**Trypanosoma brucei** undergoes antigenic variation by periodically switching the expression of its variant surface glycoprotein (VSG) genes (vsg) among an estimated 20–40 telomere-linked expression sites (ES), only one of which is fully active at a given time. We found that in bloodstream trypanosomes one ES is transcribed at a high level and other ESs are expressed at low levels, resulting in organisms containing one abundant VSG mRNA and several rare VSG RNAs. Some of the rare VSG mRNAs come from monocistronic ESs in which the promoters are situated about 2 kilobases upstream of the vsg, in contrast to the polycistronic ESs in which the promoters are located 45–60 kilobases upstream of the vsg. The monocistronic ES containing the MVAT4 vsg does not include the ES-associated genes (esag) that occur between the promoter and the vsg in polycistronic ESs. However, bloodstream MVAT4 trypanosomes contain the mRNAs for many different ESAGs 6 and 7 (transferrin receptors), suggesting that polycistronic ESs are partially active in this clone. To explain these findings, we propose a model in which both mono- and polycistronic ESs are controlled by a similar mechanism throughout the parasite’s life cycle. Certain VSGs are preferentially expressed in metacyclic versus bloodstream stages as a result of differences in ESAG expression and the proximity of the promoters to the vsg and telomere.

African trypanosomes are protozoan parasites that evade the immune response of their mammalian hosts by periodically switching the major protein on their surface, the variant surface glycoprotein (VSG)1 (for recent reviews, see Refs. 1–5). Individual VSG genes (vsg) are transcribed from 20–40 expression sites (ES), each of which is located near a chromosome telomere. The vsgs are maneuvered into the telomere-linked ESs by duplicative translocation or telomere exchange (6, 7). The switch involves either the arrival of a newly duplicated vsg in an active ES (gene conversion) or the activation of another ES already containing a vsg (*in situ* activation).

During their transmission between the tsetse fly vector and a mammalian host, African trypanosomes undergo a series of transformations and pre-adaptive changes (8, 9). The VSG is first expressed at the metacyclic stage in the salivary glands of the tsetse fly as a pre-adaptive measure for entering an unknown host and initiating an infection. The metacytic trypanosomes are a heterogeneous population expressing a small subset (15 to 20) of the vsg repertoire called the metacyclic variant antigen type (MVAT) vsgs (10, 11). After trypanosomes enter the bloodstream of their host, they continue to express the MVAT vsgs for up to 7 days and then switch to expression of a non-MVAT vsg (12). Occasionally, the MVAT vsgs are re-expressed in the bloodstream stage late in infection (reviewed in Ref. 13).

A typical bloodstream ES is composed of a 45–60 kb polycistronic transcription unit that, in addition to the telomere-linked vsg, contains a minimum of eight ES-associated genes (esag). In contrast, the MVAT vsg ESs are composed of monocistronic transcription units that are devoid of esags. A common observation about trypanosome antigenic variation is that one and only one of the ESs is usually activated at a given time in a given bloodstream trypanosome (14). This conclusion is based primarily on Northern blots of RNA from pure trypanosome clones in which only one VSG mRNA species is detected and nuclear run-on assays showing that only one vsg is transcribed in isolated nuclei of a bloodstream trypanosome clone. Yet, if a second vsg is placed in an activated ES using recombinant DNA techniques, equal amounts of both VSG molecules appear on the surface of bloodstream form trypanosomes (15). Furthermore, trypanosomes expressing two VSGs simultaneously occur naturally during the switch from one VSG to another (16, 17).

In earlier experiments, we found that about 4% of the cDNAs in a cDNA library of the MVAT4 bloodstream clone of *Trypanosoma brucei rhodesiense* encode the MVAT4 VSG (2000 out of 50,000 cDNAs screened), a result consistent with earlier estimates that the VSG and its mRNA represent 5–10% of the total protein and mRNA in bloodstream trypanosomes (6). However, partial sequence determinations of about 500 random cDNAs in this same library revealed three cDNAs that encode non-MVAT4 VSGs based on known amino acid similarities shared among VSGs (18). This result led us to re-examine the assumption that in bloodstream trypanosomes all of the telomere-linked vsg ESs are silent except for one. We initially treated either bloodstream or cultured procyclic trypanosomes with ultraviolet (UV) irradiation which enhances the amounts of some pre-mRNAs and mRNAs in trypanosomes. The molecular mechanisms underlying this enhancement are not completely understood but UV irradiation has been shown to inhibit...
Leaky Transcription of Trypanosome VSG Genes

