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An African trypanosome variant surface glycoprotein gene whose expression is not activated by duplication

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ABSTRACT

A variant surface glycoprotein (VSG) of Trypanosoma brucei is encoded by a gene whose expression is not governed by duplication-transposition. There are two copies of this gene. The 5' flanking regions of the two genes are indistinguishable by restriction mapping, although each possesses approximately 5-10 Kbp of DNA which is devoid of restriction sites. All restriction enzymes tested appeared to cut genomic DNA at a uniform distance 3' of the gene. This, coupled with the observed sensitivity of both genes to BAL 31, indicates that they lie near chromosomal termini. Length variation occurs 3' of these genes in bloodstream clones and their procyclic derivatives, although the number of length variants is conserved. This suggests that length variation alone does not control VSG switching or gene expression and that constraints exist on the extent to which 3' flanking regions can vary in length.

INTRODUCTION

Trypanosoma brucei is a protozoan that occurs as a parasite within the bloodstream and other extravascular fluids of mammals. The genome of this organism probably contains hundreds of genes (1) that encode a repertoire of glycoproteins that are serologically unrelated (2). These glycoproteins completely enshroud the cell membrane as a surface coat (3). Only one type of glycoprotein is present on the membrane of an individual cell (4,5). A clone of bloodstream trypanosomes is comprised of individuals that express an identical surface glycoprotein (5). Antibody molecules that are elicited by an inoculum of cloned trypanosomes are specific to the surface glycoprotein that distinguishes the inoculated clone from others (6). Although most of the organisms are destroyed a few survive as variants that are unrelated serologically to the original inoculum (6). Thus, T. brucei can evade antibody mediated lysis by switching from one variant surface glycoprotein (VSG) to another more rapidly than the host can respond.

So far VSG genes can be organized into two categories based upon

their modes of expression. In certain cases the expression of a VSG gene is linked to a duplication-transposition event wherein a replica of a pre-existing or basic copy gene is translocated to another region in the genome where it is expressed (7,8,9). In all cases examined this region(s) is believed to reside near the end of a chromosome (10,11,12). In clonal variants the basic copy is retained whereas the replica is usually deleted from the genome (8). Certain of these basic copy genes reside at chromosome ends (11,12). Duplicative-transposition in these cases may be associated with telomere exchange. VSG genes that fall into the second category are quite unique because their expression does not appear to be mediated by duplicative-transposition (13,8). In two cases, it has been shown that these VSG genes permanently reside at chromosome ends (14,15). Based on restriction maps of these genes and of other closely related genes it has been suggested that they arose by the duplication and transposition of a basic copy to an expression site that is located at a chromosome end (16,17).

In this study the ILTat 1.2 gene family is described. Two copies of this gene reside in the genomes of 1.2 expressors and non-expressors. These genes are virtually identical in their coding regions and for approximately 20 Kbp of their 5' flanking regions. The two genes are distinguishable only by the lengths of their 3' flanking regions and the sensitivity of one copy to digestion by DNase I in ILTat 1.2 expressors. This is presumably the transcriptionally active gene and its expression is not accompanied by duplication.

Rearrangements to the 3' side of both copies occurs in procyclic cells. As in bloodstream clones these rearrangements are coordinated. The significance of these coordinated rearrangements in cell populations that are presumed not to be of clonal origin is discussed.

MATERIALS AND METHODS

Isolation of bloodstream cells and DNA purification

The derivation of bloodstream clones is depicted in Figure 1. Bloodstream trypanosomes were obtained from Sprague-Dawley rats that were sublethally irradiated prior to infection. These rats were exsanguinated when a parasitemia of approximately 10^9 cells per ml of blood was obtained. The blood was collected in the presence of 4%(w/v) trisodium citrate + 1% D-glucose. Buffy coats were obtained by centrifugation of whole blood at 1000xg at 4°C for 10 minutes. Trypanosomes were isolated

