Co-duplication of a Variant Surface Glycoprotein Gene and Its Promoter to an Expression Site in African Trypanosomes*

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Activation of the metacyclic variant antigen type 7 (MVAT7) variant surface glycoprotein (VSG) gene in bloodstream Trypanosoma brucei rhodesiense involves a duplicative transposition of the gene. The DNA transposition unit extends from a site ~3.0 kilobases upstream of the VSG gene through the coding region and includes a 73-base pair sequence that possesses promoter activity in transient transfections. This MVAT7 promoter has 80% identity to a previously characterized promoter for the MVAT4 VSG gene. Nuclear run-on assays demonstrate that the MVAT7 promoter is active in MVAT7 bloodstream organisms and that its transcript is synthesized by an RNA polymerase resistant to α-amanitin, consistent with previously published reports regarding VSG gene transcription. The transcription start site was identified by primer extension studies and a modified rapid amplification of cDNA ends protocol. Selective mutational analysis of the MVAT7 promoter showed that two conserved trinucleotide regions are important for full promoter function. This study demonstrates that the MVAT7 VSG gene is co-duplicated with its promoter and transcribed into a monocistronic precursor RNA.

African trypanosomes are protozoan parasites that cause African trypanosomiasis, or sleeping sickness, in many tropical regions of Africa. Infective metacyclic stage trypanosomes are transmitted from the tsetse fly to the mammalian bloodstream, where they differentiate into bloodstream organisms that evade the immune response by periodically switching the major protein on their surface, the variant surface glycoprotein (VSG).1 Hundreds of different VSGs can potentially be expressed by bloodstream trypanosomes, whereas only 10–15 different VSGs are expressed during the metacyclic stage (1–3). VSGs expressed by metacyclic organisms are used to define the metacyclic variant antigen types (MVATs). Many unexpressed bloodstream VSG genes are found in minichromosomes and at internal locations of large chromosomes, whereas the MVAT VSG genes appear to be located exclusively near the telomeres of large chromosomes, independent of their expression status (4). After metacyclic parasites enter the mammalian bloodstream, they continue to express MVAT VSGs for 5–7 days and then change to the expression of a bloodstream VSG followed by periodic VSG switching. Late in the infection, MVAT VSGs are occasionally re-expressed as bloodstream VSGs. The re-expressed MVAT VSG genes are activated by either gene conversion or an unknown activation mechanism in situ (5–7).

Expressed VSG genes are invariably located near chromosomal telomeres. The telomere-linked expression sites for bloodstream VSG genes typically consume 45–60 kb and contain (in a 5′ to 3′ direction) a promoter, seven or more expression site-associated genes (ESAGs), a variable number of 70–76-bp repeats, and the VSG gene followed by subtelomeric and telomeric DNA repeats (see Refs. 8 and 9 for recent reviews). Thus, transcription of most bloodstream VSG gene expression sites yields polycistronic precursor RNAs containing eight or more protein-coding regions that are processed into mature monocistronic mRNAs. In contrast, the telomere-linked MVAT VSG expression sites do not have a full complement of ESAGs and can be devoid of ESAGs entirely. They also lack multiple copies of the 70–76-bp repeats and are transcribed into monocistronic precursor RNAs from a proximal promoter located within 2 kb of the VSG-coding region (5, 7).

Recently, we characterized a promoter for the MVAT4 VSG gene of Trypanosoma brucei rhodesiense utilizing a bloodstream trypanosome clone that re-expresses MVAT4 VSG (5). In these bloodstream organisms, the telomere-linked MVAT4 VSG gene is activated in situ without a duplication event or other detectable DNA rearrangements. This finding suggests that either (i) the promoter that functions in metacyclic organisms was reactivated in the bloodstream parasites or (ii) two promoters exist in this expression locus, one for expression at the metacyclic stage and one for the bloodstream stage. The simplest of these two possibilities is that the same promoter is responsible for transcription in both metacyclic and bloodstream organisms. Another MVAT VSG gene, encoding MVAT5 VSG, was found to be activated via duplication to a conventional bloodstream expression site whose promoter is far upstream of the VSG gene (6).