RESULTS

The Purification of the Trypanosome Clones—Interpretation of the data shown below depends heavily upon the extent to which the cloned bloodstream MVAT4, MVAT5-Rx2 and WRATat 1.1 parasites were free from contamination with parasites expressing other VSGs. To examine this question, IFA of the bloodstream trypanosomes were conducted using mAb directed against various VSGs as described under “Experimental Procedures.” In the case of the MVAT4 bloodstream parasites, whose RNA was used for the Northern blots shown in Fig. 3, more than 99% of the organisms were recognized by the MVAT4 mAb, and of the 5,000 organisms examined none were recognized by the MVAT5 or MVAT7 mAbs despite the fact that low levels of MVAT5 and 7 VSG RNAs was present. Similarly, the WRATat 1.1 parasites, whose nuclei were used for the run-on experiments shown in Fig. 2, were not contaminated with parasites expressing MVAT4, 5, or 7 VSGs at a level detectable by IFA.

Analyses of Nascent VSG Transcripts in Nuclei of Bloodstream and Procyclic Trypanosomes—Fig. 1 depicts the expression sites and transcripts for four different basic copy vsgs expressed in the WRATat serodeme of T. brucei rhodesiense. Each of the four telomere-linked vsgs is shown as it exists in the genome of WRATat 1.1 trypanosomes. The WRATat 1.1 vsg is preceded by an upstream “barren” region of 25 or more kilobases that is composed predominantly of 70-bp repeats, similar to that of several other characterized bloodstream vsg ESs. Its transcription unit appears to be very large and initiated upstream of the 70-bp repeats, consistent with the expression of two other documented bloodstream vsgs whose primary transcripts are 60 and 45 kb, respectively (30–32).

The genes for MVAT VSGs 4, 5, and 7 are typically expressed during the metacyclic stage, the final developmental stage of the parasite in its insect vector (33, 34). Previously, however, we cloned rarely occurring bloodstream parasites expressing each of these VSGs (26, 28). In the MVAT4 bloodstream clone, the MVAT4 vsg is expressed from the same site as that depicted in Fig. 1 (26). In contrast, in three independently isolated MVAT5 bloodstream clones (called MVAT5-Rx1, -Rx2, and -Rx3), the MVAT5 vsg shown in Fig. 1 is duplicated and expressed from another telomere-linked ES containing upstream 70-bp repeats similar to those in front of the WRATat 1.1 vsg (27, 28). Likewise, in an MVAT7 bloodstream clone, the MVAT7 vsg is expressed from a duplicated gene copy (35) but in this case, the duplicated segment includes the promoter shown in Fig. 1.

About 2 kb upstream of each of the three telomere-linked MVAT vsgs shown in Fig. 1 is a 70–80 bp sequence, indicated by a flag, that possesses promoter activity when placed in front of a luciferase reporter gene on a plasmid that is transfected transiently into trypanosomes (26, 35, 36). The MVAT5 and 7 vsg promoters share more than 90% identity, and the MVAT4 vsg promoter has about 50% identity with the other two (13, 36). The MVAT4 and 7 vsg promoters are used by the bloodstream trypanosomes expressing these two vsgs (26, 35). The MVAT5 promoter depicted in Fig. 1 is not used by the duplicated MVAT5 vsg in bloodstream MVAT5 trypanosomes because the upstream boundary of the duplicated segment occurs between the indicated promoter and the start codon (27). However, this MVAT5 promoter and the other two MVAT promoters indicated in Fig. 1 are thought to be active in metacyclic trypanosomes expressing that particular vsg (13, 34, 36). The promoter lying far upstream of the WRATat 1.1 vsg has not been identified.