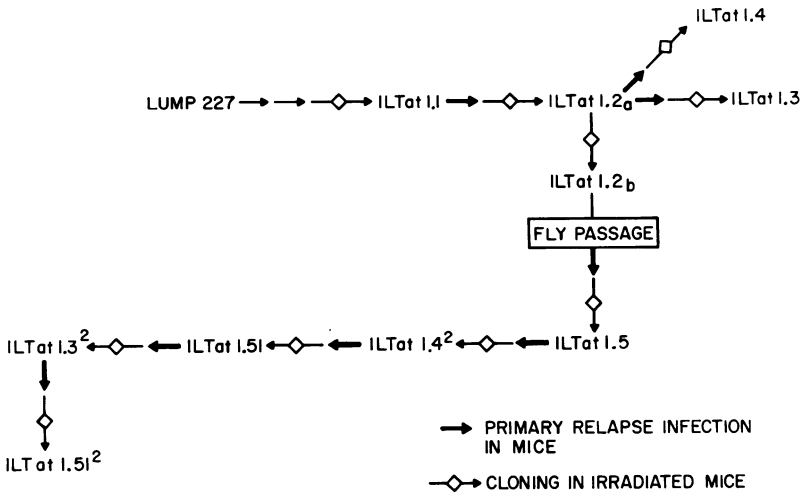

Figure 1. Derivation of clones that comprise the ILTar 1 serodeme. Superscripts indicate the number of VSG repeats, defined serologically, that have been identified. Isolation of bloodstream clones ILTat 1.1, 1.2a, 1.2b, 1.3, 1.4 and 1.5 have been described previously by others (42). Clone ILTat 1.51 was derived by inoculating ILTat 1.4 bloodstream cells into a normal mouse. A single cell was isolated from the population comprising the first patent parasitemia and inoculated into an irradiated recipient. The progeny were harvested and characterized by antibody mediated complement dependent lysis and by immunofluorescent techniques (43).

from buffy coat material by DE 52 cellulose chromatography according to the method of Lanham and Godfrey (18). After centrifugation (200 xg) and two subsequent washes in PSG (61mM sodium phosphate pH8.0, 44mM NaCl, 1% D-glucose) (18) these cells were resuspended to a concentration of 10^{10} cells per ml in PSG supplemented with 10mM DTT and 2.5 µg per ml cycloheximide. An equal volume of buffer containing 50mM Tris-acetate pH7.8, 300mM KCl, 10mM $MgCl_2$, 1 µg per ml cycloheximide was added to the suspension. Cells were then lysed in the presence of 0.5% NP-40 at 0°C during a 5 minute incubation period. Nuclei were isolated by centrifugation at 200xg for 10 minutes. The pelleted nuclei were lysed in a solution containing 10mM EDTA pH8.5 and 1% SDS. The nucleic acid was deproteinized by three successive extractions in phenol/chloroform (1:1 v/v) saturated in 10mM sodium acetate, 100mM NaCl, 1mM EDTA, pH6.0 and was followed by two chloroform extractions. The aqueous phase was adjusted to 100mM NaCl and the nucleic acid was then precipitated by the

addition of 2.5 volumes of ethanol. Precipitates were then dissolved in 10mM Tris-Cl pH8.0, 1mM EDTA (TE-buffer) and incubated successively in the presence of 50 μg/ml RNase, 100 μg/ml pronase and then re-extracted and re-precipitated. DNA samples were then centrifuged through a potassium iodide equilibrium gradient at 110,000xg for 40h (19), extracted with water saturated sec-butanol and then precipitated. Precipitates were dissolved and stored in TE-buffer.

Growth of procyclic cells in vitro and DNA isolation

Bloodstream trypanosomes were transformed to their procyclic derivatives by diluting parasitemic mouse blood, collected asceptically, in 1 ml of SM-medium (20) containing 20% heat inactivated fetal calf serum and 25mM HEPES pH7.4. Starting cultures containing cells at densities of approximately $2x10^6$ cells per ml were split at 2-3 day intervals when cell densities of $1-3x10^7$ cells per ml were obtained. All cultures were initiated in Costar cluster dish wells (17.8 x 16mm) and subsequently maintained in $25cm^2$ flat-bottom flasks at 28°C on a small gyrorotary shaker set to 50 RPM.

