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Characterization of VSG Gene Expression Site Promoters and Promoter-Associated DNA Rearrangement Events

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The expressed variant cell surface glycoprotein (VSG) gene of *Trypanosoma brucei* is located at the 3′ end of a large, telomeric, polycistronic transcription unit or expression site. We show that the region 45 kb upstream of the VSG gene, in the expression site on a 1.5-Mb chromosome, contains at least two promoters that are arranged in tandem, directing the transcription of the expression site. DNA rearrangement events occur specifically, at inactivation of the expression site, and these events delete the most upstream transcribed region and replace it with a large array of simple-sequence DNA, leaving the downstream promoter intact. Because of the placement of simple-sequence DNA, the remaining downstream promoter now becomes structurally identical to previously described VSG promoters. The downstream promoter is repetitive in the genome, since it is present at several different expression sites. Restriction fragment length polymorphism mapping allows grouping of the expression sites into two families, those with and those without an upstream transcription unit, and the DNA rearrangement events convert the expression sites from one type to the other. Deletion of the upstream transcription unit also leads to the loss of several steady-state RNAs. The findings may indicate a role for promoter-associated DNA rearrangement events, and/or interactions between tandemly arranged promoters, in expression site transcriptional control.

The African trypanosome *Trypanosoma brucei* survives in the bloodstream of its mammalian host by periodically changing the antigenic composition of its cell surface coat. This dense homogeneous coat consists of identical proteins, the variant cell surface glycoproteins (VSGs; 15, 16, 73). Antigenic variation of the VSG coat is brought about by the inactivation of the previously expressed VSG gene and the activation of a new VSG gene (for reviews, see references 8, 22, 46, 48, and 67). The expressed VSG gene is located at one of several telomeric VSG gene expression sites (19, 26, 71). Antigenic switches can result from the translocation of a new, previously silent or basic-copy (BC) VSG gene into a transcriptionally engaged expression site, generating an expression-linked copy (ELC; 27, 51, 53). Alternatively, antigenic switches can occur by the inactivation of one telomeric VSG gene expression site and the transcriptional activation of a new expression site (4, 9, 13, 40, 43, 69, 71). The mechanisms of control of expression sites switches have remained obscure, and it has been assumed that expression sites can be activated in situ, without detectable changes in their DNA sequences (76). Alternative models propose that DNA rearrangements affect transcription or that constitutive activity of expression site promoters is followed by posttranscriptional control (8, 49).

The expression sites encode large (up to 60 kb) polycistronic transcription units, with several expression site-associated genes (ESAGs) in addition to the telomerically located VSG gene (1, 18, 28, 31, 52, 55, 62, 63). The maturation of the polycistronic pre-mRNA, generated from transcription of the expression sites, involves the *trans*-splicing of a capped 39-nucleotide (nt) miniexon donor RNA onto the 5′ end of every pre-mRNA (7, 11, 14, 20, 21, 23, 26, 30, 41, 44, 45, 47, 56, 61, 64, 69, 70, 74). In contrast to the transcription of most other protein-coding genes in trypanosomes and other eukaryotes, VSG gene expression sites are transcribed by RNA polymerases that are insensitive to the drug α-amanitin at concentrations of up to 1 mg/ml (28, 29, 34, 57, 59, 63). This type of transcription is characteristic of RNA polymerase I, which in other eukaryotes transcribes rRNA genes only. Transcription of protein coding genes by RNA polymerase I may be possible in trypanosomes since the 5′ cap (24, 34, 35, 54, 65), required for mRNA stability and translation, is added in *trans* with the 39-nt miniexon.

In this report, we describe the identification of several different VSG gene expression site promoters and show that expression site promoters are highly conserved and repeated in the genome. In addition, we show that in some individual expression sites there are overlapping transcription units. We have also identified promoter-associated DNA rearrangement events, occurring at expression site inactivation, that deleted the upstream expression site transcription unit. The presence of these DNA rearrangement events and the existence of overlapping transcription units indicate that previously proposed models for in situ control may not completely capture the complexity of the transcriptional control at expression sites. Because of the repetitive nature of the downstream promoter region, it is unclear how many of the promoter repeats are active in the genome and whether these promoters are controlled at the transcriptional or posttranscriptional level.

MATERIALS AND METHODS

Trypanosomes. All trypanosomes used were of *T. brucei* stock 427-60. Variant 118 clone 1 (118 cl 1) and variant clone 8 have been described by Lee and Van der Ploeg (36); variants 118a, 221a, and 117a have been described by Cross (15); and variants 1.8c, 118a′, 118b, 1.8b, 118b′, and 1.8a have been described by Michels et al. (38–40). Variant 118 cl

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1 procyclic trypanosomes were established from variant 118 cl 1 bloodstream forms, adapted to procyclic culture (56a). Bloodstream-form trypanosomes were passaged in rats and harvested by cardiac puncture.

Genomic and chromosome-specific PFG gel electrophoresis. Chromosome-sized DNA was prepared for pulsed-field gradient (PFG) gel electrophoresis analysis essentially as described previously (71, 72). All size separations were performed in 20-cm^2 1% agarose gels in a Pharmacia LKB Pulsaphor apparatus at 15°C. Specific running conditions are noted in the figure legends.

For preparation of chromosome-specific DNA, 1% low-melting-point agarose (Bethesda Research Laboratories) PFG gels were run for 5 days at 5 V/cm at a pulse frequency of 900 s, which allowed excellent separation of band 10 (the 1.5-Mb chromosome) from chromosomes in the adjacent bands 9 and 11. After ethidium bromide staining, band 10 was cut en bloc from one lane of the gel with minimal UV exposure, trimmed to 5 by 5 by 2 mm, and washed five times in 100 volumes of 10 mM Tris (pH 8.0) for 2 h each time, with gentle shaking. Each block was then incubated in an equal volume of 2× restriction enzyme buffer solution and 50 U of the restriction endonuclease required for 12 h at 37°C. After restriction endonuclease digestion, the blocks were removed, inserted into the well of a 1% regular agarose PFG gel, and subjected to electrophoresis as described above, with the conditions described in each figure legend. Southern blotting was performed as described below.

Preparation of chromosome-specific DNA and construction of chromosome-specific libraries. Chromosome-specific DNA was purified as described above except that the equivalent of approximately 20 lanes of the 1.5-Mb chromosome-specific DNA was isolated. After washing, the low-melting-point agarose blocks were melted at 68°C in 2 volumes of 10 mM Tris (pH 8.0)–50 mM NaCl for 15 min and extracted twice with phenol, once with phenol-chloroform-isoamyl alcohol (PCI; 25:24:1), and once with chloroform-isoamyl alcohol (24:1). The DNA was then ethanol precipitated, washed once with 70% ethanol, and resuspended in 10 mM Tris (pH 8.0). The chromosome-specific DNA was used for Southern blotting as described below or used to construct chromosome-specific recombinant libraries (see below).

For the generation of chromosome-specific libraries, the DNA was digested with a 10-fold excess of the particular restriction endonuclease required (Boehringer Mannheim) for 2 h at 37°C. The DNA was next PCI extracted and coprecipitated with 100 ng of calf intestinal phosphatase-treated, restriction endonuclease-digested Bluescript or pBS vector (Stratagene). After resuspension, ligation was performed for 14 h at 16°C in a volume of 20 μl. Approximately 1 μl (1 to 5 ng of DNA) of the ligation mixture was used to transform an aliquot of MAX Efficiency DH5α competent *Escherichia coli* bacteria (Bethesda Research Laboratories). Clones were screened from duplicate filters, purified to homogeneity by sequential hybridizations, and characterized by restriction endonuclease mapping. Appropriate restriction endonuclease fragments were subcloned into the Bluescript or pBS vector and sequenced by the dideoxy-chain termination method (60) with T7 DNA polymerase (Sequenase) according to the specifications of the manufacturer (U.S. Biochemical Corp.).

PCR amplification of 1.5-Mb chromosome-specific expression site sequences. The 1.5-Mb chromosomes (band 10) were purified from PFG gels as described above, and the DNA was digested with a restriction endonuclease and ligated into the Bluescript vector. A synthetic primer was then constructed from the nucleotide sequences from the most 5′ end of previously cloned sequences; polymerase chain reaction (PCR) amplification (32) was performed as described below, using this oligonucleotide and a second oligonucleotide from vector-derived sequences. Melting of DNA hybrids was performed for 1 min at 94°C, annealing temperature was 52°C for 2 min, and *Taq* polymerase-mediated elongation was performed for 3 min at 72°C for 40 cycles. Amplified DNA was purified on low-melting-point agarose gels, ligated into the Bluescript or pBS vector, and transformed into *E. coli* DH5α, after which positively hybridizing recombinant clones were isolated as described above for the chromosome-specific libraries.

PCR amplification of cDNAs and promoter-related repeat sequences. cDNAs were prepared for PCR amplification by coprecipitating 100 ng of specific oligonucleotide primer with 20 μg of total trypanosome RNA from variant 118 cl 1; this preparation was resuspended in hybridization buffer [50% formamide, 400 mM NaCl, 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid (PIPES; pH 6.9), 1 mM EDTA], denatured at 75°C for 10 min and slowly cooled to room temperature. After precipitation, the sample was resuspended in primer extension buffer (50 mM Tris [pH 8.0], 20 mM KCl, 10 mM $MgCl_2$, 5 mM dithiothreitol), and reverse transcription was performed in the presence of 1 mM deoxynucleoside triphosphates, 25 U of RNasin (Promega), and 20 U of reverse transcriptase (Molecular Genetic Resources) for 1 h at 42°C. After completion, the cDNA was precipitated, resuspended in 0.025 N NaOH, and incubated for 10 min at 65°C. The sample was neutralized with HCl, passed over a Sephadex G-50 column, and again precipitated. The resuspended sample was then ready for PCR amplification, using the same specific primer as the one used for the reverse transcription as well as a second miniexon sense-strand primer, derived from the most 3′ 20 nt of the miniexon sequence.

