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Exposed epitopes on a *Trypanosoma equiperdum* variant surface glycoprotein altered by point mutations

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African trypanosomes are covered by a dense protein layer that is immunologically distinct on different trypanosome isolates and is termed the variant surface glycoprotein (VSG). The different VSGs are expressed in a general order, where some VSGs appear preferentially early in infection and others only later. The exposed epitopes on a late antigen, VSG 78, of *T.equiperdum* were studied by the technique of monoclonal antibody (MAb) escape selection. MAbs that neutralize trypanosomes bearing VSG 78 reacted with the VSG only when it was attached to the trypanosome surface, suggesting that the most immunogenic surface epitopes are conformational. Trypanosome clones resistant to one of the MAbs yet still expressing VSG 78 or 78^{20} were isolated *in vitro*. Two independent variants resistant to MAb H3 changed Ser192 to Arg by a single base change in the VSG gene and a variant resistant to MAb H21 had a single base change that converted Gln172 to Glu. A variant resistant to MAb H7 had several changes in the VSG gene, a gene conversion in the 5′ region and an isolated mutation in codon 220 that is proposed to be responsible for the resistance phenotype. The isotypic bias of the MAbs against VSG 78 and an analysis of the natural variants that are resistant to MAb 78H21 suggest that glycosylation plays a role in the immunogenicity of these proteins. The analysis defines some of the exposed amino acid residues and demonstrates that VSG genes are altered by mutations and small gene conversions as well as replaced by large gene conversion-like events. The results provide biological data supporting the model of VSG structure obtained by crystallographic studies.

Key words: African trypanosomes/antigenic variation/ *Trypanosoma equiperdum*/variant surface antigens/VSG epitopes

Introduction

A major virulence factor of the African trypanosomes is their ability to change the unique surface protein that covers them and acts as the primary parasite antigen. This protein, referred to as the variant surface glycoprotein (VSG), can be changed by a mechanism that inserts a copy of an inactive VSG gene into a transcriptionally active genomic region, the expression site, near a chromosome telomere. The mechanistic details of this gene conversion-like process are not understood (Donelson and Rice-Ficht, 1985; Pays and Steinert, 1988). In *Trypanosoma equiperdum* as in *Trypanosoma brucei* there is a large repertoire of VSG or VSG related genes. Capbern *et al.* (1977) demonstrated that more than 100 different surface antigens can be produced during an experimental *T.equiperdum* infection. Some of the VSGs, such as VSG 1, generally appear very early in infection while others, such as VSG 78, are usually seen only after several weeks of infection. We have previously demonstrated that the active gene for VSG 78 has a complex structure. As shown schematically in Figure 1, most of the gene is constructed from a series of segments derived from several different silent genes of the same gene family while the 3′ end of the gene is derived from an unrelated gene (Roth *et al.*, 1986 , 1989).

The sequences of different VSGs are quite variable over the N-terminal two thirds of the protein while the C-termini of the proteins are highly related and are not accessible to antibodies *in vivo* (reviewed in Donelson and Rice-Ficht, 1985). Although the crystallographic stucture of only one VSG has been determined in detail (Freymann *et al.*, 1990), several lines of evidence suggest that all VSGs have a similar structure and that only limited regions of the protein are accessible to host antibodies (reviewed by Turner, 1988; Pays and Steinert, 1988). Importantly, Pays and coworkers (Pays *et al.*, 1983, 1985) showed that the replacement of the *T.brucei* AnTat 1.1 VSG gene with another silent gene of the same family changed the ability of several polyclonal sera to kill the trypanosomes. This result, together with the observed mosaic structure in the VSG 78 gene suggested that small gene conversions might change and define exposed VSG epitopes. The recognition of such changes could be important both for understanding how the trypanosomes can expand their antigenic repertoire and for defining the portion of the VSG which is exposed on the surface of the parasite. To address these questions, monoclonal antibodies (MAbs) able to kill VSG 78 covered cells were isolated and used to select variant trypanosomes with limited alterations in the VSG. Analysis of the alterations showed that the variation was generally due to mutations and pinpointed specific amino acids in the exposed epitopes. The results are in agreement with predictions of VSG structure developed by Raman spectroscopy (Jähnig *et al.*, 1987), X-ray crystallographic studies (Metcalf *et al.*, 1987; Freymann *et al.*, 1984, 1990) and epitope denaturation studies (Clarke *et al.*, 1988). The results also clearly demonstrate that the site of VSG expression is a genetically active region undergoing both mutations and gene conversions.

