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Distinct, developmental stage-specific activation mechanisms of trypanosome VSG genes

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SUMMARY

The metacyclic form of African trypanosomes is the first to express genes for the Variant Surface Glycoprotein (VSG) and it uses an unusually predictable subset of the VSG gene repertoire. We have developed a model system for the analysis of metacyclic VSG (M-VSG) gene expression and have used this to demonstrate that, for two M-VSG genes, different modes of expression operate in the insect and mammalian phases of the life-cycle. In metacyclic-derived clones, these genes are expressed *in situ*, whereas they are routinely activated by duplication in bloodstream trypanosomes. The expression loci for both M-VSG genes studied are structurally simple and we present a model, based on this, for the maintenance of a separate M-VSG repertoire and expression system.

Key words: antigenic variation, *Trypanosoma brucei*, metacyclic VSG.

INTRODUCTION

Infective stages of *Trypanosoma brucei* are covered by a surface coat, consisting of the variant surface glycoprotein (VSG) (Vickerman, 1969; Cross, 1975). Antigenic variation, periodic switching of the VSG coat, allows the trypanosomes to evade the host immune response (reviewed by Barry, 1989). Each trypanosome has the capacity to express, one at a time, probably several hundred different variable antigen types (VATs) and this proceeds in a hierarchical, though not very predictable, order (Capbern *et al.* 1977; Barry, 1986). Each trypanosome has a repertoire of more than 1000 silent, basic copy (BC) VSG genes (Van der Ploeg *et al.* 1982), most of which are organized in tandem arrays within chromosomes, although others lie at telomeres.

In the bloodstream, expression of VSG genes occurs only at special telomeric expression sites (ES) (Kooter *et al.* 1987; Johnson, Kooter & Borst, 1987; Alexandre *et al.* 1988; Pays *et al.* 1989). For BC genes which lie within chromosomes, activation therefore entails duplication of an expression linked copy (ELC) into an ES. Telomeric BC genes may be activated by a similar gene conversion mechanism, but can also undergo telomere translocation to a transcriptionally active telomere, or can simply be activated *in situ* if they already occupy a potential ES (reviewed by Pays & Steinert, 1988). To the 5' flanks of VSG genes is a repeated series of a sequence approximately 70 base pairs (bp) long, which usually forms the 5' limit of the duplicated ELC (Liu *et al.* 1983; Aline *et al.* 1985). The number of repeats is often extensive at telomeres, giving rise to large

'barren regions' devoid of restriction sites (Michels *et al.* 1983).

In the tsetse fly phase of the trypanosome's life-cycle, VSG is not synthesized until the infective metacyclic stage develops in the salivary glands of the fly (reviewed by Barry, 1989), when a very predictable set of VSG genes – the metacyclic VAT (M-VAT) repertoire – is activated (Hajduk *et al.* 1981; Barry, Crowe & Vickerman, 1983; Esser & Schoenbechler, 1985). This predictability is surprising, as antigenic variation appears to be a system for generating diversity. The M-VAT repertoire comprises only 1–2% of the total repertoire, and from phenotypic analysis is expressed by a mechanism distinct from that used in the bloodstream (Turner, Barry & Vickerman, 1986). M-VSG BC genes occupy what seems to be a specific location in the genome, namely telomeres of the largest chromosomes. Furthermore, they are not flanked by large barren regions (Cornelissen *et al.* 1985; Lenardo *et al.* 1984, 1986; Delauw *et al.* 1987). The question arises whether there is a connexion between this specific location, the different expression system activated in the fly and the unusual predictability in M-VAT expression.

