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A Strand Bias Occurs in Point Mutations Associated with Variant Surface Glycoprotein Gene Conversion in *Trypanosoma rhodesiense*

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We previously described a bloodstream *Trypansoma rhodesiense* clone, MVAT5-Rx2, whose isolation was based on its cross-reactivity with a monoclonal antibody (MAb) directed against a metacyclic variant surface glycoprotein (VSG). When the duplicated, expressed VSG gene in MVAT5-Rx2 was compared with its donor (basic copy) gene, 11 nucleotide differences were found in the respective 1.5-kb coding regions (Y. Lu, T. Hall, L. S. Gay, and J. E. Donelson, Cell 72:397–406, 1993). Here we describe a characterization of two additional bloodstream trypanosome clones, MVAT5-Rx1 and MVAT5-Rx3, whose VSGs are expressed from duplicated copies of the same donor VSG gene. The three trypanosome clones each react with the MVAT5-specific MAb, but they have different cross-reactivities with a panel of other MAbs, suggesting that their surface epitopes are similar but nonidentical. Each of the three gene duplication events occurs at a different 5′ crossover site within a 76-bp repeat and is associated with a different set of point mutations. The 35, 11, and 28 point mutations in the duplicated VSG coding regions of Rx1, Rx2, and Rx3, respectively, exhibit a strand bias. In the sense strand, of the 74 total mutations generated in the three duplications, 54% are A-to-G or G-to-A (A:G) transitions and 7% are C:T transitions, while 26% are C:A transversions and 13% are C:G transversions. No T:G or T:A transversions occurred. Possible models for the generation of these point mutations are discussed.

African trypanosomes are protozoan parasites that spend part of their life cycle in a mammalian host and the remainder in a tsetse fly. In the mammalian bloodstream the parasite's antigenic determinants are located in the surface coat comprising about 10^7 copies of the variant surface glycoprotein (VSG) (36). Although each bloodstream organism has a single VSG species on its surface, the trypanosome genome has a repertoire of several hundred different VSG genes, many of which display little sequence similarity except in the C-terminal coding regions (10, 38). By sequentially expressing individual VSG genes, the parasites periodically change their surface determinants and evade the host immune response.

The molecular mechanisms underlying the sequential expression of VSG genes are only partially understood. The transcribed VSG gene is invariably located near a chromosomal telomere, while the silent, basic copy (BC) VSG genes can be either near telomeres or at chromosome-internal sites (6, 10, 37). There are several ways in which a VSG gene can be activated. In the best-understood examples, a BC gene is duplicatively transposed to become an expression-linked copy (ELC), an event similar to gene conversions studied in other organisms (17, 25). Evidence that an exchange of two telomeres along with their associated VSG genes can result in the activation of an already telomere-linked BC also exists (23). In other cases, telomere-linked BCs appear to be activated without detectable genomic DNA rearrangement. Virtually all of these studies have focused on the sequential activation of BC genes in the repertoire, i.e., the sequential expression of epitopes preencoded in the genome.

Measurements of the VSG switch rate suggest that it can be as high as 1 in 100 trypanosomes per generation (34). Since the trypanosome doubling time is 5 to 10 h, a repertoire of several hundred VSG genes would be exhausted within a couple of months, yet bloodstream trypanosome infections can last for many months (2, 27). Thus, late in infection trypanosomes expressing new epitopes not previously seen by the immune system have a distinct advantage. One way in which new VSG epitopes can be created is by the formation of ELCs that are mosaics of two or more closely related BC genes (2, 15, 24, 28, 33). New VSG epitopes are presumably generated either at the junctions of different BC segments or by potential reading frame switches or via alterations in the three-dimensional VSG structure imposed by the mosaic sequences.

Recently we identified another mechanism by which new epitopes can be generated (21). We examined a bloodstream clone of *Trypanosoma rhodesiense*, called MVAT5-Rx2, that cross-reacts with a monoclonal antibody (MAb) directed against the VSG expressed in metacyclic variant antigen type 5 (MVAT5), one of about a dozen MVATs expressed by this serodeme during the metacyclic stage in insects (9, 12, 19, 35). The VSG of MVAT5-Rx2 is expressed from an ELC of a single, telomere-linked BC gene in the genome. However, the duplicated ELC segment is not a faithful copy of the BC. A total of 12 point changes occur within the duplicated DNA segment, which includes upstream, VSG coding, and downstream regions of 0.9, 1.5, and at least 0.8 kb, respectively. Of the 12 differences, 1 is in the upstream region and the other 11 are in the coding region. Southern blots probed with oligonucleotides containing sequences unique to the ELC suggested that the point changes are not donated from short, similar

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sequences located elsewhere in the genome. Thus, the occurrence of these point mutations implies that the trypanosome can generate new VSG epitopes that are not preencoded in the genome.

In an attempt to study the molecular mechanism responsible for the generation of these point mutations, we examined two additional, independent *T. rhodesiense* clones, MVAT5-Rx1 and -Rx3, that were isolated by the same process as that used to obtain MVAT5-Rx2. The VSGs expressed in these two clones were found to be derived from ELCs of the same BC gene as the Rx2 VSG, but their ELCs had point mutations different from those in the Rx2 ELC. Comparison of these three sets of mutations revealed the pattern of strand bias reported here.

MATERIALS AND METHODS

Trypanosomes. The WRAT at serodeme of *T. brucei rhodesiense* is derived from stock/75/-K/18 (8). The identification and isolation of bloodstream trypanosomes, MVAT5-Rx1, -Rx2, and -Rx3, each expressing a VSG recognized by the MVAT5-specific MAb 50.5H8.3, were conducted as described previously (21). Briefly, a bloodstream trypanosome clone, WRATat1.1, was inoculated into several immunocompetent mice. After 3 to 4 months of passage through multiple animals, a small percentage (<1%) of a trypanosome population was found to react with MAb 50.5H8.3, which is directed against the MVAT5 VSG. The percentage of the MVAT5 reexpressors in this population was expanded by consecutive rounds of selective neutralization with antibodies against all expressed VSGs in the population except for MVAT5 (19). After three such rounds, the reexpressor trypanosomes were enriched to about 50%. The enriched population was then diluted, and microdrops containing a single parasite were injected into irradiated mice. Most of the mice failed to develop parasitemia, and those that did usually expressed a VSG that did not react with MAb 50.5H8.3. However, after screening of more than 100 such mice, three cloned trypanosome populations reexpressing of the MVAT5 phenotype were found and are designated MVAT5-Rx1, -Rx2, and -Rx3 (or Rx1, Rx2, and Rx3, respectively).

MAb generation and immunofluorescent assays (IFAs). Cell fusions to produce MAbs directed against each of the three bloodstream MVAT5-reexpressing trypanosome clones were conducted. The generation of MAbs against MVAT5-Rx1 is described as an example. BALB/c mice were immunized by intraperitoneal inoculation of 10^6 irradiated (60,000 rads) MVAT5-Rx1 trypanosomes. The mice were boosted 1 and 2 months later with the same number of trypanosomes. Three days following the second boost, the spleens were removed aseptically, minced to make a single cell suspension, and fused with the X63-Ag8.653 nonsecreting myeloma cell line by using polyethylene glycol to generate hybridomas (16, 18). The supernatants from the hybridoma cultures were screened by IFA as follows. Smears of mouse blood containing the MVAT5-Rx1 trypanosomes were made on microscope slides which had been divided into 30 sections with liquid embroidery paint and air dried. Different hybridoma culture supernatants were incubated on different sections of the slides for 30 min at room temperature. The slides were washed three times with phosphate-buffered saline (PBS; 130 mM NaCl, 7 mM sodium phosphate, pH 7.0) and flooded with fluorescein-labeled goat anti-mouse immunoglobulin. After another 30 min of incubation, the slides again were washed three times with PBS and air dried. They were examined with a fluorescence microscope to determine which hybridoma culture supernatants contained antibody specific for the MVAT5-Rx1 trypanosomes. Hybridoma cells from the antibody-positive wells were cloned by limiting dilution in 96-well microtiter plates at least twice. The final double-cloned hybridomas were expanded, and aliquots were stored in liquid nitrogen.