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Results

Isolation of variant clones

The complex structure of the VSG 78 gene suggested that during infection, the antigenic identity of *T.equiperdum* might be changed by the conversion of small regions of the active VSG gene with sequences from silent members of the same gene family. To examine this possibility, MAbs that react with surface epitopes as defined by their ability to kill VSG 78 coated trypanosomes were isolated and characterized. MAb clones were produced from mice infected with either BoTat 78 (VSG 78) or 78^{20} (VSG 78^{20}) trypanosomes by the protocol described in Materials and methods. The MAbs were classified with respect to their ability to kill the original target strain and the other BoTat 78 related strains. A summary of the properties of the MAbs used in this study is shown in Table I. Several interesting points can be made about these MAbs. Firstly, there is a disproportionate bias toward the IgG_3 class suggesting an involvment of carbohydrate in the selection of the responding B-cell clone (Björkland and Coutinho, 1987; Perlmutter *et al.*, 1978). Though all VSGs are glycoproteins, the bias in isotype selection is not observed in MAbs against all VSGs (Reinwald *et al.*, 1987; T.Baltz, unpublished data). Secondly, the epitope recognized by each MAb is conformational as evidenced by the lack of reactivity of the MAbs with isolated VSG. Lastly, the trypanosomes are killed in the absence of complement. A complement independent MAb that immobilizes *Trypanosoma congolense* has recently also been reported (Wei *et al.*, 1990).

Variants that had changed an epitope were selected by their resistance to killing by one of the anti-VSG 78 MAbs. In brief, the selection procedure was as follows. A single BoTat 78 trypanosome was grown to $\sim 10^7$ cells and the population was divided into six wells where it was treated with the monoclonal antibody. To counterselect against variants that survived the selection by replacing VSG 78 with an 'early' VSG, the survivors were treated with serum from a *T.equiperdum* infected rabbit that had antibodies against many early VSG variants but not against VSG 78. The surviving trypanosomes were grown and examined by immunofluorescence using polyclonal anti-VSG 78 serum. Cells were subcloned until pure clones having a polyclonal anti-VSG 78 positive and monoclonal antibody negative phenotype were obtained.

Characterization of MAb resistant clones

Clones resistant to the MAb H3 were isolated from both BoTat 78 and 78^{20} and clones resistant to MAb H7 or H21 were isolated from BoTat 78. As shown in Table I, the selected clones remain sensitive to the antibodies other than the one used in the selection. This observation is consistent with the proposition that only one epitope has been altered. In order to identify the differences between the original VSG and that of the MAb resistant variants, the genes encoding the variant VSGs were cloned and sequenced. The cDNA clones corresponding to the entire VSG gene of the VSG 78^{20} variant resistant to MAb H3 (78^{20}H3R) were sequenced and the results are summarized in Figure 2B. Three nucleotide changes were observed. Of these, two are silent (positions 402 and 603) while the third at nucleotide 576 is a G−C transversion that replaces Ser192 by Arg (Figure 3). The same nucleotide changes were also observed in a genomic clone of the gene eliminating the possibility that the change is due to a cloning artefact or post-transcriptional modification of the mRNA (data not shown).

The importance of the Ser at position 192 in the epitope defined by MAb H3 was confirmed by cloning and sequencing the 5′ region of the VSG gene in the variant 78H3R, a MAb H3 resistant variant of BoTat 78. The sequence of the first 900 nucleotides in this gene showed only one difference from that of the gene in its parent (Figure 2B). Codon 192 was changed from AGC to AGA, again substituting Arg for Ser. Thus in the two variants selected for their ability to evade killing by MAb H3, a different nucleotide change in the same codon resulted in the substitution of Ser192 by Arg. These results argue that Ser192 plays an important role in the reactivity of MAb H3 with the VSG 78 on the surface of the trypanosome. In addition, since the two H3 resistant variants remain sensitive to other MAbs such as H7, the replacement of Ser by Arg does not cause a general change in protein conformation or in the sensitivity of the cells to killing by MAbs. We conclude that Ser192 is part of the epitope recognized by the MAb H3 and is accessible on the surface of BoTat 78 and 78^{20} trypanosomes.