DNA was obtained from procyclic cells in the following manner. Cultures were harvested at densities of $2-3x10^7$ cells per ml and centrifuged at 1200xg at room temperature for 8 minutes. Cells were resuspended and washed twice in a solution containing (4.4mM sodium phosphate, 15mM $MgCl_2$, 15mM $MgSO_4$, 40mM KCl, 1mM $CaCl_2$, 0.1mM succinic acid, 0.1mM fumaric acid, 0.5mM α-ketoglutaric acid, 5mM malic acid, 25mM HEPES) pH7.4. Cells were lysed in a 0.5% N-lauroyl sarcosine solution containing 4M guanidinium thiocyanate and 25mM sodium citrate pH7.0 (21). Cell lysates were layered on top of a 5.7M CsCl cushion (22) and centrifuged at 83,000xg, 24h at 20°C. Supernatants were dialyzed extensively in TE-buffer and then digested and extracted as described above.

Restriction endonuclease digestions

Digests of DNA (1 g) were taken to completion in the presence of 10-20 units of enzyme for approximately 12-15h under conditions described by the manufacturers (Bethesda Research Labs (BRL); New England Biolabs (NBL)). Reactions were terminated in the presence of 100mM EDTA pH8.5.

Digestion by endonuclease DNase I

Nuclei were treated by DNase I essentially as described by Pays et al (9). Nuclei at estimated DNA concentrations of 0.4 or 0.5 mg/ml were digested by DNase I (P-L Biochemicals, Inc.; 1ng = 26 units) at an enzyme

concentration of 20 or 40 ng per ml. Reactions were terminated in 1% SDS, 10mM Tris-Cl pH8.0, 100mM NaCl, extracted and then their DNAs were ethanol precipitated.

Agarose gel electrophoresis and transfer to nitrocellulose filters

Restriction digests were electrophoresed through 0.6 or 0.8% agarose gels prepared in a buffer containing 40mM Tris-Acetate pH6.9 and 1mM EDTA. DNA in agarose gels were transferred to nitrocellulose filters according to the method of Southern (23). Dextran-sulphate hybridizations (24) in formamide or without it were performed as described (25). Hybridizations were terminated by washing filters for four 30 minute intervals in 0.1-0.2xSSC and 0.1% SDS at 65°C.

RESULTS

Restriction maps of ILTat 1.2 genes in two bloodstream clones that express different variant surface glycoproteins

The ILTat 1.2 gene family is defined by the ability of its members to hybridize to an ILTat 1.2 cDNA clone, pcB1352, under conditions of high stringency (26). pcB1352 contains a 1.53 Kbp insert of ILTat 1.2 coding sequence within the vector pBR322 (26). Two copies within genomic DNA hybridize to this probe (13,26). We constructed restriction maps of these two genes by using fragments of the cDNA clone as probes (Fig. 4). There is a single Hind III site within the insert of pcB1352. On either side of this Hind III site are two pairs of Pst I sites that mark the boundaries of two fragments used as probes in the following experiments.

Distal restriction sites were mapped by performing single and double digestions using thirteen restriction enzymes. Restriction maps of these genes in ILTat 1.2 and ILTat 1.51 bloodstream clones are shown in Figure 2. We found that the restriction sites 5' of both 1.2 gene copies, A and B, are identical in the ILTat 1.2 clone and are indistinguishable from their analogs, copies C and D, present in the ILTat 1.51 bloodstream clone. These regions of identity extend for at least 20 Kbp. Each of their 3' flanking sequences, however, are unique because they vary in length from 5-14 Kbp. All of these 3' flanking regions are devoid of restriction sites. Furthermore, each region may be the terminus of a DNA molecule because thirteen different enzymes appear to cut at the same location.