PCR using total RNA as a template. First-strand cDNA was synthesized by incubating 10 μg of total RNA from MVAT5-Rx1 with 0.5 μg of oligo(dT), deoxyribonucleotide triphosphates, and reverse transcriptase under standard reverse transcription conditions for 1 h at 42°C (1). This reaction mixture was diluted to 250 μl with water, and 5 μl was used in a 50-μl PCR mixture. The MVAT5-Rx1 VSG cDNA sequence was amplified with the following two primers: a 5′ primer containing the first 23 nucleotides of the spliced leader sequence, 5′-TATTATTAGAACAGTTTCTGTAC-3′ (11), and a 3′ primer complementary to the 21 nucleotides that are conserved in the 3′ untranslated region of most VSG genes, 5′-GGTGTTAAAATATATCAGAAG-3′ (see Fig. 1). PCR was conducted according to the standard protocol suggested by the supplier of *Taq* polymerase (Perkin-Elmer Corp.).

Mapping upstream (5′) boundaries of ELCs. To map the upstream ELC boundaries, PCRs were conducted using genomic DNAs (100 ng) of the three MVAT5 reexpressors as templates in 50-μl volumes. The 5′ primer in all three cases was an 18-mer (5′-CGCTCTAGAGTGTTGTGA-3′) containing 12 nucleotides found in most 70- to 76-bp repeats (32) preceded by 6 nucleotides to create an *Xba*I cloning site (primer 76 in Fig. 5). The 3′ primer used on the MVAT5-Rx1 and -Rx2 genomes was an 18-mer complementary to the sequence 839 bp upstream of the MVAT5 VSG coding region (5′-TACCTATTTATTGAATCC-3′). The 3′ primer used on the MVAT5-Rx3 genome was a 20-mer complementary to the sequence including and immediately downstream of the ATG start codon (5′-ATAAAGGCTTTTCCTATCAT-3′).

Genomic Southern blots using oligonucleotides as probes. To probe genomic Southern blots with specific VSG coding sequences (see Fig. 6), pairs of 26-mer oligonucleotides whose sequences are shown within regions I and II in Fig. 1 were synthesized. For region I, the Rx2-1 oligonucleotide corresponds to the sense strand of the MVAT5-Rx2 ELC (5′-AACCCCGGCAACAACAAGATAG CAGG-3′) and the Rx3-1 oligonucleotide corresponds to the antisense strand of the MVAT5-Rx3 ELC (5′-CCTGCTATCTTGC CGTCTTCGGGGTT-3′). For region II, the Rx2-2 oligonucleotide corresponds to the sense strand of both the MVAT5-Rx2 BC and ELC (5′-CGTAGCCGACCTTTCCGACGAGATAG-3′) and the Rx3-2 oligonucleotide corresponds to the antisense strand of the MVAT5-Rx3 ELC (5′-CTATTTCGCCGGAAAGGTCGTCT ACG-3′). The oligonucleotides were 5′ end labeled with ^{32}P by using T4 polynucleotide kinase (New England Biolabs) and used for Southern blot hybridizations as described elsewhere (30).

Other recombinant DNA techniques. Genomic DNA and RNA isolations from trypanosomes were conducted as described previously (19). The cDNA libraries of RNA from each of the three MVAT5 reexpressors were constructed with a lambda ZAPII kit (Stratagene). Southern blots, cDNA library screenings, and in situ hybridizations of bacterial colonies were conducted as described elsewhere (30). The cDNA probes used for hybridization experiments were ^{32}P labeled by using a random-priming kit (BMB). DNA sequencing was performed by the dideoxy chain termination method (14) with modified T7 DNA polymerase (Sequenase from U.S. Biochemical Corp.).

Nucleotide sequence accession number. The GenBank accession number for the sequence of the MVAT5 VSG BC gene shown in Fig. 1 is L06030.

TABLE 1. Results of IFAs of the MVAT5 reexpressor clones

Group	MAb(s)	Reactivity[a] with:		
		Rx1	Rx2	Rx3
I	114.1G3, 114.3H4, 114.6A6, 114.11B1	+	−	−
II	115.2A11, 115.3G10, 115.11H3	−	+	−
III	128.2A1, 128.2D10, 128.4B10, 128.4E2	−	−	+
IV	114.3F9	+	+	−
V	128.2B10, 129.9E9, 129.7C10, 129.9A6	−	+	+
VI	114.1E5, 114.8E2, 115.3G7, 129.2G9	+	+	+

[a] +, reactive; −, not reactive.

RESULTS

Independent bloodstream trypanosome clones reexpressing the MVAT5 VSG have different cross-reactivities with a panel of MAbs. During their metacyclic stage in the insect vector, trypanosomes express a restricted subset of 10 to 15 VSGs (9, 12, 19, 35), in contrast to the hundreds of VSGs that can be expressed during the bloodstream stage in the mammalian host. Our original objective was to examine bloodstream trypanosomes that reexpress one of the metacyclic VSGs, the MVAT5 VSG. Three such bloodstream reexpressors, designated MVAT5-Rx1, -Rx2, and -Rx3, were identified via their reactivity with a MAb specific for the MVAT5 VSG on metacyclic organisms (see Materials and Methods).

To analyze further the epitopes present on each reexpressor clone, surface-reactive MAbs were generated against each of the three MVAT5 reexpressors. These MAbs were tested against the other reexpressors by IFAs, and the properties of representative MAbs are summarized in Table 1. As expected, anti-Rx1 MAbs (numbers that begin with 114) react with Rx1 parasites, anti-Rx2 MAbs (the 115 numbers) react with Rx2 parasites, and anti-Rx3 MAbs (the 128 and 129 numbers) react with Rx3 parasites. In addition, the antibodies could be divided into six different groups based on their cross-reactivities. MAbs in groups I to III react only with the reexpressor against which they were raised. For example, MAb 114.1G3 reacts with only Rx1 parasites and not with Rx2 and Rx3 organisms. MAbs in groups IV and V react with two of the reexpressors but not the third. MAbs in group VI react with all three reexpressors, as does MAb 50.5H8.3 (not indicated), which is directed against the MVAT5 VSG on metacyclic parasites and was used to identify the reexpressors. Thus, some epitopes appear to be unique to each reexpressor, others are shared by two of the three reexpressors, and still others occur in all three.

The different antigenic cross-reactivities among the three reexpressors are associated with nucleotide differences in the VSG mRNAs. One explanation for the different MAb cross-reactivities summarized in Table 1 is that the VSGs on the individual reexpressors have similar, but nonidentical, amino acid sequences. To test this possibility, cDNA libraries of RNA from Rx1 and Rx3 bloodstream organisms were constructed and screened with a 1.1-kb *Hin*dIII-*Pst*I fragment from the previously cloned BC gene for the MVAT5 VSG (21) (see Fig. 4). Six positive cDNA clones in the Rx3 cDNA library were identified and recovered in pBluescript from their respective lambda ZAPII recombinant phage. Preliminary sequencing across the ends of these cDNAs revealed that three were independent, full-length cDNAs containing the 5′ spliced leader and a 3′ poly(A) tail. The complete sequences of each of these three full-length cDNAs were determined and found to be identical except for the lengths of the 3′ poly(A) tails and the extent of the spliced leader at the 5′ ends. In comparison with the MVAT5 BC gene, this Rx3 VSG cDNA sequence was found to have 28 point changes within the 1.5-kb coding region (Fig. 1). The 5′ and 3′ untranslated regions were identical to those of the BC gene.