A similar analysis was carried out with a strain resistant to killing by the MAb H21. When the 5′ end of the VSG 78H21 gene was sequenced, again, only a single nucleotide difference was observed. In this case, a C−G transversion at position 514 (Figure 2B) caused the Gln at position 172 to be converted to Glu (Figure 3). Though this amino acid and charge difference is consistent with an alteration of Ab binding, it is not the variation observed in VSGs 78bis and 78ter, two variants isolated in rabbit infections based simply on their production of VSG 78 and subsequently found to be resistant to MAb H21 (Table I). These two 'natural' variants differ from the sensitive ones by having Lys in place of Asn280 (Figure 3). This change removes the potential

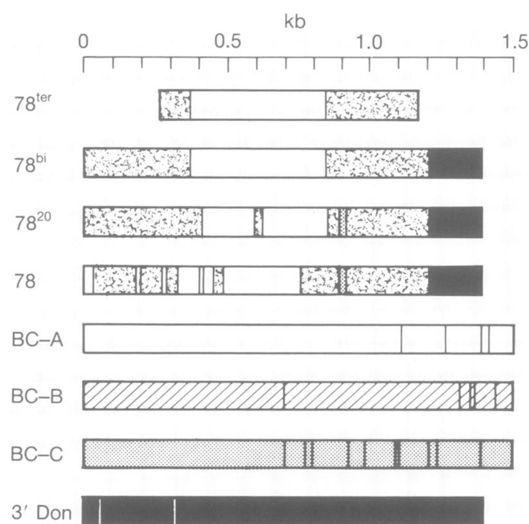


Fig. 1. Schematic representation of the VSG 78 gene family. The active VSG genes labeled 78, 78^{20}, 78 bis and the partial sequence of 78ter are shown as blocks representing regions derived from the different basic copy genes. The basic copy genes BC-A thru -C are highly related while the 3′ donor gene is only related to the other silent genes by a small region of homology around the junction point. These silent genes are present regardless of the VSG expressed while one of the active VSG 78 genes is present only in cells expressing VSG 78. The vertical lines in the schematic view of the silent genes represent stop codons, demonstrating that all of the silent genes are pseudogenes and could not independently code for a functional VSG.

glycosylation site in VSG 78 at position 280. Both changes could affect the MAb H21 epitope. Since the epitope is conformational the structure of the protein or the interaction of two monomers might bring the two distant residues into proximity with one another on the cell surface. Alternatively, Asn280 of VSG 78 might be glycosylated with a portion of that moiety involved in the epitope.

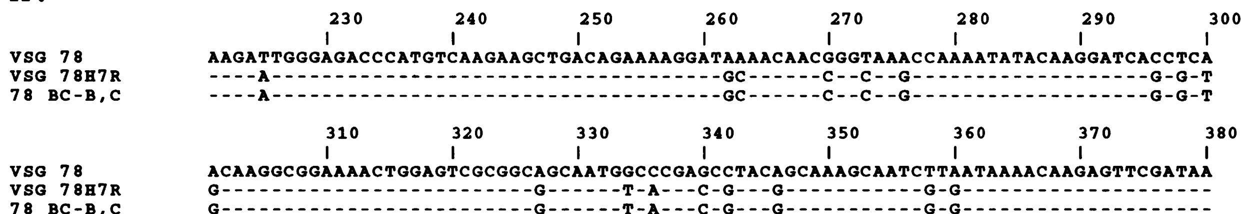
Analysis of the VSG gene in the strain resistant to MAb H7 produced a more complex result than those described above. In one region (nucleotides 220–380 and amino acids 75–120), the 78H7R VSG gene varies from the parent at a number of points (Figure 2A). The amino acid alterations resulting from these genetic changes are shown in Figure 3. All of the amino acid changes in this region, except the Leu to Val at position 120, replace the VSG 78 amino acids with residues normally found in VSG 78^{20} which is sensitive to MAb H7 (Table I and Figure 3). Thus, these amino acid changes are not sufficient to account for the difference in sensitivity of the variant to MAb H7. Another amino acid change situated outside this region (Thr220 to Ala) is caused by an A to G change at base 658 (Figure 2B). It is likely that the Thr to Ala change at position 220 is more important for the resistance phenotype.

Origin of genetic changes

Though the structure of VSG 78 is complex, the entire sequence of all the active genes can be shown to be derived from members of its silent gene family. Thus, it was of interest to determine the origin of the genetic changes observed in the different MAb resistant strains by comparing their sequences with that of the silent genes. Figure 2A shows a comparison of the region from bp 240 to 380 in 78H7R with that of the parent sequence, VSG 78, and that of the silent genes, BC-B and -C. As suggested by the similarity of the VSG 78H7R and 78^{20} amino acid sequences, all the differences between the 78H7R and 78 genes are found in the silent genes. This result indicates that the active VSG 78 gene was altered by a gene conversion-like event. In contrast to the result in the first 400 bp, the sequence difference at bp 658, which we propose to be responsible for the resistance phenotype, is unique to the active 78H7R gene, arguing that it arose from a mutation (Figure 2B).