Length variation is not a specific property of bloodstream trypanosomes

The length variation that occurs to the 3' side of the 1.2 genes in

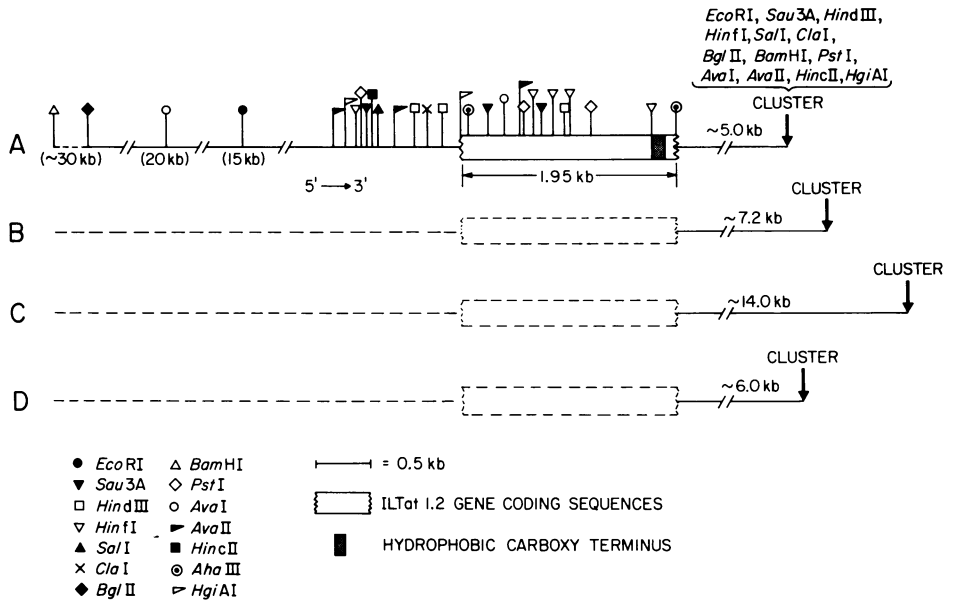

Figure 2. Restriction maps of the genomic contexts of the two ILTat 1.2 genes in ILTat 1.2 DNA (maps A and B) and ILTat 1.51 DNA (maps C and D). Map B corresponds to the DNase I sensitive VSG gene in ILTat 1.2 bloodstream cells. The 5' flanking regions in maps A-D are identical. Their 3' flanking regions vary in length from 5-14 Kbp. The 'CLUSTER' denotes a site that appears to be cut by 13 restriction enzymes.

bloodstream clones is a characteristic that is shared by several other VSG genes (8,11,12,15). It has been suggested that length variation might be induced whenever antigenic switching occurs (12) or, that the variation itself might somehow control VSG gene expression (17). We examined these possibilities by asking whether length variation can be observed in procyclic cells as these do not express VSG genes (3,27,28). The procyclic forms represent the first stage of development within the insect vector Glossina spp. (29). These cells can be obtained in vitro by introducing bloodstream forms into a medium that promotes their transformation to the procyclic stage (20). We have derived procyclic cell lines from three bloodstream clones that express different VSGs. Two of these lines are described here and are compared to their bloodstream counterparts. We have found that flanking sequence rearrangements have occurred in these two independently derived cell lines (see Fig. 3). In each procyclic cell line the contexts of both 1.2

