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Variant antigen genes of *Trypanosoma brucei*: Genomic alteration of a spliced leader orphon and retention of expression-linked copies during differentiation

(life cycle/gene regulation/RNA/parasite)

MARILYN PARSONS*, RICHARD G. NELSON*, KENNETH STUART† AND NINA AGABIAN*

*Department of Biochemistry SJ-70, University of Washington, Seattle, WA 98195; and †Issaquah Health Research Institute, 1595 N. W. Gilman Boulevard, Issaquah, WA 98027

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ABSTRACT **Variant surface glycoprotein (VSG) gene expression in *Trypanosoma brucei* involves not only the sequential activation of individual VSG genes during mammalian bloodstream stage antigenic variation but also the regulation of gene expression during cyclic transmission through alternate mammalian and insect hosts. In the bloodstream stage, transcriptional activation of many VSG genes is correlated with the appearance of an additional copy of the gene in a novel genomic location, the expression-linked copy. The parasite loses the ability to synthesize VSG during differentiation from mammalian bloodstream to insect procyclic stage. Five different bloodstream populations were individually converted to procyclic forms. In each case, the procyclic cells retained the expression-linked copy of the bloodstream parent, and it remained in the same immediate genomic context. Transcripts homologous to the VSG structural gene exon were found in bloodstream stage RNA but not in procyclic RNA. Nevertheless, transcripts containing sequences homologous to the VSG mRNA spliced leader were abundant in both procyclic and bloodstream stage cells. When the genomic organization of sequences homologous to the VSG leader was examined, a specific alteration correlated with procylic differentiation was found. These data are discussed in light of biological studies on antigenic variation.**

African trypanosomes of the *Trypanosoma brucei* subgroup are parasites requiring both mammalian and insect hosts to complete their life cycle. The mammalian bloodstream stage trypanosome is coated with a single antigenic species, the variant surface glycoprotein (VSG) (1). Antigenic variation occurs during this stage of the trypanosome life cycle by the transcriptional activation of successive VSG genes from a repertoire of 300–1,000 VSG genes per trypanosome (2). The expression of these genes is also regulated during the life cycle of these protozoa (for review, see ref. 3). The transition from mammalian bloodstream form (BSF) to insect midgut or procyclic form, initiated when the tsetse fly ingests trypanosome-infected blood, involves changes not only in parasite morphology, metabolism, and infectivity (4) but also in the cell surface. The VSG coat, which protects the trypanosome in the mammalian host, is no longer synthesized (5). After proliferation in the insect midgut, trypanosome differentiation continues with migration to the salivary glands where metacyclic trypomastigotes eventually develop. Now infective to mammals, the trypanosomes have regained a VSG surface coat (5).

Although the mechanisms regulating VSG gene expression during the insect phase of the life cycle have remained unexplored, those that guarantee the successive activation of single VSG genes during BSF antigenic variation have been the object of much research and speculation. In BSFs, the transcriptional activation of many VSG genes is correlated with the appearance of a duplicate copy of the gene in a genomic location termed the expression site (6–8). The new gene copy, called the expression-linked copy (ELC), is transcribed (9) and is usually lost after a variation event. All mature VSG mRNAs examined thus far contain the same 35-nucleotide untranslated leader sequence at their 5′ termini (10, 11). Since this leader sequence is not encoded by silent copies of VSG genes or their immediate flanking sequences, it is presumed to be encoded by the expression site(s) and joined by RNA splicing to the VSG message (10, 11). The location of the spliced leader exon relative to the transposed gene is, however, still unknown.

The study of the regulation of VSG gene expression during cyclic transmission is hampered by the lack of biological material from several of the insect stages. However, under certain culture conditions, BSFs differentiate to procyclic forms, which can be subsequently propagated (12). These procyclic culture form (PCF) cells appear morphologically and biochemically identical to the trypanosomes found in the tsetse midgut (13) and, in particular, lack the VSG protein (13, 14). Thus, the molecular mechanisms leading to the cessation of VSG synthesis in the first stage of trypanosome differentiation in the fly can be studied.