Expression site-related repeat sequences were amplified from total genomic DNA from variant 118 cl 1, using complementary primers from the downstream promoter region. All amplifications were performed in 100 μl under mineral oil in the presence of 50 mM KCl, 10 mM Tris (pH 8.3 at 20°C), 1.5 mM $MgCl_2$, 2 mM deoxynucleoside triphosphates, 500 ng of each primer, and 5 U of *Taq* polymerase (Perkin-Elmer Cetus) for 40 cycles. Samples were denatured at 94°C for 1 min, annealed at 48 to 60°C for 2 min, and extended at 72°C for 3 min.

DNA isolation and Southern blotting. High-molecular-weight genomic DNA was isolated from trypanosomes as previously described (70). DNA was digested with a 10-fold excess of restriction endonuclease (Boehringer Mannheim), size separated on 0.8 to 1.2% agarose gels, and transferred to nylon membranes (Hybond-N; Amersham). Hybridizations were performed at 65°C for 16 to 20 h in 3× SSC–10× Denhardt's solution–10% dextran sulfate–0.1% sodium dodecyl sulfate (SDS)–0.1 μg of salmon sperm DNA per ml. Probes were labeled to a specific activity of 10^9 cpm/μg of DNA (Random Primed DNA labeling kit; Boehinger Mannheim). For all blots, final washing conditions were 65°C in 0.1× SSC–0.1% SDS.

Analysis of nascent RNA and UV inactivation of trypanosomes. Nuclei were isolated and the nascent RNA run-on analysis was performed essentially as described in Rudenko et al. (58). UV inactivation of transcription was performed essentially as described by Johnson et al. (28), with the following modifications. Heparinized (15 U/ml) whole blood was collected from rats that had been infected with trypano-

some variant 118 cl 1. The density of trypanosomes was approximately 5×10^8/ml. The blood was diluted to a density of 5×10^6 trypanosomes per ml with prewarmed 37°C Baltz B medium (3) containing 10% rabbit serum and 1% glucose. These trypanosome suspensions were immediately UV irradiated as previously described (28) in a warm (37°C) room. Following UV irradiation, trypanosomes (150 ml per UV point) were incubated for 1.5 h at 37°C with vigorous shaking (125 rpm) in 250-ml Erlenmeyer flasks in a gyratory bacterial cell shaker. After incubation at 37°C, the trypanosomes were immediately lysed for nuclei isolation by passaging the cell suspension over a Stansted cell disrupter which was kept at 37°C, after which the nuclei preparations were analyzed by nuclear run-on analysis. Control sequences are described below.

Nascent RNA protection analysis. Isolated nuclei and nascent RNA were prepared as described by Rudenko et al. (58), with the following modifications. Nuclei were all preincubated in α-amanitin (1 mg/ml), extended in the presence of deoxynucleoside triphosphates and [^{32}P]UTP for 5 min at 37°C, stopped with an equal volume of 2× TES (20 mM Tris [pH 8.0], 20 mM EDTA, 1% SDS, 1 mg of proteinase K per ml) for 10 min at 50°C, passed eight times through a 21-gauge needle, PCI extracted twice, passed over a Sephadex G-50 column, PCI extracted again, and ethanol precipitated. The nascent RNA was resuspended in DNase buffer (10 mM Tris [pH 8.0], 5 mM $MgCl_2$, 0.1 mM EDTA), incubated with 25 U of RNasin and 30 U of RNase-free DNase I (Worthington) for 15 min at 37°C, PCI extracted twice, and coprecipitated with (experimental samples) or without (control samples) unlabeled antisense riboprobe prepared according to the manufacturer's specifications (T7 RNA polymerase; Promega). Each sample was resuspended in 20 μl of hybridization buffer, heated to 85°C, and then hybridized overnight at 47°C. Then 180 μl of ice-cold buffer (10 mM Tris [pH 8.0], 300 mM NaCl) and 40 U of RNase T_1 were added, and the samples were incubated at 32°C for 1 h, PCI extracted, ethanol precipitated, resuspended in loading buffer, and size separated on 6% acrylamide–6 M urea gels.

RNA isolation and Northern (RNA) analysis. Total trypanosome RNA was isolated from purified trypanosomes by LiCl precipitation as described by Auffray and Rougeon (2). For Northern analysis, RNA samples were separated in a 1.5% formaldehyde-agarose gel, transferred to nylon membranes, and hybridized with ^{32}P-labeled probes by using standard techniques (6). Following hybridization, filters were washed to a final posthybridization stringency of 0.1× SSC and 65°C.

Description of clones. (i) Recombinant clones isolated from 1.5-Mb chromosome-specific libraries. See Fig. 3 for clone locations. Clone 1 (i.e., 2.5-kb *Hin*dIII recombinant clone 1) is a 2.5-kb *Hin*dIII genomic clone, clone 4 is a 440-bp *Hin*dIII-*Ssp*I genomic clone, clone 5 is a 670-bp *Pst*I-*Spe*I genomic clone, clone 6.2 is a 600-bp *Spe*I-*Pst*I subcloned fragment, clone 6.1 is a 265-bp *Eco*RI-*Spe*I subcloned fragment, clone 10 is a 420-bp *Hin*PI-*Hpa*I subcloned fragment, and clone 11 is a 270-bp *Pst*I-*Hin*PI subcloned fragment; all of these clones are from the promoter region. Probe 1.5 MB-A is a 300-bp *Pst*I-*Kpn*I genomic clone that identifies homologous chromosomes 9 and 10 and which physical mapping shows is located at least 100 kb distant from the expression site (previously called ES 118; 25).

(ii) Other clones. VSG is the 5′ cDNA *Eco*RI-*Pst*I fragment specific for VSG gene 118 (27); ESAG is the cDNA for ESAG1 (pCE117a.01) cloned from the 1.5-Mb chromosome expression site by Cully et al. (18); pBS (pBS+; Stratagene) and pUC (pUC18; New England BioLabs) are control plasmids; 5S is a 5S rRNA clone described by Lenardo et al. (37); rRNA is ribosomal clone PR4 described by Kooter and Borst (29); TUB is the αβ-tubulin clone pTbαβ T-1 described by Thomashow et al. (66); ME is a subclone of the miniexon repeat clone pCL103 described by Laird et al. (34); and Tg118 is the 5.0-kb genomic clone TgE118.1 described by Lee and Van der Ploeg (36). PEMR subclones from the ribosomal transcription unit were essentially as described by Johnson et al. (28) except for PEMR 2.5, which contains a 850-bp *Hin*dIII-*Pst*I restriction fragment located immediately downstream of PEMR 2.

RESULTS

Isolation of VSG gene expression site promoters. Kooter et al. (31) characterized the VSG 221 expression site located on a 3-Mb chromosome in variant 221a and isolated a repetitive element with characteristics of a VSG 221 expression site promoter (28). Because of the repetitive nature of sequences in expression sites, these workers failed to isolate the 221 promoter proper and presumably obtained a related promoter repeat. We used the putative promoter sequence obtained by Kooter et al. (31) to identify homologous sequences that might encode the promoter of the VSG 118 gene in variant 118 cl 1. In this variant, the active expression site is located on a 1.5-Mb chromosome (band 10 in the PFG gels; 25, 72). To ensure isolation of VSG 118 promoter sequences, rather than repeats from elsewhere in the genome, we screened a series of libraries constructed with DNA from the isolated 1.5-Mb chromosome. Because of extreme selection against these clones in *E. coli*, several small overlapping recombinant clones had to be isolated. Figure 1 shows the physical map of the 1.5-Mb chromosome (see below for details), and Fig. 1B shows the physical map of the 5.5-kb promoter region.

Several of the recombinant clones could not be obtained directly from the chromosome-specific libraries. However, by using PCR to amplify genomic fragments, unclonable regions were isolated (Fig. 1C, clones marked by dashed lines). An outline of the method of PCR amplification as performed on 1.5-Mb chromosome DNA is given in Materials and Methods. Since PCR amplification overcame the negative selection against some of the clones, it is possible that secondary base modifications of genomic DNA affected the cloning efficiencies (5, 50).