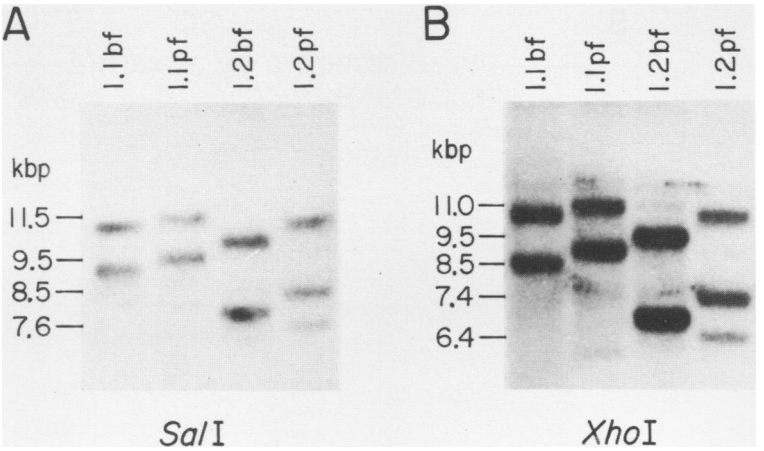

Figure 3. Rearrangements of 1.2 genes are observed in procyclic cell lines derived in vitro. DNAs obtained from bloodstream forms ILTat 1.1, 1.2 and their procyclic derivatives were digested by Sal I (panel A) or Xho I (panel B). These digests were hybridized to pcB1352. Xho I and Ava I cut at the same location in the coding region (see Fig. 2) thereby producing the 3' length variation containing fragments and an 18 Kbp 5' fragment which is not shown in panel B.

genes are clearly different from their bloodstream counterparts. Similarly flanking region length alterations of other VSG genes have occurred in these cell lines and in a third line, as well (data not shown). Three fragments are present in the ILTat 1.2 procyclic cell line. This is presumably a consequence of length variation heterogeneity within this population of cells because the upper and lower fragments are less intense than the middle one. These results show that length variation is not a specific property of bloodstream forms. Therefore, it is unlikely that length variation is induced by switching because procyclic cells lack this capacity. If antigenic switching is controlled by length variation on the other hand, it must not be the only factor involved.

Both copies of the ILTat 1.2 gene reside near a 3' flanking terminus

Our restriction maps suggested that each 1.2 gene and its corresponding 3' flanking region is the terminus of the molecule upon which it resides. We studied this possibility in more detail by asking whether the DNA exonuclease BAL 31 can specifically shorten those Hind III fragments that contain the 3' ends of the 1.2 genes. As shown