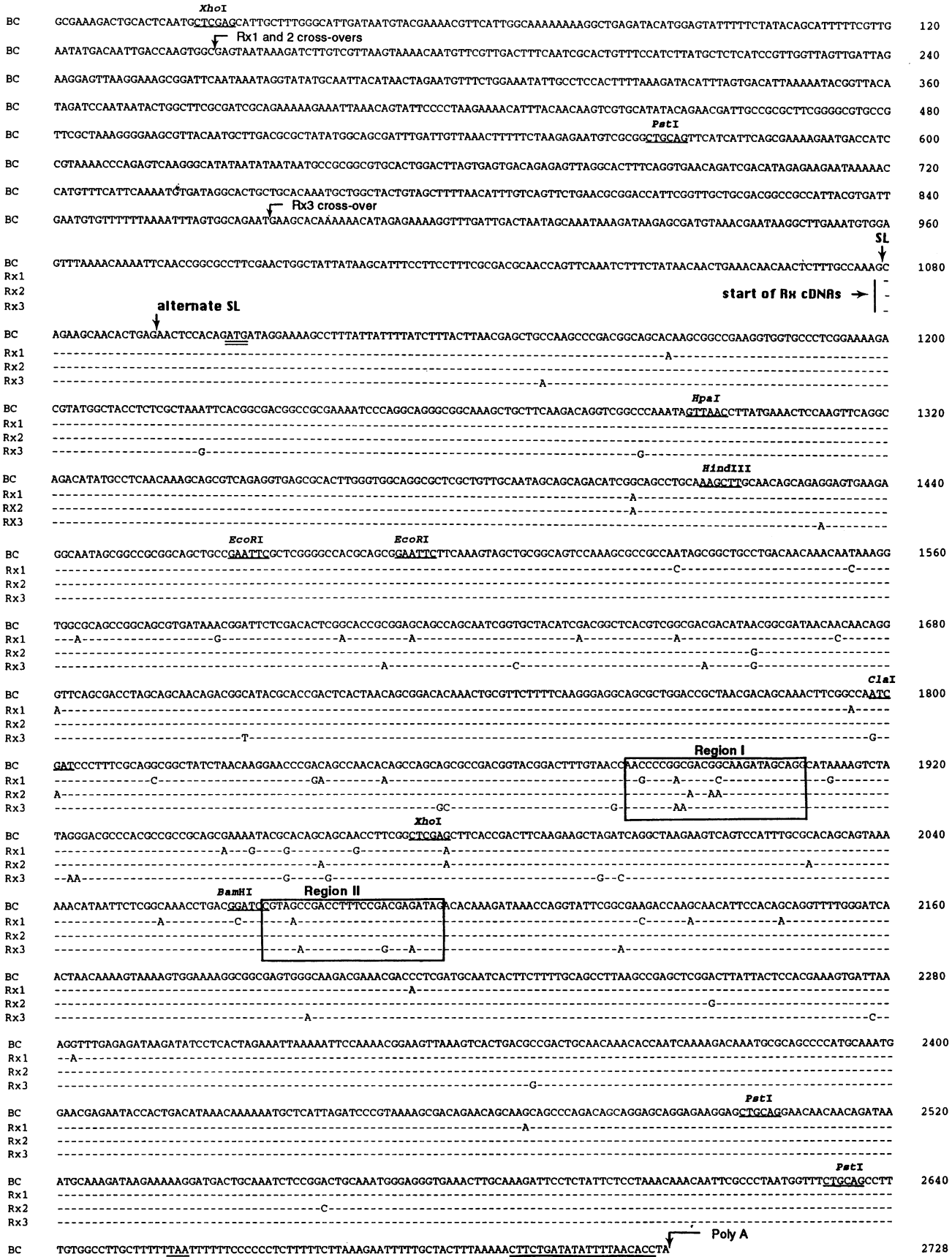
When the same 1.1-kb *Hin*dIII-*Pst*I fragment was used to screen the Rx1 cDNA library, several partial-length cDNA inserts were identified. Sequence determinations of these partial-length cDNAs revealed that they—like the Rx3 VSG cDNA—contain occasional point changes from the MVAT5 BC gene, but their composite sequences did not completely overlap to provide a full-length Rx1 VSG cDNA sequence. To obtain the complete sequence, total RNA from MVAT5-Rx1 was used as a template for first-strand cDNA synthesis by reverse transcription followed by PCR amplification. The 3′ primer for reverse transcription and PCR amplification was complementary to a sequence conserved in the 3′ untranslated region of most VSG genes (Fig. 1), and the 5′ primer in the PCR amplification contained the first 23 nucleotides of the spliced leader (11). A single PCR product of 1.6 kb was obtained and ligated into a plasmid. Bacterial transformants were screened with the 1.1-kb *Hin*dIII-*Pst*I fragment for those containing the desired insert. The PCR products in three independent colonies were completely sequenced and found to have identical sequences. This PCR sequence was identical to that of the partial-length Rx1 cDNAs in the segments where they could be compared, including the locations where the cDNAs differed from the BC gene. As shown in Fig. 1, this full-length Rx1 VSG cDNA sequence differs from the MVAT5 BC gene at 35 positions in the coding region and is identical in the 5′ and 3′ untranslated regions.

Most of the 35, 11, and 28 point mutations in the Rx1, Rx2, and Rx3 VSG cDNAs, respectively, occur in the middle one-third of the coding region (Fig. 1). These mutations result in 25, 9, and 13 amino acid replacements in the corresponding nascent VSGs (518 amino acids total) of Rx1, Rx2, and Rx3 (Fig. 2). The remaining mutations are silent and do not cause amino acid changes. These amino acid replacements are likely responsible for the differences in the MAb specificities summarized in Table 1.

Each of the three different sets of point mutations is associated with an independent duplicative transposition of the same BC gene. As described previously (21), the Rx2 VSG is expressed from a mutated ELC of the MVAT5 BC gene. To determine if the VSGs of Rx1 and Rx3 are also expressed from ELCs of the same BC gene, Southern blotting was conducted using restriction enzymes expected to detect polymorphisms caused by the point mutations. For example, the MVAT5 BC gene contains cleavage sites for *Cla*I, *Xho*I, and *Bam*HI that are affected by the different point mutations (Fig. 1). These point changes eliminate the *Cla*I site in the Rx2 and Rx3 VSG cDNAs, the *Xho*I site in the Rx1 and Rx2 cDNAs, and the *Bam*HI site in the Rx1 cDNA.

Genomic DNAs from the three MVAT5 Rx clones and from clones WRATat1.1 and MVAT4 were digested with various restriction enzyme combinations and probed in Southern blots with the 1.1-kb *Hin*dIII-*Pst*I fragment encompassing most of the MVAT5 VSG coding region (Fig. 1). WRATat1.1 is the bloodstream clone from which the MVAT5 Rx clones and the MVAT4 clone were derived (12, 19). WRATat1.1 and MVAT4 express VSGs that are not related immunologically to each other or to the MVAT5 Rx clones.

Representative examples of the blots in which the genomic DNAs are digested with these enzymes that cleave within the BC gene but do not cut within at least one of the three ELC genes are shown in Fig. 3. The Rx2 ELC, for example, can be


XhoI
Rx1 and 2 cross-overs
PstI
Rx3 cross-over
SL
start of Rx cDNAs
alternate SL
HpaI
HindIII
EcoRI
EcoRI
ClaI
Region I
XhoI
BamHI
Region II
PstI
PstI
Poly A

detected by the presence of a 1.0-kb *Pst*I-*Eco*RI fragment when the genomic DNA is triple digested with *Xho*I-*Pst*I-*Eco*RI (panel X/P/E) because the internal *Xho*I site of the BC gene is mutated in the ELC gene (lane Rx2). The corresponding segment of the BC gene is split into two smaller fragments. Likewise, in the same *Xho*I-*Pst*I-*Eco*RI digest the Rx1 ELC is apparent because it also lacks the internal *Xho*I site of the BC gene (lane Rx1). However, an Rx3 ELC cannot be detected in this digest because it retains the internal *Xho*I site and is cleaved into the same two fragments as the BC gene (lane Rx3). In the *Bam*HI-*Pst*I-*Eco*RI triple digest (panel B/P/E), only Rx1 ELC can be detected because its internal *Bam*HI site has been mutated while the ELCs of both Rx2 and Rx3 retain the internal *Bam*HI site of the BC gene. Finally, in a triple digest with *Hpa*I-*Pst*I-*Cla*I (panel Hp/P/C) the ELCs of Rx2 and Rx3 can be detected on a 1.2-kb fragment because they each lack the internal *Cla*I site of the BC gene, but the Rx1 ELC is cleaved into the same two fragments as the BC gene because it retains this *Cla*I site. Thus, all three reexpressors of the MVAT5 VSG possess an ELC of the single BC gene for this VSG. No ELC of the MVAT5 VSG BC gene was detected in trypanosome clones WRATat1.1 and MVAT4 (lanes 1.1 and 4, respectively), which express unrelated VSGs.