Here, we describe the identification and characterization of a promoter for the bloodstream expression of a third metacyclic VSG gene, the MVAT7 VSG gene. This gene and its promoter were found to be co-duplicated to a telomere-linked site, which is in contrast to both the in situ activation of the MVAT4 VSG gene and the duplication of the MVAT5 VSG gene to a conventional bloodstream expression site possessing its own promoter. Mutational studies demonstrated that at least two regions within the MVAT7 promoter are important for full promoter function. This promoter gives rise to a monocistronic VSG transcript, differing from the polycistronic transcripts of bloodstream VSG genes, but similar to the promoters for the MVAT4 VSG gene and another metacyclic VSG gene in a different trypanosome serodeme (7).
Trypanosomes—The Walter Reed Army Trypanozoon antigen type 1.1 (WRATat 1.1) clone was derived from human isolate LVH/75/US-AMR-k/18 of T. brucei rhodesiense (10). Trypanosomes expressing MVAT1–MVAT14 correspond to the WRATat 1.21–1.34 clones in standard nomenclature (11) and were obtained as described (3). The identification and cloning of a bloodstream trypanosome re-expressing MVAT7 VSG were conducted as described previously (12). Procyclic trypanosomes were established in culture by inoculating 40 ml of SM medium (13) supplemented with 3 mM *cis*-aconitate, 1 mM pyruvate, and 15% heat-inactivated fetal bovine serum with 0.5 ml of blood from trypanosome-infected rats. After incubation for 24 h at 25 °C, the red blood cells had settled, and the supernatant was transferred to another tissue culture flask. After establishment of procyclic forms, the parasites were passaged at late log phase (1 × 10^7 parasites/ml) about every 2 days.

Recombinant DNA Procedures—Trypanosome genomic DNA and total RNA were isolated as described previously (4). Southern blotting (4 μg of DNA/lane) was conducted as described previously (14). DNA probes used for hybridization experiments were 32P-labeled using a random priming kit (Boehringer Mannheim). DNA sequencing was performed by the dideoxy chain termination method (15). Oligonucleotide primers for DNA sequencing were synthesized on the basis of previously determined DNA sequences.

Plasmid Constructs—All plasmid constructs were derived from plasmid pHD-1 (16) in which the PARP (procyclic acidic repeat protein) gene promoter was removed and replaced with restriction fragments, PCR fragments, or synthetic double-stranded fragments whose sequences occur in the MVAT7 VSG locus. The MVAT7 promoter and mutants shown in Fig. 6B were generated by annealing four complementary, partially overlapping oligonucleotides (36–40 residues each) with the desired mutations incorporated. The oligonucleotides were designed such that when annealed, they formed the desired double-stranded wild-type or mutant promoter sequence. This annealed product was blunt end-ligated into the derivative of pHD-1. The resultant plasmids were recovered from *Escherichia coli* transformants, and the sequences of their inserts were determined to confirm the orientation and sequence of the inserted promoter segments.

Transient Transfections—Transient transfections of procyclic trypanosomes derived from the bloodstream forms of T. brucei rhodesiense MVAT5 Rx2 were conducted according to a protocol kindly provided by Dr. Etienne Payes and a protocol described previously (17). Briefly, cell suspensions (400 μl) were mixed with 50 μl of a solution containing 20 μg of the pHD-1 test construct and 10 μg of an internal control plasmid with the β-galactosidase gene under the transcriptional control of the PARP promoter (the kind gift of Dr. Lex Van der Floop). The mixture was pulsed two times at 1.2 kV and 25 microfarads with a Bio-Rad Gene Pulser. The cells then were transferred to 5 ml of SM medium and 15% heat-inactivated fetal calf serum and incubated at 25 °C for 20–24 h. Cells were lysed and assayed for luciferase activity as described previously (18). The same cell lysates were also assayed for β-galactosidase activity as follows. 10 ml of each cell lysate were added to 200 ml of buffer Z (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, and 50 mM β-mercaptoethanol), pH 7.5, to which 0.77 mM 4-methylumbelliferyl β-galactoside had been added. This mixture was incubated at 37 °C for 1 h. After the incubation, 2 ml of the buffer Z mixture was mixed with 2 ml of glycine carbonate solution (133 mM glycine and 83 mM Na2CO3), pH 10.7. The mixture of glycine carbonate and buffer Z was then assayed in a TRO 100 fluorometer (Hoefer Scientific Instruments).