In a preliminary analysis, we have found that the ELC of BSF variant antigen type (VAT) 11 of the IsTaR serodeme is retained in PCFs derived from VAT 11 (15). In this study the expression and genomic organization of VSG spliced leader and structural gene sequences were analyzed in five populations of PCFs (each derived from a different VAT) and their BSF parents. In every case, the ELC of the BSF parent was retained after procyclic differentiation. VSG structural gene transcripts were not detectable in PCF RNA, although spliced leader sequences were found. Analysis of the genomic organization of sequences homologous to the VSG spliced leader revealed a specific alteration correlated with procyclic differentiation.

MATERIALS AND METHODS

Trypanosomes. The construction of the IsTaR serodeme of *Trypanosoma brucei brucei* and the isolation of the VATs used here have been described (16). Total RNA and DNA were isolated from cloned antigenically homogeneous BSF trypanosomes of five different VATs (17). BSF cells from the same passage used for DNA isolation were converted to PCFs by *in vitro* culture (18). After approximately 2 months

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Abbreviations: BSF, bloodstream form trypanosome; ELC, expression-linked copy; PCF, procyclic culture form trypanosome; VAT, variant antigen type; VSG, variant surface glycoprotein; kb, kilobase(s); bp, base pair(s).

in culture, PCF cells were harvested and total RNA and DNA were isolated (17). Because heterogeneity was noted in VAT A, it was recloned before isolating DNA.

Southern and RNA blot analyses using VSG cDNA probes were carried out as described (17, 19). A 22-nucleotide synthetic probe complementary to a portion of the VSG mRNA spliced leader sequence was end labeled for use in hybridizations as detailed by Nelson *et al.* (20). The final stringency conditions of post hybridization washes for Southern blots probed with VSG cDNAs were 0.1 × SSPE (1× SSPE is 180 mM NaCl/10 mM NaH_2PO_4, pH 7.4/1 mM EDTA), 65°C; for RNA blots probed with VSG cDNAs, 0.3 × SSPE, 65°C; and for Southern and RNA blots probed with the 22-mer, 5 × SSPE, 37°C. The hybridization and washing conditions for experiments using the 22-mer have been shown to detect specific sequences in genomic DNA (20).

RESULTS

Genomic Organization of VSG Structural Genes in BSF and PCF Trypanosomes. Expression of VSGs by VATs A, 3, 5, 7, and 11 of the IsTaR serodeme is correlated with the appearance of an ELC (19). Antigenically homogenous populations of each VAT were individually converted to PCFs by *in vitro* culture. To determine the fate of the ELC after procyclic differentiation, the genomic organization of the VSG genes expressed by each of these BSF VATs was compared with that of their PCF descendents by Southern blot analyses.

Genomic DNAs from the BSF VATs and their PCF counterparts were cleaved with restriction enzymes, size fractionated by agarose gel electrophoresis, and hybridized with VSG cDNA probes after blotting to nitrocellulose (Fig. 1). Comparison of the hybridization patterns indicates that PCF trypanosomes still possess the ELC of their respective BSF parent. As shown in Fig. 1*A*, DNAs from VAT 7 and its procyclic counterpart PCF 7, as well as DNA from VAT 5 and PCF 5, were digested with both *Eco*RI and *Bam*HI and hybridized with a VSG 7 cDNA probe. The presence of an ELC in VAT 7 is evidenced by an additional high molecular weight fragment in VAT 7 DNA as compared with VAT 5 DNA. Hybridization to this fragment is weak because of slight degradation of the VAT 7 DNA. PCFs derived from VAT 7 retain the ELC. Similarly (Fig. 1*B*), hybridization of a VSG 11 cDNA probe to genomic DNA digested with *Bgl* I reveals a multigene family and an ELC in VAT 11. This ELC is also retained in PCF 11.

A slightly more complex picture is presented in the remainder of Fig. 1. It has been shown that regions 3′ to many VSG genes undergo length variation associated with trypanosome growth (21) independently of homologous gene expression (22). This variation occurs only around genes located near what appears to be a telomere (23, 24). The putative telomeric genes include both ELCs and many silent VSG genes (23, 24).