The results of Southern blots, cDNA and genomic DNA sequencing, and PCR amplifications (see below) are summarized in the restriction maps shown in Fig. 4. The telomere-linked BC gene is preceded by an expression site-associated gene (ESAGI) as determined previously. The VSG in each of the three MVAT5 reexpressors is expressed by a duplicative transposition of the single BC gene. The three ELCs are preceded by regions of at least 20 kb that at least in part are made up of 70- to 76-bp repeats found in front of many telomere-linked VSG genes (10). A different set of point mutations is associated with the coding region of each ELC, and the VSG cDNA sequences are identical to that of their respective ELCs.

The three independent VSG gene duplication events are characterized by different upstream crossover points. To map precisely the upstream boundaries of the three ELCs, PCR amplifications were conducted. The 5′ primer for the amplifications was a consensus sequence found within most 70- to 76-bp repeats (32), and the 3′ primers contained sequences complementary to segments either upstream of (Rx1 and 2) or within (Rx3) the VSG coding region. When genomic DNAs from Rx1, Rx2, and Rx3 were used as templates, multiple amplification products were detected, many of which are likely due to binding of the 76-bp-repeat-specific primer to interspersed 76-bp repeats throughout the genome. The desired PCR products within these amplification mixtures were identified by probing a Southern blot of the products with a 1.4-kb *Xho*I-*Hin*dIII fragment containing the first 300 bp of the BC and 1,100 bp immediately upstream. Fragments that hybridized were eluted from a gel and cloned, and their sequences were determined. These sequences established the crossover points within both the region upstream of the donor BC gene (Fig. 1) and the 76-bp repeats (Fig. 5).

The crossover points for ELC formations in both Rx1 and Rx2 occurred at the same position located 962 bp upstream of the VSG start codon (Fig. 1). However, within the 76-bp repeats these two crossovers took place at different locations (Fig. 5). In contrast, the crossover points for the Rx3 ELC were 234 bp in front of the start codon (Fig. 1) and at a third location within the 76-bp repeats (Fig. 5). Furthermore, the two endpoints at this 5′ crossover site in Rx3 are not directly linked at their boundary but are separated by 33 bp that are AT rich (76%) and of unknown origin. The most likely explanation for the presence of these 33 bp is that they are derived from (i) an AT-rich segment within the 76-bp repeats or (ii) the sequence in this expression site prior to arrival of the Rx3 ELC. Since AT-rich regions are known to be interspersed among the 76-bp repeats (7), we suspect that the first alternative is the more likely explanation and further hybridizations to distinguish between these two possibilities were not conducted.

Comparison of the two different crossover sites preceding the donor BC gene and the three unique crossover sites within or downstream of the 76-bp repeats revealed some modest clues about sequence motifs that might participate in the gene conversion events. Upstream of the donor BC gene, the crossovers occurred at a GAG or GAAG followed immediately by about 10 nucleotides that are AT rich. Within or downstream of the 76-bp repeats, the crossovers occurred at a mirror image motif of an AT-rich segment followed by the GAG trinucleotide. Thus, a GA(A)G sequence surrounded by AT-rich sequences may be an effective mediator of VSG gene duplications into telomere-linked sites for those donor BC genes that are not preceded by one or more copies of the 76-bp repeat.

Similar PCR amplifications were attempted to identify putative 3′ crossover points downstream of the duplicated VSG gene. These amplifications utilized 5′ primers containing sequences near the end of the VSG coding region and a 3′ primer made up of three repeats of the hexameric telomere sequence, AAATCC (4). These amplifications did not yield PCR products that hybridized to the 3′ untranslated region (not shown). The probable cause of these failures is that the hexameric telomere repeats occur too far downstream from the VSG coding region for these PCR amplifications to be effective. In the case of the Rx2 ELC, Southern blots and genomic DNA clones show that the duplication extends at least 800 bp beyond the termination codon and may go all the way to the telomere itself (21). In the Rx1 and Rx3 cases, Southern blots demonstrate that a *Hin*dIII site located 800 bp downstream of the BC gene is not present at a similar location downstream of the corresponding ELCs, suggesting that the 3′ crossover points occur before this point or that the *Hin*dIII site in the ELC is mutated.

The point mutations are probably not templated. Southern blots probed with a 1.1-kb segment of the VSG coding region (Fig. 3) indicate that a single copy of the BC gene occurs in the genome and that it serves as the donor for the different ELCs. To test whether the point differences between the BC gene and the ELCs are derived from short stretches of sequence located

FIG. 1. Sequence comparison of the BC gene for the MVAT5 VSG and cDNAs for the VSGs expressed in bloodstream trypanosome clones MVAT5-Rx1, -Rx2, and -Rx3. The complete sequence of the BC gene and upstream flanking region is shown; only point changes are shown for the three cDNAs. The upstream (5′) crossover points for the ELCs of Rx1, Rx2 and Rx3 are indicated. The sites of spliced leader addition to generate the VSG mRNAs, as detected in the cDNAs, are marked (SL). The cDNAs end with a 3′ poly(A) tail at the indicated site. Start and stop codons are indicated by double underlines. Indicated restriction sites correspond to those shown in Fig. 4. Regions I and II show the locations of the 26-nucleotide sequences used as probes in the Southern blots shown in Fig. 6. The complement of the underlined sequence at the 3′ end was used as a primer in a reverse transcriptase PCR amplification.