Nuclear Run-on Assays, Primer Extension Experiments, and RACE-derived Clone Analyses—These experimental procedures were conducted as described previously (5).

RESULTS

Identification of the Upstream Crossover Site of the MVAT7 VSG Gene Duplication—Our original characterization of the MVAT7 VSG gene was conducted on DNA and RNA from metacyclic (day 5) trypanosomes and demonstrated that, during the metacyclic stage, it exists as a single-copy telomere-linked gene (4). To examine the expression of this gene more completely, a bloodstream trypanosome clone re-expressing MVAT7 VSG was isolated using the approach documented previously (12) and grown in immunosuppressed rats. Immunofluorescence assays using a monoclonal antibody directed against MVAT7 VSG showed that at least 99% of these MVAT7 bloodstream trypanosomes express MVAT7 VSG (data not shown). Southern blotting was conducted on restriction digests of genomic DNAs isolated from this MVAT7 re-expressor clone and from the non-expressor parental WRATat 1.1 clone using the MVAT7 VSG-coding region as a probe (the Clal/BamHI fragment indicated as the M7 Probe in Fig. 1A). These blots revealed that the MVAT7 VSG gene has been duplicated in the MVAT7 re-expressor organisms (Fig. 1B). For example, in the digests with BclI, Clal, and SacII, which cut once in either the coding region or the upstream sequence, the probe hybridizes to a single fragment in WRATat 1.1 DNA and to two fragments in MVAT7 DNA. Both hybridizing fragments in the MVAT7 genome are smaller than the hybridizing fragment in the WRATat 1.1 genome. The different sizes of these fragments in the two genomes are consistent with telomere-linked locations for both the donor gene and the duplicated gene. A similar pattern is obtained with EcoRI, which cuts both upstream and within the coding region, yielding the telomere-linked fragments and a constant length 0.8-kb fragment, which is not present in Fig. 1 because it had run off the gel during the long electrophoresis time necessary to resolve the larger telomere-linked fragments. In some of the MVAT7 DNA digestions, the upper of the two hybridizing bands is weaker than the lower band. We also noticed this phenomenon with the MVAT4 VSG gene (5), and in that case, we found that the expressed telomere-linked gene gave a weaker, more diffuse signal than did the silent telomere-linked donor gene because of heterogeneity in the length of the expressed telomere, a phenomenon also observed by other investigators using trypanosomes from other serodemes (19). Thus, the larger of the two hybridizing fragments in the MVAT7 DNA likely contains the duplicated gene.