There are too few metacyclic cells available from fly salivary glands to allow direct molecular study. However, since M-VSG genes are still expressed for several days following fly transmission, experiments have been performed on trypanosomes in early bloodstream infection. From such studies it has been suggested that M-VSG genes are activated in the tsetse fly either *in situ* (Lenardo *et al.* 1986) or by duplicative transposition (Delauw *et al.* 1987). There are problems with this approach, however, due to antigenic instability of the developing infection (Le

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Ray *et al.* 1977) and the fact that M-VSG genes are activated polyclonally in the fly (Tetley *et al.* 1987): examination of individual activation events requires cloned trypanosomes. To resolve this, we have set up a model line of trypanosomes with the unusual combination of relative VAT stability and fly transmissibility, permitting isolation, in mice, of cloned metacyclic trypanosome populations fairly homogeneous in M-VAT expression. We present data from this model showing, for the first time, that M-VSG genes are activated by different routes in metacyclic and bloodstream clones and, from structural analysis of these telomeres, propose an explanation for the puzzling predictability of the M-VAT repertoire.

MATERIALS AND METHODS

Trypanosomes

A virulent, cloned line of *Trypanosoma brucei* EATRO 795 was transmitted through batches of 25–50 tsetse flies (*Glossina morsitans*) of a line with enhanced trypanosome transmissibility (Turner & Barry, 1989). Standard methods were used for growth, fly transmission, cloning and VAT-specific immunofluorescence of trypanosomes (Hajduk & Vickerman, 1981; Turner & Barry, 1989). Metacyclic trypanosomes were cloned directly from dissected fly salivary glands. With this line, clones became patent within 5 days, were monitored for VAT composition daily from patency, and yielded more than 10^8 organisms by days 6–8. The clones are identified, for convenience, by the VSG expressed: clones initiated from single metacyclic cells: 1.22i, 1.22j, 1.22v, 1.61g, 1.61h, 1.61i; 1 bloodstream trypanosome reclone of the metacyclic clone 1.22j, 1.22j'; 2 bloodstream trypanosome recluses of the metacyclic clone 1.61i, 1.61i', 1.61i''.

DNA clones

cDNA clones used as probes for VSG genes 1.22 and 1.61 were pTcV7.1–14 and pTcV7.15–21 respectively (Cornelissen *et al.* 1985). The plasmid genomic clone pMG 7.1–1 contains part of the 1.22 gene and upstream flank (Cornelissen *et al.* 1985).

Purification of trypanosome DNA, Southern blotting and hybridization

DNA was prepared from trypanosomes by standard methodology (Bernards *et al.* 1981). For the metacyclic clones, harvesting of trypanosomes as early as possible meant that little DNA was available. For routine analysis, approximately 2 μ g of DNA was digested with restriction endonucleases under the manufacturers' conditions (BRL), size fractionated by agarose gel electrophoresis and Southern blotted

Table 1. VAT composition of metacyclic clones

(VAT compositions were measured by immunofluorescence with monoclonal antibodies in bloodsmears from mice infected with single metacyclic trypanosomes on day 0. Composition is presented as no. positive/total counted. Only very low numbers were available on the earliest days of parasitaemia.)

Clone	Day			
	5	6	7	8
1.22i		50/50	95/100*	
1.22j	21/21	25/25	80/100	39/125*
1.22v			88/100*	
1.61g			5/5	26/45*
1.61h			11/11	44/88*
1.61i		17/20	78/100*	

* Denotes population from which DNA was prepared; blanks denote no trypanosomes available for analysis.

onto Nylon membrane (Hybond-N, Amersham International) (Maniatis, Fritsch & Sambrook, 1982). Radio-isotope labelled probes were prepared by preparative gel electrophoresis to separate the desired restriction fragments using low melting temperature agarose (Sigma) and radio-isotope labelling by hexanucleotide random priming (Feinberg & Vogelstein, 1983). Hybridization and washing of blots, and removal of hybridized probe, were carried out as detailed by the manufacturer (Amersham International).