Physical map of the 1.5-Mb chromosome and characterization of the recombinant clones. A single 2.5-kb *Hin*dIII fragment (recombinant clone 1 in Fig. 1C) showed significant homology to the putative promoter region of the VSG 221 expression site (31). In order to (i) demonstrate that these recombinant clones are specifically derived from the 1.5-Mb chromosome, (ii) locate these clones precisely within the VSG 118 expression site, and (iii) show that these clones are part of the single expression site on the 1.5-Mb chromosome, we hybridized these clones with restriction enzyme-digested, total genomic DNA and band 10-specific DNA that was purified from PFG gels. Figure 2A shows a representative example of a PFG gel in which *Not*I restriction enzyme-digested total genomic DNA is size separated. By using a probe specific for the VSG 118 gene, two restriction enzyme fragments can be detected (Fig. 2A, lane VSG): a 125-kb fragment specific for the VSG 118 ELC on the 1.5-Mb chromosome, and a 175-kb fragment specific for the VSG 118 BC on a 3-Mb chromosome (BC fragments were identified by hybridization with a BC-specific probe; data not shown). As expected, a probe for the repetitive ESAG1 (18)

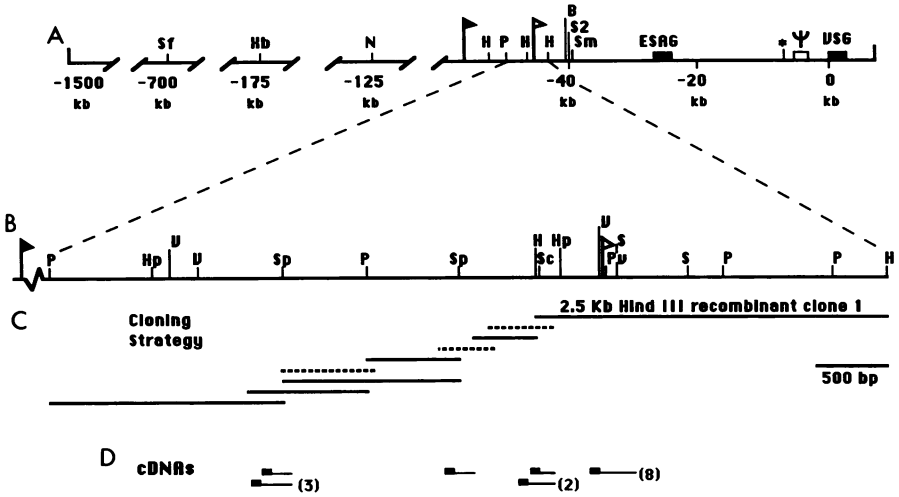

FIG. 1. Physical map of the VSG gene 118 expression site on a 1.5-Mb chromosome (band 10) in variant 118 cl 1. (A) Physical map of the 1.5-Mb chromosome; (B) physical map showing the 5.5-kb region from the promoter area that is located 45 kb upstream of the VSG gene. The white flag represents the VSG gene expression site promoter sequences (the downstream promoter); the black flag represents the upstream promoter active in variant 118 cl 1. Notation: VSG, VSG gene coding sequence; ESAG, ESAG1 (18); ψ, a VSG pseudogene; *, location of a subsidiary promoter as described by Shea et al. (62). (C) Extent of each recombinant clone. Solid lines represent recombinant clones isolated directly from chromosome-specific libraries; dashed lines represent clones isolated by PCR as described in Materials and Methods. (D) Locations of the different miniexon (■)-containing cDNAs and number of independent cDNAs isolated (in parentheses). Restriction endonuclease sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Hf, *Hin*fI; Hp, *Hpa*I; Hp2, *Hpa*II; N, *Not*I; P, *Pst*I; Pv, *Pvu*I; Rs, *Rsa*I; S, *Sal*I; Sau, *Sau*3A; Sc, *Sca*I; Sf, *Sfi*I; Sm, *Sma*I; Sp, *Spe*I; Sph, *Sph*I; S2, *Sac*II; V, *Eco*RV; Xb, *Xba*I. Only selected sites are shown in panel A.

from this chromosome detected several bands in the PFG gel, among which is the 125-kb *Not*I ELC fragment (lane ESAG). Similarly, the 2.5-kb *Hin*dIII putative promoter recombinant clone 1 (lane probe1), isolated from a 1.5-Mb chromosome-specific library, detected numerous bands in total genomic DNA, among which is the 125-kb ELC *Not*I fragment. Similar gels colocalized the VSG 118 gene, ESAG1, and the 2.5-kb *Hin*dIII clone to the same 175-kb *Xba*I and 45-kb *Pvu*I fragments but not to a 42-kb *Bam*HI or 40-kb *Sac*II fragment which contained the VSG ELC gene only (data not shown). This finding is in agreement with the notion that the 2.5-kb *Hin*dIII fragment, ESAG1, and the VSG 118 gene are confined to a 45-kb telomeric domain on the same 125-kb *Not*I fragment on the 1.5-Mb chromosome.

To prove that the putative promoter recombinant clone (clone 1) was indeed derived from the region containing the VSG 118 ELC and to pinpoint this clone on the 1.5-Mb chromosome, we purified this chromosome from PFG gels, digested it in agarose blocks with restriction enzymes, and size separated the DNA by PFG gel electrophoresis (Fig. 2B). As expected for a fragment that is present in only one copy (the ELC) on the 1.5-Mb chromosome, both the VSG 118 probe (Fig. 2B, lane VSG) and recombinant clone 1 (lane probe1) detected a single 125-kb *Not*I restriction enzyme fragment and a single 175-kb *Xba*I fragment (as expected from the previous experiment). Recombinant clone 1 also mapped to a 45-kb *Pvu*I restriction enzyme fragment (data not shown) that hybridized with the VSG 118 probe. The uniqueness of these sequences on the 1.5-Mb chromosome was confirmed in additional digests of chromosome specific DNA. For instance, a *Hin*dIII restriction enzyme digestion of the purified 1.5-Mb chromosome showed a single hybridizing restriction fragment only (Fig. 2C, lane B.10), while total genomic DNA (lane HMW) revealed the repetitive character of this putative promoter region. Additional physical mapping with purified band 10 DNA showed and further confirmed the linkage of the recombinant clones 2 to 11 with recombinant clone 1 and VSG gene 118 (Fig. 2 and data not shown). We conclude that a single copy or identical repeats of this putative promoter fragment are confined to a unique telomeric, 125-kb *Not*I restriction fragment on the 1.5-Mb chromosome that contains the active expression site.

Using PCR amplification to isolate miniexon-containing cDNAs, we obtained 16 cDNA clones derived from four different regions of the putative promoter region (indicated in Fig. 1). The nucleotide sequences of these cDNAs were identical to the sequences determined for the genomic recombinant clones, while expression site-related sequences from other chromosomes in this variant were only 95 to 99% homologous (data not shown; see also Fig. 4B). In addition, steady-state mRNA that hybridized with a subset of these recombinant clones (clones 4 and 5 in Fig. 3) could be found only in some trypanosome variants in which the VSG 118 expression site is active (see below; data not shown). Finally, a 24-mer oligonucleotide derived from clone 6 uniquely hybridized to band 10 DNA in PFG gels (data not shown). We conclude that the genomic clones obtained from the chromosome-specific libraries are derived from the VSG 118 expression site on the 1.5-Mb chromosome and that most of these clones are present in one copy in band 10-specific DNA.

Transcription of the expression site on the 1.5-Mb chromosome. To identify transcription initiation sites and to determine the extent of the transcription unit, we determined the distribution of RNA polymerases and their sensitivity to α-amanitin by performing nuclear run-on assays. Figure 3A shows the distribution of RNA polymerases extending from the most distally located VSG 118 gene (marked VSG) to ESAG1 (marked ESAG) to the putative promoter region in recombinant clone 1 up to the most proximal recombinant

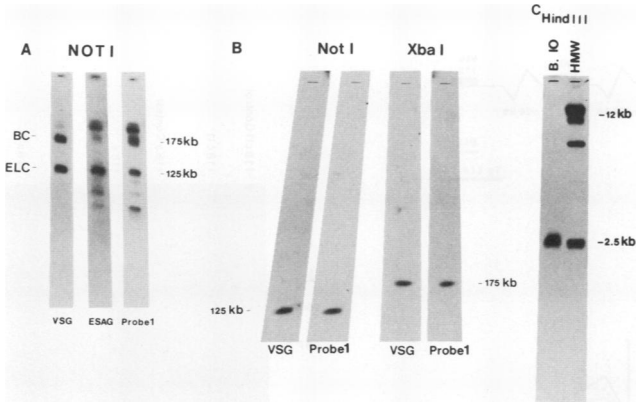

FIG. 2. Analysis of Southern blots linking promoter region clones from variant 118 cl 1 to the VSG 118 gene in the expression site on the 1.5-Mb chromosome. (A) Analysis in which genomic DNA from variant 118 cl 1 was digested with the restriction endonuclease *Not*I, size separated on a PFG gel run for 24 h at 10 V/cm at a pulse frequency of 9 s, transferred to nylon membranes, and hybridized with the probes indicated below the lanes. After hybridization, each blot was stripped by boiling in 0.1× SSC for 30 min and then reexposed before reuse. Sizes of restriction enzyme fragments are shown at the right. On the left, several of the fragments are identified; ELC is the expression-linked copy of VSG gene 118 present on the 1.5-Mb chromosome; BC is the basic copy of this gene on a large (>3-Mb) chromosome. Probe VSG is a VSG 118 gene-specific probe derived from the 5′ end of the VSG 118 gene, probe ESAG is derived from a cDNA encoding ESAG1 (18), and probe 1 is the 2.5-kb *Hin*dIII recombinant clone 1 identified in Fig. 1. (B) Analysis in which genomic DNA from variant 118 cl 1 was size separated on PFG gels, the 1.5-Mb chromosome DNA was isolated, digested with the restriction endonuclease indicated at the top, and the chromosome-specific, digested DNA was size separated in PFG gels for 36 h at 10 V/cm at a pulse frequency of 40 s. After Southern transfer, the blots were hybridized with the probes indicated at the bottom. (C). Southern blot of 1.5-Mb chromosome-specific DNA (lane B.10) and genomic DNA (lane HMW) after digestion with the restriction endonuclease *Hin*dIII. Sizes of bands are indicated on the right. The blot was probed with probe 1 (see above).

clone, clone 11. Surprisingly, every recombinant clone up to the region at ~−50 kb was transcribed in an α-amanitin-resistant manner, which did not allow the identification of a transcription initiation site (Fig. 3A). This result was in contrast with previously published work (17, 28, 76) which predicted the location of a VSG promoter in recombinant clone 1-like repeats. The nuclear run-on data therefore implied the presence of an as yet uncharacterized promoter 5′ to these regions (indicated with black flags in the physical maps in Fig. 1, 3, 4, 6, and 8).