While analyzing the VSG transcripts in nuclei of bloodstream trypanosomes expressing these VSGs, we noticed that nascent RNA from supposedly silent, telomere-linked vsgs...
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**Fig. 1.** Physical maps of the WRATat 1.1, MVAT4, MVAT5, and MVAT7 vsgs in the WRATat 1.1 genome. The black circles represent the telomeres, the jagged lines are 70-bp repeats, and the triangular flags are promoters. Gray boxes depict genes. The wavy horizontal line with an arrow indicates the primary transcript of the WRATat 1.1 vsg ES. Dashed lines with arrows depict low-level transcription from the indicated promoters in WRATat 1.1 organisms. Brackets underneath the maps labeled 1.1, U4, M4, a, etc. indicate either cloned PCR products (U4 and U7) or restriction fragments that were probed in the gels shown in Fig. 2. The indicated vsgs are -1.4 kb in length. Restriction sites are shown for HindIII (H), PstI (P), Sau3A (Sau), SphI (Sp), XhoI (X), ClaI (C), and BamHI (B).

could be detected if the bloodstream parasites were first treated with UV irradiation. The use of UV irradiation for mapping transcription units is based on the inability of RNA polymerase to traverse pyrimidine dimers created in the DNA by the irradiation. Thus, synthesis of a long RNA is more sensitive to UV inactivation than is synthesis of a short RNA (19, 31). In addition, it has been shown that UV irradiation also modifies pre-mRNA processing and inhibits mRNA decay in trypanosomes (19, 20).

An example of the use of nascent RNA from the nuclei of UV-treated parasites to probe Southern blots of different vsgs is shown in Fig. 2. The agarose gel shown in the left panel of autoradiographs (Fig. 2, top panels) derived from bloodstream WRATat 1.1 trypanosomes (top row of autoradiograms) or from procyclic trypanosomes (bottom row) derived from bloodstream trypanosome clone MVAT5-Rx2. Before nuclei isolation, the parasites were subjected to either no UV irradiation or to 50 mJ/cm² UV irradiation. In addition, the nuclei incubations with α-amanitin (200 μg/ml) were conducted in the absence or presence of α-amanitin (200 μg/ml).

The WRATat 1.1 bloodstream RNA synthesized in the absence of both UV irradiation and α-amanitin (Fig. 2, top panel, labeled 0 mJ/cm²) hybridized the genes for the WRATat 1.1 VSG and tubulin, as expected. This bloodstream run-on RNA also hybridized to the PARP gene, as has been shown previously (37, 38), even though mature PARP mRNA is not present in bloodstream trypanosomes. In addition, some hybridization to the MVAT7 vsg can be detected, and very weak signals to the MVAT4 and 5 vsgs are present upon long exposure (not shown). When α-amanitin was present during the nuclei incubation, transcription of the tubulin genes was greatly reduced, but transcription of the PARP genes and the WRATat 1.1 vsg was relatively unaffected, indicating that their transcription is mediated by an α-amanitin-resistant RNA polymerase complex, as demonstrated previously (39–41). In this particular experiment, transcription of the MVAT7 vsg in WRATat 1.1 bloodstream nuclei also appears to be reduced by α-amanitin, but this result is probably because of a flaw in the blot with no α-amanitin since in other experiments it repeatedly was unaffected by α-amanitin (see top row, second panel).

The run-on RNA isolated from WRATat 1.1 bloodstream nuclei exposed to UV irradiation displayed a different hybridization profile (Fig. 2, top panels labeled 50 mJ/cm²). Very little hybridization occurred to the WRATat 1.1 vsg, indicating that the initiation site for its transcript is located far upstream. Likewise, tubulin transcription was greatly reduced by this amount of irradiation, but PARP transcription was not, reflecting the smaller size of the primary PARP transcipts. In contrast, hybridization to the MVAT4, 5, and 7 vsgs increased relative to that of RNA synthesized without UV treatment. In addition, no hybridization occurred to fragments that lie upstream of the MVAT vsg promoters, i.e., fragments U4 and U7, indicating that the transcription was initiated at these promoters. Supporting this conclusion is the finding that the UV-enhanced transcription is relatively unaffected by α-amanitin (Fig. 2, last panel, top row), indicating that it is mediated by the same α-amanitin-resistant RNA-polymerase complex that synthesizes VSG and PARP RNAs (37, 39, 42).

The same experiments were conducted with nuclei from procyclic trypanosomes (Fig. 2, bottom row). These hybridizations showed that the α-amanitin-resistant PARP RNA was unaffected by UV treatment, similar to that seen in bloodstream nuclei. Likewise, tubulin transcription was diminished by α-amanitin, similar to that observed in bloodstream nuclei, although in this particular experiment it was reduced less by UV irradiation than in other experiments and in the bloodstream examples shown in the top row. In procyclic trypanosomes no transcription of any of the vsgs was observed under any of the conditions (all four panels in the bottom row). Thus, UV treatment does not enhance detection of RNA from the telomere-linked MVAT vsgs in procyclic organisms as it does in bloodstream trypanosomes.