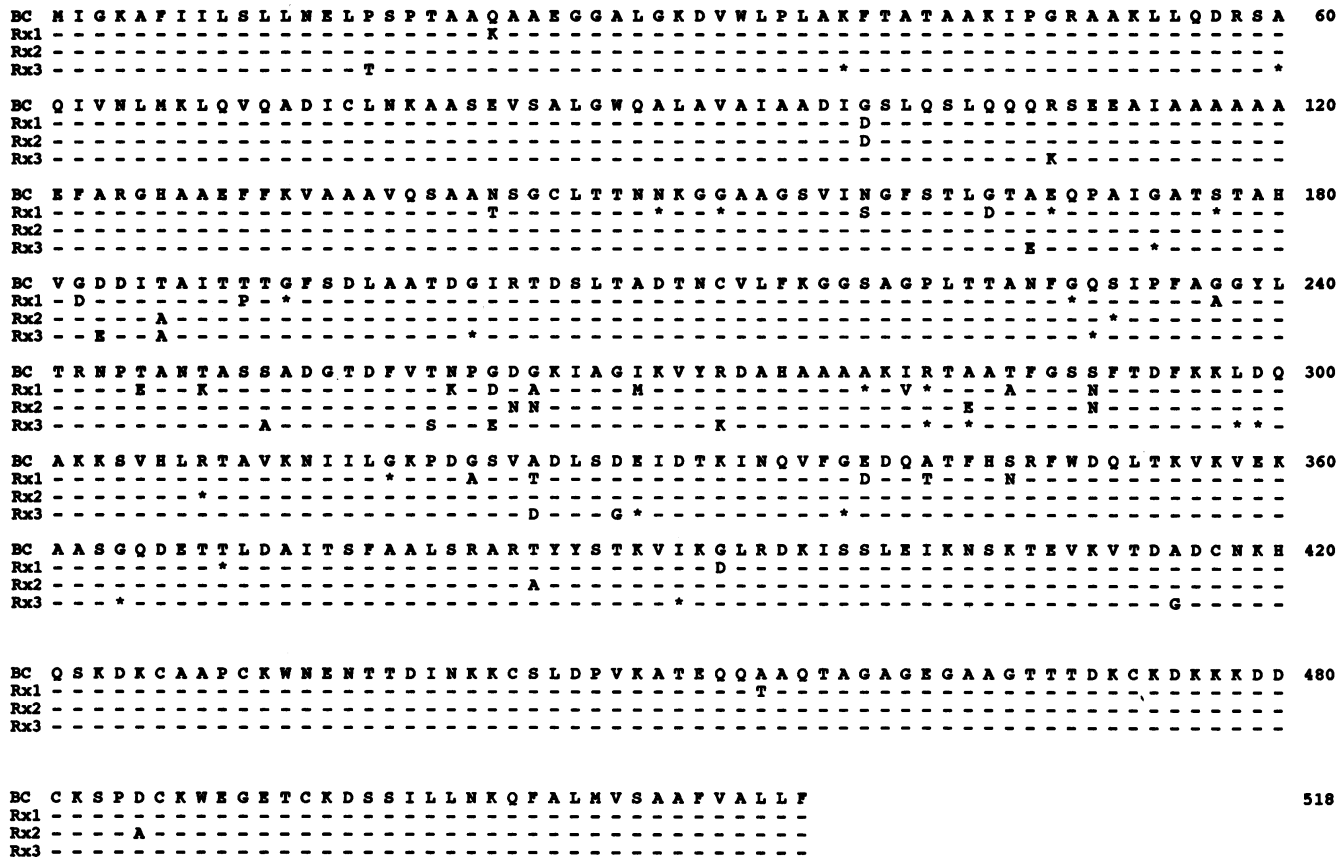


FIG. 2. Deduced amino acid sequences encoded by the MVAT5 BC gene and the cDNAs derived from MVAT5-Rx1, -Rx2, and -Rx3. Dashes indicate positions at which the sequences encoded by the Rx cDNAs are identical to that encoded by the BC. Asterisks show positions where the nucleotide changes do not cause amino acid changes.

elsewhere in the genome, Southern blots also were probed with oligonucleotides containing sequences found in the ELC but not the BC. These sequences are shown in Fig. 1 as regions I and II. Two 26-mer oligonucleotides (one specific for the Rx2 ELC and the other specific for the Rx3 ELC) were synthesized for each region and used as probes (Fig. 6).

When the Rx3-1 oligonucleotide (specific for the Rx3 ELC in region I) was used to probe restricted genomic DNAs of WRATat1.1, Rx2, and Rx3, it hybridized to only ELC fragments in the Rx3 DNA (Fig. 6, lanes 3). No hybridization to the WRATat1.1 or Rx2 DNAs was detected. Since the Rx3 ELC lacks the internal *Cla*I site because of mutation and has the internal *Xho*I site, the probe detects a 1,010-bp ELC fragment in the Rx3 digest containing *Cla*I and a 480-bp ELC fragment in the one with *Xho*I. When the same digests were probed with the Rx3-2 oligonucleotide (specific for the Rx3 ELC in region II), a similar hybridization pattern occurred. Only fragments containing the ELC in the Rx3 genome were detected, although in the *Xho*I-containing digest of Rx3 DNA, the fragment size was 530 bp, instead of 480 bp, because region II follows the *Xho*I site instead of precedes it.

Likewise, oligonucleotide Rx2-1 (specific for the Rx2 ELC in region I) hybridized only to a 1,010-bp ELC fragment in the Rx2 genome (Fig. 6, lanes 2) and did not detect fragments in the other genomes. The other Rx2 oligonucleotide (Rx2-2) hybridized to both the Rx2 ELC and the BC since the two genes are identical in region II (Fig. 1). Thus, in the *Pst*I-*Eco*RI-*Cla*I triple digest, oligonucleotide Rx2-2 detected the 1,010-bp ELC fragment in the Rx2 genome (lane 2) and the 710-bp BC fragment in all three genomes (lanes 1.1, 3, and 2). This result demonstrates that this oligonucleotide can in fact hybridize to non-ELC sequences if they are present in the genome. Similar hybridizations with oligonucleotides specific for the ELC of Rx1 were not conducted since it was discovered by IFAs that the Rx1 trypanosome population from which genomic DNA was extracted for this experiment was only 20% pure. Thus, the corresponding hybridization signal to the ELC would be only one-fifth as intense. Although surprising at the time it was discovered, this lack of stability of the Rx1 population is consistent with our previous observation that the MVAT5 clones have a high switch rate to expression of other VSGs for reasons unknown (21). All experiments using this Rx1 genomic DNA took into account the 20% purity of this Rx1 population.

DISCUSSION

The results described here demonstrate that different point mutations and 5′ crossover points are associated with each of three independent duplications of the same BC VSG gene. The generation of the mutations in the duplicated gene copy potentially provides a way for the trypanosomes to create new VSG epitopes that are not preencoded in the genome. Consistent with this possibility, 64% of the nucleotide changes found in the three duplicated coding regions cause amino acid replacements (Fig. 2). Since the three MVAT5 reexpressor

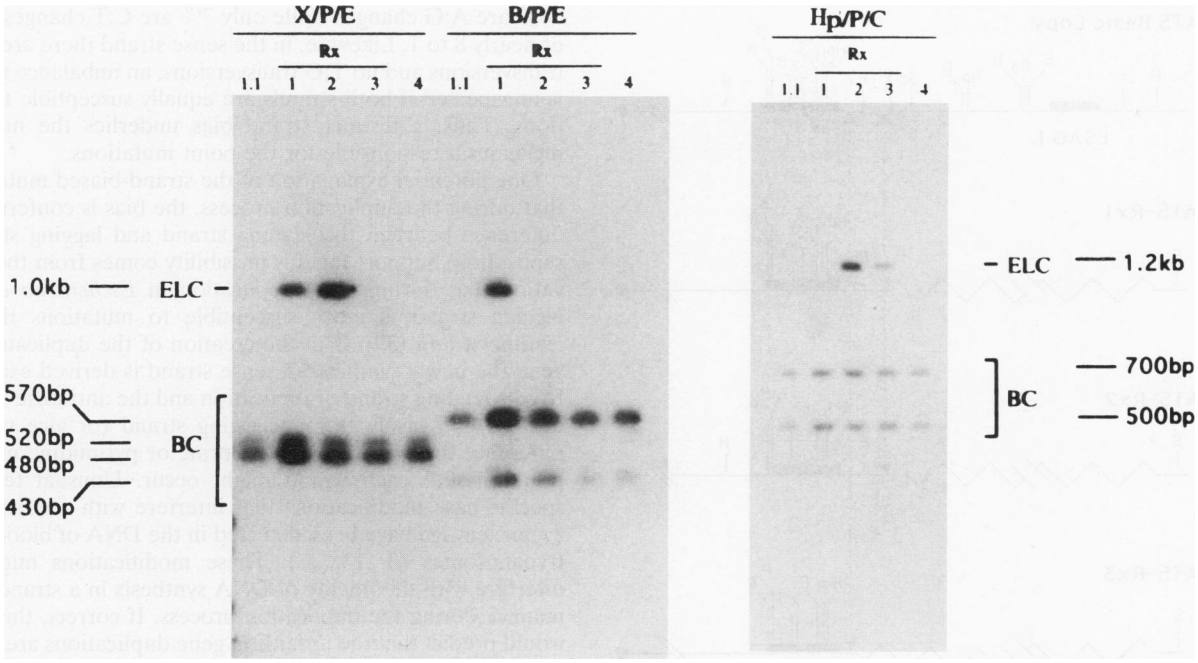


FIG. 3. Autoradiograms of Southern blots containing genomic DNAs from trypanosome clones WRATat1.1, MVAT5-Rx1, MVAT5-Rx2, MVAT5-Rx3, and MVAT4 digested with various combinations of *Xho*I (X), *Pst*I (P), *Eco*RI (E), *Bam*HI (B), *Hpa*I (Hp), and *Cla*I (C). The blots were probed with a 1.1-kb *Hin*dIII-*Pst*I fragment derived from the BC gene for the MVAT5 VSG. WRATat1.1 is the parent clone from which the other clones are derived. WRATat1.1 and MVAT4 express VSGs unrelated to each other or to the MVAT5 clones.

clones have different reactivities toward a panel of surface-reactive MAbs (Table 1), some of these amino acid replacements likely generate new epitopes because of either (i) the altered sequence itself, (ii) an altered secondary structure of the VSG, or (iii) glycosylation differences caused by the amino acid changes.