RESULTS

Trypanosome populations

From 6 separate tsetse fly transmissions, using the model trypanosome line, a total of 20 flies (9.1%) harbouring mature infections was identified. There was a characteristic pattern of VAT expression as the metacyclic clones expressing the M-VSGs ILTat 1.22 and 1.61 developed in early bloodstream infection (Table 1). As the infection became detectable (days 5–7), in most clones the VAT remained at > 99%, but by the time there were sufficient trypanosomes for isolation of DNA, purity ranged from 31 to 95%. Therefore, since VAT switching began to occur around day 7, it was important to confine studies of M-VSG activation to days 5–7 after fly feed, despite compromising the amount of material for study.

Modes of activation

Southern blots of DNA from trypanosome clones, digested with *Hind* III, were probed with either the 5' *Hind* III fragment of pTcV7.1–14 or with the entire insert of pTcV7.15–21. The former probe is

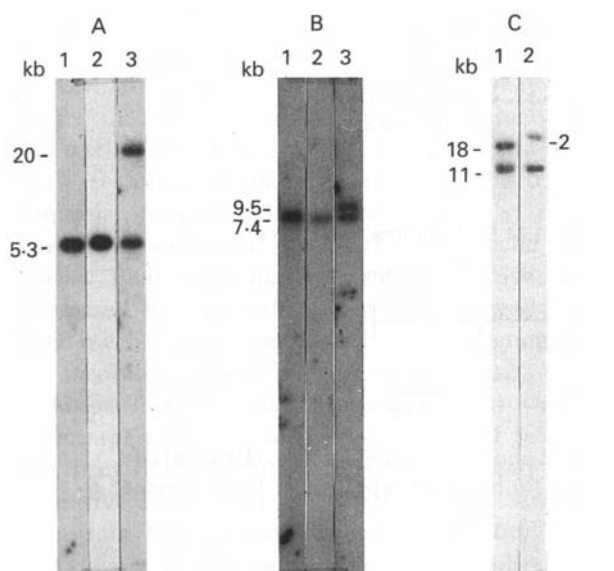


Fig. 1. Southern blot analysis of M-VSG expression in metacyclic-derived trypanosome clones. All DNAs were digested with *Hind* III. Probes used in these analyses were: Panel A, the 5' fragment of the pTcV7.1-14 cDNA clone of the 1.22 gene; Panel B, the 3' fragment of pTcV7.1-14; Panel C, the pTcV7.15-21 cDNA clone of the 1.61 gene. DNA in each lane was: A1 and B1, 1.22i; A2 and B2, 1.61i; A3 and B3, 1.22j'; C1, 1.61g; C2, 1.22i. Post-hybridizational washes were to $0.1 \times$ SSC, 65 °C. The sizes (kb) of detected bands are indicated.

specific for the 5' end of the 1.22 gene, the latter for the two 1.61 BC genes (Cornelissen *et al.* 1985). Fig. 1 shows a representative hybridization for each gene. Only a single fragment of 5.3 kb, corresponding to the BC, was observed with the 5' 1.22 probe in the metacyclic clone expressing 1.22 (Panel A, lane 1). The other two metacyclic clones, 1.22j and 1.22v, had the same pattern (data not shown). As expected, the non-expressors of 1.22 (e.g. the 1.61i clone) also showed only the 1.22 BC gene fragment (Panel A, lane 2). On the other hand, expressor trypanosome clones derived from bloodstream trypanosomes (e.g. 1.22j', Panel A, lane 3) showed 2 bands, one containing the BC gene and the other an extra ELC gene. In case an ELC fragment was present in the *Hind* III digest of 1.22i DNA, co-migrating with the BC fragment, the blot was stripped of its probe and rehybridized with the 3' *Hind* III fragment of the pTcV7.1-14 insert, which detects from the *Hind* III site within the gene to the end of the chromosome. Again, the metacyclic clones displayed only the 1 band (Fig. 1B), while the bloodstream form-derived expressor clone showed 2 bands.