The other hybridization patterns shown in Fig. 1B indicate...
that the upstream crossover site of the gene duplication occurs within a 380-bp region between the HindIII and BclI sites upstream of the basic copy donor gene (see the restriction maps in Fig. 1A). For example, if the crossover site had been upstream of the HindIII site, the hybridization pattern in the HindIII digest would have been similar to that in the BclI, CiaI, EcoRI, and SacII digests. However, in the MVAT7 genome, one of the two telomere-linked fragments obtained with HindIII is larger than that in the WRATat 1.1 genome, rather than both fragments being smaller as is the case in the other digests. This pattern could be explained either by a crossover site located downstream of HindIII and upstream of BclI site or by a single-point mutation in the duplicated copy affecting the HindIII restriction site. Further evidence that a crossover event is responsible for the difference is derived from the hybridization pattern in the BamHI digest. This BamHI pattern reveals a constant length 8-kb fragment in both genomes due to two restriction sites in the region of the basic copy gene (see Fig. 1A). If the crossover had occurred 5' to the upstream BamHI site, one would expect the probe to detect only this constant 8-kb fragment in both genomes. However, in the MVAT7 genome, both the 8-kb fragment and a much larger fragment are observed, consistent with the crossover site being located upstream of the BclI site and downstream of the BamHI and HindIII sites. Attempts to identify the precise crossover point within this 380-bp region between the HindIII and BclI sites by a variety of PCR amplifications and genomic DNA cloning experiments were not successful. In particular, PCR amplifications in which one of the primers contained various sequences of the 70–76-bp repeats did not provide evidence for the presence of these repeats at this crossover site, consistent with their absence at other VSG gene conversion sites (20). Since a determination of this exact crossover site was not crucial to the proposed experiments, its identification was not pursued.

Identification of a MVAT7 VSG Gene Promoter—Previously, we cloned a 7-kb BamHI/ClaI genomic DNA fragment containing the region upstream of the basic copy MVAT7 VSG gene and a small portion of its coding region (21) (see Fig. 1A). This fragment was derived from genomic DNA of trypanosomes collected from the bloodstream of a mouse 5 days after its infection with metacyclic organism from tsetse flies that had ingested WRATat 1.1 trypanosomes. Restriction fragments or PCR products from ~4 kb of the 7-kb genomic fragment were used to replace the PARP promoter fragment in the pHD-1 vector, as shown in Fig. 2. Background luciferase activities in extracts of cells transiently transfected with a derivative of pHD-1 in which the PARP promoter had been removed were 500–1000 units on the luminometer. Subcloned fragments from upstream of the MVAT7 VSG gene that stimulated at least 50 times more luciferase activity than this background level were scored as potentially positive for promoter activity and were selected for further analyses. The smallest fragment that scored positive in these preliminary analyses was a 490-bp PvuII/RsaI fragment whose presence stimulated >100,000 luciferase units. All of the fragments shown in Fig. 2 yielded only background luciferase units when inserted in the reverse orientation (data not shown).

The sequence of this 490-bp PvuII/RsaI fragment was determined and found to have a 70-bp region that possesses striking similarity to the promoter for the MVAT4 VSG gene that we have previously characterized (5). About 80% sequence identity occurs between these two 70-bp regions (see Fig. 6A). The sequence conservation extends into the mapped transcription start site of the MVAT4 VSG gene, allowing us to predict the location of a potential transcription start site for the MVAT7 VSG transcript. This putative promoter is located 1.5 kb upstream of the MVAT7 VSG-coding region, whereas the MVAT4 promoter is 2.0 kb upstream of its VSG-coding region.

A sequence of 4.8 kb surrounding this putative MVAT7 promoter was determined and is shown in Fig. 3. This sequence includes the 1420-bp coding region of the basic copy donor MVAT7 VSG gene (shaded segment). No sequence similarities to the known ESAGs were found in the 4.8-kb segment or in the partial sequence obtained on the remainder of the 7-kb BamHI/ClaI genomic fragment (data not shown). Likewise, no translation open reading frames of any substantial length were found other than the VSG-coding sequence.

In addition to determining this genomic DNA sequence, the MVAT7 VSG mRNA was amplified by reverse transcription-PCR using total RNA from MVAT7 trypanosomes as the template. Oligonucleotide primers complementary to the spliced leader sequence and to a sequence within the MVAT7 VSG-coding region were used to PCR-amplify the first three-fourths of the MVAT7 VSG mRNA. A second set of oligonucleotide primers was used to PCR-amplify the last one-third of the sequence. The PCR products were cloned, and their complete sequences were determined. This sequence analysis revealed the 5'-spliced leader and 3'-poly(A) addition sites and confirmed that the cDNAs were derived from an mRNA template rather than the result of genomic DNA contamination. As indicated in Fig. 3, the splice acceptor site is located 26 bp downstream of the start codon, and the polyadenylation site is 79 bp downstream of the stop codon. The sequences of these amplified VSG cDNAs are identical to the corresponding regions of the basic copy donor MVAT7 VSG gene. Thus, no point changes occur within the VSG-coding regions of the donor gene and the
duplicated gene, in contrast to the multiple nucleotide replacements that we observed earlier in three independently derived duplicated copies of another bloodstream-expressed metacyclic VSG gene, the MVAT5 VSG gene (6, 12, 22) (see "Discussion").