The structures of the N-terminal variable domains (about 380 amino acids) of two immunologically distinct VSGs, MITat1.2 and ILTat1.24, have been determined by X-ray crystallography and found to be strikingly similar, despite very little sequence identity (5). This finding suggests that all VSGs, including those of MVAT5, possess similar three-dimensional structures. When the sequences of the three MVAT5 VSGs are aligned with the MITat1.2 and ILTat1.24 VSG sequences, the amino acid differences among the MVAT5 VSGs are located in regions that are both distal and proximal to the cell membrane. Yet only four amino acid replacements occur in the N-terminal 140 amino acids (two of which are in the signal peptide region), the region of the VSG that forms two large, antiparallel α-helices. The paucity of amino acid replacements in this region may suggest that such changes are deleterious to the proper folding of the VSG. Likewise, very few changes occur near the C terminus, a region whose conformation is not known from the crystallographic studies. Amino acid replacements in this region also may be poorly tolerated because they interfere with the protein's conformation near the cell membrane. In contrast, a hot spot of frequent amino acid changes (two to three differences in each reexpressor occur in the 6 residues from positions 259 to 264 [Fig. 2]) is located in a region equivalent to loop p that protrudes from outer surface of the VSG (5). This region is probably both readily accessible to the host immune system and not crucial for the protein's overall conformation. Finally, it is not clear if the limited number of amino acid replacements among the MVAT5 VSGs is sufficient for the parasites to evade the host's immune response to an earlier expression of the MVAT5 BC gene. It is worth noting, however, that influenza viruses require only a dozen or so changes on their surface to escape neutralization from antibodies raised in the previous infection (40).

What is the molecular mechanism underlying the generation of the point mutations, and why have the mutations not been detected earlier? Several possibilities for the mutation generator exist. Perhaps the simplest explanation is that the MVAT5 VSG BC gene is not the only template participating in the gene duplication and that the mutations are created in a manner similar to that of the generation of mosaic ELCs from two or more closely related BC genes (2, 15, 24, 28, 33). In this case, the mutations would be templated, not from VSG isogenes, but from much smaller stretches of related DNA sequences located elsewhere in the genome. However, genomic Southern hybridizations using 26-mer oligonucleotide probes (Fig. 6) failed to reveal the existence of templates for such small partial gene conversion events. Furthermore, the large battery of different point mutations detected in the three gene duplications would have to be derived from an equally large number of small partial gene conversions. Thus, although we cannot completely discount the possibility that the point changes are templated from segments of the genome shorter than 26 bp, this alternative seems unlikely and is difficult to test experimentally.

If the MVAT5 VSG BC gene is indeed the only template used to generate the ELC gene in the three reexpressors, then another possibility is that a DNA polymerase and/or mismatch repair system involved in the VSG gene conversions lacks fidelity. In a conventional gene conversion (17, 25), both strands of the donor BC gene are thought to serve as templates for synthesis of a new strand. Thus, in either strand the number of purine transitions (A to G [A:G] or G:A) and pyrimidine

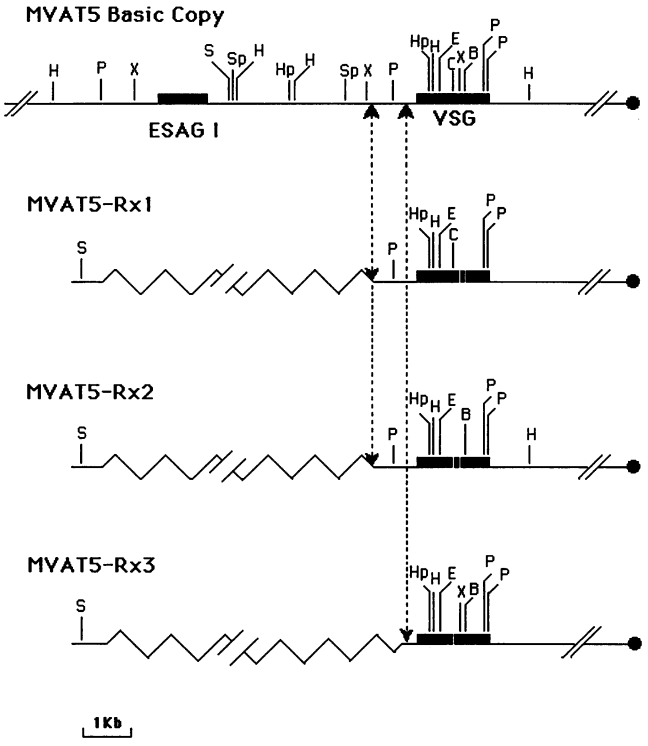


FIG. 4. Restriction maps of the BC gene and the duplicated ELC genes expressed in trypanosome clones MVAT5-Rx1, -Rx2, and -Rx3. Black rectangles denote the coding regions for the VSG and an expression site-associated gene (ESAGI). White vertical lines in the ELC genes indicate point mutations that destroy restriction sites in the BC gene. Zigzag lines upstream of the ELC genes show regions of at least 20 kb thought to be composed predominately of 70- to 76-bp repeats. Vertical dotted lines show the upstream (5′) crossover points in the generation of the ELC genes. Closed circles indicate telomeres. Restriction sites are shown for *Hin*dIII (H), *Pst*I (P), *Xho*I (X), *Hpa*I (Hp), *Bam*HI (B), *Sac*I (S), *Sph*I (Sp), *Eco*RI (E), and *Cla*I (C). Two closely spaced *Eco*RI sites are indicated by a single E.

transitions (T:C or C:T) should be about the same, since an A:G transition in the sense strand is equivalent to a T:C transition in the antisense strand and vice versa. However, as illustrated in Table 2, one of the most distinctive features of the total of 74 nucleotide changes in the three duplicated VSG coding regions is that in the sense strand the numbers of pyrimidine and purine transitions are not equal. In this strand, 54% are A:G changes while only 7% are C:T changes, a ratio of nearly 8 to 1. Likewise, in the sense strand there are 19 A:C transversions and no T:G transversions, an imbalance that also is unexpected if both strands are equally susceptible to mutations. Thus, a distinct strand bias underlies the molecular mechanism responsible for the point mutations.

One potential explanation of the strand-biased mutations is that during the duplication process, the bias is conferred by a difference between the leading strand and lagging strand of replication. Support for this possibility comes from the observation that during DNA replication in *Escherichia coli*, the lagging strand is more susceptible to mutations than the leading strand (39). If in the creation of the duplicated VSG gene the newly synthesized sense strand is derived exclusively from a leading strand of replication and the antisense strand is derived exclusively from a lagging strand (or vice versa), a difference in the frequency of purine or pyrimidine misincorporation into each strand might occur. Unusual telomere-specific base modifications that interfere with restriction enzyme cleavage have been detected in the DNA of bloodstream trypanosomes (3, 13, 22). These modifications might also interfere with the fidelity of DNA synthesis in a strand-biased manner during the duplication process. If correct, this model would predict that the unfaithful gene duplications are derived only from telomere-linked BC VSG genes containing base modifications and not from internal BC VSG genes lacking the telomere-associated base modifications. Since most ELCs studied are gene conversions of internal BC genes, this reasoning provides an explanation for why point mutations in ELCs have only rarely been detected previously (15, 26).