Fig. 1*C* presents the results for *Sst* I-digested DNA hybridized with a VSG 5 cDNA probe. Since the VSG 5 cDNA contains an internal *Sst* I site, two hybridizing fragments are expected and are observed for the ELC. The ELC fragment that contains 5′ sequences appears identical in size in PCF 5 and VAT 5, while that containing 3′ sequences is larger in PCF 5 than in VAT 5. In addition to this variation 3′ to the ELC, another larger fragment containing 3′ VSG 5 sequences also shows variation. Not only does the latter variable fragment differ in size between different VATs, it also differs in size between BSFs and their PCF counterparts, a phenomenon we have observed with other silent telomeric VSG genes (15). Thus the 12.4-kilobase (kb) fragment found in VAT 5 is increased to 13.4 kb in PCF 5 (where it comigrates with another, stable gene).

Similarly, a VSG A cDNA probe hybridized with genomic DNA digested with *Ava* I reveals one constant and one variable fragment in each sample (Fig. 1*D*). The VSG A cDNA probe also detects an ELC in VAT A that is retained, but it resides on a larger restriction fragment in PCF A. The variation associated with both genes was localized to sequences 3′ to the genes. Hybridization to the ELC is fainter in PCF A, a finding that could be explained by gradual loss of the ELC during the 2 months of procyclic culture or by heterogeneity introduced by telomeric variation. The latter hypothesis is favored because some telomeric heterogeneity was noted in the original VAT A used to generate PCF A (VAT A was recloned prior to these analyses).

Finally, the gene family revealed by the VSG 3 cDNA probe in *Pvu* II-digested DNA is large and, with the exception of one member, stable. The general picture is the same