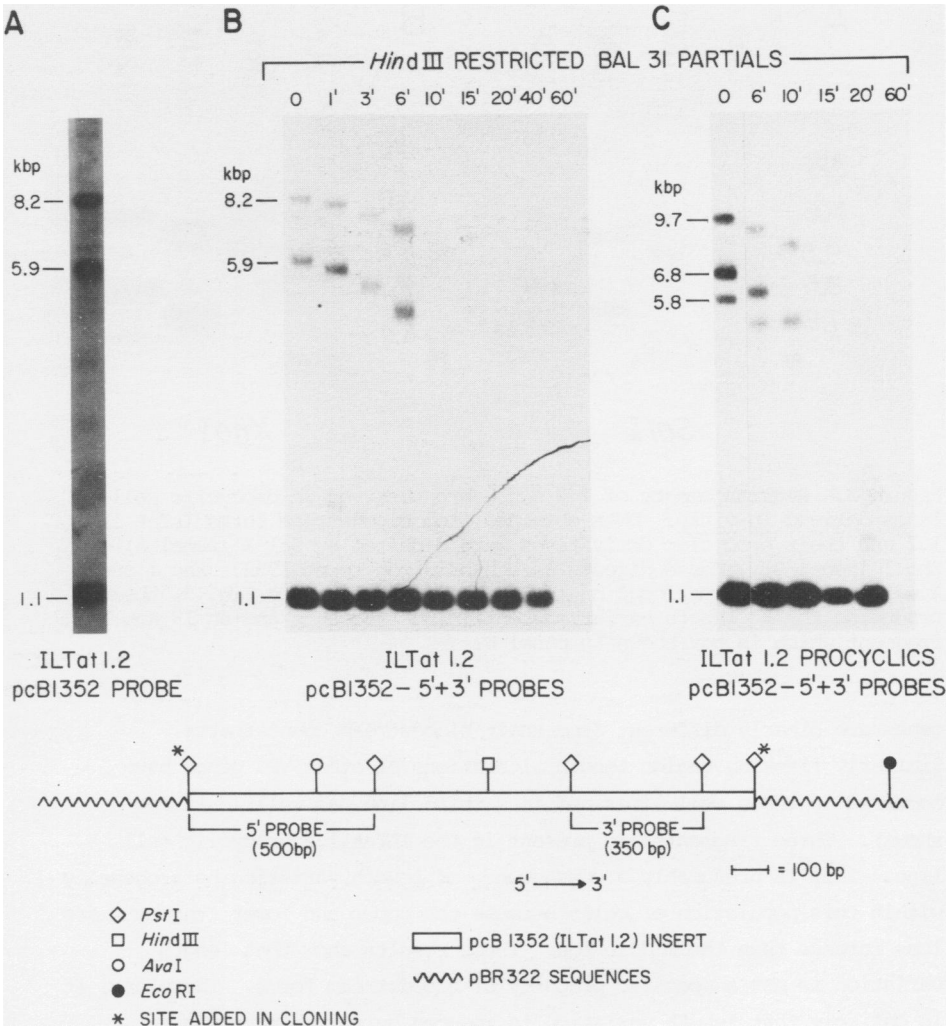

Figure 4. Evidence that VSG genes are located near the ends of DNA molecules. Panel A shows the ILTat 1.2 VSG genes as they occur in ILTat 1.2 bloodstream trypanosome DNA that was digested by Hind III alone. In panels B and C DNAs derived from bloodstream forms and their procyclic derivatives respectively were digested by Bal 31 for varying incubation periods. Reactions were terminated by extraction in phenol-chloroform. Samples were digested subsequently by Hind III.

in Figure 4 (B and C) 3' Hind III fragments are more sensitive than their 5' counterparts. These results are concordant with our observation that 13 restriction enzymes appear to cut in the same vicinity 3' of the 1.2

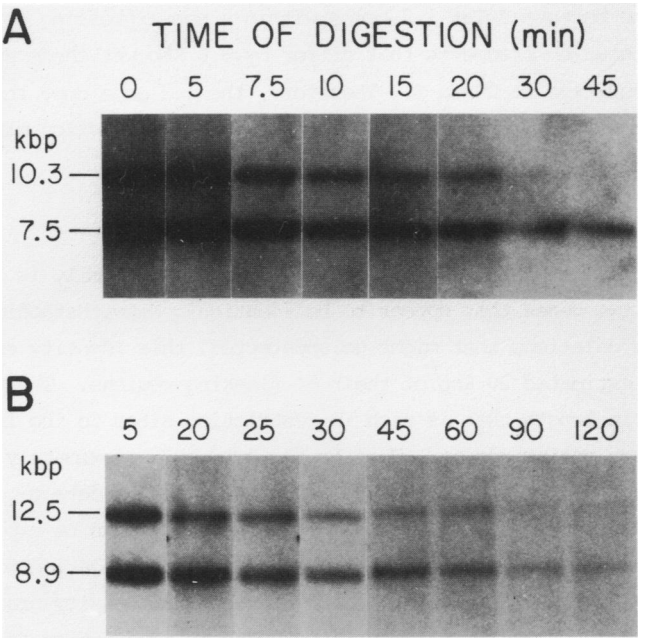

Figure 5. Identification of the transcriptionally active 1.2 gene. Nuclei derived from ILTat 1.2 (panel A) and their procyclic derivatives (panel B) were digested by DNase I as described in Materials and Methods and subsequently by Sal I. Digests were hybridized to the pcB1352 cDNA plasmid.

genes. It is likely that this region does not contain recognition sites of restriction endonucleases but is a natural break in the DNA molecule and therefore, possibly a chromosome end.