Still another possibility is that mutations occur not during but after the duplication event, perhaps in a transcriptionally related manner. Examples of transcription-mediated, strand-biased mutations have been observed in biological systems as diverse as humans, *Saccharomyces cerevisiae*, and *E. coli*. In these other organisms lesions in the transcribed (antisense) strand interfere with transcription and are repaired more rapidly than lesions in the nontranscribed (sense) strand. Thus, more mutations first occur in the sense strand of a transcribed region (20, 31). In a corresponding trypanosome scenario the RNA polymerase might temporarily stall at different positions of a faithfully duplicated ELC gene. The stalled RNA polymerase could recruit unknown molecules or factors to these pause sites that would damage or replace some DNA nucleotides in a strand-biased fashion. The nucleotide modifications mentioned above and a specific telomere-associated chromatin conformation are features that might cause the RNA polymerase to pause. The mutations could also be a unique feature

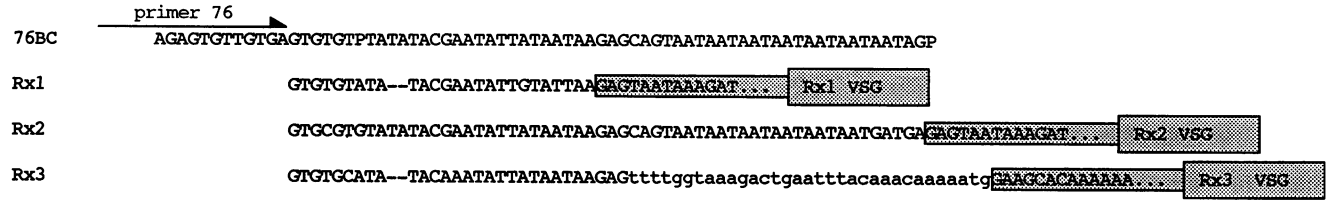


FIG. 5. Comparison of the segments of a 70- to 76-bp repeat that lie immediately upstream of the 5′ crossover points for the ELCs in the genomes of MVAT5-Rx1, -Rx2, and -Rx3. A consensus 76-bp repeat (76BC) is shown at the top (32); P indicates a purine. Primer 76 (arrow) was used as the 5′ primer in PCRs to amplify the regions surrounding the 5′ crossover sites in the three ELCs. Each of these three regions is shown below the consensus sequence. The portions of the amplification products that correspond to the 76-bp repeat are aligned with the consensus sequence, and dashes are introduced to maximize homology. The nucleotides in the box are the first 13 nucleotides immediately downstream of the 5′ crossover region in each of the ELCs. In Rx3, 33 nucleotides (76% AT) of unknown origin that occur between the 70- to 76-bp repeat segment and the region upstream of the VSG gene are shown in lowercase.

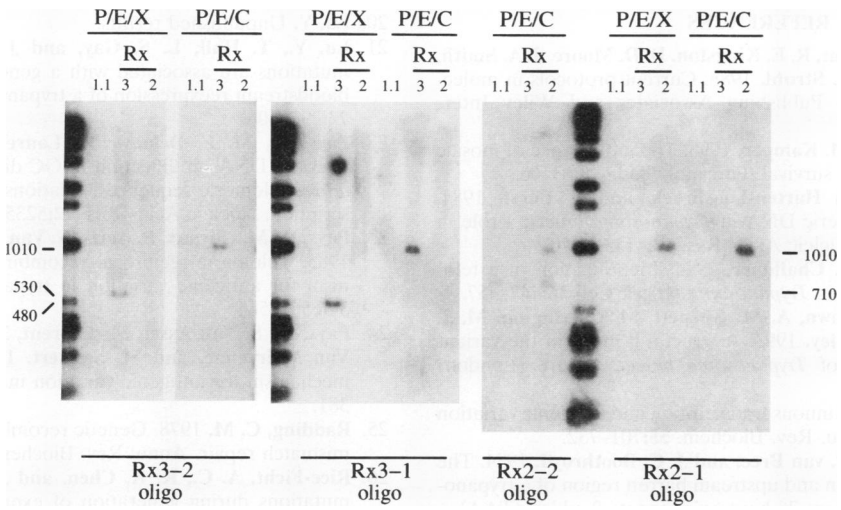


FIG. 6. Autoradiograms of Southern blots of genomic DNAs from WRATat1.1, MVAT5-Rx2, and MVAT5-Rx3 probed with four different oligonucleotides. The 26-nucleotide oligonucleotides are specific for the ELCs of Rx2 (oligonucleotides Rx2-1 and Rx2-2) or Rx3 (oligonucleotides Rx3-1 and Rx3-2) and are derived from sequences in region I (Rx2-1 and Rx3-1) or region II (Rx2-2 and Rx3-2) shown in Fig. 1. The genomic DNAs were triple digested with *Pst*I (P), *Eco*RI (R), and *Xho*I (X) or *Cla*I (C). Unmarked lanes contain labeled, standard length markers. The sizes (in base pairs) of genomic DNA fragments that hybridize to the oligonucleotide probes are indicated on the left and right.

of the special α-amanitin-resistant RNA polymerase that transcribes the genes for VSGs, procyclin, and a few other proteins (29), of which only the VSG genes are telomere linked. An attractive feature of this transcription-coupled model of mutation is that it predicts that mutations will accumulate with time as the MVAT5 ELC gene is continuously transcribed. This prediction can be tested by passaging the MVAT5 reexpressor clones in immunocompromised mice for several months, followed by periodic recloning of the MVAT5 parasites to see if additional point changes have occurred in the duplicated gene.

A related possibility for the mutation generator is that some ELCs are derived from their BC genes through an RNA intermediate. A rare transcript of a normally silent, telomere-linked BC gene could serve as a template for the synthesis of the duplicated gene copy. Mutations might occur either during the initial transcription step or during potential reverse transcriptase-mediated steps and integration events that follow. The identification of a sequence in front of the MVAT5 VSG BC gene that can serve as a promoter in reporter gene assays (20a) provides support for the occurrence of low-level, monocistronic transcription of this gene. A disadvantage of this model, as with the others above, is that it does not offer a clear explanation for why in the cases of the Rx1 and Rx2 ELCs the mutations do not occur as frequently in the 1-kb duplicated upstream segment as they do in the VSG coding region.

On an evolutionary scale, some BC isogenes could be remnants of mutated ELCs retained in the genome after their inactivation. If so, the nucleotide differences among genes of a BC isogene family should reflect a strand bias as well. To test this possibility, the sequences of the four BC genes of *T. brucei* involved in the generation of mosaic ELC genes described by Kamper and Barbet (15) were examined. Since it is not clear which, if any, of the four BC genes is the progenitor gene, the individual sequences were compared with a hypothetical sequence compiled by selecting the most common nucleotide at each position. These comparisons revealed a similar strand-specific nucleotide bias in the differences among these four coding regions. In the sense strand the ratio both of the A:G transitions to C:T transitions and of the A:C transversions to T:G transversions is 3:1. Even more dramatically, a comparison of the two BC genes that participated in the generation of the mosaic VSG gene expressed in the *Trypanosoma equiperdum* clone BoTat20 (33) reveals a ratio in the sense strand of 6:1 for the A:G transitions to C:T transitions and a ratio of 7:1 for the A:C transversions to T:G transversions (33). Thus, nucleotide changes among members of two different families of BC isogenes display a strand bias consistent with that of mutations found in the ELC genes of the MVAT5 reexpressors. The fact that these two VSG isogene families are in different trypanosome species and the MVAT5 clones are in still another trypanosome subspecies suggests that the mechanism responsible for generating strand-biased mutations in telomere-linked VSG genes is more common than was previously appreciated.