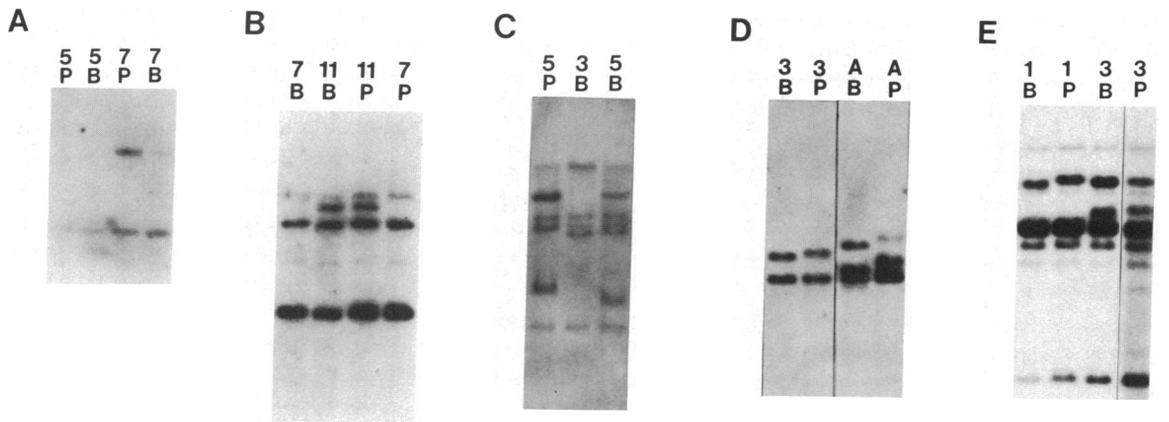

FIG. 1. Southern blot hybridization analyses of VSG genes of bloodstream stage cells (lanes B) and their procyclic counterparts (lanes P). Genomic DNA cleaved with restriction enzymes was separated on agarose gels, transferred to nitrocellulose filters, and hybridized with ^{32}P-labeled VSG cDNA probes (19). The restriction fragments containing the ELCs are marked by arrows and those containing silent telomeric genes, by asterisks. Hash marks indicate positions of the four largest DNA fragments produced by *Hin*dIII cleavage of phage λ DNA: 23 kb, 9.6 kb, 6.6 kb, and 4.4 kb. (*A*) VSG 7 ELC. DNA digested with *Eco*RI and *Bam*HI was hybridized with the 800-bp VSG 7 cDNA probe pTb1.7-c1. The probe contains no *Eco*RI or *Bam*HI cleavage sites. (*B*) VSG 11 ELC. *Bgl* I-digested DNA was hybridized with the 5′-terminal 750-bp VSG 11 cDNA probe pTb1.11-c4. The VSG 11 structural gene contains two *Bgl* I sites; the probe detects only sequences 5′ to these sites. (*C*) VSG 5 ELC. *Sst* I-digested DNA was hybridized to the VSG 5 cDNA pTb1.5-c1, which contains an *Sst* I site. Restriction fragments containing the 5′ and 3′ portions of the ELC are marked. (*D*) VSG A ELC. *Ava* I-digested DNA was hybridized with the 600-bp VSG A cDNA pTb1.A-c1. None of the VSG A genes contain an *Ava* I site, although all have *Ava* I sites immediately 5′ to the coding regions. (*E*) VSG 3 ELC. *Pvu* II-digested DNA was hybridized with a fragment of the VSG 3 cDNA pTb1.3-c2. The VSG 3 structural gene contains a *Pvu* II recognition site, and the 700-bp probe detects only sequences 3′ to the site.

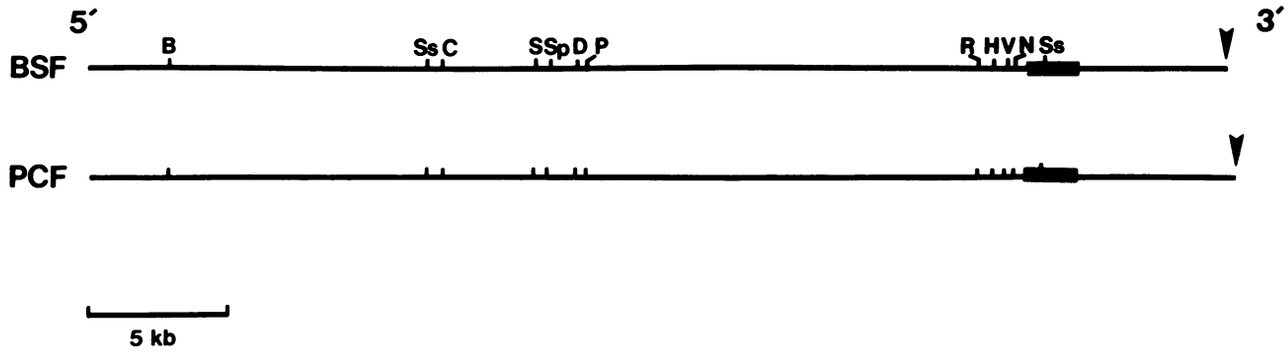

FIG. 2. Genomic restriction map of the regions flanking the VSG 5 ELC in VAT 5 BSFs and PCFs. The arrow marks the point where several restriction enzymes appear to cleave. In BSFs this point is 6.5 kb from the *Sst* I site in the VSG 5 coding sequence, while in PCFs it is 6.85 kb. B, *Bam*HI; C, *Cla* I; D, *Hin*dIII; H, *Hin*cII; N, *Bst*NI; P, *Pst* I; R, *Rsa* I; S, *Sal* I; Sp, *Sph* I; Ss, *Sst* I; V, *Pvu* II. The coding sequence is designated by the thickened line.

as that observed with the VSG A cDNA probe: the ELC is retained, but the size of the restriction fragment on which it is located increases. This variation also occurs in sequences 3′ to the gene.

To compare the ELCs more carefully, we examined the genomic environment of the VSG 5 ELC in BSF and PCF trypanosomes by restriction enzyme mapping (Fig. 2). We found that all restriction sites 3′ to the VSG 5 ELC mapped to the same point, located 6.5 kb from the internal *Sst* I site in the BSF ELC and 6.85 kb from this site in the PCF ELC. There are several restriction sites in the 2 kb immediately 5′ to the coding sequence, and these are in the same location in the PCF and BSF ELCs. This region contains the DNA segment that was duplicated and cotransposed with the structural gene during the formation of the ELC (unpublished results). Further 5′, a large region of DNA devoid of restriction sites is present in both PCF and BSF DNAs, followed by a region of "normal" DNA. Thus, the genomic regions 5′ to the ELC of the VSG 5 gene appear identical in PCF 5 and VAT 5. When the genomic regions containing other ELCs were compared in PCFs and BSFs, no alteration in sequences 5′ to the genes was observed, although variation 3′ to the genes was always detected.