Similar experiments using nuclei from other bloodstream trypanosome clones yielded results similar to that shown in the top row of Fig. 2. For example, run-on RNA prepared from UV-treated bloodstream MVAT4 nuclei hybridized to the MVAT5 and MVAT7 vsgs, and run-on RNA from UV-treated bloodstream MVAT7 nuclei hybridized to the MVAT4 and 5 VSG genes (not shown). However, these UV-treated run-on...
RNAs from the MVAT trypanosome clones did not hybridize to the WRATat 1.1 vsg, as expected since UV irradiation decreases the amount of its very long transcript (Fig. 2, top panel).

Thus, UV irradiation of bloodstream trypanosomes increases the amount of run-on RNA from those unexpressed, telomere-linked MVAT vsgs whose promoters are located about 2 kb from the start codon. The simplest explanation of this observation is that the promoters are close enough to these genes that UV treatment enhances their RNA abundance, rather than diminishes it as is the case for the WRATat 1.1 VSG RNA whose promoter is far upstream. Therefore, in the bloodstream stage, the monocistronic ESs appear to retain a low level activity even though they are not fully activated.

**Northern Blots of RNAs Derived from Silent VSG Genes**—Since the nuclear run-on experiments indicated that the monocistronic telomere-linked MVAT vsgs undergo a low level transcription in bloodstream trypanosomes expressing other vsgs, we looked for these rare transcripts on Northern blots. Fig. 3 shows a representative blot of MVAT4 bloodstream RNA probed with the MVAT4, 5, and 7 VSG coding sequence fragments (Fig. 1, M4, M5c, and M7). The autoradiograms of the M5c and M7 blots were exposed for 20 h and the autoradiogram of the M4 blot was exposed for 2 h. The same filters were stripped and reprobed for rRNA and tubulin RNAs to confirm equal loading in each lane (not shown). When either the M5c or M7 probe was used, a weak signal corresponding to a transcript of about 1.5 kb was detected which is the expected size of a mature VSG mRNA. Neither of these two probes encode the C-terminal homology region of the VSG (Fig. 1), eliminating...
Although the possibility of cross-hybridization with the MVAT4 VSG mRNA. These M5c and M7 signals increased by 2–3-fold (as scanned by densitometry) when the RNA was isolated from MVAT4 bloodstream parasites subjected to UV treatment (lanes labeled 50 mJ/cm²), consistent with the increased signal intensity observed in the nuclear run-on experiments of Fig. 2 when the RNA was isolated from UV-treated nuclei. As expected, the M4 probe gave a much stronger signal (about 100-fold more intense by densitometry) than either of the other two vsg probes since the RNA was isolated from trypanosomes expressing the MVAT4 VSG. A shorter exposure of the M4 blot indicated that UV treatment did not substantially increase the amount of the MVAT4 VSG mRNA, in contrast to the 2–3-fold increase seen in the low amounts of the MVAT5 and 7 mRNAs. None of the vsg probes hybridized to RNA from procyclic organisms (the P lanes), indicating, as did the nuclear run-on experiments, that no VSG RNA is synthesized at this developmental stage.

Similar results were obtained when these Northern blots were conducted with RNAs isolated from bloodstream WRATat 1.1 or MVAT5-Rx2 trypanosomes (not shown). WRATat 1.1 RNA contained low amounts of MVAT4, 5, and 7 VSG RNA, whereas MVAT5-Rx2 RNA contained low amounts of MVAT4 and 7 VSG RNA. However, no WRATat 1.1 VSG RNA was detected in the RNAs from non-WRATat 1.1 organisms, again probably because the promoter for the WRATat 1.1 vsg lies far upstream.

**MVAT4 cDNA Library Screenings**—To confirm that RNAs from supposedly silent, telomere-linked vsgs are transcribed, we used the M7 probe to screen about 50,000 recombinant cDNA clones in the MVAT4 bloodstream trypanosome cDNA library containing 4% MVAT4 VSG cDNAs. Three clones were detected with this M7 probe. These three phage DNAs were isolated and the sequences of their cDNA boundaries determined. All three cDNAs were found to encode the MVAT7 VSG and to contain a 5′ poly(A), and one was found to have a 5′ SL, collectively demonstrating that they are derived from mature MVAT7 VSG mRNAs. Thus, the ratio of cDNAs for the MVAT4 VSG versus the MVAT7 VSG in this cDNA library is about 200:1 to 1. This result is roughly consistent with the Northern blots shown in Fig. 3. About 100,000 clones in the same bloodstream MVAT4 cDNA library were also probed with an MVAT5 VSG cDNA and no positive clones were detected, suggesting that the MVAT5 vsg may be transcribed to a lesser extent in MVAT4 bloodstream organisms than is the MVAT7 vsg.