The 1.61 gene gave similar results, showing only the 2 BCs in both expressor (1.61g) and non-expressor (1.22i) metacyclic clones (Fig. 1C) as well as in the 2 other metacyclic clones 1.61h and 1.61i (data not shown). We conclude that trypanosomes activating these M-VSG genes in the fly do not use

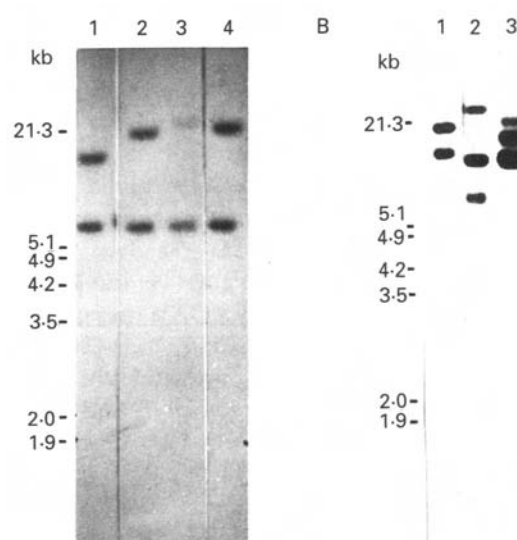


Fig. 2. Southern blot analysis of 1.22 and 1.61 expression in bloodstream form-derived trypanosome clones. (A) The 5' *Hind* III fragment of the TcV7.1-14 cDNA clone was hybridized with a Southern blot of *Hind* III digested DNA from the 1.22a, 1.22c, 1.22d, and 1.22j' clones (lanes 1-4 respectively). (B) The insert of TcV7.15-21 cDNA clone was hybridized with a Southern blot of *Hind* III digested DNA from the 1.61g, 1.61i' and 1.61i'' clones (lanes 1-3 respectively). Post-hybridizational washes were to $0.1 \times$ SSC, 65 °C.

a duplicative activation mechanism, but rather are activated either *in situ* or by translocation of an unusually long segment of the BC telomere to another telomere. The latter could not be checked by pulsed field gel electrophoresis, due to there being only small amounts of DNA available.

In the above experiment, bloodstream form-derived trypanosome clones exhibited activation of 1.22 and 1.61 by duplicative transposition rather than *in situ* activation. To determine if this was a routine mode of bloodstream activation for the two M-VSG genes, a Southern blot of *Hind* III-digested DNA from a set of 8 bloodstream trypanosome clones which had individually activated the 1.22 gene was probed with the 5' fragment of pTcV7.1-14. All 8 displayed the single BC gene and an expression linked copy. Four of these hybridizations are shown in Fig. 2A. Analysis of 2 similar clones expressing the 1.61 gene also showed activation by duplicative generation of ELCs (Fig. 2B).

Structure of expression sites

Fig. 3 shows a map of the basic copy telomeres for the 1.22 and 1.61 VSG genes which are used as expression sites in the metacyclic cells. In comparison with bloodstream ES, these ES are very simple: they contain very short 70 bp repeat regions (DNA sequencing reveals 1-2 repeats in each) and very little other sequence repetitive in the genome (Matthews *et al.* 1990). The brevity of the 70 bp