In the case of resistance to MAb H3, strain 78H3 was altered by a single nucleotide change at bp 576, while the 78^{20}H3R variant had three nucleotide changes. BC-A contains the sequence in the VSG 78 gene and though BC-B and -C have sequences different from those sequences,

A.



B.

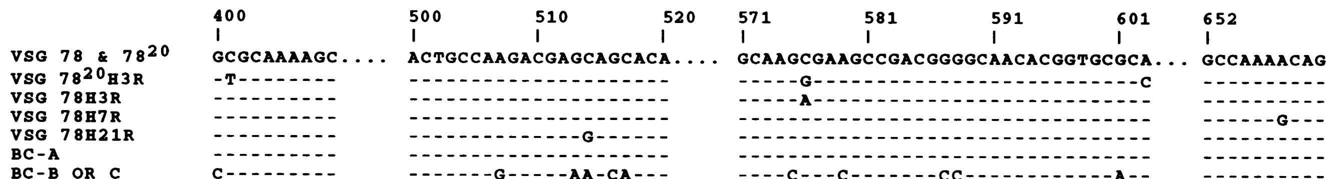


Fig. 2. Comparison of the nucleotide sequences of VSG 78 genes in MAb sensitive and resistant variants. (**A**) The 5′ end of the VSG 78H7R gene was altered by a gene conversion. The nucleic acid sequence of the parent, BoTat 78, and MAb H7 resistant variant VSG genes are compared with those of the silent BC-B and -C genes over the region where the differences can be attributed to a gene conversion. (**B**) Alterations in the middle of the gene are unique to the MAb resistant variant VSG genes. In the region indicated by a dotted line the sequence in the variants does not differ from that of the parents. The numbering for both (A) and (B) begins at the codon for the first amino acid in the mature VSG 78 protein, the 18th amino acid from the initiating methionine. A dash indicates that the nucleotide is the same as that listed for VSG 78. The sequence of the VSG 78 and 78^{20} genes is the same in the region shown in (B); likewise, those of the BC-B and -C genes in both (A) and (B) are the same. The sequences of VSG 78, 78^{20} and BC-A thru -C are from Roth *et al.* (1989).

Table I. Reaction of anti-VSG 78 monoclonal antibodies with the family of BoTat 78-like strains of *T.equiperdum*.

Group	Antibody	Strain																Isotype
		78		78^{20}		78bis		78ter		78H3R		78H7R		78H21R		78^{20}H3R		
		F	N	F	N	F	N	F	N	F	N	F	N	F	N			
I	H3 and H4	+	+	+	+	+	+	+	+	–	–	+	+	+	+	–	–	G3,G3
II	H5, H6, H8	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	M,G3,G3
III	H7	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	G3
IV	H21	+	+	+	+	–	–	–	–	+	+	+	+	–	–	+	+	G3