**ESAG 6 and 7 Gene Families**—The monocistronic transcription unit of the MVAT4 vsg ES does not contain any of the esags that are typically downstream of the promoters of the polycistronic ESs and are transcribed in concert with the active vsg. However, many different members of esag families are expressed in the bloodstream MVAT4 trypanosome clone. We have previously reported that the locations and expression of the esag family members in these organisms are not always linked to the vsg ESs (43). In addition, we found that multiple members of the esag family are transcribed in the bloodstream MVAT4 organisms (not shown).

The ESAG 6 and 7 genes encode two closely related subunits of a heterodimeric transferrin receptor located at the trypanosome flagellar pocket (44–46). Since the bloodstream trypanosomes take up iron in the form of transferrin from the blood of their mammalian hosts, the esag 6 and 7 products are likely to be essential for the survival of the parasites (22). Despite the fact that the MVAT4 vsg ES does not contain either esag 6 or 7, Steverding and Overath (23) have shown with Western blots and uptake assays that MVAT4 trypanosomes express functional transferrin receptors derived from esag 6 and 7. These genes are typically found in tandem immediately downstream of the promoters of the polycistronic vsg ESs and are expressed along with the vsg. In contrast to the esag 1 family, Southern blot analysis and screening of a bacteriophage P1 genomic DNA library indicate that esag 6 and 7 are only found in polycistronic vsg ESs.\(^2\) This observation raises the question of whether the transferrin receptor subunits in MVAT4 trypanosomes are derived from esag 6 and 7 of one or more partially active polycistronic ESs.

We first examined whether transcription of esag 6 and 7 in MVAT4 trypanosomes is resistant to α-amanitin, similar to that of vsgs. Subcloned fragments containing the coding regions of an esag 6 and an esag 7, kindly provided by P. Borst, were probed with radioactive RNA from MVAT4 nuclei incubated in the absence or presence of α-amanitin (Fig. 4). We found that, in contrast to the esag 1 family (43), transcription of esag 6 and 7 is resistant to α-amanitin. In addition, UV irradiation experiments indicated that transcription of esag 6 and 7 is initiated from nearby promoters (not shown), similar to that of esag 6 and 7 family members known to be located in the polycistronic ESs. These data are consistent with the possibility that multiple ESs are partially active in the MVAT4 trypanosome clone.

To determine if multiple esag 6 and 7 are expressed in MVAT4, the coding region of esag 6 was used to screen the MVAT4 cDNA library. About 200,000 cDNA clones were

\(^2\) G. Rudenko and C. Clayton, personal communication.
screened, resulting in identification of 243 positive clones of which 12 were randomly picked and sequenced. Eight of the cDNAs were found to encode ESAG 6 and four to encode ESAG 7. In the ESAG 6 group, four of the cDNAs have identical sequences, a representative of which, ESAG 6-a, is shown in Fig. 5B. An alignment of the nucleotide sequences of these 12 clones revealed that there are four different cDNAs in each group (Fig. 5).

Strikingly, one cDNA from each group is identical to the sequence of the corresponding gene in the AnTat 1.3A ES (5). This observation suggests that an AnTat 1.3A-like vsg ES is partially active in the MVAT4 trypanosomes. Compared with the MVAT4 cDNAs encoding members of the esag 1 and 3 families (43), the nucleotide sequences of the MVAT4 ESAG 6 and 7 cDNAs show a higher degree of homology to each other. This similarity is most evident in an alignment of their deduced amino acid sequences (Fig. 6). This comparison also indicates a region of hypervariability at amino acid positions 151–169, an observation also noted by Borst et al. (47) for other ESAG 6 and 7 amino acid sequences.

