapping, VATs (Van Meirvenne et al., 1975; Robinson et al., 1999). Therefore, the lack of correlation between switch-off and switch-on VATs is probably even more significant than is apparent from our analysis.

One of the VATs we have studied, ILTat 1.71, diverged from the others in having discrepancy between transcript presence and onset of antibody in both cattle (Fig. 1) and unusually broad range of antibody onset times in the mouse infections (Fig. 2(C)). A possible explanation is that each activation of 1.71 creates a different sequence, due to the mosaic *VSG* gene phenomenon. We have shown previously that there appears to be only one 1.71 silent gene in the EATRO 795 genome (Robinson et al., 1999). We have isolated genomic DNA clones that hybridise strongly with a 1.71 probe, and have found that the sequence of the *VSG* has 43 mismatches with the 1.71 cDNA sequence over the 285 nucleotides running from the ATG start codon. As this gene appears to have an array location, it is likely to be a pseudogene or a partial gene (El Sayed et al., 2003; http://www.sanger.ac.uk/Projects/T_brucei/), such that the expressed copy is a mosaic with other coding sequences. It follows that there is a strong likelihood that all activations of 1.71 in the cattle and mice will differ from each other, and from the reference clone (expressing the 1.71 sequence corresponding to the cDNA sequence) with which we have detected anti-1.71 immune responses. The antibody data for this VAT are thus unreliable.

There has been little study of switching patterns in other parasites. Recently, however, a novel variant-specific steady-state RNA assay was applied to switching of *var* genes in *P. falciparum* (Horrocks et al., 2004). The assay has the significant benefit that it reveals, for the first time, switch-off rates. It was found that each variant has distinct switch-off and switch-on rates, which differ from each other and are reproducible between independent clones. Furthermore, an influence of switch-off variant on ensuing variants was reported. It is often assumed that parasite antigenic variations systems are similar at this level, with the differences occurring between underlying genetic mechanisms. Trypanosomes and *Plasmodium* appear to share the feature that variant types have discrete activation probabilities. It is not known, however, if trypanosome VATs have discrete switch-off probabilities, similar to *P. falciparum var* variants. The mRNA assay developed for *Plasmodium* cannot be applied to trypanosomes, because it requires a switch rate near the highest achieved in *T. brucei*, but trypanosomes switching at this natural rate do not grow for the necessary period in vitro. A main difference between our findings and those of (Horrocks et al., 2004) is that we demonstrate no influence of switch-off VAT on its successors, whereas it is reported that, in *P. falciparum*, the ability to switch to certain variant types might depend on the antigenic switching history of the parasite. We believe we have been able to discriminate clearly between these possibilities because of the large number of infections analysed. Nevertheless, differences between the systems in the two parasites may be real. *var* genes are activated in situ and so retain characteristics of their genomic environment. Given that there are differences in switch-on probability, which may be dictated by an element of that environment, for example in the gene flanks, then it is easy to imagine that there will also be influence on switch-off rate, and possibly also on which particular variant ensues. On the other hand, activation of VSG genes early in infection involves duplication via conserved flanks, independently of the coding sequence, and the transposed, active copy inherits much of its flanking sequence from the expression site. It seems unlikely that the active gene would carry the information for its switch-off rate, or indeed for helping determine what variant follows it.

The one-step model concurs with much that is known about VSG switching. Early in infection, telomeric genes are activated (Pays et al., 1983; Young et al., 1983; Myler et al., 1984; Liu et al., 1985; Robinson et al., 1999), usually by an event that duplicates them, from the set of imperfect 70-bp repeats some 1–2 kb upstream of the coding sequence to apparently the end of the chromosome. We have speculated that the duplication of the whole telomere in this way is mediated by break-induced replication, but little is known about its mechanics (Barry and McCulloch, 2001). In rapidly switching trypanosomes, duplication of a telomere appears to operate independently of RAD51, a major activity in the main pathway of homologous recombination (Burton, P., McCulloch, R. and Barry, J.D., unpublished data). Until it is known what sequences are directly involved in initiation of the event, it is unlikely that we shall be able to determine how each gene achieves its particular activation probability. Later in infection, however, the situation changes. As the host accumulates antibodies against more and more VATs that are easily activated, trypanosomes expressing VSGs encoded by array genes

proliferate. Most such genes are dysfunctional and, if they are to be expressed, can be activated only by participating in the creation of intact VSGs in expression sites (Thon et al., 1990; Barbet and Kamper, 1993). The critical difference in this type of activation is that it relies on interaction via homology in the coding sequences. It is therefore likely that, for these genes, there is a considerable deterministic influence of the switch-off VAT on its successors. The different locus types are likely to be expressed as overlapping sets of VATs; we have examined only samples of the earliest VATs in each of these groups. In our analysis, the transcriptional switch between BESs occurs at the head of the locus type hierarchy, but caution must be exercised as only one gene in this category was analysed, and activation of this particular site happens consistently but transiently in a subpopulation, with the previously active BES regaining dominance in the population (Robinson et al., 1999). In contrast, slowly switching laboratory line trypanosomes indulge in this type of switching more commonly, albeit at a low overall rate (Liu et al., 1985). The phenomenon of transcriptional switching in rapidly switching trypanosomes in vivo requires further study.

With the completion of the genome sequencing project, it will become possible to examine the extent of mosaic gene formation in chronic infection and the degree to which it establishes order in that phase. The dominant role of minichromosomes in establishment of order might be tested if a mutant that lost those chromosomes became available. A less direct way of testing the role of minichromosomes is by examination of events in *T. vivax*, which has very few of them (Dickin and Gibson, 1989) and for which a genome sequencing project is in progress.

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