Identification of the 5'-End of the Nascent MVAT7 VSG Transcript—Since the transient transfections and sequence similarity described above provided an approximate location of the transcription start site of the expressed MVAT7 VSG gene, primer extension experiments were performed to identify this start site more precisely. An oligonucleotide complementary to the sequence 130 nucleotides downstream of the putative transcription start site and 1.4 kb upstream of the spliced leader addition site was 5'-end-labeled with 32P and used in a primer extension experiment on total RNA as the template. The results shown in Fig. 4A indicate that the transcription start site maps to an adenosine residue in the nontranscribed strand.

To confirm this transcription start site, a modification of the RACE technique was used to determine the sequence at the 5'-ends of cDNAs derived from nascent MVAT7 VSG RNAs. Briefly, an oligonucleotide complementary to a sequence near the presumed 5'-end of the nascent RNA was used to synthesize a first-strand cDNA. A second oligonucleotide, whose 3'-end was blocked, was ligated to the 3'-end of the newly synthesized first-strand cDNA. The cDNA was PCR-amplified using oligonucleotide primers used to PCR-amplify fragment 2 are indicated by arrow A and B.

FIG. 3. Sequence of the basic copy MVAT7 VSG gene and upstream region. The coding region for MVAT7 VSG is shaded. The start and stop codons are indicated (***). The splice acceptor and polyadenylation sites of the mRNA are denoted by ovals with SL and An, respectively. The 73-bp promoter for the MVAT7 VSG gene is indicated by the box from nucleotides –67 to +6. Nucleotide +1 in the MVAT7 promoter is the mapped transcription start site. Restriction sites used in the Southern blot analysis (Fig. 1) and for cloning of fragments for transient transfections (Fig. 2) are indicated. Oligonucleotide primers used to PCR-amplify fragment 2 are indicated by arrows A and B.
oligonucleotides complementary to the blocked oligonucleotide and to the RNA, and the resultant PCR products were cloned for subsequent analysis. Fig. 4B shows the sequence determination at the boundary between the oligonucleotide and the transcript sequence of one such cloned PCR product of the nascent MVAT7 VSG RNA. The 5' end of this RNA occurs at the same adenosine residue as was identified by the primer extension experiment shown in Fig. 4A. Furthermore, the nascent RNA of the MVAT7 VSG gene that served as a template for these experiments does not possess a spliced leader at its 5'-end.

Characterization of the MVAT7 Promoter in Vivo—Nuclear run-on assays were performed using nuclei isolated from MVAT7 bloodstream trypanosomes. Nascent RNAs synthesized from the nuclei were used to probe fragments flanking the identified MVAT7 promoter, including the MVAT7 VSG-coding region (Fig. 5). The results of these nuclear run-on assays demonstrate that the promoter identified above is active in MVAT7 bloodstream trypanosomes. A 1.6-kb PCR fragment (fragment 2 in Fig. 5) located ∼60 bp upstream of the identified promoter did not hybridize to the labeled nascent RNA. This fragment contains at least 1.1 kb of sequence downstream of the duplication crossover point that occurs between the HindIII and BclI site. Thus, if this region is transcribed in the expression site containing the duplicated VSG gene, it should hybridize to the labeled nascent RNA. The fact that it is not recognized by the nascent RNA indicates that, in MVAT7 bloodstream trypanosomes, transcription of the MVAT7 VSG gene begins downstream of this fragment, as expected if the identified promoter is the site at which the transcription is initiated. Also as expected, a 2.3-kb fragment (fragment 1 in Fig. 5) located between 4.0 and 6.3 kb upstream of the basic copy VSG-coding region is not transcribed. However, a fragment located ∼200 bp downstream of the promoter (fragment 3) and one containing the MVAT7 VSG-coding region (fragment 4) hybridize strongly to the nascent RNA, demonstrating active transcription of this region (the weak upper band in lane 4 is likely due to incomplete digestion of the plasmid). The apparent start of transcription is consistent with the presence of a monocistronic transcript for the MVAT7 VSG gene since there are no extensive open reading frames in the 1.5 kb between the promoter and the MVAT7 VSG-coding region. These results and the Southern blot data (Fig. 1) collectively indicate that a co-duplication of a promoter and a VSG gene has occurred, leading to the gene's activation.