**DISCUSSION**

The most likely reason that the low-level transcription of supposedly "silent" telomere-linked vsgs was not detected in earlier studies is that the vsgs previously examined are transcribed from polycistronic ESs in which the promoters are located 45 kb or more upstream. In contrast, the promoters for telomere-linked MVAT4, 5, and 7 vsgs studied here are situated only about 2 kb upstream of the vsg start codons. The close proximity of the MVAT vsgs to their promoters also provided us with an experimental way to enrich for the VSG transcripts by UV irradiation. Since most if not all of the 15–20 metacyclic vsgs in the trypanosome genome (33, 48, 49) appear to be telomere-linked and expressed as monocistronic precursor RNAs from nearby promoters (26, 36, 50, 51), it is possible that low levels of at least 15–20 different VSG mRNA species are present in bloodstream trypanosomes.

At least five different non-MVAT4 VSG mRNAs occur at low levels in MVAT4 bloodstream trypanosomes, i.e. the MVAT5 and 7 mRNAs detected in the Northern blots shown in Fig. 3, and the mRNAs responsible for the three different poly(A)-
containing, non-MVAT4 VSG cDNAs detected by sequencing random cDNAs in the MVAT4 cDNA library (18). A reasonable prediction is that these other three VSG-encoding RNAs are also transcribed from promoters located a relatively short distance upstream of their respective \( vsg \)s. Southern blot analysis demonstrated that all of these \( vsg \)s are located near telomeres (not shown), but it is not known if they are expressed from monocistronic ESs or if their VSGs are normally expressed during the metacyclic stage. Likewise, the nuclear run-on experiments shown in Fig. 2 suggest that WRATat 1.1 bloodstream trypanosomes possess a low level of transcripts for the MVAT4, 5, and 7 VSGs. Thus, trypanosomes expressing a VSG from an ES with a far upstream promoter, such as the WRATat 1.1 \( vsg \) ES, also have low levels of other VSG RNAs. These data collectively support the notion that, in addition to the full activation of a specific ES promoter, many ES promoters are active at a low level in a given bloodstream trypanosome.

Two independent lines of evidence indicate that the low level transcripts of these supposedly silent \( vsg \)s are not due to small numbers of contaminating parasites expressing these other VSGs. First, the IFAs indicate that contaminating parasites are fewer than 1 in 5000, and they may be even fewer since it was not reasonable to inspect a larger number of parasites via this visual approach. Second, UV irradiation resulted in a 2–3-fold increase in the rare RNA from supposedly silent \( vsg \)s but not a corresponding increase in the abundant RNA from the active \( vsg \). If these rare VSG RNAs were derived from rare contaminating parasites expressing that VSG, then the rare VSG RNAs and the abundant VSG RNA should be equally affected by the UV irradiation. We have not formally eliminated still a third possibility that individual trypanosomes express only one or two minor VSG mRNAs whereas the cloned population as a whole expresses many minor VSG mRNAs, but there seems no reason to invoke this alternative scenario.

Further support for our interpretation of the data is provided by independent studies from several laboratories. Rudenko et al. (52) alluded to the detection of two non-221 \( vsg \) ES-derived cDNAs from trypanosomes that appear to be exclusively 221 expressors by IFA, and they speculated that a minor percentage of this 221 population might be a double expressor for another ES. Navarro and Cross (53) have shown that in the bloodstream trypanosome clone 221a the insertion of a drug-resistant gene 1 kb downstream of silent ES promoters confers a low level of drug resistance to the parasites. These investigators concluded that short-range transcription could be achieved from supposedly silent ESs. Vanhamme and Pays (54) reported that in bloodstream trypanosomes, reverse transcriptase-PCR experiments using primers specific for the con-

FIG. 6. A comparison of the deduced amino acid sequences encoded by the ESAG 6 and 7 cDNAs indicates a region of hypervariability. Comparison of the deduced amino acid sequences of the indicated esag 7 (panel A) and esag 6 (panel B) cDNAs. Dots indicate identical amino acids. Asterisks show the positions of the stop codons. Dashes depict gaps introduced to maximize homology. The brackets highlight a hypervariable region.
served regions at the beginning of the known polycistronic ESs resulted in a major and several minor PCR products. About 95% of the PCR products were derived from the active ES, and 5% of the PCR products came from other ESs. Finally, Ansorge et al. (55) report that in the bloodstream trypanosome clone expressing the polycistronic 222 vsg ES, the major ESAG 6 transcript is derived from the active ES, but 2–3 other minor ESAG 6 transcripts are derived from other ESs. The results described here are consistent with all of these observations and suggest that all bloodstream trypanosomes are actually low expressors of many vsg ESs and high expressors of one ES.