The Antibody column lists the names of the monoclonal antibodies and F and N in the columns headed by the VSG names refer to the ability of the antibody to produce a positive immunofluorescence reaction or neutralize the the indicated strain. A '+' sign indicates a positive reaction or the ability to neutralize the strain. G3 indicates IgG_3 and M indicates IgM. The grouping is based on the reactivity of the MAb with the different trypanosome strains; it is not known if the MAbs in group two react with the same epitope.

```
                     10         20         30         40         50         60
VSG 78        AVNDNAAEFR VFCDILGMDT RVETISITLD TAKATEVLEE LAYLNLTAAS DEWLKDKNGE
VSG 78H3R     ---------- ---------- ---------- ---------- ---------- ----------
VSG 78H7R     ---------- ---------- ---------- ---------- ---------- ----------
VSG 78H21R    ---------- ---------- ---------- ---------- ---------- ----------
VSG 78^20     ---------- ---------- ---------- ---------- ---------- ----------
VSG 78^20H3   ---------- ---------- ---------- ---------- ---------- ----------
VSG 78bis     ---------- ---------- ---------- ---------- ---------- ----------

                     70         80         90        100        110        120
VSG 78        LADENGTDKK TERQDWETHV KKLTEKDKTT GKPKYTRITS TRRKTGVAAA MARAYSKAIL
VSG 78H3R     ---------- ---------- ---------- ---------- ---------- ----------
VSG 78H7R     ---------- ----E----- -------A-- --------SA A--------- -S-P-G---V
VSG 78H21R    ---------- ---------- ---------- ---------- ---------- ----------
VSG 78^20     ---------- ----E----- -------A-- --------SA A--------- -S-P-G----
VSG 78^20H3R  ---------- ----E----- -------A-- --------SA A--------- -S-P-G----
VSG 78bis     ---------- ----E----- -------A-- --------SA A--------- ----------
VSG 78ter                 ---E----- -------A-- --------S- ---------- ----------

                    130        140        150        160        170        180
VSG 78        IKQEFDKLTD QVTAAKAKAT SEIKSALYGD GKATFDKGAL ESTPAKNCQD EQHNKNAGKC
VSG 78H3R     ---------- ---------- ---------- ---------- ---------- ----------
VSG 78H7R     ---------- ---------- ---------- ---------- ---------- ----------
VSG 78H21R    ---------- ---------- ---------- ---------- ---------- -E--------
VSG 78^20     ---------- ---G------ ---------- ---------- ---------- ----------
VSG 78^20H3R  ---------- ---G------ ---------- ---------- ---------- ----------
VSG 78bis     ---------- ---G------ ---------- ---------- ---------- ----------
VSG 78ter     ---------- T--G------ ---------- ---------- ---------- ----------

                    190        200        210        220        230        240
VSG 78        VAWDFLCLCT ASEADGATRC AHGANGGQLA DPSSLETAKT AFTTIKNSCP QKPANKALTA
VSG 78H3R     ---------- -R-------- ---------- ---------- ---------- ----------
VSG 78H7R     ---------- ---------- ---------- ---------A ---------- ----------
VSG 78H21R    ---------- ---------- ---------- ---------- ---------- ----------
VSG 78^20     ---------- ---------- ---------- ---------- ---------- ----------
VSG 78^20H3R  ---------- -R-------- ---------- ---------- ---------- ----------
VSG 78bis     ---------- ---------- ---------- ---------- ---------- ----------
VSG 78ter     ---------- ---------- ---------- ---------- ---------- ----------

                    250        260        270        280        290        300
VSG 78        DEIFGTIASF ESLLGRQSAG QTNAPNHYIF GKPHSDGTCN GSNAEGMCVN YKTQLSKGGS
VSG 78H3R     ---------- ---------- ---------- ---------- ---------- ----------
VSG 78H7R     ---------- ---------- ---------- ---------- ---------- ----------
VSG 78H21R    ---------- ---------- ---------- ---------- ---------- ----------
VSG 78^20     ---------- ---------- ---------- ---------- ---------- ----------
VSG 78^20H3R  ---------- ---------- ---------- ---------- ---------- ----------
VSG 78bis     ---------- ---------- ---------- ---------K ---------- ----------
VSG 78ter     ---------- ---------- ---------- ---------K ---------- ----------
```

Fig. 3. The deduced amino acid sequences of the VSG 78 related genes. The sequences starting at the first amino acid in the mature VSG 78 protein and extending to amino acid 300 are shown in single letter code. A dash indicates that the amino acid is the same as in VSG 78, amino acid differences are indicated in single letter code. Potential N-glycosylation sites in the VSG 78 sequence are underlined.

they do not contain the nucleotide found in the 78H3R gene (Figure 2B). Likewise, the difference at bp 576 and the two silent changes are unique to the 78^{20}H3R variant gene. These results again argue that the variations in this region of the VSG 78 and 78^{20} genes resulted from mutations in the active gene copy. To verify that the nucleotide variation did not originate as a mutation in BC-A that was subsequently passed to the active gene by gene conversion, the silent gene was recloned and the region sequenced; no difference from the original sequence was observed (data not shown). In the case of 78H21R the C–G transversion is also unique to the VSG gene even though there are numerous differences between BC-B or -C and the VSG 78 gene around nucleotide 514.

```
VSG 1 aa     L   T   T   L   L   A   G   N   D   A
VSG 1       CTA ACA ACG CTA CTA GCG GGA AAC GAT GCG(810)
VSG 1B      --- --- -G- --- --- --- --- --- --- ---
VSG 1B aa    -   -   R   -   -   -   -   -   -   -
                   (263)
```

Fig. 4. Differences in the nucleotide and deduced amino acid sequences of the VSG 1 and MAb 1B resistant genes. The nucleic acid sequences between nucleotides 781 and 810 of the VSG 1 and the MAb 1B resistant variant genes are aligned with the derived amino acid sequences. The nucleotides or amino acids in the MAb resistant variant gene that are the same as the cognate in the VSG 1 gene are indicated by a dash. The figure uses the first AUG in the major ORF as the starting point for numbering both the nucleotide and amino acid sequences because the first amino acid in the mature protein is not known.