Transcription of other bloodstream VSG genes has been shown to be α-amanitin-resistant, a characteristic of transcription catalyzed by RNA polymerase I (23). To determine whether the RNA polymerase that initiates transcription at the MVAT7 promoter is also resistant to α-amanitin, nuclear run-on experiments were performed with and without α-amanitin (compare the α-amanitin and (+) α-amanitin panels in Fig. 5). Consistent with other reports, the RNA polymerase that transcribes the MVAT7 VSG gene is resistant to α-amanitin. The negative effect of α-amanitin on synthesis of a presumed RNA polymerase II transcript is apparent in lane T, containing the tubulin gene.

Effects of Directed Mutations within the MVAT7 Promoter—In addition to the MVAT7 promoter described here, our laboratory has previously identified promoters for two other metacyclic VSG genes, encoding ANTat 1.3A, VSG 118, and VSG 221, from another trypanosome serodeme (5, 24). The promoters for three bloodstream VSG genes, encoding ANTat 1.3A, VSG 118, and VSG 221, from another trypanosome serodeme have been reported by other laboratories (25–29). Fig. 6A shows a comparison of these six known promoters for VSG genes. The three MVAT VSG gene promoters characterized in our laboratory display 42% sequence identity over a 77-bp region, with the
MVAT4 and MVAT7 promoters sharing 77% identity. The three bloodstream VSG promoters have a more dramatic 93% identity over a 75-bp region. When all six promoters are aligned, only 21% sequence identity is observed (CONSENSUS OF BOTH in Fig. 6A), with the largest identical stretches being two trinucleotides (CCA and AAA, respectively, in Fig. 6A). In view of this rather limited sequence identity, we targeted mutations to these two trinucleotide regions and a few other nearby random positions to assess their importance in promoter function.

Overlapping oligonucleotides were used to generate a 73-bp synthetic double-stranded MVAT7 promoter fragment (WT in Fig. 6B) and various mutant versions of this “wild-type” sequence. These fragments were ligated into the plasmid containing the luciferase gene as described for the experiments shown in Fig. 2. Each plasmid construct was tested for luciferase activity in a minimum of three independent transient transfection experiments, with some constructs tested in 10 or more separate transfection experiments (Fig. 6B). The activity of the 73-bp wild-type promoter was similar to that of the 5′IRES fragment shown in Fig. 2 and was designated as 100%. Mutations in either of the two conserved trinucleotides reduced promoter activity to about one-fourth (22–27%) of the 73-bp wild-type promoter (compare PM-M1 and PM-M2 with WT in Fig. 6B). Mutations in both trinucleotide regions had an additive effect, decreasing promoter activity to 7% of the wild-type promoter (PM-M3). In contrast, mutations at several random locations outside of the two conserved regions had a minimal effect on promoter activity, yielding values ranging from 81 to 130% of the wild-type promoter (compare PM-M4 through PM-M9 with WT in Fig. 6B). Unfortunately, none of these random mutations occurred at any of the locations of single-nucleotide conservation within the six promoters, so the significance of these positions could not be assessed. However, the results do clearly demonstrate the importance of the two trinucleotide segments in the activity of the MVAT7 promoter.