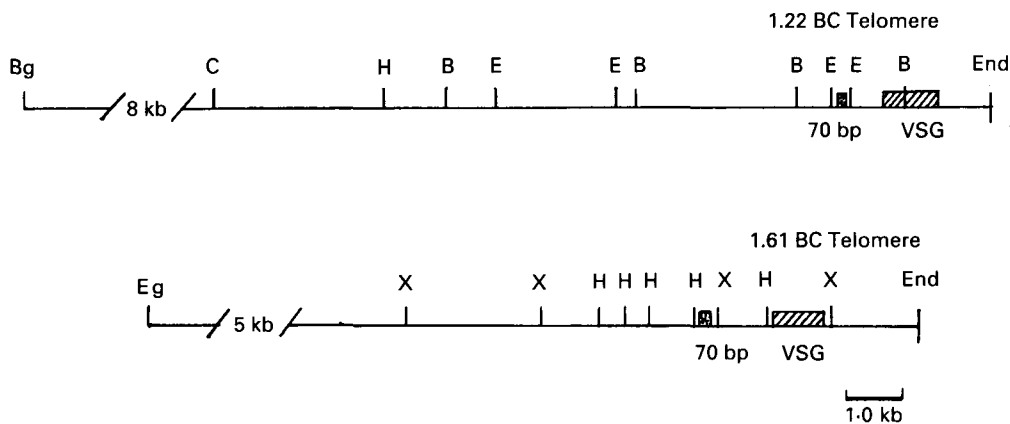


Fig. 3. Restriction maps of the two basic copy telomeres for the 1.22 and 1.61 M-VSG genes. (B) *Bam*H I; (E) *Eco*R I; (H) *Hind* III; (C) *Cla* I; (Bg) *Bgl* II; (X) *Xba* I. 70 bp, 70 base pair repeat unit; VSG, variant surface glycoprotein gene region.

repeat regions may suggest a low probability of participation in ELC formation, but our results with the bloodstream expressors of 1.22 and 1.61 show that this is not the case: both telomeres can act frequently as donor telomeres in gene conversion events.

DISCUSSION

When trypanosomes enter the tsetse fly, transcription of VSG genes ceases (Overath *et al.* 1983) and is resumed only upon development of the metacyclic form, a transient stage which develops to mammalian forms probably within hours of being transmitted from the fly (Brun *et al.* 1984). The brief existence and the paucity of metacyclic cells in tsetse flies thwart direct experimental analysis. Fortunately, however, indirect experimental methods are possible, because M-VSG expression continues for several days in the trypanosome population multiplying in the mouse, despite the change to bloodstream forms (Barry, Hajduk & Vickerman, 1979). Although this similarity in VSG expression has sometimes engendered the erroneous belief that the metacyclic stage itself persists for several days in the mammal, we believe that the mechanisms used in the fly are still extant. For example, phenotypic analysis shows that M-VSG expression in this early bloodstream phase proceeds in a manner distinct from, and taking precedence over, that typical of antigenic variation in chronic bloodstream infection (Barry, Crowe & Vickerman, 1985; Turner *et al.*, 1986).

There are disadvantages inherent in the indirect approach described above. Metacyclic populations are polyclones with respect to the expression of individual VSGs (Tetley *et al.* 1987) and fly-transmitted trypanosomes undergo antigenic variation several orders of magnitude more frequently than do the abnormal rodent-adapted lines generally used in studies on antigenic variation, so stably expressing clones are not readily available (Turner &

Barry, 1989). Our model system circumvents these problems: we use a trypanosome line with the unusual combination of fly-transmissibility and rodent virulence which does provide clones sufficiently stable for molecular analysis. Two previous studies of putative M-VSG genes have contrastingly reported, respectively, *in situ* and duplicative activation in fly-derived populations. Neither studied activation of the same genes in bloodstream infection. In the first study (Lenardo *et al.* 1984), trypanosomes present in mice 5 days after fly transmission were enriched for major M-VATs by antibody treatment and revealed only the basic copy gene for the major M-VSG. However, the polyclonal origin of each VAT means that analysis by that approach could not exclude, for example, the presence of multiple ELCs within the population, each with a different fragment size in Southern blot analysis and therefore at an undetectable level. The second study utilized trypanosome cloned a few days after fly transmission and reported ELC production as the expression mode (Delauw *et al.* 1987). It is apparent, however, that the clones were raised to high levels by the conventional method, involving syringe-passaging in mice, which yield, as our experiments show, organisms which have switched from the *in situ* to the ELC mechanism.