Variation in an early gene

The results in the previous section demonstrate that a gene encoding the late variant, VSG 78, can be immunologically altered by mutations. Since the genes that code for the VSGs appearing early in infection generally do not have a complex structure like those that arise later (Longacre and Eisen, 1986), it was of interest to determine whether their immunological properties also are altered by single nucleotide changes rather than larger gene conversions. To investigate this question a derivative of BoTat 1, an early variant, was selected with the MAb 1B which specifically kills BoTat 1. The gene encoding the variant VSG 1 was examined by cloning a cDNA representing the active antigen gene and comparing its sequence with that of the gene in BoTat 1. The result of this study is summarized in Figure 4. Only one nucleotide difference was found between the original VSG 1 gene and the gene of the MAb resistant variant; nucleotide 788 was changed from C to G. This transition changes amino acid 263 in the protein from threonine to arginine. Unfortunately, the sequences of the silent genes related to the VSG 1 gene are not known, thus it could not be determined if any of them contain a G at the corresponding position. Nevertheless, the occurrence of a single base change suggests that the variation resulted from a mutation. Finding a variation of the immunological identity of VSG 1 by a point variation rather than a larger gene conversion is consistent with the observation of Longacre and Eisen (1986) demonstrating that several VSG 1 genes from independent isolates were all identical and came from a single silent gene.

Discussion

The main mechanism used by African trypanosomes to evade elimination by the host immune system is antigenic variation, which allows the parasite to present a continually varying immunological identity. This defense mechanism implies that it is advantageous for the trypanosome to have a large reservoir of antigen genes and/or the ability to alter those genes. The genes encoding the VSGs are generally members of mutigene families that can include pseudogenes. In *T.equiperdum*, we have observed the formation of complex VSG genes created from parts of several related pseudogenes which combined in the expression site to form a functional VSG gene (Roth *et al.*, 1989). This ability to create mosaic genes suggested that gene conversion of the active VSG gene by the related silent genes might be a source of antigenic variation. To determine whether specific VSG epitopes could be altered we used the antibody selection technique previously used successfully to study the surface epitopes on viruses (Wiley *et al.*, 1981; Sherry *et al.*, 1986). Monoclonal antibodies recognizing the VSG on the trypanosome were isolated and used to select new variants of the VSG resistant to killing by the specific MAb.

The MAbs that specifically killed the BoTat 78 family of trypanosomes had several interesting features. They killed the trypanosomes in the absence of complement and their isotype was strongly biased. Six of the seven MAbs studied were of the IgG_3 class (Table I). The reason for the isotype bias is not known but is generally associated with the glycosylation of antigens (Perlmutter *et al*, 1978; Björklund and Coutinho, 1983). This bias is not observed with all VSGs and might be related to the type or position of the protein glycosylation. The observation that trypanosomes expressing VSG 78 are agglutinated by concanavalin-A, while VSG 1 expressing trypanosomes are not agglutinated and that the anti-VSG 1 MAbs do not show an isotype bias is in accord with this idea (Baltz *et al.*, 1976, 1977). The mechanism by which the trypanosomes are killed is another unknown. The fact that complement is not required suggests that the mechanism might affect a general property such as flagellar movement.

The VSGs of three of the four MAb resistant variants differed from the parent VSG by only a single amino acid and these changes appear to result from mutations rather than gene conversions. The two variants selected with MAb H3 changed Ser192 in the mature VSG to an Arg (Figure 3). The variant resistant to MAb H21 also had a single amino acid alteration, Gln172 to Glu. In contrast, the variant resistant to MAb H7 had numerous amino acid changes of which all but one were the result of a partial gene conversion of the VSG with sequences from an inactive pseudogene, either BC-B or -C (Figure 2A). The other nucleotide changes in this and the other variants are not found in any of the silent VSG 78 related genes and are most easily explained as mutations. In the case of variant 78^{20}H3R, there were three nucleotide changes over a distance of 202 bp. This is a high rate of mutation when one considers that in each of the four 'VSG 78' genes in isolates derived from infections, every nucleotide could be accounted for by the sequence in the silent gene copies (Roth *et al.*, 1989 and this paper). This observation argues that the active VSG gene is very susceptible to mutations as well as gene conversions. The high rate of mutation and gene conversion in these genes might result from a high incidence of accidental DNA breaks that are repaired by the general recombination machinery of the cell as proposed by Borst and Greaves (1987). Though we cannot formally rule out the possibility that the nucleotide changes are derived from gene conversions using genes with very small regions of homology with the VSG gene, the observation that Ser192 was changed in two variants by different nucleotide changes argues against this possibility.