Since at least some of the low level VSG RNAs possess a 5' SL and 3' poly(A), it is likely that their respective VSGs are also synthesized at low levels. Thus, the trypanosome must cope with the presence of minor species of VSG. Less clear is whether these minor VSGs actually acquire a glycolipid anchor and reach the surface of trypanosomes to be integrated into the VSG coat. Since this coat is composed of closely packed VSG homodimers (15, 56, 57), an occasional heterologous VSG molecule might cause localized distortion of the homogeneous VSG array and need to be discarded. In this regard, bloodstream trypanosomes possess an abundant glycosylphosphatidylinositol phospholipase C in the endoplasmic reticulum whose biological function(s) is not known (58). One of the possible roles for this enzyme might be to release from the glycosylphosphatidylinositol anchor any heterologous VSGs that do not assemble into a homodimer in the endoplasmic reticulum, resulting in the intracellular degradation of the heterologous VSGs.

It is not clear how far the low level transcription extends downstream of the promoters in the large polycistronic vsg ESs. Nonetheless, it is likely that this low level transcription rarely if ever reaches the vsg 45–60 kb downstream. In support of this view, we did not detect any WRATat 1.1 VSG RNAs in non-WRATat 1.1 bloodstream organisms. Furthermore, the expression profile of the different esag families in MVAT4 trypanosomes argues against far extended transcription from the polycistronic ES promoters. First, in the MVAT4 cDNA library, the cDNAs encoding ESAGs 6 and 7 are 2- and 7-fold more abundant than those of ESAGs 1 and 3, respectively. Second, while transcription of esag 6 and 7 is resistant to α-amanitin (500 μg/ml), transcription of the esag 1 family is sensitive to α-amanitin (200 μg/ml). Finally, insertion of a drug-resistant gene close to the vsg of a silent ES does not result in any detectable expression, in contrast to an insertion immediately downstream of the promoters (53, 59).

We have combined the findings of our laboratory and several others into a model outlining the regulation of both monocistronic and polycistronic ESs throughout the T. brucei life cycle (Fig. 7). In procyclic organisms, the promoters of large polycistronic ESs are active but the transcription is aborted a short distance from the promoters (Fig. 7). In procyclic organisms, the promoters of large polycistronic ESs are active but the transcription is aborted a short distance from the promoters (short dotted lines in the top box). In contrast, transcription of the short monocistronic ESs is highly repressed in procyclic organisms. This observation suggests that a form of telomere silencing may be in place during this phase. As trypanosomes transform into the metacyclic forms, the tight repression of monocistronic metacyclic vsg ESs is lifted, leading to the reexpression of these ESs. The model indicates that the transcription of ESs is regulated at the level of gene expression, and that the expression of ESs is coordinated with the developmental stage of the parasite.

**Table: ES Promoter Activity and References**

| Activity | References |
|----------|------------|
| −        | This paper, accompanying paper, and 51 |
| +        | 52         |
| +        | This paper, accompanying paper |
| +        | This paper, 38, 43 |
| +/-      | This paper |
| +/−      | This paper, 53, 54, 55 |

**Fig. 7. A model for the regulation of both mono- and polycistronic ESs throughout the T. brucei life cycle.** Boxes depict single trypanosome cells from the indicated developmental stages. Each box shows four representative ESs, two monocistronic and two polycistronic. Black circles represent the telomeres. Small rectangles depict genes, with the one closest to the telomere being the vsg and the others representing esags. Flags depict promoters. Short dotted lines without arrowheads show abortive transcription. The wavy lines with arrowheads depict the primary transcripts of the fully active ESs, whereas the dotted lines with arrowheads indicate either basal low level transcription (+/−) or partial activation (++) at other ESs. The curved arrow denotes that in the case of bloodstream trypanosomes re-expressing the MVAT4 VSG, the relative expression of the different ESs mimics that which we suspect to occur in metacyclic organisms.
to a heterogeneous population of trypanosomes, each expressing a specific MVAT vsg at a high level and other MVAT vsgs at low levels (Fig. 7, middle box). Thus, different monocistronic metacyclic ESs are fully activated in different cells, although the event(s) that fully activates only one metacyclic ES and nearly, but not completely, represses the others is not known. The monocistronic ESs also become partially activated resulting in expression of RNAs encoding many different ESAGs 6 and 7, and perhaps additional ESAGs (dotted lines with arrows). This situation provides the metacyclic parasites with a significant advantage in initiating a successful infection when they enter the bloodstream of an unknown host because they are expressing diverse products of the esag(s) (e.g., the different transferrin receptor products of esag 6 and 7). After entering the bloodstream of a mammalian host, trypanosomes transform to the rapidly dividing bloodstream forms and after 6–7 days, they switch to expression of a specific polycistronic ES. This switch appears to be host-specific and related to the specific sets of the esag repertoire in given ESs (2, 22, 60). Recent reports by Gerrits et al. (60) suggest that this process is driven by the host’s immune response selecting against trypanosomes with low-affinity transferrin receptors. As a result, one ES is expressed at high levels, whereas others retain a low level of activity. Once again, the underlying molecular mechanism(s) ensuring full expression of a single ES is not known. Occasionally, a switch to a monocistronic ES occurs (e.g. MVAT4), but the resulting parasites are less adept at surviving for the reasons mentioned above.