However, the relative efficiency of transcription throughout this region was not constant and increased drastically, by about 10-fold, when recombinant clones 1 and 2 were compared with clones immediately upstream. This finding suggested the presence of an additional promoter in this region in a manner analogous to what has been found for previously described subsidiary promoters (1, 62). Recent work (17, 28, 49, 76) relied on UV inactivation of transcription for promoter mapping. We therefore mapped the promoter region in our expression site by UV inactivation of transcription, which relies on the fact that UV-induced thymidine dimer formation will prevent RNA polymerase from traversing the DNA template. The distance of a recombinant clone from its promoter is therefore inversely proportional to its UV sensitivity, the most UV-resistant clones being closest to the promoter. For some promoter-containing fragments, an initial increase of the nascent RNA signal can result from exposure to a low dose of UV (12, 49). Performing UV inactivation of transcription, we obtained indirect evidence for the presence of a promoter sequence in recombinant clone 1 (Fig. 3B): (i) the efficiency of nascent RNA transcription at recombinant clone 1 initially increased at low UV doses; (ii) at higher UV doses, the transcription of recombinant clone 1 remained resistant to UV inactivation of transcription and overall behaved identically to the fragment containing the rRNA gene promoter (Fig. 3B; compare clone PEMR 1 with recombinant clone 1); and (iii) clones immediately upstream of recombinant clone 1 (clones 4, 5, 6.1, and 6.2) as well as those located downstream (VSG and ESAG) were all far more sensitive to UV inactivation of transcription. These data therefore suggested that recombinant clone 1 may contain a transcription initiation site.

If this fragment indeed encoded a promoter, it should be possible to locate the 5′ ends of primary RNA transcripts, determining the sites of transcription initiation in nascent RNA. We isolated ^{32}P-labeled nascent RNA, protected this RNA with antisense riboprobes (see map in Fig. 3), and determined the resistance of the nascent RNA-RNA hybrid to digestion by RNase T_1. We focused on the region upstream of a *Sal*I restriction enzyme site, where the transcription initiation site should be located (as determined by the step-up in nuclear run-on transcription and the resistance of this region to UV inactivation of transcription; Fig. 3B and C and data not shown). Three nascent RNAs with 5′ ends located between nt 80 and 88 upstream of the *Sal*I restriction enzyme site could be identified (Fig. 3C, bands marked between 80 and 88 nt). The nascent RNA analysis, however, does not determine the presence of the predicted triphosphate or cap structure at the 5′ ends of these RNAs. Additional experiments are therefore needed to confirm that the 5′ ends of these RNA molecules map to the transcription initiation site of this VSG promoter.

Comparison of this region with predicted promoter regions of diverged trypanosome stocks described previously (31, 52, 76) showed significant homology between the physical maps of these expression sites and their nucleotide sequences (Fig. 4). To address the significance of the base substitutions in the promoter region of the VSG 221 and VSG 118 expression sites, we cloned several homologous repeats and determined their DNA nucleotide sequences (Fig. 4B). Comparison of these repetitive elements showed that they can be grouped into several classes, sharing permutations of a few base substitutions only.

The DNA nucleotide sequence conservation between the potential VSG promoter regions, the nascent RNA and UV inactivation studies, and the mapping of 5′ ends on nascent RNA transcripts all make it plausible that a transcription initiation site is located 80 to 88 nt upstream of a *Sal*I restriction enzyme site in recombinant clone 1 (white flags in Fig. 1, 3, 4, 6, and 8). We will refer to this promoter as the downstream promoter. In variant 118 cl 1, however, this promoter is part of a larger transcription unit, and RNA polymerases that have initiated upstream appear to read throughout this promoter region (Fig. 3A). In support of this hypothesis are the findings that (i) gaps could not be detected in the nuclear run-on analysis, (ii) all fragments were transcribed in an α-amanitin-resistant manner, (iii) several cDNAs (with miniexons at their 5′ ends) were isolated that spanned the downstream promoter (Fig. 1 and 4B), and (iv) these cDNAs and additional steady-state RNAs