This study differs from other published studies in the selective pressures used to isolate the variants. The variants were selected to resist killing by a single MAb and to retain at least some of the other epitopes recognized by a polyclonal antiserum. It was hoped that this procedure would select variants with limited alterations in the VSG gene rather than those that had greatly changed the gene. This selective pressure may favor mutations rather than gene conversions, since the latter may have a high probability of altering multiple epitopes even though the amino sequence encoded by the silent gene is very similar to that of the VSG. For example, Pays *et al.* (1983, 1985) isolated a variant *in vivo* that was created from different members of the same gene family but produced an immunologically different VSG (AnTaTs 1.1 and 1.10). These variants could switch back and forth by gene conversion-like events that involved nearly the entire gene. Thus, while the selection procedure used in our study is not truly representative of the physiological situation, it more directly examines the ability of the Africain trypanosomes to alter specific epitopes. This property makes the procedure important for examining the exposed regions of other VSGs and trypanosome surface proteins in general.

Several studies have examined the structure of purified VSG molecules. Both the crystallographic (Feymann *et al.*,

1984, 1990; Metcalf *et al.*, 1987) and the Raman spectrographic (Jähnig *et al.*, 1987) studies suggest a basic VSG structure that contains two large α-helical segments connected by a more loosely structured region. It has been proposed from these studies that the loosely structured region is the part of the protein exposed on the surface of the trypanosome. The epitopes we have identified on VSG 78 lie in the region between amino acids 170 and 220 of the mature VSG. The only detailed structural study of a VSG is the crystallographic study of Freymann *et al.* (1990) showing that at the 2.9 Å level: Phe180, Leu189, Met210, Tyr214 and Val217 are exposed on the surface of VSG MITat 1.2. Though there is no direct evidence that the structure of VSG 78 is the same as that of MITat 1.2, our biological data are in agreement with their proposal that amino acids in this region are exposed. Additionally, in MITat 1.2 the carbohydrate at Asn263 appears to be packed against the surface of the molecule. This observation could correlate with a presumptive role for a carbohydrate moiety on Asn280 of VSG 78, both in the reactivity of MAb H21 and the isotypic selection in the MAbs that kill BoTat 78 trypanosomes. Although the exact structure of VSG 78 must await physical studies of the protein, the correlation between our observations and those of the published X-ray crystallography demonstrates the complementarity of the two approaches, one at the biological level and the other at the physical level.

The question arises as to the physiological significance of mutations and small gene conversions in the expression linked copy of a VSG gene since this copy generally exists for only a relatively short time before being replaced by a new gene. However, Laurent *et al.* (1984) have shown that in some cases the expression linked copy of the VSG gene is not lost when another VSG gene is activated, rather the previous VSG gene is simply inactivated *in situ*. In this case, the inactivated VSG gene is preserved and is reactivated in subsequent infections. Any changes, point mutations or gene conversions, in the preserved expression linked VSG gene will become part of an expanded VSG repertoire. Additionally, these alterations of the expression copy may be important in laboratory strains continually selected to express the same VSG. As seen in Figure 1, the strain BoTat 78 that was isolated by Capbern *et al.* (1977) and has been subcloned many times has the most complex VSG 78 gene structure. The very recently isolated strains, BoTats 78bis and 78ter, have relatively simple gene structures. In the active VSG gene of these latter strains, the region in BC-B containing a mutation is replaced by the homologous region of BC-A. The structure of the active VSG gene in BoTat 78^{20} is intermediate between these extremes and the strain has been subcloned an intermediate number of times. We suggest that part of the complexity in the VSG 78 gene has developed after its initial isolation.

Materials and methods

Trypanosomes and VSG preparation

Trypanosoma equiperdum variants BoTats 1, 20, 78 and 78^{20} have been described previously (Capbern *et al.*, 1977) and 78bis and 78ter were isolated in the same manner using a polyclonal anti-VSG78 antiserum to identify the strains. The VSGs were purified by previously described procedures (Baltz *et al.*, 1977).