VSG Structural Gene Transcripts. PCF trypanosomes neither possess a surface coat nor synthesize VSG yet retain the ELC. If any VSG mRNA is transcribed in PCFs it would most likely be that encoded by this ELC. Hence, we assessed the presence of the appropriate VSG gene transcripts in BSFs and PCFs by RNA blot analysis. The hybridization patterns of a VSG 11 cDNA probe with total RNA isolated from VAT 11 and PCF 11 are shown in Fig. 3. A VSG transcript of approximately 1.8 kb is detected in VAT 11 RNA. No VSG 11 transcripts were observed in PCF 11 RNA (nor in PCF or BSF RNAs derived from other VATs), even on prolonged autoradiography. Similarly, VSG 5 mRNA was abundant in VAT 5 cells but undetectable in PCF 5 cells (data not shown).

VSG Spliced Leader Transcripts. We then examined these cells for VSG spliced leader transcripts, to evaluate the relationship between leader and structural gene transcripts. After BSF cells were converted to PCFs, more than 50 generations elapsed before the isolation of PCF RNA, allowing substantial dilution of any unusually stable BSF RNAs. A 22-nucleotide probe homologous to a portion of the VSG leader (20) was used in RNA blot analyses such as those described above. As shown in Fig. 4, the 22-mer probe hybridizes to a continuous size spectrum ranging from approximately 0.7 to 4 kb in both PCF and BSF cells. These hybridizing sequences are sensitive to base hydrolysis, confirming they are RNA and not DNA. Several discrete bands of hybridization are visible, at least some of which are common to both samples. The prominent 1.9-kb species, however, is observed in BSF RNA only. Rehybridization of the same blot with the appropriate VSG cDNA probe also revealed a 1.9-kb RNA. Thus, although no VSG structural gene transcripts are observed in PCF cells, sequences homologous to the VSG spliced leader continue to be highly transcribed.

Genomic Organization of Sequences Homologous to the VSG Spliced Leader. We have shown that the DNA sequences homologous to the VSG mRNA spliced leader are highly reiterated in the trypanosome genome (20). Most members of this sequence family reside in a tandemly repeated 1.4-kb unit. However, a few homologous sequences are dispersed from the tandem repeat (20). We have examined

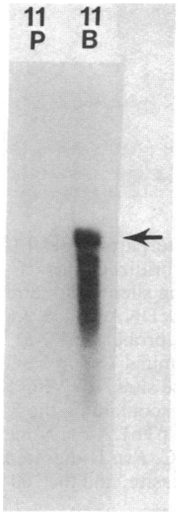

FIG. 3. RNA blot analysis of bloodstream stage (lane B) and procyclic stage (lane P) RNAs with the VSG structural gene probe. Ten micrograms of total RNA isolated from BSF 11 and PCF 11 was electrophoresed in a formaldehyde/agarose (1.4%) gel, transferred to a nitrocellulose membrane, and hybridized to the 630-bp VSG 11 cDNA pTb1.11-c1. This cDNA corresponds to the penultimate 3′ portion of the structural gene. The 1.8-kb VSG 11 mRNA is indicated by an arrow.

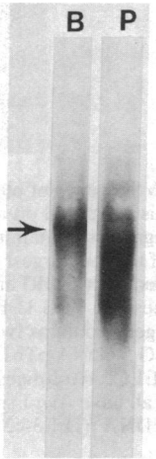

FIG. 4. RNA blot analysis of bloodstream stage (lane B) and procyclic stage (lane P) RNA with the VSG spliced leader probe. Nitrocellulose blots of total RNA (10 μg) isolated from VAT 3 and PCF 5 were prepared as described in Fig. 3. The blots were hybridized with an end-labeled 22-nucleotide synthetic probe complementary to the VSG mRNA leader sequence. The arrow indicates the position of the 1.9-kb VSG 3 mRNA found in 1.3 BSF RNA on rehybridization of this blot with a VSG 3 cDNA probe. Similar experiments using a synthetic 18-mer probe (homologous to another trypanosome sequence of interest) do not show hybridization to multiple RNA species, even at lower stringencies.