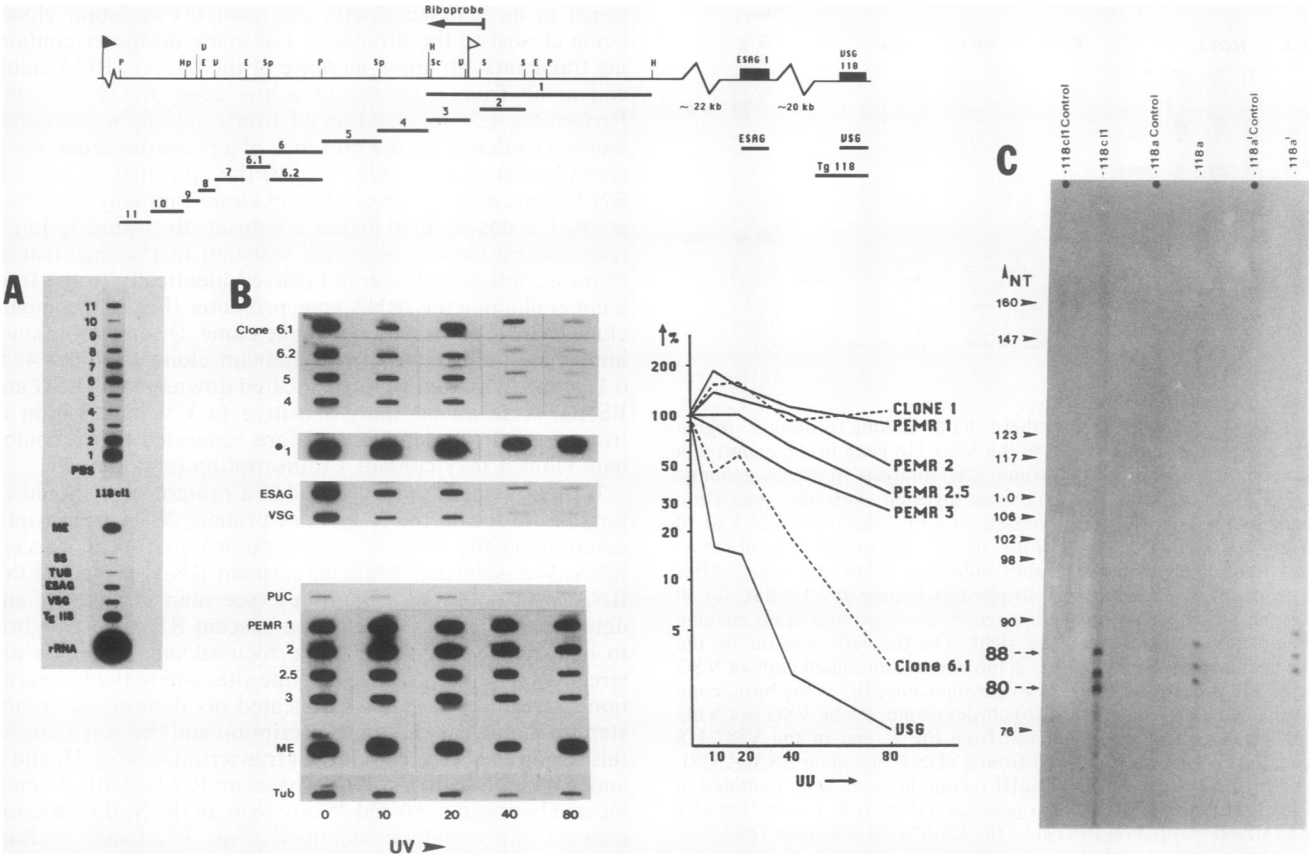

FIG. 3. Identification of promoter sequences in the expression site on the 1.5-Mb chromosome in variant 118 cl 1. At the top is a physical map of the promoter region in variant 118 cl 1 showing the locations of a series of numbered recombinant clones (used in panels A and B). The location of a riboprobe used for promoter analysis (C) is also highlighted (riboprobe arrow = 500 bp). (A) Nascent RNA run-on analysis of the promoter region of the active expression site of variant 118 cl 1, using recombinant clones 1 to 11 as identified in the physical map and performed in the presence (A) or absence (not shown) of the drug α-amanitin (1 mg/ml). We have previously published the comparison and quantitation of α-amanitin sensitivity of transcription of the genes tested here (57–59, 62, 63), and to save space we have chosen not to present these data here. However, all α-amanitin sensitively transcribed genes were inhibited by at least 90% upon the addition of α-amanitin (data not shown). DNA was slot blotted onto nitrocellulose filters and hybridized with ^{32}P-labeled nascent RNA from variant 118 cl 1. PBS, Vector control; ME, the miniexon repeat unit; 5S, 5S rRNA gene; TUB, αβ-tubulin; ESAG, ESAG1; VSG, 5′ end of the coding sequence of VSG gene 118; Tg118, a 5-kb genomic clone from the 3′ region of this expression site; rRNA, rRNA repeat. The relative efficiency of transcription per base pair differed by at most twofold in the entire region covered by clones 3 to 11. Variation in the absolute intensity of the hybridization in the region covered by clones 3 to 11 could differ slightly between individual experiments (see Fig. 6). (B) UV inactivation of transcription in variant 118 cl 1. Trypanosomes were exposed to increasing doses of UV light (given in 100 ergs/mm^2) as indicated on the bottom, and their nuclei were isolated and used in a nuclear run-on assay to measure the distribution of nascent RNA. The bottom panel shows slot blots of subclones of different control genes: PUC, plasmid vector control; PEMR 1, 2, 2.5, and 3, clones spanning the rDNA transcription unit (28, 58). The top panel shows the VSG 118 expression site-derived clones. The black dot marks the 2.5-kb *Hin*dIII recombinant clone 1, which is resistant to UV inactivation of transcription. The right panel shows quantitation of the UV inactivation of transcription data for selected clones. This is a semilogarithmic graph of the relative transcription (signal after UV dose/signal with no UV × 100%; see also Materials and Methods) plotted against the UV dose for subclones from some of the control genes (PEMR 1, 2, 2.5, and 3 and VSG [solid lines]) as well as clones from the promoter region of the active expression site (clones 1 and 6.1 [dashed lines]). The graph corresponds exactly (without correction) to the hybridization data on the left. (C) Nascent RNA protection studies of the downstream promoter region in closely related variants 118 cl 1, 118a, and 118a′. ^{32}P-labeled nascent RNA was isolated from nuclei of each variant, hybridized with the antisense riboprobe indicated on the map, digested with RNase T_1, and size separated on a 6% denaturing polyacrylamide gel. A control incubation without added riboprobe is shown in every adjacent lane, as indicated. Size markers (in nucleotides [NT]) are indicated at the left. See legend to Fig. 1 for abbreviations. Note the three protected bands between nt 80 and 88 that map an initiation site to the downstream promoter region (white flag on map) both in variants with (118 cl 1 and 118a) and in those without (118a′) the upstream transcription unit (for details, see text). The 500-bp band, corresponding to the fully protected riboprobe, that is expected in variants 118 cl 1 and 118a could not be visualized because of the technical limitations of the nascent RNA protection technique. This fragment could, however, be demonstrated easily in S1 nuclease protection studies with steady-state RNA (data not shown).

could be identified only in variants that actively transcribe the VSG 118 expression site on band 10 (see below; data not shown). The simplest interpretation of these data is that the expression sites analyzed thus far have very similar downstream promoter sequences. However, the expression site in variant 118 cl 1 is organized differently, and its downstream promoter is located within a larger transcription unit.

Significance of the upstream VSG expression site transcription unit. To understand the developmental control of expression sites, we compared their transcription patterns

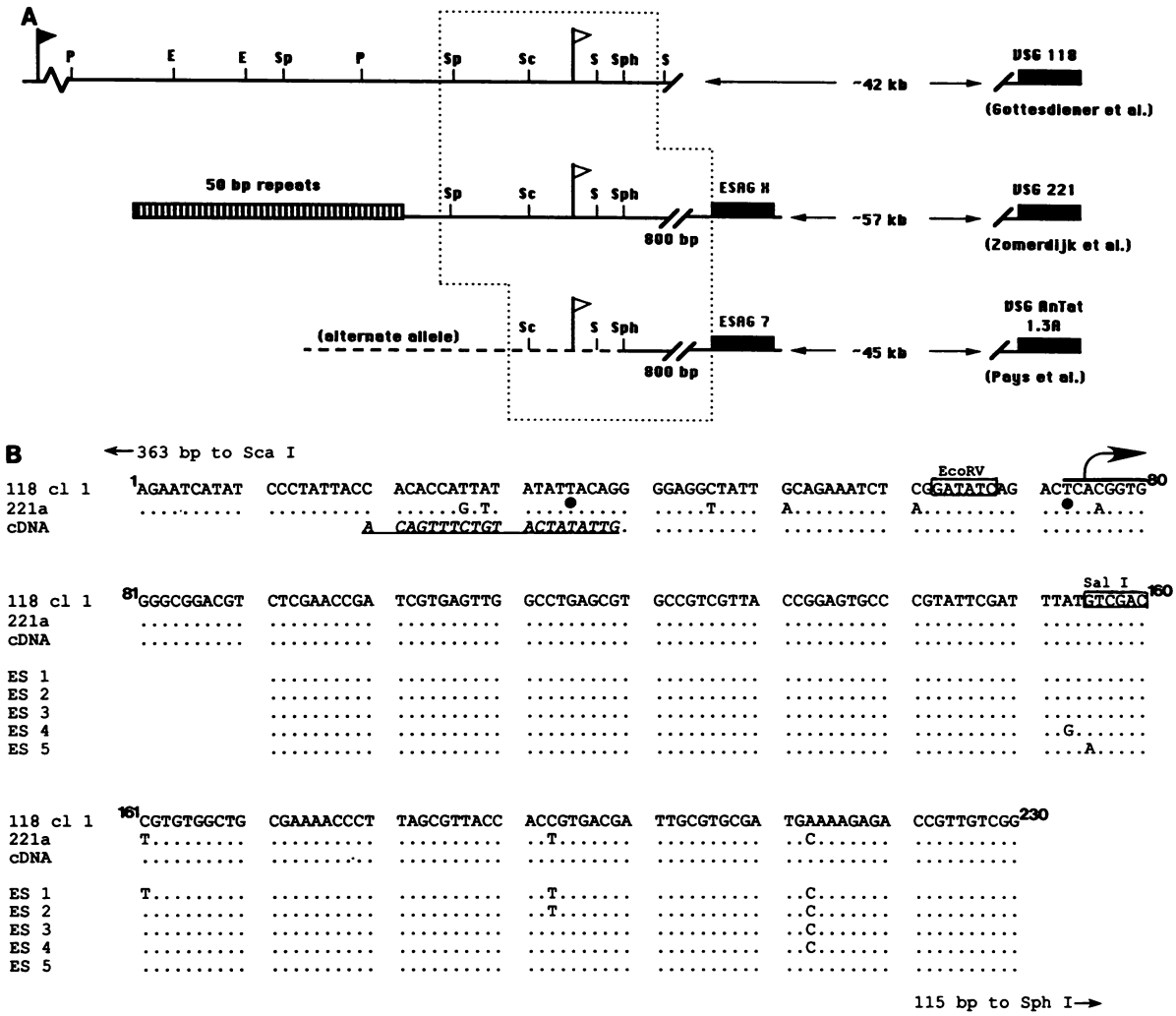

FIG. 4. Comparison of VSG expression sites. (A) Restriction enzyme maps of expression sites for variant 118 cl 1 on a 1.5-Mb chromosome (VSG 118; this report), variant 221a on a 3-Mb chromosome (VSG 221; 76) and variant AnTat 1.3A on a 200-kb chromosome (VSG AnTat 1.3A; 52). Protein-coding regions are indicated by black boxes; letters refer to restriction enzyme sites (see Fig. 1 for abbreviations); ESAG, ESAG1. The white flag (downstream promoter) and black flag (upstream promoter) are discussed in the text. Regions of >95% homology between sequences of these three expression sites are boxed by the dotted line. (B) Sequence comparisons in the region of the downstream promoter (white flag; see above). The top line shows the nucleotide sequence from the expression site on the 1.5-Mb chromosome of variant 118 cl 1, the second line shows the nucleotide sequence from the expression site in variant 221a, and the third line shows the nucleotide sequence from a miniexon-containing cDNA derived from RNA from variant 118 cl 1. ES 1 to 5 are the DNA nucleotide sequences from promoter-related repeats, purified from variant 118 cl 1. Identical base pairs are indicated by small dots. The tentative locations of the three downstream promoter initiation sites in variant 118 cl 1 are indicated by the black line above the sequences and the large arrow (bp 72 to 80, which are 80 to 88 nt 5′ to the *SalI* restriction enzyme site); note the lack of splice acceptor sites in this region to account for the three transcripts mapped. The two initiation sites identified by Zomerdijk et al. (76) in variant 221a are indicated by large dots. Restriction enzyme sites are boxed and labeled; nucleotides are arbitrarily numbered for convenience.

among closely related variants. We studied two different, independently obtained series of antigenic switches in which the VSG 118 gene in the expression site on the 1.5-Mb chromosome is active (variants 118a and 118b) and then becomes inactivated (variants 1.8c and 1.8b) and is maintained as a lingering nontranscribed ELC, which is subsequently reactivated in variants 118a′ and 118b′ (Fig. 5). The control panels (containing miniexon, 5S rRNA, tubulin, ESAG, VSG, and rRNA sequences) in Fig. 6E show the pattern of transcription at the VSG gene and ESAG1 of the expression site on band 10. As expected, the VSG 118 gene and ESAG1 are inactive in variants 1.8b and 1.8c. The transcription patterns in the promoter region compared among variants 118 cl 1, 118a, and 118b appeared identical (Fig. 6D). Since the physical maps of these expression sites were identical in the promoter region (Fig. 6A and data not shown), we conclude that the expression sites in variants 118 cl 1, 118a, and 118b are organized similarly. However, in variants 1.8c and 1.8b, in which these VSG 118 expression sites were inactivated, the regions located upstream of recombinant clone 1 were no longer transcriptionally active (Fig. 6D). The sequences immediately 3′ to the downstream promoter, however, remained transcribed in an α-amanitin-resistant manner (compare transcriptional efficiencies of recombinant clones 1 and 2 with those of recombinant clones 3 to 11). This hybridization can be explained by the signifi-