Cell fusion and selection of monoclonal antibody producing hybridomas

Balb/C mice (CERJ-St Berthevin) were immunized by several cycles of infection and cure as follows. Eight to ten week old mice were injected intraperitoneally (i.p.) with 10^4 trypanosomes and cured 2 days later by treatment with 0.5 mg/mouse of Berenil (Hoechst Pharmaceuticals). Two booster injections of 2×10^8 trypanosomes administered i.p. were followed 6 h later by Berenil treatment. The first boost was 3 weeks after the initial infection and the second boost was 1 month later. The spleens were removed 4 days after the second boost and the cells were fused to Sp2/0 myeloma cells and the hybrids were selected in HAT medium (Fazekas de Saint-Groth). Aliquots of medium were assayed for the presence of VSG specific antibodies by several tests: *in vitro* neutralization of living trypanosomes, indirect immunofluorescence assay (IFA), and enzyme-linked immunosorbant assay (ELISA). Hybridomas from positive wells were cloned by dilution and cultured either *in vitro* or injected (10^6 cells) into primed Balb/c mice for the preparation of ascite fluid.

The isotype of each monoclonal antibody was identified by ELISA using biotinylated isotype specific antisera (1/100 in PBS) and streptavidin peroxidase conjugate (Amersham, 1/1000 in PBS) after precipitation of the Ab with polyethylene glycol (PEG).

Assays for antibodies against VSGs

In vitro neutralization assay. Equal volumes (100 μl) of freshly isolated trypanosomes (10^5 parasites/ml) in culture medium supplemented with heat inactivated serum (Baltz *et al.*, 1985) and hybridoma culture supernatant were mixed and incubated at 37°C in an atmosphere containing 7% CO_2. The presence of neutralizing antibodies resulted in either a rapid immobilization or lysis of the trypanosomes.

IFA. Acetone fixed trypanosomes were assayed as previously described (Baltz *et al.*, 1985). Bound antibodies were detected with fluorescein-conjugated goat anti-rabbit immunogobulin (Pasteur Institute Diagnostics).

ELISA. These tests were performed using peroxidase conjugated rabbit anti-mouse IgG (Cathy and Raykndalia, 1989). The plates were coated by a solution of the appropriate VSG (5 μg/ml in 20 mM bicarbonate buffer, pH 9.6).

In vitro selection of monoclonal antibody resistant BoTat 78 variants

BoTat 78 trypanosomes were grown in culture and subcloned as previously described (Baltz *et al.*, 1985). Selection was performed by the addition of 50 μl ascites fluid of either the appropriate MAb and 50 μl rabbit serum obtained from a BoTat 1 infected animal 1 week before the expression of variant 78. The few remaining cells in each well were grown to $\sim 10^6$ cells/ml and tested by IMF for the presence of parasites with VSG 78 specificity with a polyclonal monospecific rabbit anti-VSG 78 serum diluted 1/100 in PBS.

Cloning and sequencing of the ELC gene

The VSG gene for VSG 78^{20} H3R and VSG 1 resistant to MAb B were cloned as cDNA using the kit of BRL (Rockeville, MD, USA) from poly(A) selected RNA. The genomic copy of the 5′ end of the 78^{20} H3R gene was cloned as a *Pst*–*Bgl*II fragment and includes the coding region for amino acids 1–241. The 5′ region of the 78H3R, 78H7R and 78H21R VSG genes were cloned as a 2.5 kb *Bam*HI fragment in pUC13. The 78H21 gene was also cloned by PCR of the cDNA using oligonucleotides specific for the 35mer region and for the 3′ end of the VSG 78 gene. Genomic clones hybridizing with VSG 78 cDNA were selected and the 1.3 kb *Pst*–*Bam* fragment was either subcloned into M13 or directly sequenced in the plasmid. The sequences were determined by the dideoxynucleotide termination technique (Sanger *et al.*, 1977) using VSG 78 specific oligonucleotides as primers. The DNA sequences were recorded and analyzed with the DNA Strider program (Marck, 1988). The complete nucleic acid sequences of VSGs 78, 78^{20}, 78bis, BC-A, BC-B and BC-C are published and listed in GenBank (Roth *et al.*, 1989). The sequences of the VSG 1 and VSG 78ter genes have been submitted to the EMBL database.

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