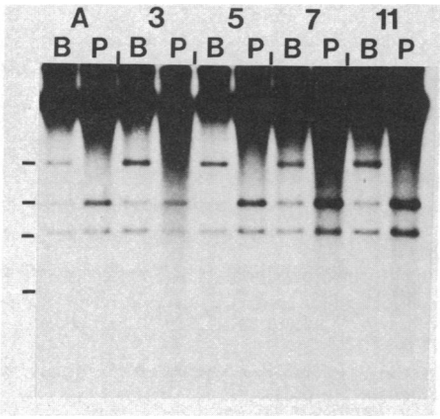

FIG. 5. Southern blot analyses of bloodstream stage (lanes B) and procyclic stage (lanes P) DNA with the VSG spliced leader probe. One microgram of *Eco*RV-digested DNA from each of the indicated VATs and their PCF descendents was fractionated on an 0.8% agarose gel, transferred to a nitrocellulose membrane, and hybridized to the end-labeled VSG leader probe as described by Nelson *et al.* (20). The hash marks indicate the positions of the 12-kb, 7.4-kb, 5.8-kb, and 3.5-kb VSG spliced leader orphons. Hybridization to the 3.5-kb fragment was weak.

the genomic organization of these orphon spliced leader sequences after differentiation of BSF trypanosomes to PCFs. As shown in Fig. 5, *Eco*RV-digested DNA from PCFs and their BSF counterparts hybridized with the VSG spliced leader probe. Since the 1.4-kb repeat unit does not contain an *Eco*RV site, the majority of the hybridizing sequences remain tandemly linked, migrating near the top of the gel. Orphon leader sequences are also revealed by *Eco*RV digestion. In BSF DNAs, four smaller fragments that hybridize to the leader probe are observed, with sizes of 12, 7.4, 5.8, and 3.5 kb (hybridization to the 3.5-kb fragment is weak). In PCF DNAs, only the latter three fragments are found. Thus, in each case, the 12-kb *Eco*RV spliced leader orphon found in BSFs disappears on procyclic differentiation.

DISCUSSION

The mechanisms controlling trypanosome VSG gene expression are complex, involving not only antigen switching in the mammalian bloodstream stage but also the regulation of surface antigen expression during cyclic transmission. Metacyclic populations introduced into the mammalian host by the fly are heterogeneous with respect to the VSG expressed, but the constellation of metacyclic antigens expressed by a given trypanosome stock appears similar regardless of which VAT was used to infect the fly (25). Prior immunization with a metacyclic population protects animals from homologous challenge, further suggesting that the metacyclic repertoire of VSGs is limited and essentially constant (26, 27). Since these animals are not protected against challenge with bloodstream populations derived from the same parasite clone, it appears that the bloodstream stage VSG repertoire differs substantially from the metacyclic repertoire. The first post-metacyclic populations to appear in the new host (5–7 days after fly bite) are considerably biased toward expression of the VAT originally ingested by the fly (28). What mechanisms provide for the cessation of VSG expression during the procyclic stage and activation of alternative VSG genes during the metacyclic stage, yet still allow the trypansome to retain a memory of prior bloodstream antigenic identity? In an initial effort to answer this complex question, we examined the fate of the ELC of five different bloodstream VATs after differentiation to PCFs.

We show that in each case the ELC is retained. This phenomenon is not restricted to the IsTaR serodeme, as the VSG 2 ELC of the IaTaR serodeme is also retained but not transcribed after procyclic differentiation (29). In contrast to the report of Pays *et al.* (7) showing that PCFs derived from AnTat VAT 1.1 have lost the 1.1 ELC, the retention of the ELC after procyclic differentiation appears to be a frequent event.

The genomic restriction fragments containing the PCF ELCs are slightly larger than those containing the homologous BSF ELCs. Preliminary experiments indicate that the telomeric fragments continue to increase in size during procyclic growth (unpublished results). This alteration occurs 3′ to the duplicated VSG gene, near a chromosome end. In other systems, sequences 3′ to genes have been shown to directly affect transcriptional activity (30). However, the distance between the VSG gene and the telomere varies among the ELCs of different subclones expressing the same antigen (19). In addition, such alterations occur continuously during BSF growth in the absence of antigenic variation (21). Since these telomeric size alterations occur in BSFs without obviously affecting VSG gene expression, the similar if not identical alterations we have observed in regions flanking PCF ELCs are probably not responsible for the inactivation of VSG genes.