A single 1.2 gene is active in ILTat 1.2 bloodstream cells

Unlike certain other VSG genes that have been described (7-9,11, 12,30), the expression of an ILTat 1.2 gene is not associated with gene duplication which is an event that often makes it possible to distinguish the silent and transcriptionally active gene forms. As the trypanosome genome contains two indistinguishable copies of the ILTat 1.2 gene, we resorted to digestion by DNase I to identify the transcriptionally active homologue. Transcriptionally active chromatin is known to exhibit increased sensitivity to this enzyme (31-33). In Figure 5A there are two fragments within ILTat 1.2 bloodstream DNA that differ by 2.4 Kbp. Of these fragments it is the larger one that is more sensitive to digestion by DNase I. The greater sensitivity of this fragment cannot be due to

its size alone because ILTat 1.2 genes within a non-expressing cell line (Figure 5B) occur on fragments that differ by 3.6 Kbp yet these appear to be equally sensitive to DNase I. Therefore, the 1.2 gene copy that is presumably transcriptionally active corresponds to restriction map B in Figure 2.

DISCUSSION

Our restriction maps show that the ILTat 1.2 gene family is comprised of two genes that appear to be identical. Notwithstanding small insertions or deletions that might go undetected, this identity extends to within an estimated 20 Kbp of their 5' flanking regions. There is a region 1 Kbp in length that is rich in restriction sites to the immediate 5' side of each coding region. This is followed by approximately 20 Kbp of DNA that is nearly devoid of sites. Our inability to detect additional restriction sites in this very large region can be explained by the absence of recognition sequences or by base modification of these sequences. Recently it has been shown by equilibrium density gradient centrifugation that a telomere-containing VSG gene restriction fragment appears to be associated exclusively with DNA that contains a novel base modification (34).

The only features that distinguish these 1.2 genes are their 3' length variation regions and the greater sensitivity of one of these genes, copy B, to digestion by DNase I. Both 1.2 gene copies are presumably located at telomeres because a large set of enzymes appear to cut at the same location at the 3' ends of these length variation regions. This interpretation was substantiated by the ability of BAL 31 to degrade specifically the 3' ends (Fig. 4). In summary, these two ILTat 1.2 genes and their surrounding genomic environs appear to be conserved in expressor and nonexpressor bloodstream clones as well as in procyclic tissue culture forms.

Are the characteristics of the ILTat 1.2 genes explicable by current models of variant surface glycoprotein gene expression and evolution? The expression of certain VSG genes is controlled by duplicative transposition wherein a copy of a pre-existing gene and 1-2 Kbp of 5' flanking sequence is translocated to the end of a chromosome (8,11, 12,35,36). A promotor region is presumed to be located somewhere near the telomere because promotor-like sequences have not been detected in the transposed element (37). This transposition event usually alters the

3' end of the coding sequence (38-40). Regions that are nearly devoid of restriction sites occur to the 5' side of the point of insertion and to the 3' side of the coding sequence. The 1.2 genes, on the other hand, do not undergo duplicative transposition, yet they are similarly flanked by regions that have few restriction sites (Fig. 2). Recently, two identical VSG genes whose expression are not linked to duplication have been described (17). These genes are surrounded by restriction site-free regions as well. In this case, it is believed that one of these copies evolved from the other by duplicative transposition to a telomeric region where it has been maintained in the genomes of all bloodstream clones that comprise the ILTar 1 serodeme. In this event 1-2 Kbp of 5' flanking sequence was transposed.

The Iltat 1.2 genes described here might also have arisen by the duplication of an ILTat 1.2 basic copy gene based upon the fact that they appear to be identical, reside at telomeres and are present within many ILTar 1 bloodstream clones and procyclic cell lines. This event must have occurred recently as we are unable to detect any unique restriction sites on either side of each gene copy. This would be difficult to explain otherwise without invoking a mechanism that prevents these genes from diverging. The transposition units of those VSG genes whose expression is controlled by duplication include no more than 2 Kbp of 5' flanking sequence. If duplicative transposition can explain the origins of two ILTat 1.2 gene copies, then this is by far the longest unit of duplication characterized to date. Insertion into an identical 5' flanking region might have occurred but again, this would have required the conservation of approximately 20 Kbp of 5' flanking sequence. By these same arguments it is difficult to explain expression of the 1.2 copy B gene (which is not linked to duplication) merely by transposition.