It seems clear, from our analysis of 6 metacyclic-derived clones, that both M-VSG genes are activated *in situ* in the fly. Although it cannot be ruled out that a more complex process occurs, such a non-duplicative translocation of a very long segment of DNA, study of B-VSG genes has amply documented *in situ* activation as a common means of VSG switching (reviewed by Pays & Steinert, 1988). In the bloodstream, only duplicative activation of both genes was observed in the 10 clones examined. Moreover, for another two M-VSG genes we have studied, activation in bloodstream trypanosomes also occurred only by gene duplication (Cornelissen *et al.* 1985). Therefore, our data strongly suggest separate,

life-cycle stage-specific expression mechanisms, as we have predicted previously (Barry, Crowe & Vickerman, 1985; Turner *et al.* 1988). It may be that these mechanisms are mutually exclusive; an interesting possibility is that, in the fly and during the early phase of mammal infection, the trypanosome is incapable of generating ELCs, thereby permitting exclusive use of the metacyclic mechanism. That individual genes can be activated and expressed by such different developmentally regulated mechanisms is novel and perhaps demonstrates the flexibility of gene control in parasites.

These findings, taken in conjunction with the structural simplicity of the 1.22 and 1.61 telomeres (i.e. brevity of the 70 bp repeat region and lack of repetitive sequences) (Matthews *et al.* 1990) have important implications regarding stability of M-VSG genes and the surprising predictability of M-VAT expression. (1) Lack of homology with other telomeres probably results in increased stability of unique M-VSG gene telomeres (e.g. the 1.22 telomere), including the VSG gene, by decreasing the frequency of reciprocal recombination and non-specific conversion with different chromosomes (Kooter *et al.* 1988). Indeed, amongst the conserved metacyclic repertoire of this serodeme, 1.22 (known also as GUTat 7.1) is the most stable VAT (Barry *et al.* 1983). At the molecular level, the only difference involving 1.22 noted so far between different trypanosome stocks is an apparent duplication of the whole telomere, rather than any internal rearrangement (Cornelissen *et al.* 1985). (2) We can now offer a possible explanation for the strange characteristics of the M-VSG repertoire – a mixture of VATs which is very predictable in content. The mixture is probably required for trypanosome survival because, in the field, trypanosomes are transmitted from flies into partially immune reservoir hosts which possess antibodies against many of the VSGs in the trypanosome's repertoire. What is more puzzling is the predictability, which seems inappropriate for an infective stage. We propose that simultaneous expression of the mixture is achieved through a metacyclic-specific mechanism and that the predictability results unavoidably from this mechanism. To permit expression of the mixture, the metacyclic-specific mechanism would have to differ from the bloodstream mechanism, which involves highly preferential activation of one or just a few expression sites (Liu *et al.* 1983; Longacre & Eisen, 1986; Delauw *et al.* 1987; Myler *et al.* 1988). The metacyclic expression system would operate via random activation within the unique pool of telomeres (Tetley *et al.* 1987) discussed above (telomeres of the largest set of chromosomes) which would act as ES in the fly, and would be simpler than that used in the bloodstream: VSG switching is not required in the fly. Predictability of the M-VSG repertoire would be the inevitable consequence of the com-

ination of *in situ* expression and recombinational isolation of each of this pool of telomeres: the linked VSG gene would be conserved and be expressed only *in situ* in the metacyclic stage. Some M-VSG telomeres, such as those described by Lenardo *et al.* (1984), could be devoid of 70 bp repeat regions and not participate in normal ELC formation while others, such as 1.22 and 1.61, would have a very small number, restricting their interactive role with bloodstream VSG telomeres merely to that of donor (Matthews *et al.* manuscript in preparation). It is important, however, to note that, especially in such a large and dynamic system as this, not all M-VSG genes need conform to this picture, as this theory requires only that a reasonable number belong to a separate pool. Generation of a VSG switching mechanism would only become necessary a few days after transmission from the fly. If this theory is correct, then there is a basic conflict in the trypanosome, with the pressure for antigenic heterogeneity outweighing the pressure for antigenic unpredictability: some parasite evasion mechanisms may be more precarious than at first suspected.

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