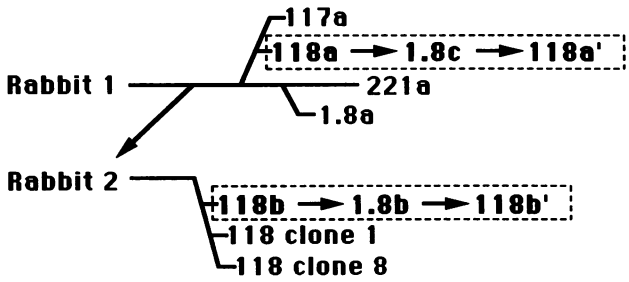

FIG. 5. Lineage of antigenic variants in *T. brucei* stock 427-60. The lineages of trypanosomes derived from two rabbits are shown. Numbers refer to individual cloned trypanosome variants that are described Materials and Methods. Connecting lines represent different times of parasitemia at which trypanosomes were isolated from the rabbits. Trypanosomes derived from single relapse switches (switches in which each resulting clone is a direct descendant of the parent clone) are indicated by arrows, and the two independent lineages studied are boxed for convenience. Variants 118 cl 1, 118a, 118a', 118b, 118b', and 117a all have an active expression site on the 1.5-Mb chromosome.

cant sequence homology of the downstream promoters of several expression sites (Fig. 4 and data not shown) or by the constitutive activity of the downstream promoter region. However, in the latter case a posttranscriptionally occurring controlling mechanism must prematurely terminate transcription, to prevent expression of the VSG gene and ESAG1 at the expression site in band 10 in variants 1.8b and 1.8c. However, we consider it more plausible that the VSG 1.8 expression site promoter regions in variants 1.8b and 1.8c share sequence homology with the VSG 118 downstream promoter on the 1.5-Mb chromosome, thus explaining the hybridization.

Surprisingly, reactivation of the VSG 118 expression site on the 1.5-Mb chromosome in variants 118a' and 118b' was associated with a pattern of transcription different from that seen in the original variants 118a and 118b. The upstream regions, represented by clones 3 to 11, were not transcribed, and transcription initiation occurred only at the downstream promoter sequences (Fig. 6D). This alteration in the transcription pattern compared among closely related variants using the same expression site, suggesting that the lingering ELC on the 1.5-Mb chromosome may have been activated through a different mechanism, involving exclusive utilization of the promoter represented by recombinant clone 1.

To confirm this result, we compared the locations of the 5' ends of nascent RNA from the downstream promoter in variants 118a and 118a' and variant 118 cl 1 (Fig. 3C) and variant 1.8c and procyclic trypanosomes (data not shown). The 5' ends of nascent RNA molecules map 80 to 88 nt upstream of the *Sal*I restriction enzyme site in all variants in which these repeats are transcribed (Fig. 3C). This matches with the location of the last restriction enzyme fragment (clone 2 in Fig. 6C) that still hybridizes in the nuclear run-on assay in variants 118a' and 118b' (Fig. 6D). These results thus predict the presence of an initiation site just upstream of the *Sal*I restriction enzyme site in variants 118a, 118a', 118b, 118b', and 118 cl 1. However, protection with nascent RNA from variants 1.8c and procyclic trypanosomes, which transcribe these promoter repeats or other promoterlike repeats but which do not transcribe the expression site on the 1.5-Mb chromosome, gave identical protection results.

We conclude that (i) the expression sites on the 1.5-Mb chromosomes in variants 118 cl 1, 118a, and 118b are

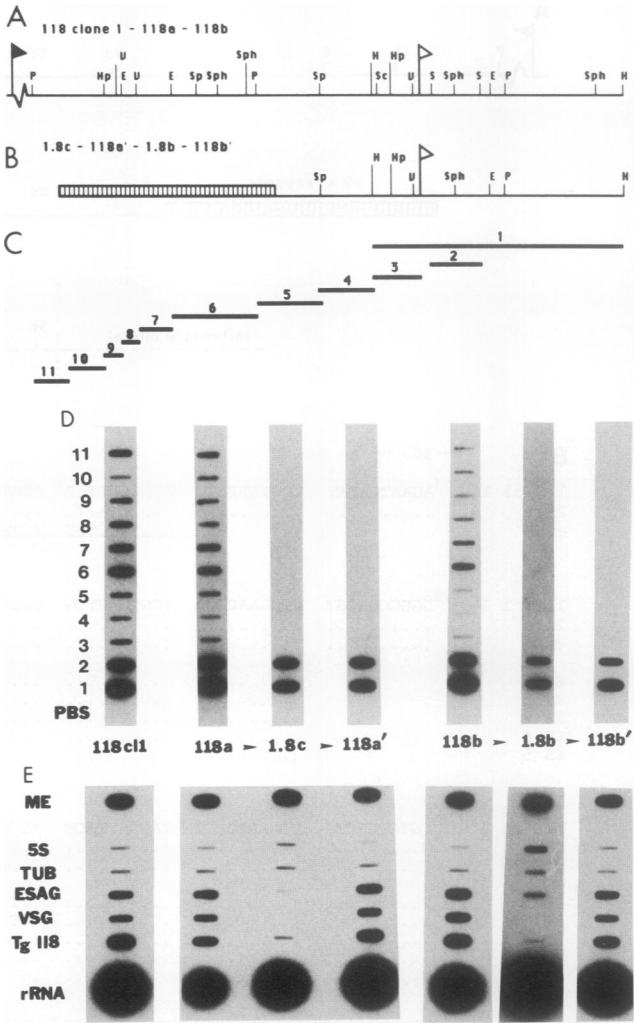

FIG. 6. Nascent RNA run-on analysis in the promoter region of closely related variants. (A) Physical map representing the promoter region of the active expression site on the 1.5-Mb chromosome in variants 118 cl 1, 118a, and 118b; (B) physical map of the same expression site in the four variants in which this expression site has undergone rearrangement events (see text for details); (C) locations of a series of recombinant clones (1 to 11) used for promoter analysis; (D) slot blots hybridized with ^{32}P-labeled nuclear run-on RNA from the variants indicated below the panels; (E) controls for each of these nuclear run-on assays (see legends to Fig. 1 and 3 for abbreviations). Nuclear run-on assays were performed in the presence (shown) and absence (not shown) of the drug α-amanitin (1 mg/ml). Single-relapse-derived lineages are indicated by arrowheads. DNA was slot blotted onto nitrocellulose filters, hybridized with nascent RNA, and washed to a final stringency of 0.1× SSC and 65°C.

organized similarly; (ii) the downstream promoters in variants 118 cl 1, 118a, and 118b are likely to be active and may function as subsidiary (internal) promoters; (iii) variants 1.8b and 1.8c, which transcribe a different expression site, are likely to have downstream promoter regions that are similar to the expression site on the 1.5-Mb chromosome, explaining their nuclear run-on and nascent RNA protection patterns; (iv) reactivation of the expression sites on the 1.5-Mb chromosome in variants 118a' and 118b' is associated with the exclusive use of the downstream promoter; (v) procyclic

trypanosomes have similar, transcriptionally active promoter repeats but do not appear to transcribe other sequences from the expression site; and (vi) because of the repetitive nature of these promoter repeats, it is difficult to prove that any particular promoter repeat is active or inactive. Control could be exerted at the level of transcription initiation (obscured by the repetitive nature of the promoter) or by differential attenuation that begins shortly downstream of the initiation site, at constitutively active promoter repeats.

Promoter-associated DNA rearrangement events. To understand the differential transcription of the upstream region, we searched for potential DNA rearrangement events that could explain their altered patterns of transcription. We size separated chromosomal DNA from these different trypanosome variants by PFG gel electrophoresis and hybridized the PFG gels with probes from the VSG 118 expression site promoter region. Surprisingly, all probes that were located upstream of fragment 5 (see Fig. 3 for probe location) failed to detect the 1.5-Mb chromosome in variants 1.8b, 1.8c, 118a′, and 118b′ (Fig. 7A to C), while probe 5 and all probes located downstream hybridized normally to the 1.5-Mb chromosome in these variants (data not shown). Further mapping studies revealed this deletion of upstream DNA sequence in five of eight variants. Examples of Southern blots used to define this DNA rearrangement are shown in Fig. 7D and E. *Hpa*II and *Hpa*I restriction enzyme digestions of total genomic DNA were hybridized with a probe from the deleted region (probe 10; see Fig. 3 for probe location). The 3-kb *Hpa*II band and the 4-kb *Hpa*I band (marked with asterisks) are present in only some trypanosome variants (i.e., 118a, 118b, 221, and 118 cl 1). Other gels, using probe 5 (present in all variants), allowed fine mapping of the upstream limit of the deletion. The region adjacent to the deleted sequences in the rearranged variants contained a large stretch of simple-sequence DNA that could not be cut with any of the restriction enzymes. For instance, probe 5, which is not deleted in variants 1.8b, 1.8c, 118a′, and 118b′, showed the apparent loss of a 1.8-kb *Hpa*II fragment in regular electrophoresis (Fig. 7G, variants 118 cl 1 and 118b versus 1.8b). However, in PFG Southern blots of restriction enzyme-digested DNA, an 80-kb *Hpa*II fragment is now seen (Fig. 7F, band marked with an asterisk in lane 1.8b). This 80-kb band has undergone secondary rearrangement events in variant 118b′: the 80-kb fragment detected in variant 1.8b now presumably comigrates with a repetitive, 75-kb *Hpa*II fragment (as judged from the relative intensities of the restriction enzyme fragments). These latter rearrangement events, however, did not occur in the region near the downstream promoter, but further upstream (either within or 5′ to the simple-sequence DNA). In other variants, the simple-sequence DNA could not be mapped precisely but was larger than the resolution of standard agarose electrophoresis (>20 kb). The physical maps of seven different trypanosome variants and the partial physical maps of two additional variants (clone 8 and variant 1.8a) are shown in Fig. 8. It is clear from these mapping data that DNA rearrangement events, involving large deletions, had occurred independently in several different trypanosome variants.