Contrasting reports exist on the effects of mutations at the actual transcription start sites of other trypanosome promoters. Mutations in the start site of the trypanosome rRNA promoter reduced its activity dramatically (30), whereas similar mutations had negligible effects on the activity of the PARP gene promoter (26). Transcription of both of these genes is resistant to α-amanitin. Because of these differences, the effect of eliminating the MVAT7 start site was also investigated. Overlapping oligonucleotides were used to generate a 64-bp MVAT7 promoter that lacks the mapped transcription start site. When inserted into the expression plasmid, this 64-bp version possessed 21% of the activity of the 73-bp wild-type promoter (PM-M10 Fig. 6B). The vector sequence (GGGCGTCAC) that replaced the final 9 bp of the MVAT7 VSG gene promoter (GCCAGAAGAAA) does not contain a purine residue in the mapped transcription start site, but instead has a cytosine. Thus, the native transcription start site is not absolutely essential to retain promoter activity above background, and a neighboring purine is likely used as the transcription start site.

Mutations in the conserved trinucleotides of the 64-bp wild-type sequence (PM-M10) had effects similar to those of the same mutations in the 73-bp promoter, i.e. mutations in the trinucleotides caused promoter activity to decrease to 15–25% of their respective wild-type promoters (compare PM-M1 and PM-M2 with WT and PM-M11 and PM-M12 with PM-M10 in Fig. 6B). Mutations in the conserved region combined with the transcription start site deletion had an additive effect on promoter activity (PM-M11, PM-M12, and PM-M13 in Fig. 6B). Furthermore, mutations in the conserved regions of the 73-bp wild-type promoter had a similar effect on promoter activity as deleting the transcription start site (compare PM-M1, PM-M2, and PM-M10 with WT in Fig. 6B). These results clearly demonstrate the importance of both the conserved trinucleotides and the transcription start site for full promoter function.

**DISCUSSION**

Previously, we have examined the bloodstream re-expression of two other single-copy MVAT VSG genes in the same trypanosome serodeme. The MVAT4 VSG gene was found to be activated in situ and transcribed into a monocistronic precursor RNA (5). The MVAT5 VSG gene was shown to be duplicated and transposed downstream of 70–76-bp repeats at a conventional bloodstream VSG gene expression site, where it is transcribed as part of a large polycistronic precursor RNA (6). Here, we describe the bloodstream activation of the single-copy MVAT7 VSG gene, which is co-duplicated with its own promoter to another telomere-linked site, where it is transcribed into a monocistronic precursor RNA. These different bloodstream re-expression events for the three MVAT VSG genes are summarized in Fig. 7. To our knowledge, only one other example of the cotransposition of a VSG gene and a promoter has been reported (31). In that case, the duplicative transposition of a 6.5-kb fragment containing VSG 118 to a telomere-linked expression site appeared to activate a cotransposed promoter on the same fragment that was located ~3.5 kb upstream of the VSG gene. Subsequent studies, however, indicated that this cotransposed promoter was “subsidiary” to two tandem promoters already in the expression site at locations ~45 kb upstream of the transposed VSG gene (27, 32). In the MVAT7 case described here, the nuclear run-on experiments (Fig. 5) demonstrate that no transcription occurs immediately upstream of
the cotransposed promoter, indicating that only this promoter is active at this expression site.