We have considered alternative explanations to account for the presence of two copies of the same gene. Because each gene copy appears to reside in the vicinity of a telomere, they might be located at each terminus of the same chromosome or they may occur on separate chromosomes. Two chromosomes bearing identical genes might have their origins in an aberrant event during cell division in which sister chromatids failed to segregate into daughter cells. One of these daughter cells, therefore, might have received two chromosomes, each bearing a 1.2 gene. This event of non-disjunction would have had to occur in a progenitor cell of the ILTar 1 serodeme. We wish to stress

that this is speculative. Although it is not impossible to accommodate the evolution or expression of the 1.2 genes into presently available models of VSG gene evolution, we feel that other possibilities ought to be considered because the dynamic properties of these genes remain obscure.

By comparing multiple bloodstream clones, Williams et al. were the first to observe that regions flanking certain VSG genes to their 3' side can vary in length (13). These length variation regions were later shown to be associated specifically with those VSG genes that are located at telomeres (14-17). Because unique length variants of a particular VSG gene were found among bloodstream clones expressing different VSGs, it was concluded that length variation could not be correlated with the expression of individual VSG genes. However, the possibility remained that the mechanism(s) governing 3' length variation might be a developmental-specific signal that permits any VSG gene to be expressed in bloodstream and metacyclic cells. Metacyclic cells represent the last developmental stage within the vector Glossina spp. and they are the first to express variant surface glycoprotein genes (3,27). We approached the question of the significance of 3' length variation by comparing the genomic contexts of the ILTat 1.2 genes in different bloodstream clones and in their procyclic derivatives. Our results show that 3' length variation does occur in procyclic cells. We have also observed analogous length variations of certain members of the ILTat 1.3 and ILTat 1.4 gene families (16) in the cell lines described here and in a third procyclic cell line derived from the ILTat 1.51 bloodstream clone (unpublished data). Thus, 3' length variation is not a developmentally specific phenomenon of VSG genes.

Several laboratories have observed that among multiple bloodstream clones that produce the same variant surface glycoprotein the length variation region of the expressed gene is consistently longer than in clonal variants that express different VSG genes (11,17). Accordingly, it has been suggested that insertions or telomere exchange might regulate expression (17). Yet, as depicted in Figure 3, the expressed 1.2 gene, copy B, in the ILTat 1.2 bloodstream clone occurs on a fragment that is shorter than one of two fragments in the ILTat 1.1 bloodstream clone or procyclic cell line. Furthermore, a longer fragment can be found in procyclic cells that were derived from the ILTat 1.2 bloodstream clone. It is unlikely, therefore, that 3' length variation alone, controls the

expression of the ILTat 1.2 genes.

Bernards et al., have shown very recently that 3' flanking region length variations of bloodstream form trypanosome telomeres occurs by a process that is most likely associated with DNA replication (41). Our results support their data.. In bloodstream clones and in their procyclic derivatives, for the most part, the same number of hybridizable fragments are observed. This is expected if identical gene rearrangements occurred in all cells during their morphological transformation or growth in vitro. Yet, it is difficult to explain by an insertion-deletion or telomere exchange model (12,17,40) how fragments of identical size can be generated by the majority of cells in an unselected population without invoking a mechanism that places constraints on the lengths of the segments involved in these events. At this time we cannot exclude the possibility that these cells were cloned inadvertently during their transformation or propagation in vitro. Again, three different bloodstream clones were transformed individually by introducing approximately 10^6 trypanosomes into 1 ml of medium. Their transformation efficiencies were not determined. Thus, if each cell line had been derived from a single cell, then this would have had to occur on three separate occasions. On the other hand, if each of these cell lines represent multiple, independent developmental transformations of individual bloodstream cells, then we can suggest that the limited number of length variants observed in each cell line might be due to the elongation or contraction of 3' ends in a manner that proceeds at a constant rate among the majority of cells.

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