The mechanism of the DNA rearrangement events is still obscure. However, they appear to be nonrandom: hybridizations of total genomic DNA with probe 5, which is present on at least eight different chromosomes, shows that all promoter repeats fall into two families in an *Rsa*I-*Spe*I restriction enzyme digestion (Fig. 9). The DNA rearrangements have converted the expression site from one with a 0.95-kb *Rsa*I-*Spe*I restriction fragment length polymorphism (RFLP) into one with a 0.45-kb *Rsa*I-*Spe*I RFLP, the latter being characteristic of downstream promoter repeats flanked by simple-sequence DNA (Fig. 8 and 9). It is thus tempting to speculate that these RFLPs indicate the existence of two families of expression sites that may, by DNA rearrangement events, convert from the one into the other. Further evidence for this hypothesis comes from the analysis of other expression sites: the 221 promoter in variant 221a, analyzed by Zomerdijk et al. (76), has the 0.45-kb RFLP, while the putative promoter repeat analyzed by Kooter et al. (31) has the 0.95-kb RFLP (data not shown).

Steady-state mRNA encoded in the deleted region and presence of open reading frames. To address the significance of the deletions, we compared the steady-state RNA expression patterns among the different variants, using probes from the deleted region. Several steady-state mRNAs are no longer present after the deletion of the upstream transcription unit (Fig. 10; compare RNA in variant 118a with RNA in variant 118b′), and similar results were obtained with the other variants (data not shown). We therefore conclude that these variant-specific RNAs are derived from the expression site on band 10. We have thus far obtained about 4 kb of the upstream transcription unit, and this domain encoded several mRNAs. The difficulty in obtaining these regions in clones has thus far prevented us from assessing the coding potential of the entire region. UV inactivation of transcription suggests that it may still be >5 kb from the upstream promoter.

Several short open reading frames are present in the sequences analyzed thus far. One of these, a 100-amino-acid open reading frame, shared 33% identity with the *E. coli dicBs* gene, which encodes a 62-amino-acid protein (10, 33). Expression of the *dicBs* gene inhibits division of *E. coli* either by affecting functioning of the *E. coli* beta subunit of RNA polymerase or by exerting translational control (33).

DISCUSSION

We have characterized promoters of VSG gene expression sites. The isolation of recombinant clones from chromosome-specific libraries and careful linkage of these clones to the VSG 118 gene have made it possible to characterize the VSG promoter region described here, rather than an unrelated repeat from elsewhere in the genome. The repetitive nature of these promoters and the difficulty in obtaining these repeats as recombinant clones have led other investigators to study related repeats (31, 53) or to use indirect arguments for the characterization of the promoter regions (76).

As shown, there is a high level of homology among the different expression site promoters, and only a few base substitutions can be identified in the regions immediately flanking the transcription initiation sites (Fig. 4B). The sequences directly upstream of the proposed promoters did not reveal significant DNA nucleotide sequence homology with either the α-amanitin resistantly transcribed procyclic acidic repetitive protein (PARP) gene promoters (12, 58), the rRNA gene promoter (75), or a previously identified subsidiary VSG gene promoter (62). DNA transfection experiments of these promoter regions fused to the chloramphenicol acetyltransferase (CAT) reporter gene have shown CAT activity for the PARP (58), rRNA (59a), and previously

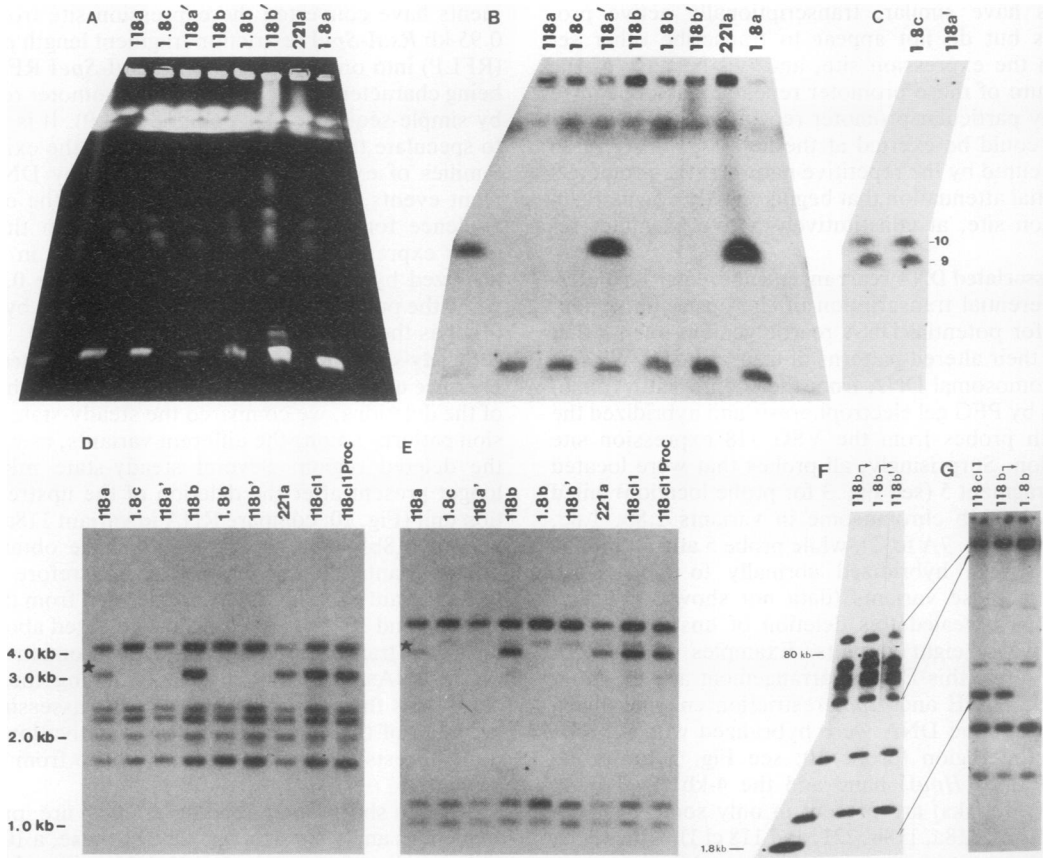

FIG. 7. Analysis of DNA rearrangements and deletions in the region 5′ to the downstream promoter of the expression site on the 1.5-Mb chromosome. (A) Ethidium bromide-stained PFG gel, run for 5 days at 5 V/cm at a pulse frequency of 900 s. Each of the variants from which DNA was size separated is shown above each lane. Individual chromosomes are numbered to the right according to Van der Ploeg et al. (72). (B) Southern blot of this gel after hybridization with probe 6.1 (see Fig. 3 for probe location). Note that hybridization to band 10 (indicated at the left) is seen only in variants 118a, 118b (active expression site on band 10, the 1.5-Mb chromosome), and 221a (active expression site on a larger, 3-Mb chromosome; the unrearranged, inactive expression site on the 1.5-Mb chromosome appears to be present). (C) Control rehybridization of two lanes from the blot in panel B. The blot was probed with probe 1.5Mb-A, a fragment isolated at random from a band 10, chromosome-specific library, which identifies chromosomes 9 and 10. Because of the curvature of the PFG gel, there is apparent misalignment of band 10 when panels B and C are compared. (D and E) Southern blots of genomic DNA from the variants identified above the lanes after digestion with *Hpa*II (D) and *Hpa*I (E). Hybridization of each Southern blot was performed with probe 10, which originates from sequences that are deleted from the upstream promoter region in several variants (see physical map in Fig. 3 for location of the probe). Sizes of restriction enzyme fragments are indicated at the left. 118c11Proc is an insect-form trypanosome line derived from bloodstream-form variant 118 cl 1. Bands marked with stars are present in variants 118a, 118b, 221a, and 118 cl 1 only. (F) Southern blot of genomic DNA from the variants identified above the lanes after digestion with restriction endonuclease *Hpa*II and hybridization with probe 5 (see physical map in Fig. 3 for probe location). Probe 5 is present in all variants and is located close to the recombination breakpoint. Note the 1.8-kb band, present in the unrearranged variants 118 cl 1 and 118b, that disappears in variant 1.8b while the band at the top of the gel increases in intensity of hybridization. This result is further clarified in panel G, which shows a Southern blot of a PFG gel with *Hpa*II-digested, genomic DNA from the trypanosome variants identified above the lanes. Note that the 1.8-kb band (marked by an asterisk in lane 118b) disappears in variant 1.8b in association with the appearance of a new 80-kb band (marked by an asterisk in lane 1.8b). This 80-kb band hybridized more weakly than its 1.8-kb counterpart because it was visualized in whole-trypanosome DNA maintained in solution, rather than in agarose blocks, to prevent the occurrence of possible partial digestion products. Limited shearing of very large DNA in solution resulted in a more faint appearance of the 80-kb band. Hybridization of additional large bands is more intense because of the repetitive nature of several of the large bands representing more than one copy (data not shown). Solid lines are used to align bands in the two blots.

characterized subsidiary VSG (36a) gene promoters. Recent findings by Zomerdijk et al. (76), who used the related VSG 221 promoter in transient expression assays in insect-form trypanosomes, showed that CAT activity could be detected once a 79-nt stretch from the promoter for one of the PARP genes (active in insect-form trypanosomes) and its 3′ splice acceptor site were fused to the VSG 221 promoter. The control and functional activity of the downstream promoter are currently being tested in our laboratory, with a focus on a comparison of the significance of transcription from episomes and endogenous loci. Importantly, the fact that the rRNA promoter fused to the PARP 3′ splice acceptor site produced CAT activity at levels comparable to that of the PARP promoter (59a) indicates that the α-amanitin resistantly transcribed VSG gene expression sites theoretically could be transcribed by RNA polymerase I.