Although the telomere-linked MVAT4 and MVAT7 VSG genes both give rise to monocistronic precursor RNAs, there are differences in their expression sites. An ESAG I is located 5 kb upstream of the MVAT4 VSG gene, whereas a corresponding ESAG is not present in the 7 kb upstream of the basic copy MVAT7 VSG gene. The promoter for the MVAT4 VSG gene was already at its bloodstream expression site prior to its activation, whereas the promoter for the MVAT7 VSG gene arrived at its bloodstream expression site along with the gene. Computer analyses of the sequences of the two expression sites demonstrate that they do not share substantive sequence similarities other than those in the promoters (Fig. 6 A) and the 3′-ends of the VSG genes (data not shown). Despite considerable effort by a number of laboratories, the actual molecular mechanisms that activate the promoter in one telomere-linked expression site and silence all other telomere-linked expression sites remain a mystery. In bloodstream trypanosomes, these mechanisms are likely unrelated to whether the primary transcript of the expression site is polycistronic or monocistronic. Instead, DNA transfection/integration experiments suggest that a form of telomere silencing, similar to that studied in yeast and other organisms, may be a dominant factor regulating VSG gene expression (33, 34). Thus, the main function of VSG gene rearrangements near telomeres may be to place a VSG gene in an “on deck” location downstream of a sequence that can serve as a promoter when the chromatin structure at that telomere is such that transcription is permitted.

Sequence alignment of the six reported promoters for VSG genes led to the identification of a consensus “core” sequence (Fig. 6A). An earlier consensus sequence of bloodstream VSG gene promoters and the PARP promoter, derived by Van der Ploeg and co-workers (26), consisted of two conserved regions located at positions 270 to 260 and positions 222 to 210 upstream of the transcription start site. The importance of these two segments was shown experimentally by linker scanning mutagenesis of the PARP promoter. Deletion of either of the two conserved regions had dramatic effects on promoter activity as measured by transient transfection analysis. Similarly, we found that two segments in the MVAT7 promoter, located at positions 249 to 247 and positions 218 to 216, are both necessary for full promoter function. These results are also consistent with experiments in which different segments of the PARP and rRNA promoters were exchanged, indicating that two regions are important for the functioning of the promoters used by the 15-amanitin-resistant RNA polymerase(s) in trypanosomes (35). However, the directed mutations in the MVAT7 promoter (Fig. 6B) did not give the same results as those obtained when 10-bp linker scanning mutagenesis was conducted on the MVAT4 promoter (5). In these earlier linker scanning studies, the replacement of each of several different 10-bp segments in the 73-bp MVAT4 promoter with a linker resulted in a loss of 95% of promoter activity. Although the reason for this difference in the outcomes of directed mutagenesis versus linker scanning of the MVAT promoters is not clear,
replacing 10-bp segments may have altered additional important sequences in the MVAT4 promoter that were not affected in the directed mutagenesis experiments on the MVAT7 promoter. Thus, the precise introduction of mutations into the trypanosome promoters is likely to be the preferred approach in the future for identifying nucleotides crucial for VSG gene promoter activity.

Finally, no nucleotide differences were observed when the MVAT7 VSG cDNA sequence was compared with the genomic sequence of the single-copy MVAT7 VSG gene in trypanosomes expressing other VSGs. Thus, the duplicated MVAT7 expression-linked copy (ELC) gene is a faithful copy of its basic copy donor gene. This scenario differs dramatically from the bloodstream re-expression of the MVAT5 VSG gene, in which each of three independently expressed ELC genes was found to have point mutations in the coding region compared with its donor gene (6). It is not clear why the duplicative transposition of one telomere-linked donor MVAT VSG gene would be accompanied by point mutations, whereas a similar duplication of another such gene would not, but several possible explanations exist. Perhaps the simplest alternative is that VSG genes can be duplicated by point mutations, whereas a similar duplication of another VSG gene (6). It is not clear why the duplicative transposition of one telomere-linked donor MVAT VSG gene would be accompanied by point mutations, whereas a similar duplication of another such gene would not, but several possible explanations exist. Perhaps the simplest alternative is that VSG genes can be duplicated by point mutations, whereas a similar duplication of another such gene would not, but several possible explanations exist. Perhaps the simplest alternative is that VSG genes can be duplicated by point mutations, whereas a similar duplication of another such gene would not, but several possible explanations exist. Perhaps the simplest alternative is that VSG genes can be duplicated by point mutations, whereas a similar duplication of another such gene would not, but several possible explanations exist. 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Co-duplication of a VSG Gene

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