The genomic restriction map of the VSG 5 procyclic ELC is identical to that of its BSF parent ELC for >22 kb 5′ to the gene. In *Trypanosoma equiperdum*, which does not undergo cyclic transmission (and therefore has no procyclic stage) (31), and more rarely in *T. brucei* (ref. 6; unpublished results), a previously expressed ELC may occasionally be retained in an inactive state after an antigen switch. Like the genomic maps of homologous BSF and PCF ELCs, the restriction maps 5′ to the homologous expressed and residual ELCs are identical (31). The mechanism by which these residual BSF ELCs are inactivated is not known. If there were only one bloodstream stage VSG expression site, it would appear that the residual BSF ELC has been physically removed from this site and replaced by a new, transcriptionally active VSG gene. Unfortunately a definitive molecular description of the expression site(s) remains elusive; for example, no sequences encoding the VSG mRNA spliced leader have been found linked to the ELC (20, 32). As with the residual BSF ELCs, the mechanism by which PCF ELCs are inactivated is not known. In any case, the mere presence of an ELC is not sufficient for VSG expression.

Although there are no stable VSG structural gene transcripts in PCF cells, the spliced leader sequence is transcribed in both BSF and PCF cells. Since the sequence encoding the spliced leader is highly repeated in the trypanosome genome, the significance of this finding is unclear. The leader may also be used on other structural genes. For example, a sequence immediately 5′ to the *T. brucei* α-tubulin gene shows substantial homology with the 35-nucleotide VSG mRNA leader (unpublished results). Like the VSG leader, this α-tubulin leader sequence is transcribed but not translated. Thus, although the presence of leader sequences in PCF RNA may reflect abortive VSG transcriptional starts or alternative RNA processing pathways, it cannot yet be determined whether the VSG leader itself [i.e., that leader sequence(s) postulated to reside in the VSG expression site(s)] is actually transcribed in PCFs. Alternatively, the leader sequences observed in PCF RNA may reflect the use of this sequence by non-VSG genes that are transcribed during insect stages of the life cycle.

Our demonstration of a specific alteration of an orphon containing VSG leader sequences on procyclic differentiation is intriguing. In each of the five cases examined, the 12-kb *Eco*RV leader orphon apparent in DNA from BSF cells was lost in their PCF descendents. Although the arguments outlined above do not allow us to indict the alteration as the causative agent of VSG gene inactivation, the complete cor-

relation is impressive. In other studies, we have shown that BSF trypanosomes of the IsTaR serodeme that possess an active ELC invariably possess the 12-kb *Eco*RV orphon, while cells expressing VSG genes by an alternative mechanism not linked to gene duplication often do not (20). The nature of the genomic alteration that results in the loss of the 12-kb *Eco*RV orphon in PCFs is not yet known. Its occurrence in each of the five separate procyclic differentiation events rules out point mutation, but it might be explained by deletion, genomic rearrangement, or DNA modification. Regarding the latter, no DNA modifications affecting *Eco*RV activity have been described, but trypanosomes may have unique modification systems.

It is worthwhile to consider the data reported here in light of previous biological findings on cyclic transmission and antigenic variation. When introduced into the mammalian host by the fly, metacyclic VATs rapidly convert to bloodstream stage VATs, with a high probability of expressing the VAT originally ingested by the fly (28). Our findings suggest that this results from reactivation of a bloodstream stage VSG gene that remained in the expression site but was not expressed during the insect phase of the life cycle. An extension of this hypothesis is that the genes encoding metacyclic VSGs use a separate expression site. For ELC type genes, entry to the bloodstream stage expression site is hypothesized to involve limited regions of homology between the entering sequence and that currently residing in the site (33). A distinct metacyclic expression site presumably could have different sequence requirements for entry, which may explain the constant and limited VSG gene repertoire of metacyclic trypanosomes.

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