Unlike other expression site promoter regions described thus far, the VSG gene expression site analyzed in variants 118 cl 1, 118a, and 118b consisted of overlapping transcription units with transcriptional readthrough into the down-

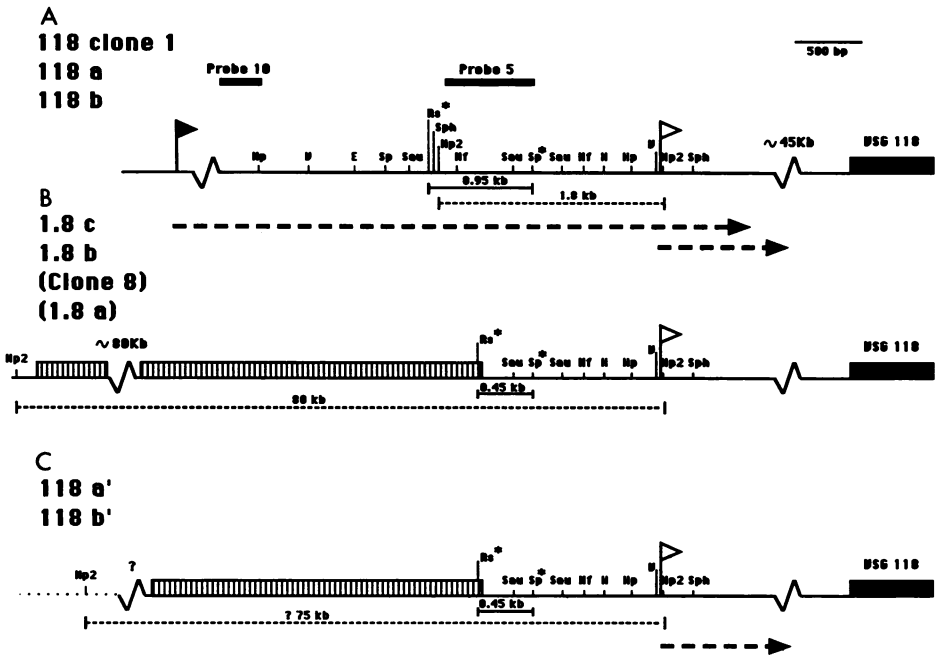

FIG. 8. Physical maps of the 1.5-Mb chromosome-derived expression sites in closely related variants, showing promoter-associated DNA rearrangements. (A) Physical map of the active expression site in the unrearranged variants (indicated at the left), with transcribed regions indicated by dashed arrows. The black flag (upstream promoter) and white flag (downstream promoter) are discussed in the text. The locations of probes 5 and 10 are shown above the physical map. The *Rsa*I and *Spe*I restriction enzyme sites (*) are discussed in the text. The sizes of the *Rsa*I-*Spe*I-generated RFLPs in every variant are shown below and marked with a solid line (0.95- and 0.45-kb fragments). *Hpa*II sites are discussed in the text and in the legend to Fig. 7F; the sizes of these fragments are highlighted by the light, dashed lines. Abbreviations for restriction endonuclease sites are as given in the legend to Fig. 1. (B) Physical map of the inactivated, rearranged expression site in the variants indicated at the left; clones in parentheses have been characterized with a few relevant restriction enzymes only. Simple-sequence DNA (▥) may consist entirely of 50-bp repeats. (C) Physical map of the rearranged, reactivated expression sites in the variants indicated. The direction of transcription is shown by the heavy dashed arrow. Question marks indicate uncertainties as to fragment size.

stream promoter region. The repetitive nature of the downstream promoter region has made it difficult to determine whether the downstream promoter is indeed active in the expression site on the 1.5-Mb chromosome in variants 118 cl 1, 118a, and 118b. However, independent of the activity of the downstream promoter in these variants, it is clear that a highly conserved internal (downstream) promoter element is part of the expression site on the 1.5-Mb chromosome.

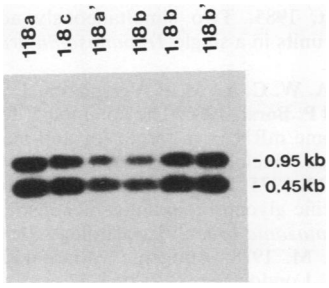

FIG. 9. Southern blot showing that repeated elements of the recombination breakpoint allow grouping of expression site-derived repeats into only two families. Genomic DNA from different trypanosome variants (indicated above the lanes) was double digested with the restriction endonucleases *Spe*I and *Rsa*I, size separated on an agarose gel, blotted to a nylon membrane, and hybridized with probe 5 (see Fig. 8 for probe location). Note the existence of unique 0.95- and 0.45-kb bands, each representing multiple expression sites.

Following a DNA rearrangement event, the downstream promoter becomes the major expression site promoter element and becomes structurally comparable to the previously described VSG 221 promoter (76).

We identified promoter-associated DNA rearrangement events that had not been previously observed (52, 76). These DNA rearrangement events went unnoticed because only a small portion had been obtained in recombinant clones and thus the DNA sequences 5′ to the internal (downstream) promoter could not be thoroughly analyzed. Creative use of PCR technology played a key role in overcoming this problem. These DNA rearrangement events specifically occurred at inactivation of the expression site in band 10 and resulted in the generation of a structurally altered expression site which, when reactivated, used only the downstream promoter. Several observations hint at the potential role of the DNA rearrangement events in transcriptional control: on the basis of RFLP mapping, we conclude that the DNA rearrangement events converted the expression site from a family of expression sites with an upstream transcription unit into one without. The role of the DNA rearrangement event may therefore be in affecting the existing pattern of transcription at the expression site as well as in affecting the mechanism by which the expression site can be reactivated following antigenic switches.

The mechanism by which the DNA rearrangement events occur during an antigenic switch is unclear. However, in the activation of VSG 1.8 in the switch from variant 118b to variant 1.8b, the 1.8 gene, located on band 8, was duplica-

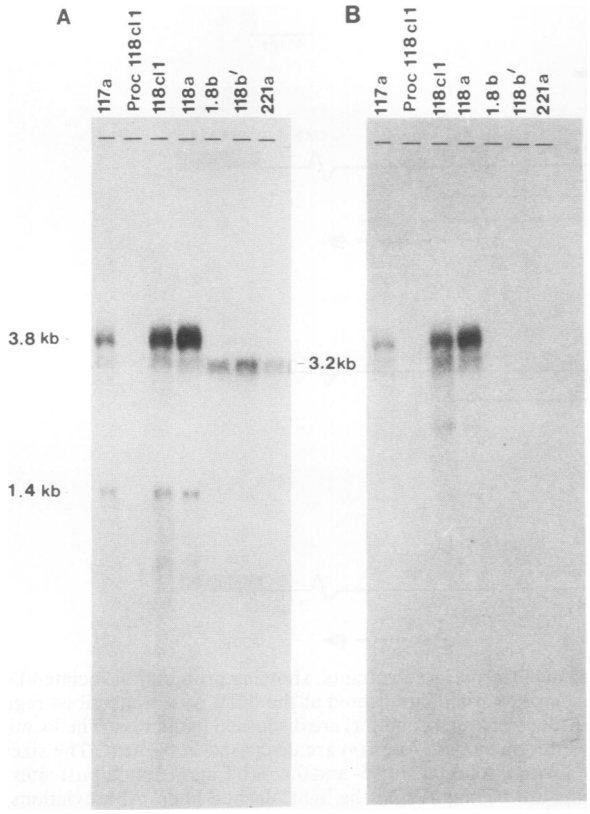

FIG. 10. Northern blots of steady-state RNA identified by probes from the deleted, upstream transcription unit. Steady-state RNA from the variants shown above the lanes was blotted to nylon membranes and hybridized to probes 10-11 (A) and 6.1 (B). Proc 118cl1 represents variant 118 cl 1 insect-form trypanosomes; variant 117a has an active expression site on the 1.5-Mb chromosome that is organized similarly to the expression site of variant 118 cl 1. Sizes of the RNA molecules are indicated on the left (see Fig. 3 for probe location). Probe 10-11 (A) is a fragment that encompasses both probes 10 and 11. Note that only probe 11 identifies the 3.2-kb, cross-hybridizing message present in all variants, while the other probes (probes 4 to 10 [Fig. 3]; data not shown) do not identify any steady-state RNA in variants that have a rearranged expression site (as exemplified by probe 6.1 [B]).

tively translocated on a 90-kb stretch of DNA to a chromosome in PFG band 5 (69). Sequences on at least three different chromosomes (chromosomes in PFG bands 5, 8, and 10) were thus involved in the switch from VSG 118b to VSG 1.8b (68, 69). It is tempting to speculate that these recombinational events, at three different chromosomes, occurred in a coordinated manner rather than independently. The control of expression sites therefore appears more complicated than stated in the previously proposed models of in situ activation and inactivation (76). The relevance of the DNA rearrangement events may also be at several other levels at which control can be exerted: (i) some expression sites can be preferentially activated early in the infection while others are preferentially used late, indicating that different types of expression sites indeed exist; (ii) some expression sites, in both the early and late types of expression sites, are preferentially used; and (iii) inactivation of expression sites in insect-form trypanosomes can occur without obvious DNA rearrangement events (25a), indicating that a different control mechanism operates in insect-form trypanosomes.

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