TABLE 2. Nucleotide differences in the sense strand between the BC gene and the Rx1, Rx2, and Rx3 duplicated genes

Nucleotide changes	No. of changed positions				% of total changes
	In Rx1	In Rx2	In Rx3	Total	
A↔G	19	8	13	40	54
C↔T	1	0	4	5	7
A↔C	10	3	6	19	26
T↔G	0	0	0	0	0
C↔G	5	0	5	10	13
T↔A	0	0	0	0	0
Total	35	11	28	74	100

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Siedman, and K. Struhl.** 1988. Current protocols in molecular biology. Greene Publishing Associates and Wiley Interscience, New York.
2. **Barbet, A. F., and S. M. Kamper.** 1993. The importance of mosaic genes to trypanosome survival. Parasitol. Today **9**:63–66.
3. **Bernards, A., N. van Harten-Loosbroek, and P. Borst.** 1984. Modification of telomeric DNA in *Trypanosoma brucei*; a role in antigenic variation. Nucleic Acids Res. **12**:4153–4170.
4. **Blackburn, E., and P. Challoner.** 1984. Identification of a telomeric DNA sequence in *Trypanosoma brucei*. Cell **36**:447–457.
5. **Blum, M. L., J. A. Down, A. M. Gurnett, M. Carrington, M. J. Turner, and D. C. Wiley.** 1993. A structural motif in the variant surface glycoprotein of *Trypanosoma brucei*. Nature (London) **362**:603–609.
6. **Borst, P.** 1986. Discontinuous transcription and antigenic variation in trypanosomes. Annu. Rev. Biochem. **55**:701–732.
7. **Campbell, D. A., M. P. van Bree, and J. C. Boothroyd.** 1984. The 5′-limit of transposition and upstream barren region of a trypanosome VSG gene: tandem 76 base-pair repeats flanking $(TAA)_{90}$. Nucleic Acids Res. **12**:2759–2774.
8. **Campbell, G. H., K. M. Esser, B. T. Wellde, and C. L. Diggs.** 1979. Isolation and characterization of a new serodeme of a *Trypanosoma rhodesiense*. Am. J. Trop. Med. Hyg. **28**:974–983.
9. **Crowe, J. S., J. D. Barry, A. G. Luckins, C. A. Ross, and K. Vickerman.** 1983. All metacyclic variable antigen types of *Trypanosoma congolense* identified using monoclonal antibodies. Nature (London) **306**:389–391.
10. **Donelson, J. E., and A. C. Rice-Ficht.** 1985. Molecular biology of trypanosome antigenic variation. Microbiol. Rev. **49**:107–125.
11. **Dorfman, D. M., and J. E. Donelson.** 1984. Characterization of the 1.35 kilobase DNA repeat unit containing the conserved 35 nucleotides at the 5′-termini of variable surface glycoprotein mRNAs in *Trypanosoma brucei*. Nucleic Acids Res. **12**:4907–4920.
12. **Esser, K. M., M. J. Schoenbechler, and J. B. Gingrich.** 1982. *Trypanosoma rhodesiense* blood forms express all antigen specificities relevant to protection against metacyclic (insect form) challenge. J. Immunol. **129**:1715–1718.
13. **Gommers-Ampt, J., J. Lutgerink, and P. Borst.** 1991. A novel DNA nucleotide in *Trypanosoma brucei* only present in the mammalian phase of the life-cycle. Nucleic Acids Res. **19**:1745–1751.
14. **Hattori, M., and Y. Sakaki.** 1986. Dideoxy sequencing method using denatured plasmid templates. Anal. Biochem. **152**:232–238.
15. **Kamper, S. M., and A. F. Barbet.** 1992. Surface epitope variation via mosaic gene formation is potential key to long-term survival of *Trypanosoma brucei*. Mol. Biochem. Parasitol. **53**:33–44.
16. **Kearney, J. F., A. Radbruch, B. Liesegang, and K. Rajewsky.** 1979. A new mouse myeloma cell line that lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. J. Immunol. **123**:1548–1550.
17. **Klar, A. J. S.** 1989. The interconversion of yeast mating type: *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, p. 671–691. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
18. **Kohler, G., and C. Milstein.** 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature (London) **256**:495–497.
19. **Lenardo, M. J., A. C. Rice-Ficht, G. Kelly, K. M. Esser, and J. E. Donelson.** 1984. Characterization of the genes specifying two metacyclic variable antigen types in *Trypanosoma brucei rhodesiense*. Proc. Natl. Acad. Sci. USA **81**:6642–6646.
20. **Lommel, L., and P. C. Hanawalt.** 1993. Increased UV resistance of a xeroderma pigmentosum revertant cell line is correlated with selective repair of the transcribed strand of an expressed gene. Mol. Cell. Biol. **13**:970–976.

20a. **Lu, Y.** Unpublished results.

21. **Lu, Y., T. Hall, L. S. Gay, and J. E. Donelson.** 1993. Point mutations are associated with a gene duplication leading to the bloodstream reexpression of a trypanosome metacyclic VSG. Cell **72**:397–406.
22. **Pays, E., M. F. Delauw, M. Laurent, and M. Steinert.** 1984. Possible DNA modification in GC dinucleotides of *Trypanosoma brucei* telomeric sequences; relationship with antigen gene transcription. Nucleic Acids Res. **12**:5235–5247.
23. **Pays, E., M. Guyaux, D. Aerts, N. Van Meirvenne, and M. Steinert.** 1985. Telomeric reciprocal recombination as a possible mechanism for antigenic variation in trypanosomes. Nature (London) **316**:562–564.
24. **Pays, E., S. Van Assel, M. Laurent, M. Darville, T. Vervoort, N. Van Meirvenne, and M. Steinert.** 1983. Gene conversion as a mechanism for antigenic variation in trypanosomes. Cell **34**:371–381.
25. **Radding, C. M.** 1978. Genetic recombination: strand transfer and mismatch repair. Annu. Rev. Biochem. **47**:847–880.
26. **Rice-Ficht, A. C., K. K. Chen, and J. E. Donelson.** 1982. Point mutations during generation of expression-linked extra copy of trypanosome surface glycoprotein gene. Nature (London) **298**:676–679.
27. **Ross, R., and D. Thomson.** 1910. A case of sleeping sickness studied by precise enumerative methods. Regular periodic increase in the parasites disclosed. Proc. R. Soc. Lond. (Biol.) **82**:411–415.
28. **Roth, C., F. Bringaud, R. E. Layden, T. Baltz, and H. Eisen.** 1989. Active late-appearing variable surface antigen genes in *Trypanosoma equiperdum* are constructed entirely from pseudogenes. Proc. Natl. Acad. Sci. USA **86**:9375–9379.
29. **Rudenko, G., D. Bishop, K. Gottesdiener, and L. H. Van der Ploeg.** 1989. Alpha-amanitin resistant transcription of protein coding genes in insect and bloodstream form Trypanosoma brucei. EMBO J. **8**:4259–4263.
30. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
31. **Selby, C. P., and A. Sancar.** 1993. Molecular mechanism of transcription-repair coupling. Science **260**:53–58.
32. **Shah, J. S., J. R. Young, B. E. Kimmel, K. P. Iams, and R. O. Williams.** 1987. The 5′ flanking sequence of a *Trypanosoma brucei* variable surface glycoprotein gene. Mol. Biochem. Parasitol. **24**:163–174.
33. **Thon, G., T. Baltz, and H. Eisen.** 1989. Antigenic diversity by the recombination of pseudogenes. Genes Dev. **3**:1247–1254.
34. **Turner, C. M., and J. D. Barry.** 1989. High frequency of antigenic variation in *Trypanosoma brucei rhodesiense* infections. Parasitology **99**:67–75.
35. **Turner, C. M., J. D. Barry, I. Maudlin, and K. Vickerman.** 1988. An estimate of the size of the metacyclic variable antigen repertoire of *Trypanosoma brucei rhodesiense*. Parasitology **97**:269–276.
36. **Turner, M. J.** 1985. The biochemistry of the surface antigens of the African trypanosomes. Br. Med. Bull. **41**:137–143.
37. **Van der Ploeg, L. H. T.** 1991. Control of antigenic variation in African trypanosomes. New Biol. **3**:324–330.
38. **Van der Ploeg, L. H. T., D. Valerio, T. de Lange, A. Bernards, P. Borst, and F. G. Grosveld.** 1982. An analysis of cosmid clones of nuclear DNA from *Trypanosoma brucei* shows that the genes for the variant surface glycoproteins are clustered in the genome. Nucleic Acids Res. **10**:5905–5923.
39. **Veaute, X., and R. P. P. Fuchs.** 1993. Greater susceptibility to mutations in lagging strand of DNA replication in *Escherichia coli* than in leading strand. Science **261**:598–600.
40. **Wiley, D. C.** 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. Annu. Rev. Biochem. **56**:365–394.