A major theme of this model is that both mono- and polycistronic ESs are regulated by an epigenetic mechanism, as proposed by a number of other investigators for polycistronic ESs (53, 54, 61), rather than being sequence-specific. This interpretation differs from that of Barry and colleagues (51, 62) who, based on the study of the ESs for the two metacyclic vsgs 1.22 and 1.61 in another trypanosome serodeme, have proposed that metacyclic ESs are regulated by life cycle stage-specific control mechanisms, and that in the procyclic organisms, the main level of this control is exerted via cis-acting promoter sequences. It is worth recalling that in general the studies of vsg activation in metacyclic trypanosomes have been hampered by technical limitations, mainly an insufficient number of cells available for molecular analysis and the inherently high VSG switch rates of the tsetse fly transmissible lines (62). In order to obtain a sufficient number of cells, these investigators used “metacyclic-derived” cells amplified in the bloodstream of laboratory animals, taking advantage of the fact that the fly-transmitted trypanosomes, despite having differentiated to bloodstream forms, continue to express the metacyclic vsgs for as long as a week. Nonetheless, these rapidly changing early bloodstream trypanosomes may not reflect the molecular events in the metacyclic stage. It is also worth noting that the initial characterization of the MVAT vsgs in our laboratory was conducted using metacyclic-derived amplified bloodstream cells (33, 34). In fact, our search for the bloodstream re-expressors of the MVAT vsgs started as a way of avoiding the technical problems discussed above. As suggested by Barry et al. (62), the development of single-cell reporter techniques will be necessary to overcome these technical difficulties and permit a direct examination of true metacyclic organisms.

When Graham et al. (63) placed the promoter of the metacyclic vsg 1.22 at a chromosome internal position or onto an episcopal vector in bloodstream trypanosomes, it was highly active. Thus, the main difference between our observations (26, 35, 36) and those of Barry and colleagues (51, 62) is the relative activity of MVAT vsg promoters demonstrated in procyclic trypanosomes in transient transfection experiments. In our hands, the MVAT4, 5, and 7 vsg promoters are highly active in procyclic organisms (as much as 200-fold above background), compared with vector sequences alone, when presented on plasmids introduced by transient transfection. In contrast, in similar experiments, the 1.22 and 1.61 vsg promoters appear to have relatively low levels of activity (about 5-fold above background). One possible explanation for this difference is that these promoters represent somewhat different sets of metacyclic promoters (62). Another possibility may involve differences in experimental procedures/conditions or, perhaps, trypanosome serodemes. A direct comparison of the activities of the MVAT4, 5, and 7 vsg promoters with those for 1.22 and 1.61 vsgs under the same experimental conditions is necessary to resolve this question.

Our data demonstrate that in bloodstream trypanosomes, the MVAT4 vsg is expressed without DNA rearrangements from the same monocistronic ES as in the metacyclic stage (26, 33, 34) and that de/activation of this ES occurs in a sequence-independent manner. Furthermore, in the bloodstream trypanosomes the other supposedly silent MVAT vsg ESs retain low levels of expression. Therefore, our findings argue against a strict life cycle stage-specific expression of the metacyclic vsgs. Instead, they collectively suggest that the MVAT vsg ESs are under similar control mechanisms as those of polycistronic ESs. Thus, the high level repression of the MVAT vsg ESs in the procyclic stage, their selective activation in the metacyclic stage, and their low level expression in the bloodstream stage are all likely controlled by an epigenetic mechanism and the close proximity of their promoters to the vsgs and telomeres.

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